Memory facilitation and stimulation of endogenous nerve growth factor synthesis by the ACh releaser PG9

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Memory Facilitation and Stimulation of Endogenous Nerve Growth Factor Synthesis by the Acetylcholine Releaser PG-9

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ABSTRACT—The effects of PG-9 (3α-tropyl 2-(*p*-bromophenyl)propionate), the acetylcholine releaser, on memory processes and nerve growth factor (NGF) synthesis were evaluated. In the mouse passive-avoidance test, PG-9 (10–30 mg/kg, i.p.), administered 20 min before the training session, prevented amnesia induced by both the non selective antimuscarinic drug scopolamine and the M_1-selective antagonist *S*-(−)-ET-126. In the same experimental conditions, PG-9 (5–20 μg per mouse, i.c.v.) was also able to prevent antimuscarine-induced amnesia, demonstrating a central localization of the activity. At the highest effective doses, PG-9 did not produce any collateral symptoms as revealed by the Irwin test, and it did not modify spontaneous motility and inspection activity, as revealed by the hole-board test. PG-9 was also able to increase the amount of NGF secreted in vitro by astrocytes in a dose-dependent manner. The maximal NGF contents obtained by PG-9 were 17.6-fold of the control value. During culture, no morphological changes were found at effective concentrations of PG-9. The current work indicates the ability of PG-9 to induce beneficial effects on cognitive processes and stimulate activity of NGF synthesis in astroglial cells. Therefore, PG-9 could represent a potential useful drug able to improve the function of impaired cognitive processes.

Keywords: PG-9, Learning, Memory, Nerve growth factor, Cholinergic system

Cholinergic activity has long been associated with memory processes. Morphological and neurochemical studies of Alzheimer's disease, the major type of dementia, revealed marked decreases in the cholinergic innervation of the cortex and hippocampus (1–3). Drugs involving cholinergic stimulation alleviated cognitive dysfunctions in Alzheimer's disease (1) and in particular unselective muscarinic agonists (oxotremorine, thiopilpcarpine), M_1-selective agonists (McN-A-343, AF102B, xanomeline) and acetylcholine (ACh) releasers (SM-21, linopirdine, ondansetron) may represent a treatment strategy in this pathology (2–5).

Nerve growth factor (NGF) is a protein that is required for the survival and differentiation of cholinergic neurons of the basal forebrain in the central nervous system (6). Pathophysiological studies and animal experiments have suggested the possible use of NGF as a therapeutic tool for degenerative neuronal disorders such as Alzheimer's disease (7–10).

Since PG-9 has been reported to be able to enhance ACh release (11) and NGF is important for the functionality of the cholinergic system, we thought it worthwhile to investigate its capability of ameliorating cognitive processes in vivo and ability of enhancing NGF production in vitro.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (23–30 g) from Morini breeding farm (San Polo d'Enza, Italy) were used. Fifteen mice were housed per cage. The cages were placed in the experimental room 24 hr before the test for acclimatization. The animals were kept at 23±1°C with 12-hr light/dark cycle, light at 7 a.m., with food and water ad libitum. All experiments were carried out according to the "Guiding Principles for the Care and Use of Laboratory Animals".

I.c.v. injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anesthesia using isotonic saline as solvent, according to the method described by Haley and McCormick (12) for mice. Briefly, during anesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4-mm external diameter hypodermic needle attached to a 10-μl syringe was inserted perpendicularly through the skull at a depth of no more than 2 mm into the brain, where 5 μl of drug solution was then administered. The injection site was 1.5 mm from either side of the middle of a line drawn through to the anterior base of the ears. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice were i.c.v. injected with 5 μl of diluted 1 : 10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was 95.

In vivo studies

Passive-avoidance test: The test was performed according to the step-through method described by Jarvik and Kopp (13). The apparatus consists of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. Mice, as soon as they entered the dark compartment, received a punishing electrical shock (0.5 mA, 1 sec). The latency times for entering the dark compartment were measured in the training test and after 24 hr in the retention test. For memory disruption, animals were injected with the amnesic drugs scopolamine and *S*-(−)-ET-126, administered immediately after termination of the training session. PG-9 was injected 20 min before the training session. The maximum entry latency allowed in the training and retention session was 60 and 180 sec, respectively. The memory degree of received punishment was expressed as latencies recorded in the training and retention sessions.

Hole-board test: The hole-board test consists of a 40-cm square plane with 16 flush mounted cylindrical holes (diameter of 3 cm) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and left to move about freely for a period of 5 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animals on the surface of the plane. Miniature photoelectric cells, in each of the 16 holes, recorded the exploration of the holes (head plunging activity) by the mice. The test was performed 15 min after PG-9 injection.

Irwin test: The test was performed according to the method described by Irwin (14).

In vitro studies

NGF synthesis stimulatory activity: Astrocytes were cultured from a few-day-old ICR mouse (Japan SLC, Shizuoka) brain as described previously (15, 16). In short, the cells were grown to confluency in a 5% CO_2 atmosphere at 37°C in 10-cm culture dishes (Falcon, Oxnard, CA, USA) containing Dulbecco's minimum essential Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Then the cells were brought into the quiescent phase by being cultured for another one week in the same medium except that 0.5% bovine serum albumin (BSA) was substituted for the FCS. The medium was changed every 3 days, and the cells were then used for the experiments. PG-9 was prepared at threefold varing concentrations from 0.3 μg to 1 mg/ml in DMEM containing 0.5% BSA and added to the culture. The culture media were collected following 24-hr culture, and their NGF contents determined by a sensitive enzyme immunoassay (EIA) for mouse NGF as described (17, 18). Epinephrine was used as a positive compound with NGF stimulatory activity (19, 20).

Drugs

The following drugs were used: PG-9 (3α-tropyl 2-(*p*-bromophenyl)propionate) racemate and *S*-(−)-ET-126 (*S*-(−)-α-(hydroxymethyl)benzene-acetic acid 1-methyl-4-piperidinyl ester) were prepared in the Department of Pharmaceutical Sciences of the University of Florence, according to Gualtieri et al. (21) and Gualtieri et al. (22), respectively; scopolamine hydrobromide, physostigmine hemisulfate, epinephrine (Sigma, St. Louis, MO, USA); oxotremorine (Fluka, Buchs, Switzerland); D-amphetamine sulfate (Recordati, Milan, Italy); Dulbecco's minimum essential Eagle's medium (Nissui, Tokyo); fetal calf serum (Irvine Scientific, Santa Ana, CA, USA); bovine serum albumin (Nacalai Tesque, Kyoto). All drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml/kg by the intraperitoneal (i.p.) route or 5 μl per mouse by the i.c.v. route.

Statistical analyses

Results are given as the mean±S.E.M.; analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) procedure for post-hoc comparison, was used to verify the significance between two means of behavioral experiments. The significance between experimental values and the control value obtained without drug in in vitro experiments was evaluated by the Cochran-Cox test; P values of less than 0.05 were considered significant. Data were analyzed with the StatView for the Macintosh computer program (1992).

RESULTS

In vivo experiments

Antiamnesic activity of PG-9: Pretreatment with PG-9 (10–30 mg/kg, i.p.; 5–20 μg per mouse, i.c.v.), injected 20 min before the training session, prevented the amnesia induced by scopolamine (1 mg/kg, i.p.; Fig. 1) and *S*-(−)-ET-126 (0.1 μg per mouse, i.c.v.; Fig. 2) in the mouse passive-avoidance test. PG-9 enhanced the entrance latency up to a value comparable to that produced by control animals (Figs. 1 and 2). PG-9, at 1 mg/kg, i.p. and 1 μg per mouse, i.c.v., was completely ineffective (Figs. 1 and 2). The antiamnesic effect of PG-9 was equal to that produced by the cholinesterase inhibitor physostigmine (0.2 mg/kg, i.p.) and the nootropic drug piracetam (30 mg/kg, i.p.) (data not shown).

PG-9, when given alone, at the highest doses used, had no effect on the mouse passive-avoidance test in comparison with saline-treated mice, nor were there any differences in the entrance latencies for each group in the training session of the passive-avoidance test (Figs. 1 and 2).

Evaluation of the PG-9 effect on mice behavior: The spontaneous motility and inspection activity of mice was unmodified by pretreatment with PG-9 (30 mg/kg, i.p.; 20 μg per mouse, i.c.v.) in comparison with saline-treated mice as revealed by the hole-board test (Fig. 3). In the same experimental conditions, D-amphetamine (1 mg/kg, s.c.) increased both evaluated parameters (Fig. 3).

PG-9, unlike oxotremorine and physostigmine, prevented scopolamine- and *S*-(−)-ET-126-induced amnesia without causing the typical cholinergic symptomatology evaluated by the Irwin test (Table 1).

In vitro experiments

Effect of PG-9 on NGF synthesis on cultured mouse astrocytes: We used quiescent astrocytes in culture to evaluate NGF synthesis stimulatory activity for the following reasons: 1) NGF synthesis is regulated in a growth-dependent manner in cultured astrocytes (15); 2) most of the astrocytes in in vivo brain are in the quiescent phase and do not express NGF gene. Figure 4 shows that PG-9 and epinephrine obviously increased the amount of NGF secreted by the cells in a dose-dependent manner. The maximal NGF contents obtained by PG-9 and epinephrine at their optimal concentrations were 16.7- and 5.9-fold, respectively, of the control value. During culture, no morphological changes were found at effective concentrations of both drugs. However, slight cell toxicity or morphological changes were observed at 1 mg/ml of PG-9, which might cause an abrupt decline of the NGF content.

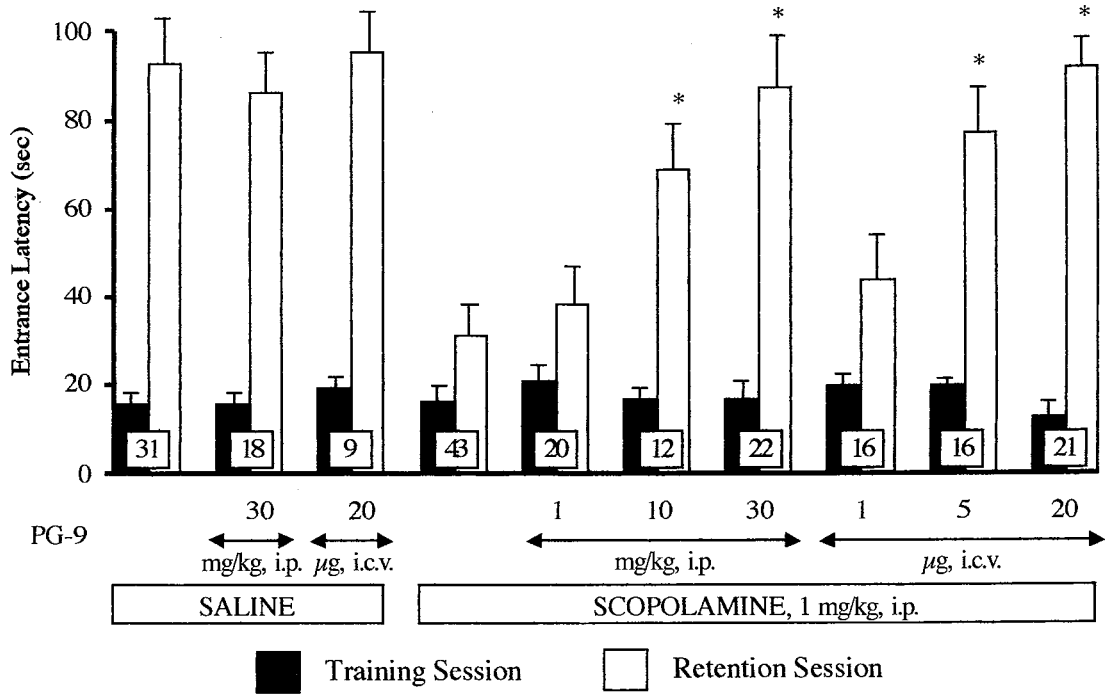

Fig. 1. Effect of injected PG-9 on scopolamine-induced amnesia in mouse passive-avoidance test. PG-9 was injected 20 min before the training session and scopolamine was administered immediately after the training session. *$P<0.01$, in comparison with the scopolamine-treated mice. The number of mice is inside the columns. Vertical lines represent S.E.M.

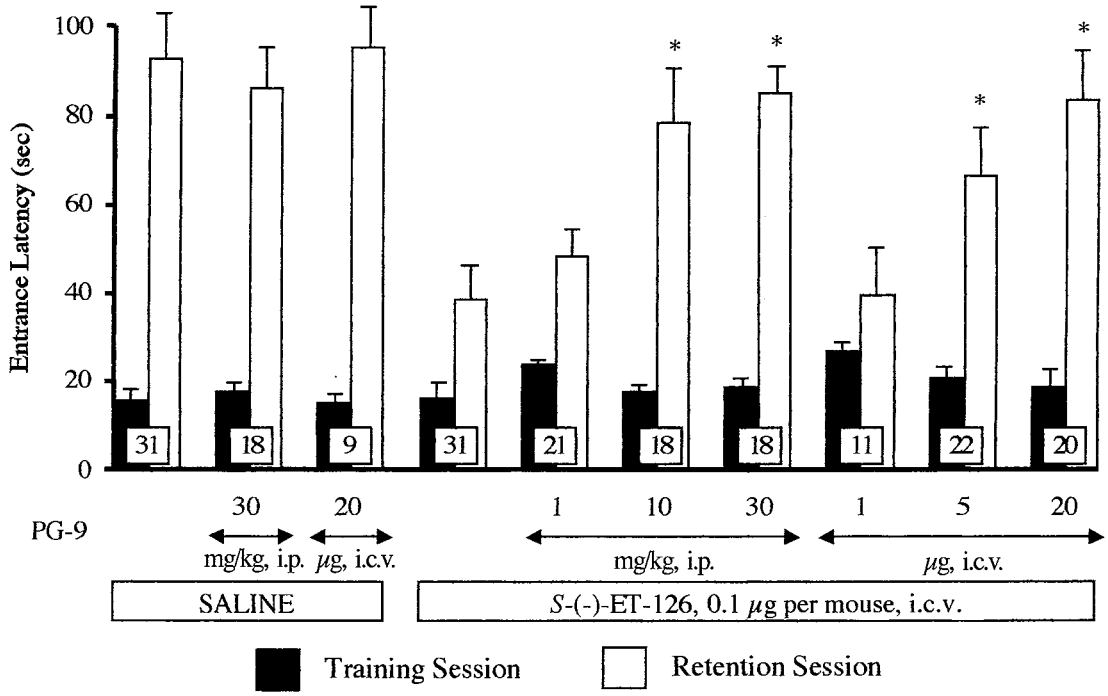

Fig. 2. Effect of PG-9 injected on *S*-(−)-ET-126-induced amnesia in mouse passive-avoidance test. PG-9 was injected 20 min before the training session and *S*-(−)-ET-126 was administered immediately after the training session. *P < 0.01, in comparison with *S*-(−)-ET-126-treated mice. The number of mice is inside the columns. Vertical lines represent S.E.M.

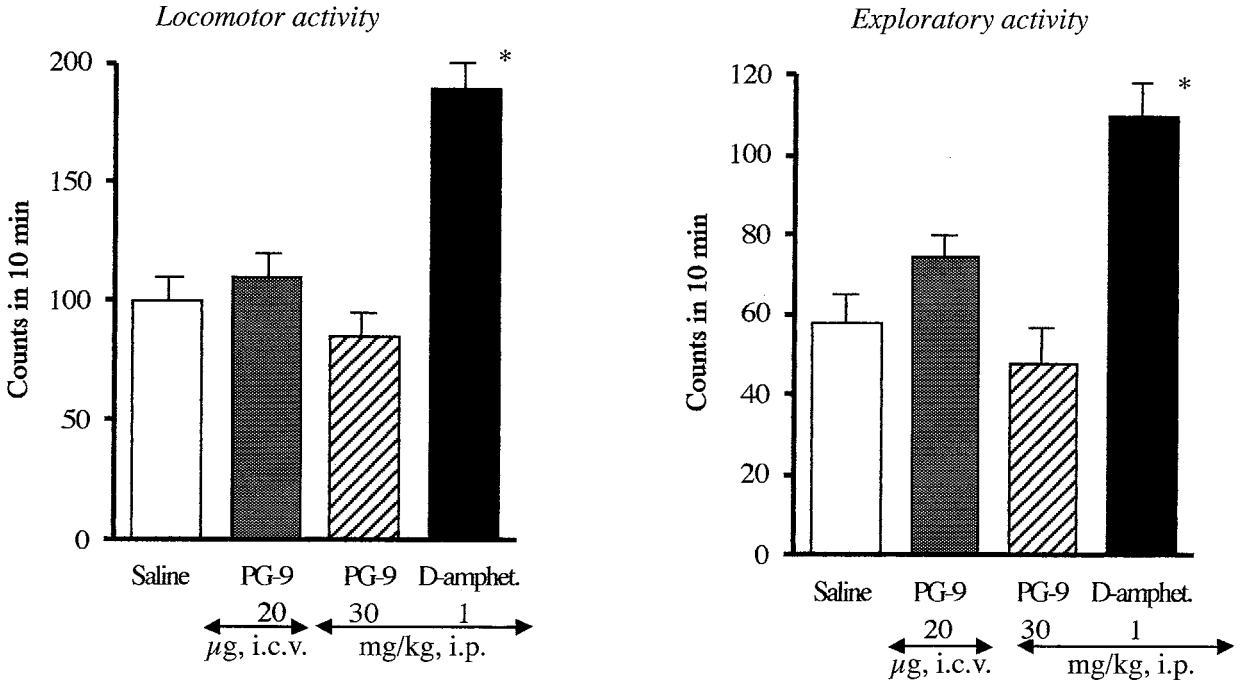

Fig. 3. Lack of effect of PG-9 on hole-board test in comparison with D-amphetamine. The responses were recorded 15–25 min after drug administration. Each column represents the mean of 10 mice. *P < 0.01, in comparison with saline controls.

Table 1. Effect of PG-9 in comparison with oxotremorine and physostigmine in the Irwin test

Treatment		Tremors	Salivation	Lacrimation	Diarrhea	Abdominal tone	Spontaneous motility
Saline	s.c.	0	0	0	0	4	4
PG-9	30 mg/kg, i.p.	0	0	0	0	4	4
PG-9	20 μg, i.c.v.	0	0	0	0	4	4
Oxotremorine	40 μg/kg, s.c.	4	4	+	+	0	2
Physostigmine	200 μg/kg, s.c.	2	6	+	+	2	0

Tremors: absent=0, maximum score=8; Salivation: absent=0, maximum score=8; Lacrimation: absent=0, present=+; Diarrhea: absent=0, present=+; Abdominal tone: flaccid abdomen=0, normal=4, abdomen board-like=8; Spontaneous motility: absent=0, normal=4, maximum socre=8. Each value represents the mean of 5 mice. Spontaneous motility was evaluated by Animex test.

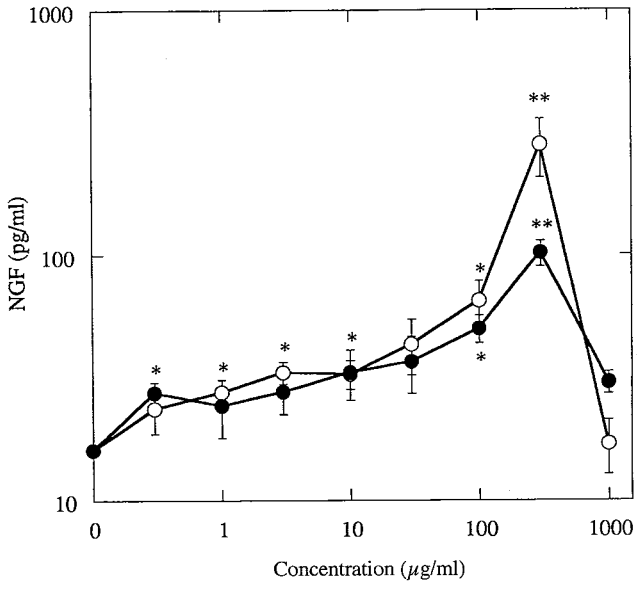

Fig. 4. Effect of PG-9 and epinephrine on NGF content in conditioned medium of mouse astrocytes. Mouse astrocytes in the quiescent phase were cultured for 1 day in DMEM supplemented with 0.5% BSA which contained PG-9 or epinephrine at three-fold varying concentrations. The culture media were collected and provided for the EIA to determine NGF content. NGF content in the media was calculated from a standard curve of known concentrations of NGF purified from the mouse submaxillary gland according to Zafra et al. (35), *$P<0.05$, **$P<0.01$, in comparison with control values. ○: PG-9, ●: epinephrine.

DISCUSSION

The present results indicate that PG-9 was able to prevent impairment of the acquistition of a passive-avoidance response induced by antimuscarinic drugs. The PG-9 antiamnesic effect was obtained without visibly modifying animal gross behavior. Moreover, PG-9-treated mice showed a normal spontaneous motility and inspection activity as revealed by the hole-board test. Amnesia was induced by using the unselective antimuscarinic drug scopolamine and the M_1-selective antagonist *S*-(−)-ET-126 (23) since it has been reported that the muscarinic receptor subtype mainly involved in the regulation of memory processes is the M_1-receptor subtype. M_1-selective agonists have been indicated as a useful strategy for the treatment of Alzheimer's disease (2−5). On the other hand, treating animals with the M_1-selective antagonists pirenzepine (24), dicyclomine (25) and *S*-(−)-ET-126 impaired the passive avoidance learning in mice (26−28).

PG-9 exerted its antiamnesic effect by acting centrally. It was in fact, possible to prevent antimuscarinic drug-induced amnesia by injecting directly into the cerebral ventricles doses (5−20 μg per mouse) of PG-9 that were about fifty times lower than those needed parenterally. Thus the possibility that the memory facilitating effect depends on a retrodiffusion of the drug from the cerebral ventricles to the periphery can be ruled out.

The prevention by PG-9 of the action of the M_1-antagonist *S*-(−)-ET-126 injected i.c.v. further supports the hypothesis of a central mechanism for the PG-9 antiamnesic effect.

Since it has long been known that stimulation of the cholinergic system improves cognitive processes (29), it is reasonable to suppose that the antiamnesic effect induced by PG-9 could be related to its ability to activate the cholinergic system. A presynaptic cholinergic mechanism for PG-9 can be hypothesized on the bases of previous results demonstrating, by microdialysis studies, an increase in ACh release from rat cerebral cortex induced by PG-9 administration (11). This effect occurred in the same range of doses in which PG-9 exerted the facilitation of memory processes. The increase in ACh extracellular levels is also responsible for other pharmacological activities of PG-9 such as antinociception (30).

It should be noted that PG-9 is able to prevent the amnesic effect of antimuscarinic drugs, but it is unable to ameliorate cognitive functions in unimpaired animals.

PG-9 stimulated NGF synthesis of cultured astrocytes to a much higher extent than epinephrine, a well-known stimulator of NGF synthesis. The question has been

raised about whether the compound can stimulate NGF synthesis in in vivo brain. There is a case of 4-methylcatechol, one of the low-molecular-weight compounds with the potent stimulatory activity on cultured astrocytes (16, 20). Intraperitoneal administration of 4-methylcatechol at a dosage much lower than that effective in vitro enhanced NGF synthesis in the rat peripheral nervous system (18). Repetitive administration of the compound caused marked enhancement of tyrosine hydroxylase activity in the sympathetic ganglia, showing that the NGF excessively produced is physiologically active (18). I.c.v. (31) and peripheral (32) administration of 4-methylcatechol has been proved to cause an elevation of NGF synthesis in the adult rat brain.

NGF and brain-derived neurotrophic factor (BDNF), another member of the neurotrophin family of neurotrophic factors, are dominantly synthesized in neurons of projection areas of basal forebrain magnocellular cholinergic neurons such as the hippocampus and cortex (33, 34). Depolarizing stimulations such as treatment with kainic acid enhance NGF and BDNF mRNA expression in neurons of the hippocampus and cerebral cortex in vivo and in vitro (35). The activation of the cholinergic system but not the stimulation by other neurotransmitters enhances BDNF mRNA in cultured hippocampal neurons (35). It is also suggested that septal cholinergic input is involved in the regulation of hippocampal BDNF and NGF mRNA levels (36).

Our in vitro observations suggest that PG-9 might stimulate NGF synthesis also in in vivo brain and that the excessively produced NGF could act on basal forebrain cholinergic neurons, a well-known target of NGF. PG-9 is active in the behavioral paradigm when injected 20 min before the test. At this administration time, PG-9 also reaches its maximum effect on ACh release (11). The effect of PG-9 on NGF production is measured after 24 hr because at this time NGF is accumulated and easily detectable as previously reported (37). This discrepancy in the time courese between the in vivo and in vitro studies could be explained by the fact that the retention test is performed 24 hr after the training session when the stimulatory activity on NGF is already obtained. Therefore, we can suppose that ACh release by PG-9 may be involved in functional facilitation of the central cholinergic system via enhancement of neuronal NGF synthesis. This activity could explain the protection exerted by PG-9 against antimuscarine-induced amnesia. However, it is also plausible that the NGF stimulatory activity produced by PG-9 should cause a rather lately-evoked action such as increase in choline acetyl transferase activity, independent from the increase in ACh release.

The current work indicates that the ACh releaser PG-9 is a compound endowed with beneficial effects on cognitive processes and stimulating activity of NGF synthesis in astroglial cells. Therefore, PG-9 may represent a useful drug able to ameliorate neuronal functions and may be a potential treatment for cognitive disorders.

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