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Specific cholinergic destruction in the basal magnocellular nucleus and impaired passive avoidance behavior of rodents

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Key words: Memory; Alzheimer's disease; Cholinergic system; Basal forebrain; Selective lesion; Nerve growth factor diphtheria toxin

A nerve growth factor (NGF)-diphtheria toxin conjugate (NGDT) was found to selectively abolish or depress the activity of NGF receptor-bearing cholinergic neurons of the basal magnocellular nucleus (BMN). Bilateral cortical injections of NGDT impaired the retention of passive avoidance behavior in mice. A memory deficit was also revealed when cortical injections of NGDT were administered after the acquisition of a passive avoidance response. Thus, retrograde destruction of BMN cholinergic neurons by the cortical injection of NGDT interfered with both learning and memory processes. The animal model outlined here should be useful in analyzing the pathogenesis of Alzheimer's disease and the functions of the cholinergic system in the BMN.

INTRODUCTION

The selective loss of acetylcholine from the cerebral cortex is one of the best established neurochemical findings in Alzheimer's disease[1,4,17,29], and depletion of acetylcholine has been used in several attempts to produce animal models of this disorder[3,5,11,14,18,23,24,28]. The most common technique has been to electrically produce a lesion of the basal nucleus of Meynert, which is the main source of cholinergic fibers in the cerebral cortex[11,14,23]. This method, however, has the disadvantage of also destroying noncholinergic neurons in the nucleus and nerve fibers passing through it. A similar lack of specificity has been observed when toxic substances such as quinolic acid[15], kainic acid, and ibotenic acid have been used in place of an electrical current. Kainic acid or *N*-methyl-D-aspartic acid (NMDA) injected into the cerebral cortex can destroy cholinergic neurons in the basal nucleus[3,6,24] by a mechanism that has been attributed to transneuronal retrograde degeneration[25]. However, changes also occur in non-cholinergic neurons in the BMN and extensive degeneration develops in the cerebral cortex and probably in other areas which project to this region of the brain. Ventricular injection of a selective cholinergic neurotoxin, ethylcholine mustard aziridinium ion (AF64A), is also unable to produce a useful model, since this compound affects almost all cholinergic neurons in the central nervous system[28]. One way to limit the damage to cholinergic neurons in the basal nucleus might be to inject AF64A directly into the basal forebrain, but the destruction of non-cholinergic neurons and nerve fibers passing through the basal nucleus would still occur and the possibility of AF64A diffusing into other brain regions would exist.

Accordingly, we recently developed a method for the selective destruction of cholinergic neurons in the basal forebrain area as a new animal model of Alzheimer's disease[10,22]. The presence of NGF and its receptors has been demonstrated in the central nervous system[9,21,23,26]. There is agreement that NGF receptors are localized only on the basal forebrain cholinergic neuron group (Ch1–4)[8,26], while other cholinergic and non-cholinergic neurons lack NGF receptors in adult animals. Recent findings have demonstrating that NGF receptors have a role in binding, internalizing and transporting NGF from the nerve terminal to the soma[16,27]. In addition, NGF injected exogenously into the cerebral cortex has been shown to be specifically accumulated in the cholinergic soma of the basal forebrain[19,20].

Injection of an NGF-diphtheria toxin (DT) conjugate

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(NGDT) into the cerebral cortex of rats has been shown to result in a marked ipsilateral reduction of cholinergic neurons in the horizontal limb of the diagonal band and BMN. No adverse effects due to this conjugate were detected in the cholinergic neurons of other regions of the brain or in the catecholaminergic neurons[10]. The selectivity of the damage to the cholinergic neurons in the basal forebrain suggested that this animal model could be very useful in analyzing the pathogenesis of Alzheimer's disease. In the present study, to further establish the value of our model of Alzheimer's disease, we examined passive avoidance learning behavior in mice injected with the NGDT-conjugate.

MATERIALS AND METHODS

Preparation of NGDT

Forty μl of 20 μM 2.5S-NGF (Collaborative Res.) were mixed with 7 μl of 63 μM diphtheria toxin (59 kDa) in a siliconized plastic tube. Following the addition of 40 μl of 1-ethyl-3-(3-L-dimethyl aminopropyl) carbodiimide-HCl (100 mg/ml; Sigma, U.S.A.), the mixture was allowed to react for 12 h at room temperature, and then dialyzed against 0.02 M phosphate-buffered saline (PBS) for 24 h at 4 °C. The production of NGDT and contamination by unconjugated free toxin were checked by SDS–polyacrylamide gel electrophoresis with silver staining and by HPLC.

Preparation of primary cultured cells

The basal forebrain of a rat embryo (gestational age: 17–19 days) was removed and dissected into small pieces with scissors under sterile conditions. The dissected tissue was then digested at 37 °C for 10 min in a solution of 180 U of papain (Sigma, U.S.A.), 0.02% DL-cysteine-HCl, 0.02% bovine serum albumin (Sigma Type V, U.S.A.), 0.5% glucose, and 0.1% deoxyribonuclease (Sigma, U.S.A.) in PBS (pH 7.4). A precipitate was obtained by centrifugation at 900 rpm and was washed with Dulbecco's modified Eagle's medium (DMEM; Gibco, U.S.A.). The precipitate was resuspended in DMEM containing 10% fetal calf serum (Sigma, U.S.A.) and triturated using a fire-polished pipette. Cells were counted using a Thoma's hemocytometer and were placed on a Flexiperm Mikro 12 (Heraus Biotek, F.R.G.) with a poly-D-lysine-(M.W. 30,000–70,000; Sigma, U.S.A.) precoated coverglass (1×10^5 cells/well). Cultured cells were maintained at 37 °C in DMEM containing 10% fetal calf serum and 100 ng/ml NGF with a 95% CO_2/5% O_2 atmosphere[7].

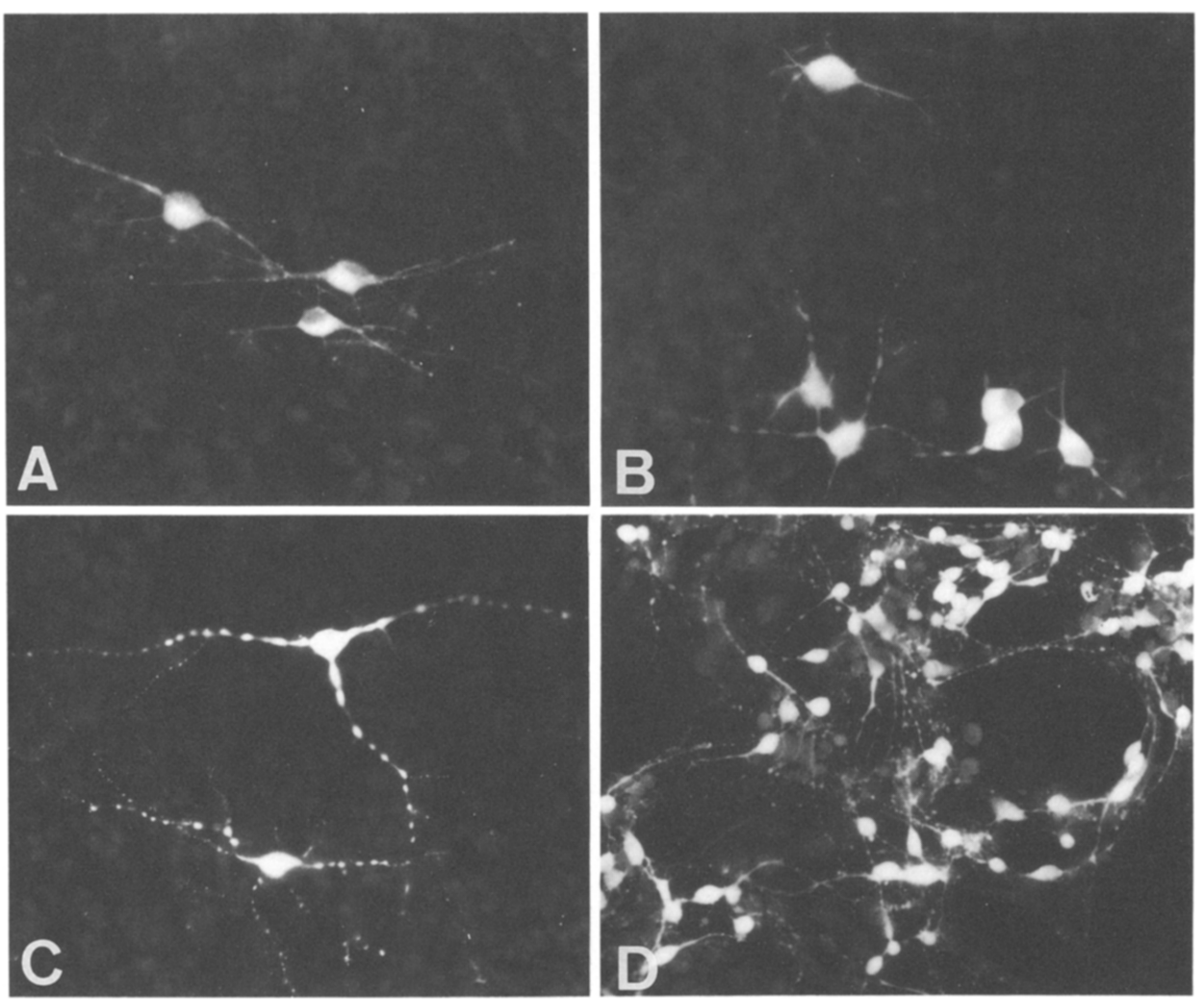

Fig. 1. Immunostaining of cultured neurons using (A) anti-NGF-receptor antiserum, (B) anti-choline acetyltransferase antiserum, (C) anti-tyrosine hydroxylase antiserum, and (D) anti-neuron-specific enolase antiserum.

Assessment of the toxicity of NGDT for cultured cells

To observe the effect of NGDT on cultured cells, NGDT in PBS was added to culture wells at final concentrations of 5, 10, 20, and 50 ng/ml. DT in PBS was added at concentrations of 3, 6, 12, 30, 60, 120, and 300 ng/ml to observe the non-selective effect of DT on cultured cells. As a control, PBS alone was added to the wells.

After one week, cultured cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH. 7.4) for 5 h at 4 °C. Cells were then rinsed 3 times in PBS for 10 min each time, followed by rinsing in PBS containing 3% bovine serum albumin and 0.3% Triton X-100 for 30 min. Immunocytochemical analysis was then undertaken for NGF receptors (NGF-R; donated by Professor Hatanaka, Osaka University), choline acetyltransferase (ChAT; Chemicon), tyrosine hydroxylase (TH; Eugine Tech) and neuron specific enolase (NSE) as described elsewhere.

Cell counts and data analysis

Cultured cells were observed and photographed under a Nikon TMD interference microscope. After immunostaining was carried out by an immunofluorescent technique[2] and cells were photographed under a Nikon fluorescent microscope, immunofluorescent cells were counted and recorded as a percentage with respect to the number of immunofluorescent cells of the control (no NGDT added). Experiments were performed 5–7 times, the data were pooled, and were subjected to analysis by Student's two-tailed *t*-test.

Histochemical assessment of the selectivity of NGDT toxicity for cholinergic neurons of the BMN

Male Wistar rats weighing 150–170 g were used in this study. NGDT solution (0.5 μl; 0.4 μg/μl) was stereotaxically injected into the cerebral cortex on one side as described previously[10]. After 5 days, the animals were perfused transcardially with 500 ml of a 4% paraformaldehyde-phosphate buffer solution under pentobarbital anesthesia. The brains were quickly removed and tissue sections were subjected to indirect immunofluorescence staining using ChAT. Photographs of the medial septal nucleus, the horizontal limb of the diagonal band, and the BMN were processed with an image analyzer (Argus-100, Hamamatsu Photonics, Japan). Cells with a fluorescence intensity above 85 as determined by the Argus-100 were designated as 'high intensity' cells, and cells with a fluorescence intensity below 85 were designated as 'low intensity' cells. The number of high intensity and low intensity cells in each section was compared between the NGDT-injected side and the PBS-treated (control) side. Data obtained using 11 sections from 3 rats were pooled and analyzed using Student's two-tailed *t*-test.

Passive avoidance learning test

Animals. Male ddY mice weighing 30 g (Shizuoka Laboratory Animal Center, Japan) were housed individually in a temperature-controlled room (20–22 °C) with a 12-h light/dark cycle, and were allowed free access to food and water.

Injection of NGDT. Under anesthesia, 0.2 μl of the NGDT solution (0.4 μg/μl) was stereotaxically injected bilaterally into the frontal and parietal cortex using a Hamilton syringe. Control animals were given a similar injection of 0.2 μl of saline instead of NGDT.

ChAT immunoreactivity and assessment of lesions induced at the NGDT injection site. Several animals were taken from each group and post-injection changes in the brain were investigated immunocytochemically. ChAT immunoreactivity was used as a marker for cholinergic neurons.

The NGDT-injected area was immunostained using antisera for neuropeptide Y, somatostatin, and substance P to determine changes in the interneurons of the cerebral cortex. The brains of some of the animals were embedded in paraffin and stained with hematoxylin–eosin to determine the histological architecture of the injected area.

Passive avoidance learning test. A one-trial step-through passive avoidance task was set. In experiment 1, two groups of 26 mice underwent the injection of either saline or NGDT into the cerebral cortex. Three days after the injections were given, training commenced with the mouse being placed in a small illuminated chamber (7 × 9 × 14 cm). The guillotine door was immediately raised, and when the animal had moved completely into a large dark adjoining chamber (14 × 14 × 14 cm), a 3-s AC current (0.5 mA) was delivered via the metal grid floor. Immediately following the electric shock, the mice returned to the illuminated chamber. Animals displaying an initial latency period of more than 120 s before re-entering the dark chamber were selected for further experimentation. The remaining mice were subjected to repeated trials and those still not exceeding the 120 s mark were excluded from further involvement in this study.

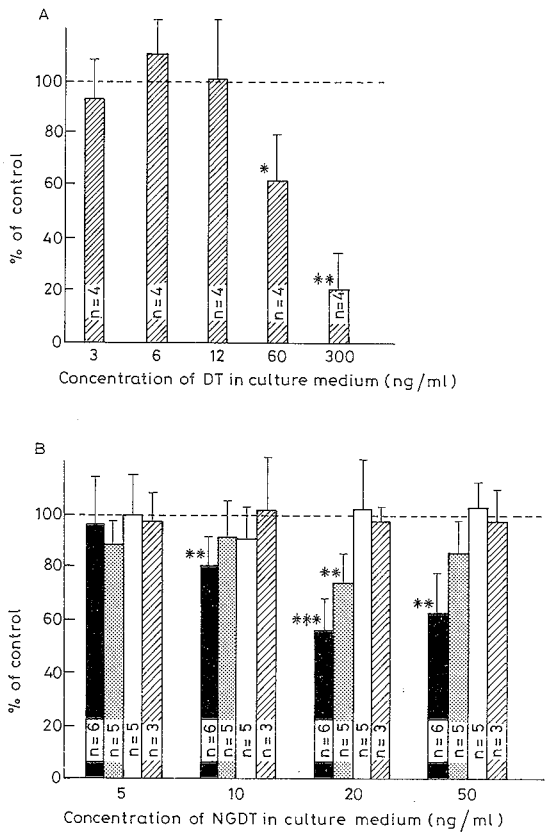

Fig. 2. A: concentration-dependent decrease in the number of NSE immunoreactive cells in DT-supplemented culture medium. B: decrease of neurons immunoreactive for NGF-R (black bars) and ChAT (dotted bars), but not those immunoreactive for NSE (shaded bars) and TH (open bars) in NGDT-supplemented culture medium. Bars denote the mean ± S.D. Significant difference from the control: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Animals were then returned to their home cage and a retention test was given 8 days later. Retention latency was measured in a manner similar to that described above, except that an electric shock was not applied and the guillotine door was always open. Two kinds of latency were recorded, according to whether the body from the head to the first lumbar level (L1) (upper body latency) or from the head to the hind limbs (whole body latency) entered the dark room. If the latency period was longer than 300 s, the experiment was stopped and a value of 300 s was recorded. In experiment 2, two groups of 29 untreated mice were first put through the acquisition trials and then immediately treated by an injection of either saline or NGDT into the cerebral cortex. Eight days after the operation, these animals were subjected to the retention test.

Data analysis for the passive avoidance learning test. The mean latency period (measured in seconds) before animals entered the

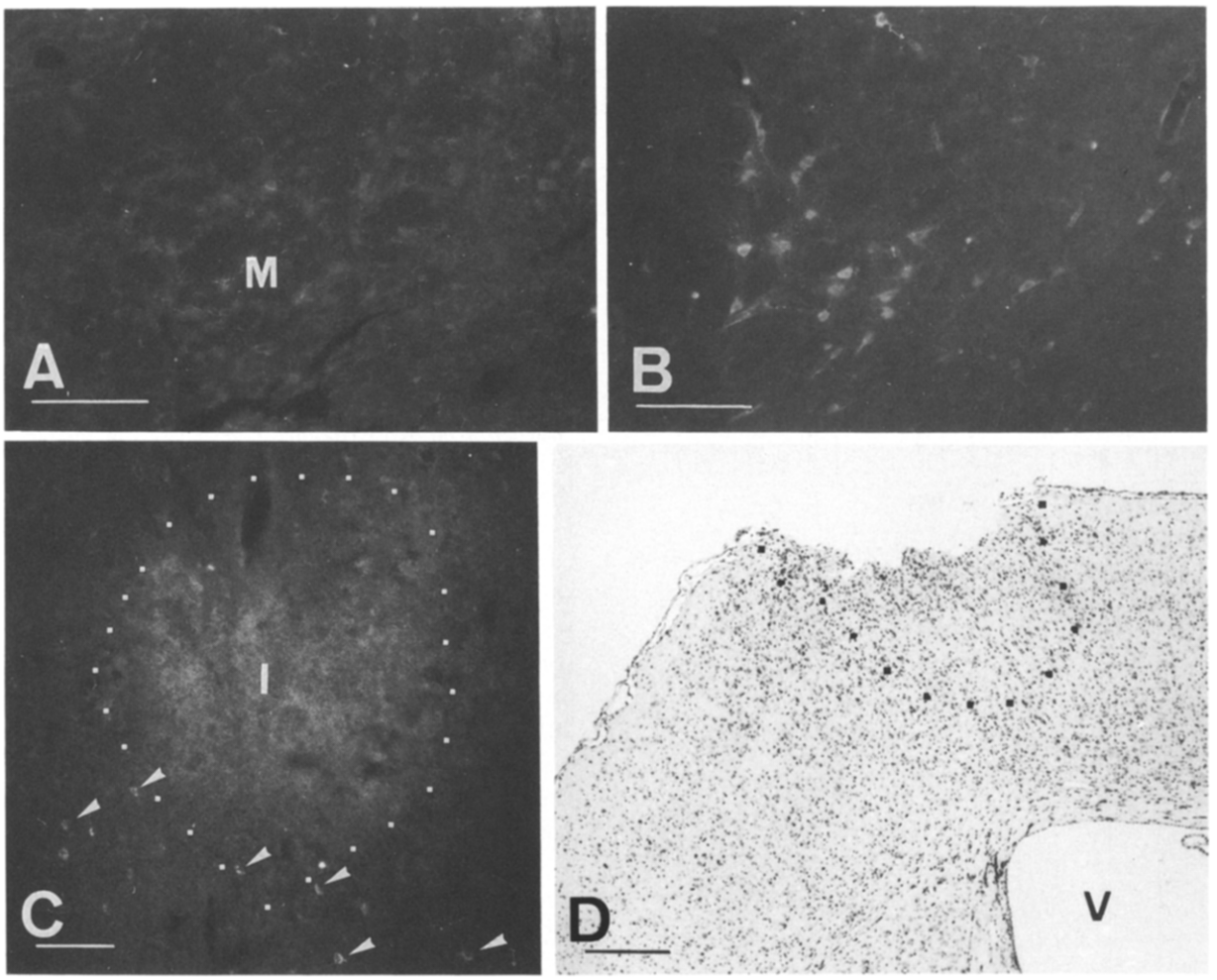

Fig. 3. Fluorescence photomicrographs showing changes in choline acetyltransferase-immunoreactive neurons in the BMN of NGDT-injected (A) and control (B) mice. Immunoreactive neurons showed weaker fluorescence and were less numerous in the NGDT-injected group. C: fluorescence photomicrograph showing somatostatin-immunoreactive neurons in the cerebral cortex. A number of immunoreactive cells were found close to the injection site (surrounded by a broken line). (D) Bright-field photomicrograph showing hematoxylin–eosin staining of one of the NGDT-injection sites (surrounded by a broken line). Abbreviations: I, injection site; M, basal nucleus of Meynert; V, lateral ventricle. Scale bars = 100 μm (A–C) and 300 μm (D).

dark room during the retention test was compared between the NGDT-injected and saline-injected groups (n = 26 for experiment 1 and n = 29 for experiment 2 in both groups) using the Mann–Whitney U-test (two-tailed). Fisher's exact probability test was employed for the analysis of latency periods of longer than 300 s.

RESULTS

Selective effect of NGDT on cultured NGF-R and ChAT immunoreactive neurons

Before assessing the effects of NGDT, the toxicity of unconjugated DT for cultured rat forebrain cells was determined. Phase contrast photography revealed that DT had no effect on the cultured cells at concentrations of up to 12 ng/ml. However, at 60 ng/ml there was an obvious decrease in cell numbers (data not shown). An immunocytochemical study using NSE antiserum was performed to determine the total number of neuronal cells among the cultured forebrain cells (Fig. 1D). Figure 2A shows the number of NSE-immunoreactive cells in cultures performed in media with and without DT. Cell numbers were not reduced by 12 ng/ml of DT in the culture medium, but were significantly reduced at DT concentrations of 60 ng/ml (40% decrease) and 300 ng/ml (80% decrease) (Fig. 2A). The non-specific toxic effect of DT was obviously reduced by conjugating it with NGF, because cell numbers remained unaffected when 5–50 ng/ml of NGDT was added to the culture medium (NSE immunoreactivity in Fig. 2B).

The effect of NGDT on cells immunoreactive to NGF-R, ChAT, TH, and NSE (a marker for all neuronal cells) was investigated using primary cell cultures and immunocytochemistry (Fig. 1). At NGDT concentrations of 10, 20, and 50 ng/ml, NGF-R-immunoreactive cells

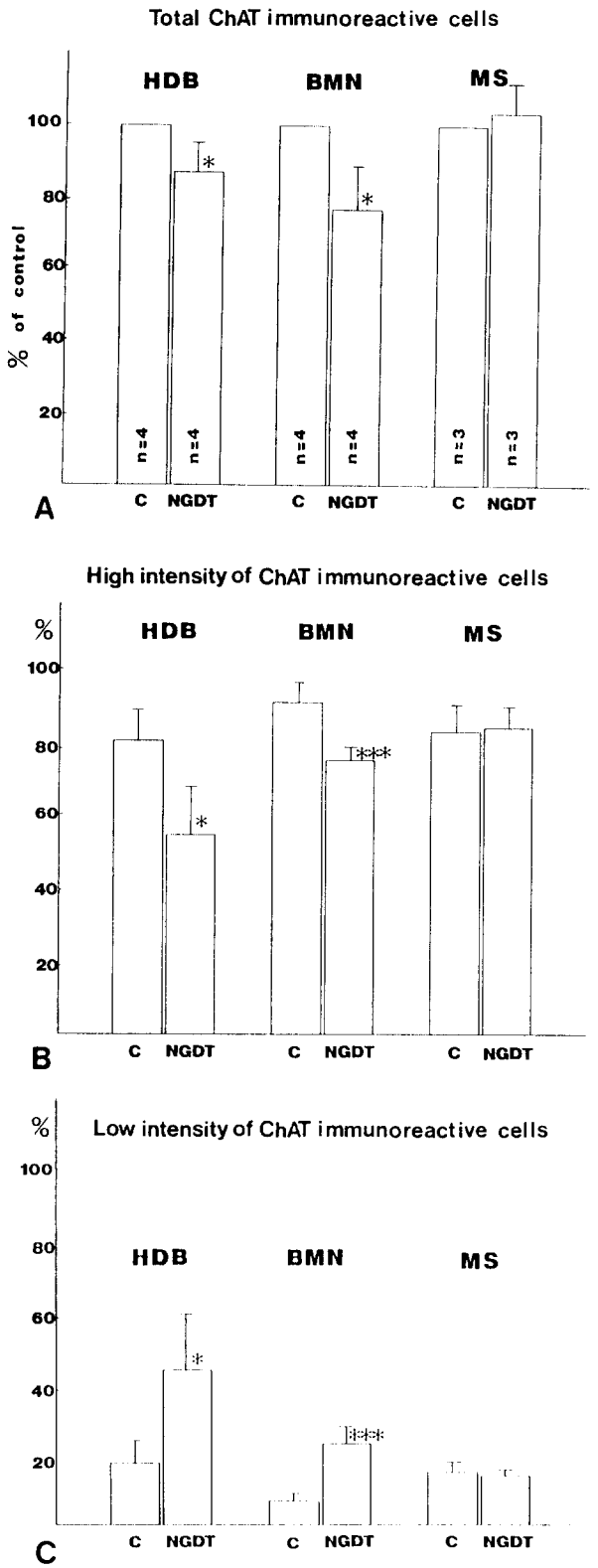

Fig. 4. Image analysis of ChAT-immunoreactive cells in the basal magnocellular nucleus using the Argus-100 image analyzer (A–C). A decrease in ChAT-immunoreactive cells was noted on the NGDT-injected side in the horizontal limb of the diagonal band (HDB) and the basal magnocellular nucleus (BMN), but not in the medial septal area (MS) (A). A decrease in the percentage of high-intensity cells (B) and an increase in the percentage of low-intensity cells (C) was noted in the HDB and BMN, respectively. Bars denote the mean ± S.D. Significant difference from the control: $^{*}P < 0.05$ and $^{***}P < 0.001$.

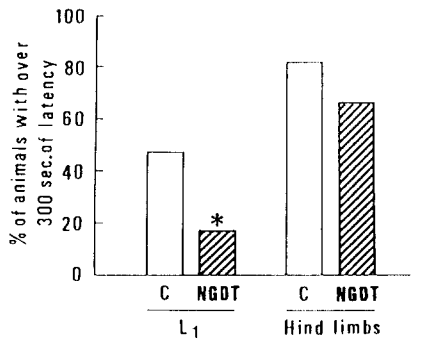

Fig. 5. During retention testing of the control group, a latency of 300 s or more was seen in 48.3% or 82.8% of the animals depending on whether the end of latency was determined by entry into the dark chamber as far as L1 (the first lumbar level) or the hind limbs. NGDT treatment before the behavior acquisition trial caused these percentages to decrease (L1, $P < 0.05$; hind limbs, $P = 0.1149$).

were significantly decreased by 19%, 44%, and 36%, respectively ($P < 0.01$, $P < 0.001$, and $P < 0.01$), as compared to the control culture without NGDT. ChAT-immunoreactive neurons reduced by 25% in cultures with 20 ng/ml of NGDT ($P < 0.01$) (Fig. 2B). Thus, NGDT was revealed to inhibit the growth of NGF-R- and ChAT-immunoreactive cells from the basal forebrain and not that of TH- and NSE-immunoreactive cells.

Histochemical assessment of the selective toxicity of NGDT after injection into the cerebral cortex

Unilateral injection of NGDT into the cerebral cortex resulted in a significant decrease in ChAT immunoreactive neurons in the ipsilateral BMN in rats and mice (Fig. 3A,B). However, little or no reduction occurred in the number of ChAT-immunoreactive Ch1–2 neurons (septal region and vertical limb of the diagonal band), which project to the hippocampus or the olfactory bulb and the occipital cortex[10,13]. Also, no decrease was noted in the

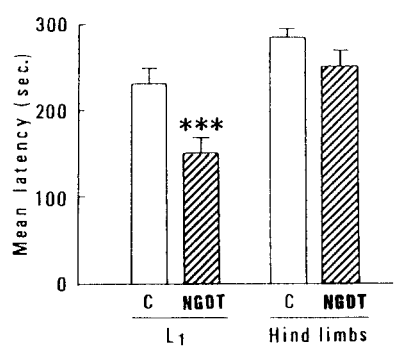

Fig. 6. Mean latency periods before entrance into the dark chamber during retention testing for control (C) and NGDT-lesioned mice. NGDT was injected just after the behavior acquisition trial. The bars represent the mean ± S.E.M. for 26 animals. Retention latency in the NGDT-treated mice decreased for both the L1 ($P < 0.001$) and hind limb ($P = 0.0525$) latencies.

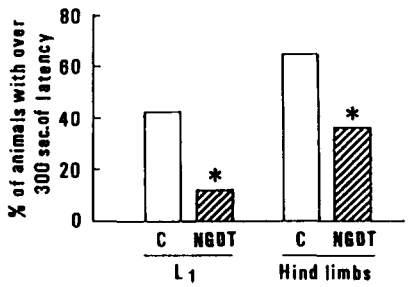

Fig. 7. Retention testing of the control group (treatment after behavior acquisition) showed a latency of longer than 300 s in 42.3% of the animals when entry as far as the L1 level (upper body) was used as the criterion for the end of latency and in 65.4% when entry as far as the hind limbs (whole body) was employed. These percentages were significantly with NGDT after avoidance behavior acquisition.

laterodorsal tegmental cholinergic neurons which project to the frontal lobe of the cerebral cortex and display no NGF-R immunoreactivity, in the intrinsic ChAT-immunoreactive cortical neurons which have no NGF-R immunoreactivity, and in the intrinsic striatal neurons[10]. ChAT-positive cell numbers in the basal forebrain and the fluorescence intensity of positive cells were measured using an Argus-100 image analyzer. In the horizontal limb of the diagonal band and the BMN, a significant decrease of immunoreactive cell numbers was seen for both all cells and high-intensity cells (Fig. 4A,B), while the number of low-intensity cells in both nuclei was significantly increased (Fig. 4C). However, in the medial septal area, there were no changes.

Figure 3D shows a hematoxylin–eosin-stained section of an NGDT injection site. The cerebral cortex exhibited a normal architecture except at the injection site where gliosis was found.

Neuropeptide Y, somatostatin, and substance P immunoreactive interneurons showed no decrease in immunoreactivity, even close to the NGDT injection site (Fig. 3C).

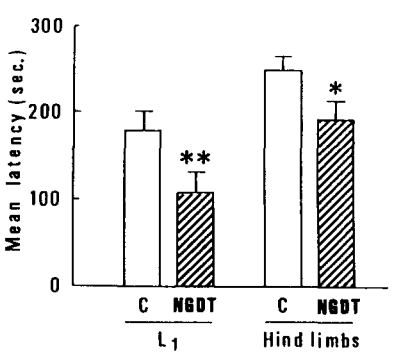

Fig. 8. Mean latency periods before entrance into the dark chamber during retention testing for control (C) and NGDT-lesioned mice. Injection of NGDT was performed 3 days before the behavior acquisition trial. The bars represent mean retention latencies ± S.E.M. for 29 control and 29 NGDT-treated animals. Retention latency in the NGDT-treated mice was reduced when either penetration into the dark room as far as L1 ($P < 0.01$) or the hind limbs ($P < 0.05$) was used as the criterion for the end of the latent period.

Effect of bilateral NGDT injection on the retention of passive avoidance learning behavior

NGDT was bilaterally injected into the cerebral cortex of mice prior to the acquisition trial (experiment 1) or after the acquisition trial (experiment 2). No changes in gross behavior were observed in either the control or NGDT-treated animals. Two mice were selected from both the control and NGDT-treated groups, and the activity of these animals was measured with a spontaneous motor activity measuring device (Nippon Medical & Chemical Instruments, Japan). No changes between the control and NGDT-treated mice were observed (data not shown). By the histochemical analysis, the ChAT-positive cell number in the basal forebrain of NGDT injected mice was significantly decreased by 40% ($P < 0.01$) as compared to that of control mice.

Experiment 1

NGDT- and saline-injected mice were habituated to a two-chamber test apparatus, so that upon being placed in the illuminated compartment they promptly entered the dark compartment. After receiving an electric shock in the dark, the mice were inhibited from re-entering the dark room when subsequently placed in the illuminated chamber.

When entrance of the upper body into the dark was used as the criterion (L1 = the first lumbar vertebra in Figs. 5–8) for determining the retention latency, 48.3% of the control mice did not enter for 300 s or more. With entrance of the whole body as the criterion ('hind limbs' in Figs. 5–8), 82.8% of the control mice did not re-enter for at least 300 s (Fig. 5). Pre-treatment with NGDT resulted in a marked decrease to 17.2% of animals displaying a greater than 300-s hesitation to place the upper body in the dark room, and a slight decrease to 65.5% in those waiting for more than 300 s to enter it completely (Fig. 5).

The mean L1 and hind limb latencies (± S.E.M.) for the retention test in the control group were 231.3 ± 14.4 s and 285.0 ± 7.9 s ($n = 26$), respectively. NGDT treatment ($n = 26$) caused a decrease in the latency period during the retention test (Fig. 6). This tendency was more marked for L1 latency (151.9 ± 16.9 s) than for hind limb latency (256.2 ± 14.5 s).

Experiment 2

In this experiment, NGDT was administered immediately after the acquisition trial and retention was tested 8 days later. The percentage of control animals staying

out of the dark for 300 s or more was 42.3% or 65.4% depending on whether latency was determined for the upper body or the whole body. The respective values in the NGDT-treated group were significantly lower (11.5 and 38.5%) (Fig. 7).

As shown in Fig. 8, NGDT treatment immediately after the acquisition trial resulted in a shortening of both latencies, and particularly of the L1 latency. The mean L1 and hind limb latencies for the control group were 179.2 ± 22.7 s and 252.3 ± 15.3 s (n = 29), respectively, while in the NGDT-treated group respective values were 105.0 ± 20.7 s and 192.1 ± 19.8 s (n = 29) (Fig. 8).

DISCUSSION

Quantitative analysis using primary cell cultures clearly demonstrated that NGF-R- and ChAT-immunoreactive neurons were selectively affected by NGDT. In addition, free DT (if present) was shown to have little or no toxicity at a concentration of less than 12 ng/ml. Histochemical studies showed a decrease in the number and intensity of ChAT-immunoreactive cells in the horizontal limb of the diagonal band and the BMN following NGDT injection. Thus, both the quantitative analysis of cell cultures and the histochemical study showed that NGF-R- and ChAT-containing neurons of the BMN could be selectively affected by NGDT injection into their terminal field.

In comparison with animals given a saline operation, NGDT-treated animals had a shorter retention latency. Thus, in the NGDT-pretreated mice, the retention of passive avoidance behavior was impaired, a result consistent with many previous studies, suggesting that the cholinergic neuron system extending from the basal forebrain area to the cortex is closely involved with learning and memory[1,11,18].

It was also found that mice injected with NGDT just after the acquisition trial exhibited a shorter retention latency period than did control animals. This finding suggests that destruction of the cholinergic neuron system in the basal forebrain area was accompanied by impaired memory or impaired retention of previously learned behavior patterns.

Studies have shown that memory impairment occurs in various animal models of Alzheimer's disease constructed by electrolytic or neurotoxic lesioning[5,6,11,14,18] (for a review, see Smith[23]). In these animals, cortical acetylcholine levels were reduced, and since a major source of cortical acetylcholine is the cholinergic neuron system in the basal forebrain, the memory disturbance was attributed to a decrease in acetylcholine levels following the loss of cholinergic neurons in this area of the brain. However, it was not made clear in such studies (as mentioned in the Introduction) whether the loss of cholinergic fibers projecting from the basal forebrain to the cerebral cortex was really related to memory impairment, because there was associated destruction of non-cholinergic neurons and fibers passing through the basal forebrain, as well as the loss of cholinergic neurons located in various other regions of the brain (cholinergic fibers in the cerebral cortex include intrinsic cholinergic neurons and neurons arising in the laterodorsal tegmental area, in addition to those of the basal forebrain). In the new animal model of Alzheimer's disease presented here, the cholinergic neurons of the basal forebrain were selectively destroyed while the non-cholinergic system in this region and the other cholinergic neurons of the brain remained unaffected. Memory loss was still detected, indicating that the basal cholinergic system is closely involved in the retention of memory.

Profound reductions in cortical acetylcholine levels together with the degeneration of cholinergic neurons in the basal forebrain area have been reported in patients with Alzheimer's disease[4,17]. A similar loss of the cholinergic neurons of the basal forebrain and impairment of learning and memory occur in mice injected with NGDT, suggesting that this model is more suitable for Alzheimer's disease than those produced previously.

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