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Acetylcholine-Secreting Cells Improve Age-Induced Memory Deficits

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The present study examined whether aged rats with naturally occurring cognitive deficits in spatial learning and memory would benefit from local chronic supplementation of acetylcholine. Aged impaired and aged unimpaired rats were pretested in the water maze to characterize the extent of age-induced cognitive impairment. Groups were matched for extent of deficits. The animals subsequently received implants of either acetylcholine-releasing cells or control cells into the cortical and hippocampal target regions of the basal forebrain. One week postgrafting, spatial learning and memory were retested using the same behavioral procedure. All aged groups acquired the platform position more slowly than young controls. However, aged impaired rats grafted with acetylcholine-releasing cells performed significantly better than aged impaired rats with control grafts, and they did not differ from aged unimpaired groups. A spatial memory probe test revealed that memory for the escape platform location of the acetylcholine-grafted rats was significantly better than that of rats with control grafts and matched the performance of young controls. *In vitro,* biochemical and electrophysiological analyses of the engineered cells confirmed choline acetyltransferase activity and showed quantal release of acetylcholine from the transduced cells. *In vivo,* RT-PCR of microdissected grafts indicated that the engineered cells expressed the choline acetyltransferase transgene for up to 40 days postgrafting. These results indicate that locally restricted supplementation of acetylcholine into the two major target regions of the cholinergic basal forebrain of aged impaired rats ameliorates some age-related cognitive deficits.

Key Words: acetylcholine, choline acetyltransferase, aging, basal forebrain, nucleus basalis, medial septum, neocortex, hippocampus, learning, memory, water maze, engineered cells

INTRODUCTION

The two major cholinergic basal forebrain (CBF) nuclei, the medial septum (MS) and the nucleus basalis magnocellularis (NBM), project to the hippocampus and the neocortex, respectively, and both are affected in the course of Alzheimer's disease (AD) [1–4]. In particular, choline acetyltransferase (ChAT), the biosynthetic enzyme for acetylcholine (ACh), is most prominently reduced by 60–90% in the hippocampus and the cerebral cortices of AD brains [5,6]. Although multiple neurotransmitter pathways (e.g., serotonergic cells in the dorsal raphe and noradrenergic neurons of the locus coeruleus) are disrupted in the course of the disease and cholinergic deficiencies do not account for all of the functional deficits observed in AD, one of the most promising therapeutic approaches thus far is aimed at restoring cholinergic functioning [7].

As with normal human aging and AD, rodents and monkeys show an age-dependent cognitive decline associated with neurodegenerative changes and cell death both in the CBF nuclei and in its target areas [8–11]. More importantly, in tasks of spatial learning and memory that are sensitive to both septohippocampal and basal–neocortical damage, such as the Morris water maze, the degree of age-dependent cognitive decline is correlated with the

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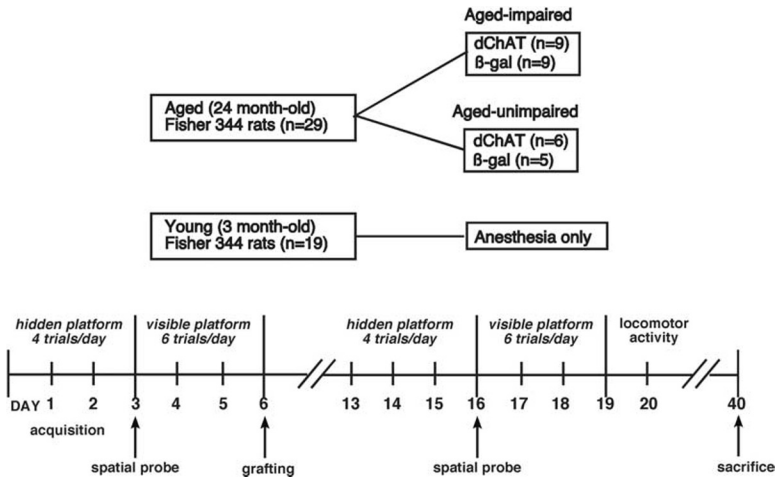

FIG. 1. Temporal design of the study. Animals were screened for spatial memory performance in the Morris water maze (hidden and visible platform) prior to surgery. Next, aged animals were subdivided into groups of cognitively impaired and unimpaired animals based on their performance during the acquisition of the hidden platform segment of the water maze testing. The aged impaired and aged unimpaired groups were balanced so that the groups had similar swim path lengths, before they were then randomly assigned to receive either dChAT or β-Gal grafts. One week postgrafting, all groups were retested in the hidden and visible platform water maze tests using similar testing procedures. After completion of water maze testing, general locomotor activity and habituation were assessed. All animals were decapitated at the end of the third week for further molecular and morphological analyses.

magnitude of degenerative changes in the CBF system [12,13]. Thus, aged rats represent a particularly suitable model for the *in vivo* assessment of strategies aimed at enhancing functional recovery of the CBF system.

Different strategies to supplement cholinergic function have been used to improve age-related functional deficits, e.g., systemic administration of compounds stimulating cholinergic synaptic neurotransmission throughout the brain [14]. Alternatively, intracerebral infusions of exogenous trophic factors such as nerve growth factor (NGF) have repeatedly been demonstrated to have beneficial effects on both morphology and behavior in age-related cognitive impairment in rats [13,15–20]. The delivery of NGF remains the most effective long-term strategy to structurally repair the aged brain. The morphological effects of NGF are most prominent in the cholinergic system, and thus it is assumed that the NGF-induced alleviation of age-related deficits is mediated through an enhanced cholinergic response.

Initial transplantation studies using grafts of cholinergic-rich fetal tissue into the hippocampus and/or neocortex showed a reversal of the cognitive deficits produced by aging or acute brain damage in models similar to those used to examine the effects of NGF [20–28].

However, several crucial questions remain unanswered: is the functional recovery observed with NGF due to the effects of NGF on the cholinergic system and, if so, are the results due to local effects or brain-wide systemic effects? Furthermore, because fetal grafts are composed of a variety of cell types, only less than 5% of which are cholinergic, are the effects of fetal tissue grafts on cognitive performance due to the cholinergic cells or to some other combination of cells and factors in the grafts?

Recently, genetically modified cells have been used as a strategy to answer some of these questions [26]. Neural and nonneural cells engineered to produce NGF have been shown to minimize age-related neuronal degeneration and cognitive deficits [18,29]. Furthermore, intracerebral transplantation of cells genetically modified to produce and release ACh has successfully reversed behavioral impairments in young animals with CBF lesions [30,31]. In particular, cortical and/or hippocampal grafts of genetically modified fibroblasts bearing the ChAT transgene and releasing ACh were sufficient to ameliorate deficits in animals with cholinergic lesions induced either by excitotoxic or by aspirative lesions of the NBM or the fimbria–fornix, respectively. Thus, functional recovery was obtained after acute CNS damage by grafting cells that secrete ACh locally in an unregulated, constitutive manner.

The present study (summarized in Fig. 1) was designed to explore the effect of this strategy in the cognitively impaired, aged rat. In addition, we sought to determine whether local ACh delivery to restricted target areas of the hippocampus and neocortex is by itself sufficient to induce significant cognitive recovery from a broad range of age-related deficits. *In vitro* biochemical and electrophysiological characterization of engineered fibroblasts carrying the ChAT transgene and *in vivo* molecular and functional assessment of aged impaired rats were used to evaluate the restorative mechanisms of ACh-releasing cells grafted into aged impaired rats.

RESULTS

In Vitro Analysis of ChAT Activity

We confirmed expression of *Drosophila* ChAT (dChAT) within transduced fibroblasts prior to grafting by *in vitro* analyses of ChAT activity in cells obtained from sister flasks to those used for transplantation. The dChAT fibroblasts showed significantly higher levels of ChAT activity (292.0 ± 51.7 nmol ACh/h/mg protein, $P < 0.05$) compared to control β-Gal fibroblasts, which showed almost no detectable ChAT activity (0.9 ± 0.6 nmol ACh/h/mg protein).

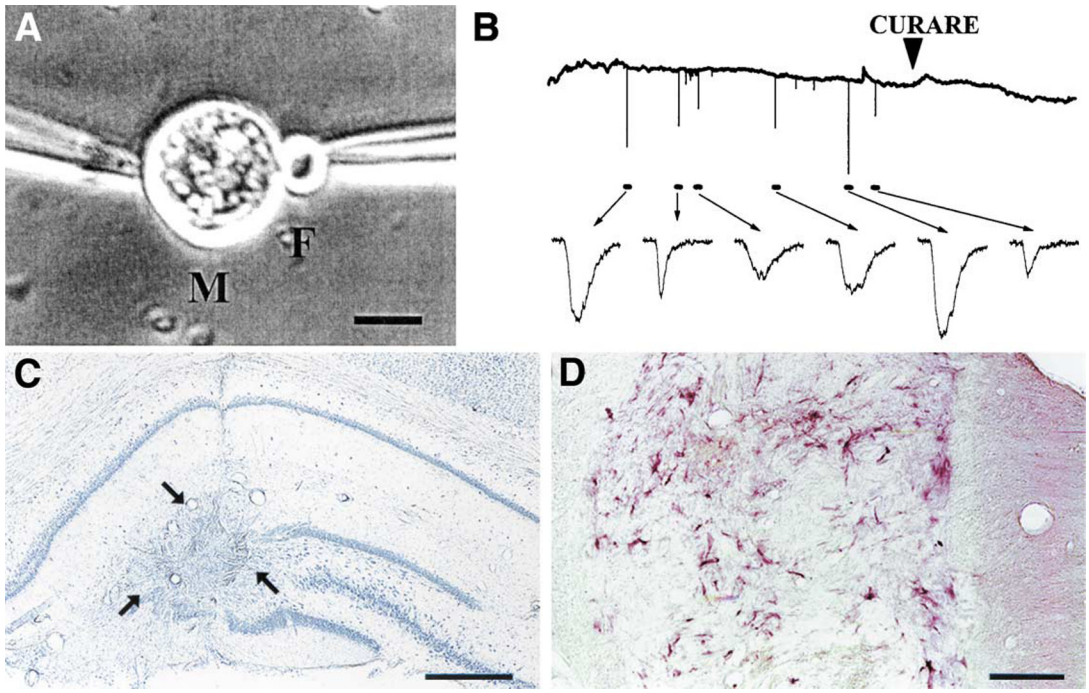

FIG. 2. Quantal release of ACh from dChAT fibroblasts and placement of hippocampal grafts. (A) Phase-contrast image of dChAT fibroblast (F) manipulated into contact with a whole-cell clamped myocyte (M) in a 1-day-old *Xenopus* culture. The myocyte was voltage clamped at −70 mV. Scale bar, 15 μm. (B) The upper continuous trace depicts membrane currents in a voltage-clamped myocyte (V_c = −70 mV) after a dChAT fibroblast was manipulated into contact. Samples of spontaneous currents resembling end-plate currents in the myocyte at various times are shown below at a higher time resolution (filtered at 2.5 kHz). The arrowhead marks the effect of bath application of d-tubucurarine (final concentration of 0.5 mM). Scales, 200 pA and 30 s (top) and for higher time resolution 80 ms (bottom). (C) Coronal section of representative graft in the hippocampus stained for Nissl through the hippocampus of an aged impaired rat and (D) a section with β-Gal immunoreactivity. Arrows indicate the border of the graft. Note the circular shape of the hippocampal graft.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of dChAT Gene Expression

We prepared pooled hippocampal and cortical grafts microdissected 40 days postgrafting from a subset of behaviorally characterized animals (based on percentage improvement from pre- versus postgrafting acquisition) for RT-PCR of dChAT mRNA to assess the expression of the dChAT transgene *in vivo*. The primers, which were specific for the *Drosophila* ChAT transcript within the viral vector, revealed higher amounts (approximately 2.9-fold) of dChAT mRNA from the dChAT animal [relative band density (RBD) = 0.0982], with robust improvement compared to the grafted dChAT animal with no behavioral improvement (RBD = 0.0591) or an exacerbated impairment (RBD = 0.0188; similar to nongrafted controls). These results are consistent with previous findings that dChAT fibroblasts grafted in the deafferented neocortex express the dChAT transgene for extended periods [30].

Electrophysiological Analysis of ACh Release by dChAT Fibroblasts

We detected the frequent release of ACh after a cultured *Xenopus* myocyte was manipulated into close contact with the dChAT fibroblasts (Fig. 2A). Whole-cell recordings from the myocyte revealed transient inward currents resembling miniature end-plate currents (Fig. 2B). These currents reflect pulsatile release of ACh from the dChAT fibroblasts, since they were totally abolished by application of d-tubucurarine (0.5 mM; Fig. 2B). No currents were found in myocytes in contact with control fibroblasts without expression of dChAT. The secretion appeared to result from the accumulation and subsequent exocytosis of ACh by vesicles in the constitutive secretion or membrane recycling pathways [39]. The currents are very similar in amplitude and time course to those seen after loading endocytotic compartments of fibroblasts with exogenous ACh, either via pressure injection using a conventional glass micropipette or via a brief incubation in a solution containing ACh [40].

Histological Analysis of Grafts

Histological assessment of the grafts, which we conducted 40 days postgrafting, revealed that almost all rats analyzed had well-positioned and viable grafts in the appropriate CBF target areas. One animal of the dChAT-unimpaired group had to be excluded based on the viability of the graft. Hippocampal grafts consisting of either dChAT or β-Gal fibroblasts were located at the dorsal leaf of the

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TABLE 1: Assessment of nonspatial Morris water maze performance and motor activity

Group (*N*)	Morris water maze: Visible platform[b] pregrafting (cm)	Morris water maze: Visible platform[b] postgrafting (cm)	Morris water maze: Swim speed[c] (cm/s)	Motor activity[a] (Counts ± SEM)
Young controls (19)	132.0 ± 6.5*	108.3 ± 4.3	27.2 ± 0.7*	148.8 ± 14.4*
β-Gal–unimpaired (5)	227.3 ± 34.3	142.5 ± 11.0	20.7 ± 1.3	90.9 ± 11.3
dChAT–unimpaired (6)	175.9 ± 22.5	165.5 ± 15.4*	22.9 ± 0.8	76.5 ± 11.6
β-Gal–impaired (9)	197.4 ± 13.5	226.3 ± 20.8*	20.7 ± 1.3	94.1 ± 9.4
dChAT–impaired (9)	193.2 ± 9.2	170.9 ± 21.0*	22.8 ± 1.2	105.5 ± 12.4

[a]Activity was examined during 2 consecutive days when animals were exposed to a novel environment. Data are expressed as averaged activity counts (infrared beam breaks, mean ± SEM) across day 1 and day 2 over two 40-min periods ($F_{4,41} = 4.1$, $P < 0.007$). *Significant difference from all aged groups, $P < 0.05$ (dChAT-impaired and β-Gal-unimpaired) and $P < 0.01$ (dChAT-unimpaired and β-Gal-impaired).

[b]Data represent the average swim path length (mean ± SEM) to find the marked and elevated platform during 2 days of testing (six trials/day). Significant differences in swim path length during both testing periods (pregrafting $F_{4,43} = 8.2$, $P < 0.0001$; postgrafting $F_{4,43} = 12.4$, $P < 0.0001$). Pregrafting: *significant difference from aged groups, $P < 0.05$. Postgrafting: *different from young, $P < 0.05$.

[c]Data represent the average swim speed (mean ± SEM) derived from the pre- and postgrafting period during the acquisition. Significant difference in swim speed among groups ($F_{4,43} = 12.0$, $P < 0.0001$). *Significant difference from aged groups, $P < 0.001$.

dentate gyrus in a well-defined cluster (Fig. 2C). Cortical grafts were typically found in area 2 of the frontal cortex and the ventral portion of parietal areas 1 and 2 in sections at the level of the ventral hippocampal commissure. Cortical grafts appeared more compact than hippocampal grafts. Sections from β-Gal grafts showed strong immunoreactive labeling for β-Gal protein (Fig. 2D), indicating good cellular survival and transgene expression at the conclusion of testing.

Behavioral Assessment

Swim speed. During the pre-and postgrafting testing periods, there was a significant difference in mean swim speed during all phases of water maze testing. The averaged swim speeds (mean ± SEM, in cm/s) of each group across both testing periods are summarized in Table 1 ($F_{4,43} = 12.0$, $P < 0.0001$). Young controls swam up to 31% faster than all aged groups throughout the experiment. Because within-group differences in swim speed were observed in the aged animals, swim path length was used to evaluate water maze performance.

Pregrafting spatial performance. The young and aged rats were tested in the Morris water maze pregrafting. Aged rats were identified as "impaired" if the swim distance during the last day of the pregrafting acquisition exceeded the value of 2 SD from the mean performance of the young group [24]. Over 3 days, animals (young, $n = 19$; aged, $n = 29$) learned to find the hidden platform as indicated by decreasing escape distances ($F_{2,90} = 6.3$, $P < 0.003$; Fig. 3A, left). Based on our 2 SD criterion, we found impairment in performance compared to young animals in about 62% ($n = 18$) of the 24-month-old rats. This group was designated the "aged impaired" group. The remaining aged rats ($n = 11$) performed within the range of the 2 SD criterion. This latter group of rats was designated "aged unimpaired." After assignment to the groups, analysis of acquisition (group × days) showed a significant group effect ($F_{2,45} = 13.7$, $P < 0.0001$) and group-by-day interaction ($F_{4,90} = 12.0$, $P < 0.0001$). Aged impaired rats showed significantly longer escape distances than aged unimpaired and young controls ($P < 0.0001$ aged impaired versus both young and aged unimpaired). The averaged escape distances (mean ± SEM, in cm) across all trials for the young (440.0 ± 29.3) and aged unimpaired (416.3 ± 23.8) rats were significantly shorter than those of the aged impaired rats (592.5 ± 21.1; $P < 0.0001$).

Reanalysis of the pregrafting acquisition performance based on the future surgical assignment confirmed the differences during acquisition ($F_{2,86} = 5.0$, $P < 0.009$), the significant group effect ($F_{4,43} = 6.2$, $P < 0.0005$), and the group-by-day interaction ($F_{8,86} = 5.6$, $P < 0.0001$; Fig. 3B, left). The future β-Gal impaired ($n = 9$) and dChAT impaired ($n = 9$) groups were matched (Fig. 3B, left) and showed significantly longer escape distances than the β-Gal unimpaired ($n = 5$), dChAT unimpaired ($n = 6$), and young controls ($P < 0.0001$). In addition, the averaged escape distances (mean ± SEM, in cm) across all trials were significantly shorter for young controls (440.0 ± 29.3), β-Gal unimpaired (430.4 ± 37.5), and dChAT unimpaired (404.5 ± 31.1) rats compared to the β-Gal impaired (587.9 ± 29.4) and dChAT impaired (597.1 ± 30.8) rats (for both impaired groups, $P < 0.0001$).

After the completion of the acquisition and prior to the grafting, the spatial acuity of young control, aged impaired, and aged unimpaired animals was tested in one 40-s probe trial, in which the platform was removed. The percentage of swim path length that animals spent in the platform quadrant was taken as a measure of spatial acuity. Thus, a random swimming pattern would generate approximately 25% of the total swim path in each of the four quadrants. Young control animals spent significantly

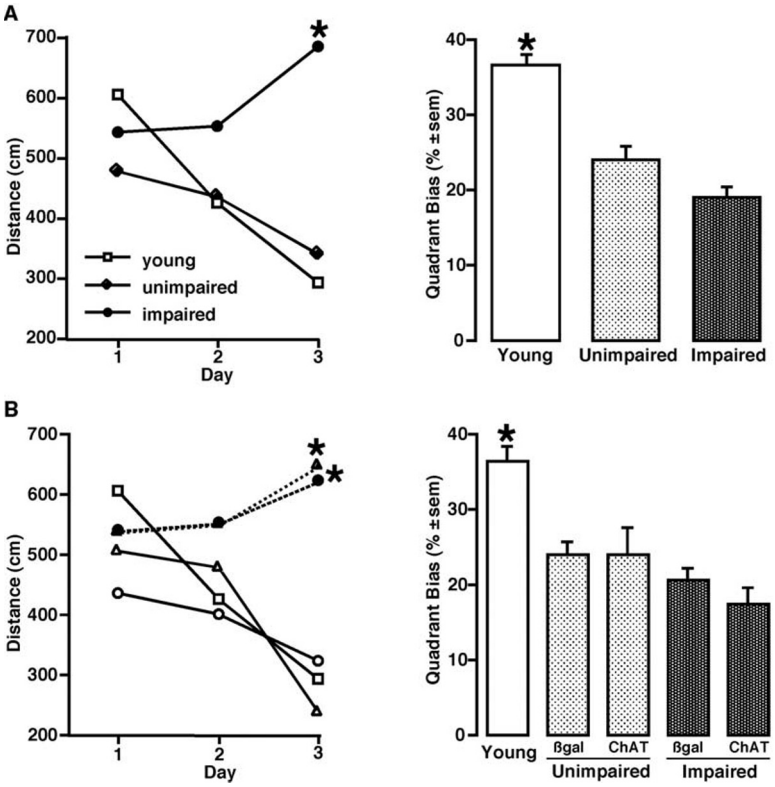

FIG. 3. Pregrafting spatial navigation screen in the Morris water maze. (A) Left: Pregrafting spatial navigation screen in the Morris water maze. Young (3 months old) and aged (24 months old) animals learned the position of the platform, as indicated by the decrease in swim path length over time. According to the criterion of 2 standard deviations from the mean performance of the young group [27], aged animals were subdivided into aged impaired ($n = 18$) and aged unimpaired ($n = 11$) animals, with the latter performing at the level of young controls ($n = 19$). Each point represents the average of four trials (*$P < 0.0005$). Right: Averaged percentages of total distance swum in platform quadrant (mean ± SEM, in %) during spatial probe. Young control animals clearly performed above chance, whereas all aged animals spent significantly less time in the platform quadrant and were not different from each other. *Significant difference from the aged groups, $P < 0.0001$. (B) Left: Reanalysis of the pregrafting acquisition performance based on the future surgical assignment: young controls (open squares; $n = 19$), β-Gal unimpaired (open circle; $n = 5$), and dChAT unimpaired (open diamond; $n = 6$) performed significantly better compared to β-Gal impaired (closed circle; $n = 9$) and dChAT impaired (closed triangle; $n = 9$) (*$P < 0.0005$). Right: Averaged percentages of the five groups again based on the future surgical assignment for the total distance swum in platform quadrant (mean ± SEM, in %) during spatial probe. Young animals clearly performed above chance, whereas all four aged groups spent significantly less time in the platform quadrant; however, they were not different from each other. *Significant difference from the aged groups, $P < 0.00001$.

longer searching for the platform in the appropriate quadrant compared to both aged impaired and aged unimpaired animals, which performed at or below chance, respectively (Fig. 3A, right; $F_{2,45} = 22.5$, $P < 0.0001$; $P < 0.0002$, young control animals different from both aged groups). Reanalysis of the spatial probe based on the future surgical assignment confirmed this result (Fig. 3B, right; $F_{4,43} = 11.1$, $P < 0.0001$; $P < 0.0002$, β-Gal impaired and dChAT impaired different from young control, β-Gal unimpaired, and dChAT unimpaired, respectively). These results suggest that there may be a dissociation between spatial acquisition and memory in aged animals. The spatial probe revealed an underlying deficit that was not apparent during acquisition trials, allowing the separation of rats based only on the performance during acquisition.

During the following 2 days, animals were taught to escape to the visible platform. This portion of the task measures sensorimotor and visual deficits. Performance of all groups (again based on the future surgical assignment) improved significantly with each trial ($F_{11,473} = 6.1$, $P < 0.0001$) and all groups learned at a similar rate ($F_{44,473} = 1.7$, $P > 0.05$). There was a significant effect between groups ($F_{4,43} = 8.2$, $P < 0.0001$; Table 1), indicating that all aged impaired animals performed at an equal level but swam significantly longer to the marked platform than young controls (Table 1). These results suggest that all aged groups were equally able and motivated to learn the procedure and to escape to the trial-by-trial relocated, but visible, platform.

At the end of the pregrafting testing period, both the impaired and the unimpaired aged rats were balanced so that the groups had similar acquisition performances before being randomly transplanted with either dChAT or β-Gal fibroblasts. The number of rats in each group (see Fig. 1) was as follows: young controls ($n = 19$), β-Gal unimpaired ($n = 5$), dChAT unimpaired ($n = 6$), β-Gal-impaired ($n = 9$), and dChAT impaired ($n = 9$).

Postgrafting spatial performance. One week postgrafting, all rats were retested for 4 days using the same pregrafting testing protocol in the water maze. Escape performance during the postgrafting reacquisition onto the newly positioned platform is presented in Figs. 4A and 4B. A two-way ANOVA (group × day) of swim path length revealed significant effects for group ($F_{4,43} = 14.0$, $P < 0.0001$) and day ($F_{3,129} = 18.1$, $P < 0.0001$) but no interaction between groups and trials ($F_{12,129} = 1.1$, $P > 0.05$). Young controls (mean ± SEM across all trials, in cm; 268.4 ± 22.6) showed significantly shorter escape swim path lengths to the relocated platform during the reacquisition period compared to all aged groups, independent of graft type ($P < 0.005$). The performance of aged, behaviorally impaired rats with β-Gal grafts (mean ± SEM across all

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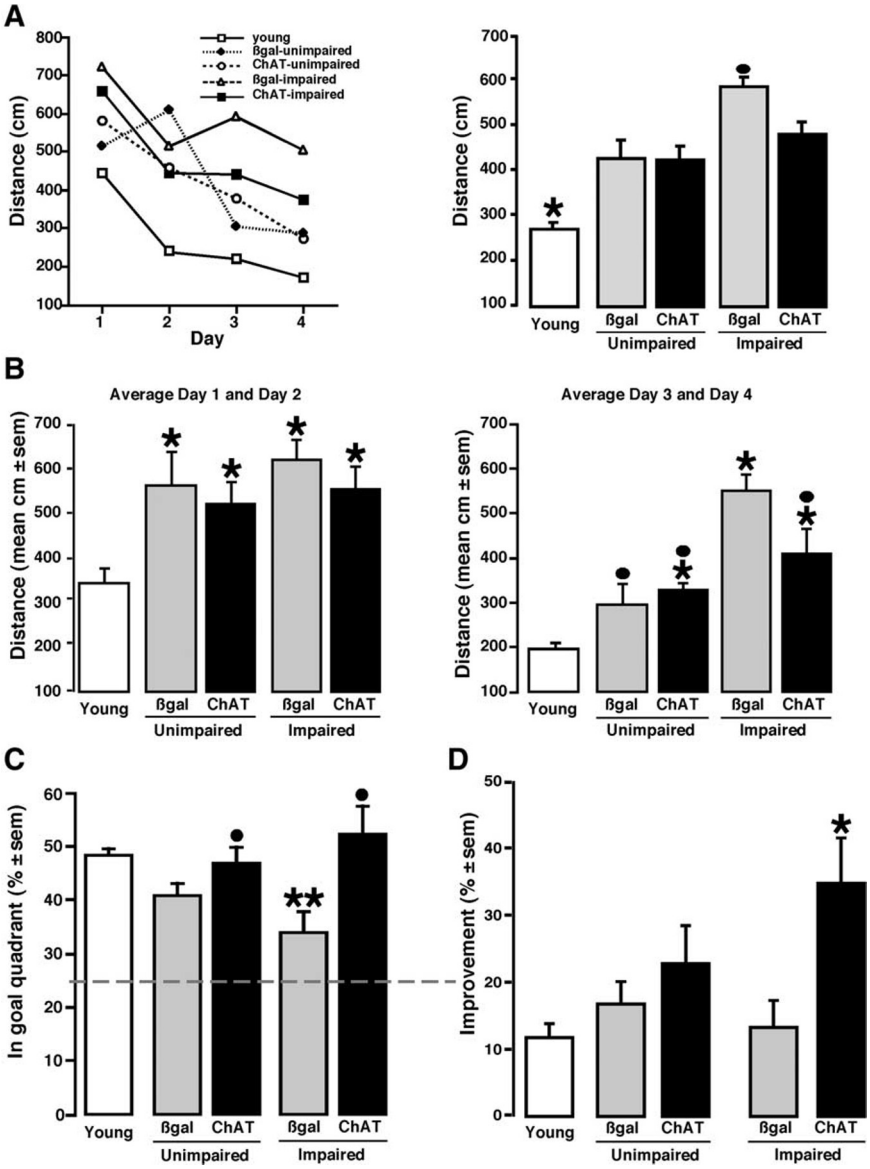

FIG. 4. Postgrafting performance in the Morris water maze. dChAT grafts improve spatial navigation in aged impaired rats during reacquisition (A and B) and spatial probe (C). (A) One week postgrafting, animals had to escape to a relocated submerged platform. Overall repeated-measures ANOVA indicated a significant group effect during the 4 days of reacquisition ($F_{4,43} = 14.0$, $P < 0.0001$). Young controls showed significantly shorter escape distances than all aged groups (*$P < 0.005$). The escape performance of impaired animals with β-Gal grafts was significantly worse than all other aged groups (· $P < 0.05$). Thus dChAT grafts mitigated spatial impairment compared with control grafted impaired rats. (B) Averaged swim path length of the initial half of the reacquisition period (left, days 1 and 2) separated from the second half (right, days 3 and 4). During the initial 2 days, the performance of young controls was significantly better compared to all aged groups (left, *$P < 0.001$, for all comparisons), but there were no differences among the aged animals. During the second half of the reacquisition, however, the aged impaired rats with β-Gal grafts swam significantly longer than both the aged impaired and the aged unimpaired rats with dChAT grafts (right, *$P < 0.05$ different from young; · different from β-Gal impaired). (C) Averaged percentages of the five groups (means ± SEM, in %) postgrafting for the total distance swum in the platform quadrant. All animals were able to memorize the newly acquired location of the platform, which is clearly indicated by the bias toward the platform quadrant greater than 25% for all animals (broken line). There was a significant difference between groups ($F_{4,43} = 3.6$, $P < 0.05$). β-Gal impaired animals were significantly impaired compared to the young controls and β-Gal unimpaired animals (**$P < 0.05$). More importantly, aged impaired and aged unimpaired rats with ChAT grafts searched significantly longer for the platform in the correct location compared to the corresponding animals with β-Gal grafts (· $P < 0.05$). (D) Improvement in performance comparing pre- and postgrafting percentage bias for the target quadrant during spatial probe. Data are presented as averaged differences (means ± SEM, in %) of the spatial bias for the target quadrant across the post- and pregrafting periods. The spatial acuity of dChAT impaired animals improved significantly more than all other groups across pre- and postgrafting spatial probe. *dChAT impaired animals differed from young controls ($P < 0.0005$), β-Gal impaired ($P < 0.005$), and β-Gal unimpaired ($P < 0.05$).

trials, in cm; 582.8 ± 30.8) was significantly worse than that of both aged unimpaired groups (β-Gal unimpaired, 427.6 ± 51.5; dChAT unimpaired, 422.2 ± 37.5; $P < 0.05$, for all comparisons). More importantly, the aged impaired animals with dChAT grafts (mean ± SEM across all trials, in cm; 479.2 ± 39.2) performed significantly better than the corresponding aged impaired group with β-Gal grafts (582.8 ± 30.8; $P < 0.05$). Figure 4B presents the averaged swim path length (mean ± SEM, in cm, across 2 days) of the initial half of the reacquisition period (Fig. 4B, left, days 1 and 2) separated from the second half (Fig. 4B, right, days 3 and 4). Although all animals were familiar with the testing procedure during the initial 2 days, they had to acquire a new location of the hidden platform. During this phase, the performance of young controls was significantly better compared to all aged groups ($P < 0.001$, for all comparisons; Fig. 4B, left), but there were no differences among the aged animals. During the second half of the reacquisition, however, the aged impaired rats with β-Gal grafts swam significantly longer than both the aged impaired and the aged unimpaired rats with dChAT grafts ($P < 0.05$; Fig. 4B, right). The behavioral outcomes of day 3 and day 4 suggest that the acquisition of a new platform position is significantly affected by locally supplying ACh to both hippocampi and frontoparietal cortices.

One day after the last reacquisition block, a spatial probe trial in which the platform was not present was again conducted to assess the spatial acuity postgrafting. Despite all animals being able to memorize the newly acquired location of the platform, which is clearly indicated by the bias toward the platform quadrant (Fig. 4C, broken line; >25% for all animals), there was a significant difference between groups ($F_{4,43} = 3.6$, $P < 0.05$; Fig. 4C). Aged impaired and aged unimpaired rats with dChAT grafts searched significantly longer for the platform in the correct location compared to the corresponding animals with β-Gal grafts ($P < 0.05$; Fig. 4C). It is interesting to note that the performance of animals with dChAT grafts matched the acuity of young controls (Fig. 4C). This result establishes that the topographically restricted supply of ACh to age-related, compromised target regions of the cholinergic basal forebrain is sufficient to reverse deficits in spatial memory.

One day after the postgrafting probe trial, all animals were retested to locate the trial-by-trial newly positioned, visible platform. This second visible portion of the task measures whether the ability of the aged animals to swim and to locate the platform by using intramaze cues was affected by the grafting procedure (Table 1). All transplanted animals were equally able to locate the platform (trial, $F_{11,473} = 8.2$, $P < 0.0001$; group-by-trial interaction, $F_{44,473} = 1.0$, $P > 0.05$), demonstrating that none of the aged groups was compromised by the grafting surgery in their ability to escape to the marked platform postgrafting. Similar to the visible portion of the water maze testing during the pregrafting period, young controls swam more efficiently ($F_{4,43} = 12.4$, $P < 0.0001$; $P < 0.007$, young different from dChAT impaired, dChAT unimpaired, and β-Gal impaired).

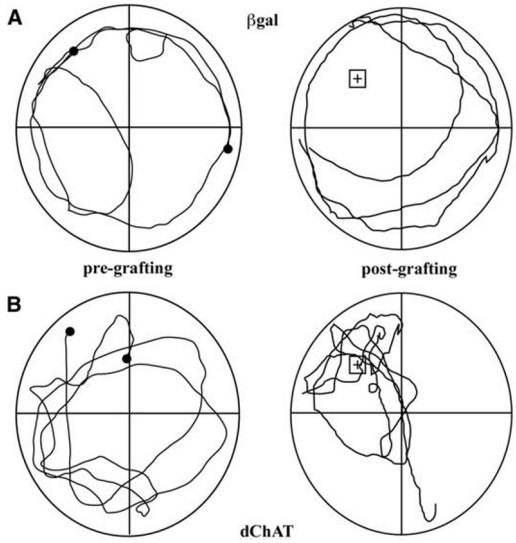

FIG. 5. Spatial probe swim path in the Morris water maze. Representative swim paths of (A) a β-Gal impaired and (B) a dChAT impaired animal during the pre- (left) and postgrafting (right) spatial probe trials. Note that both the β-Gal impaired and dChAT impaired rats showed a random search pattern pregrafting, whereas only the dChAT impaired rat focused its search around the previous platform location postgrafting.

Comparison between pre- and postgrafting spatial orientation in aged rats. We analyzed the difference in the percentage bias during the spatial probe trial between the post- and the pregrafting periods for all groups (Fig. 4D). There was a significant group effect for this comparison ($F_{4,42} = 4.2$, $P < 0.007$). The spatial acuity of aged impaired rats with dChAT grafts improved significantly more than all other experimental groups, indicating that the magnitude of the graft-induced recovery is most pronounced in animals with grafts of ACh-releasing cells ($P < 0.0005$ from young controls, $P < 0.005$ from β-Gal impaired, and $P < 0.05$ from β-Gal unimpaired). Examination of swim paths during the probe conducted in pre- and postgrafting periods revealed that impaired rats with ChAT grafts showed remarkable improvements in spatial bias. This improvement was not observed in impaired rats with β-Gal grafts (Fig. 5).

Locomotor activity and habituation. One day after completion of the water maze testing, all groups showed active exploratory behavior when placed into the testing chamber. One-way ANOVA of averaged activity counts across both test sessions revealed a significant group difference (group analysis; across days 1 and 2, $F_{4,41} = 4.1$, $P < 0.007$; day 1, $F_{4,41} = 3.8$, $P < 0.05$; day 2, $F_{4,41} = 3.1$, P

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< 0.05). Young control animals crossed the photoelectric beams significantly more often than did all aged groups combined (Table1, $P < 0.05$). However, there was no significant difference in locomotor activity between the aged animals with ChAT and those with β-Gal grafts. This heightened activity in the young animals decreases over time, reflecting habituation to the novel stimulus (block analysis; across days 1 and 2, $F_{15,615} = 30.5$, $P < 0.0001$; day 1, $F_{7,287} = 49.8$, $P < 0.0001$; day 2, $F_{7,287} = 27.8$, $P < 0.0001$). The consecutive reintroduction to the same surroundings typically results in a lower motor activity compared to the first exposure and can thus be used as an index of memory of the environment. However, there was no group-by-block interaction, indicating that all groups habituated similarly to the new environment despite the fact that young animals crossed the photoelectric beams more often (group-by-block interaction; across days 1 and 2, $F_{60,615} = 0.6$, $P > 0.05$; day 1 separate, $F_{28,287} = 0.5$, $P > 0.05$; day 2 separate, $F_{28,287} = 0.7$, $P > 0.05$).

DISCUSSION

Aged impaired animals with dChAT grafts performed significantly better than the corresponding aged impaired group with β-Gal grafts during both acquisition and the spatial probe postgrafting. Moreover, there was a significant improvement between the pre- and postgrafting performances in spatial navigation, both for the acquisition and for the probe trial of the aged animals with dChAT grafts, but not with β-Gal grafts. These results indicate that age-related cognitive deficits in spatial learning and memory were corrected to the level of young controls by site-specific enhancement of ACh in the hippocampus and the neocortex. Although numerous previous studies have used acquisition performance in the water maze as a criterion for identifying age-induced cognitive impairments, it is important to note that ChAT-expressing fibroblasts grafted to the cortex and the hippocampus are capable of improving both acquisition and retention deficits of aged animals in this task. One potential interpretation of these findings is that improved acquisition enhances recall performance [13,18,24,41]. Thus, our results extend previous observations that cholinergic-rich fetal grafts into the hippocampus (derived from the medial septum) reversed age-related deficits in learning and memory [41]. Our data suggest that this recovery is achieved regardless of whether other neurotransmitter systems or neuroanatomical pathways contribute to the age-related cognitive impairment.

In contrast to previous results with septal grafts placed into the hippocampus [41], in this study dChAT fibroblasts were transplanted within both compromised target areas of the CBF—the hippocampus and the neocortex—in aged rats. Thus, the data leave open the question of whether the improvement in aged rats is exclusively linked to a role for ACh in the hippocampal or neocortical circuitry. In addition, since aging processes functionally disrupt both the septal–hippocampal and the basal–neocortical projections, it remains to be clarified whether dChAT graft-induced cognitive improvements in the water maze reflect a combined or separate effect on cognitive components such as learning and memory or attention.

Recent findings indicate that, as with naturally occurring age-related deficits, site-specific delivery of ACh is also sufficient to attenuate water maze deficits produced by acutely induced, nonspecific CBF damage in young rats using either excitotoxic NBM lesions or transections of the fimbria–fornix [30,31]. After hippocampal deafferentation via fimbria–fornix transection, however, only intrahippocampal dChAT grafts, and not cortical transplants, were able to induce functional recovery postlesioning.

Chronic infusions of NGF into the lateral ventricle of aged, cognitively impaired rats resulted in a significant improvement in spatial learning and memory, accompanied by an enhancement of multiple cholinergic parameters of the CBF [13,15–17]. Due to the mode of NGF administration into the cerebrospinal fluid via intracerebroventricular cannulas, however, the infused NGF may have been taken up by both major cholinergic CBF subpopulations, the medial septum and the NBM. Thus, it remained unclear whether the enhanced functioning of the septal–hippocampal or the basal–neocortical efferents accounted for the functional recovery. Recently, Gustilo *et al.* [20] confirmed the ameliorative effects of intraventricular NGF in aged rats. This study described morphological changes caused by NGF on the cholinergic cell bodies and in the terminals and concluded that the effects of NGF on cognitive performance are likely due to an increase in cholinergic terminals in the limbic terminal field of the cortex and hippocampus. However, this study confirmed concerns raised in previous studies that NGF may cause an impairment in various learning and memory paradigms in young animals [16–18].

To restrict the access of NGF locally to one of the CBF nuclei, primary fibroblasts modified to secrete NGF were implanted into a region adjacent to the NBM of aged, memory-impaired rats [18]. Although a significant amelioration of the spatial memory impairment was observed after transplantation, the functional consequences of NGF-secreting fibroblasts grafted solely into the MS of aged, cognitively impaired rats have not yet been explored. However, CNS-derived, immortalized neural progenitor cells engineered to secrete NGF were grafted into close proximity of either the NBM and the MS or the NBM only of aged impaired rats. Both experimental groups showed an almost complete reversal of the spatial memory deficit of the cognitively impaired animals [29]. Since damage to either the NBM or the MS can affect the performance of animals in a spatial learning task, it is possible that spatial deficits in rodents may be ameliorated by

either hippocampal or cortical cholinergic supplementation.

During the pregrafting acquisition, the rats were designated as "impaired" or "unimpaired" if their mean swim path length on the last day of testing on the hidden platform task was greater or less than 2 SD above young controls. Interestingly, both impaired and unimpaired groups performed equally well during the pregrafting spatial probe trial. This dissociation between acquisition and spatial probe of aged impaired animal has been observed previously [13] and raises the question as to whether the learning of new information is more affected by aging than is short-term recall. The percentage of impaired rats in this study (62% in 24-month-old rats) was similar to previously observed percentage impairments of 45% in 18-month-old and 90% in 30-month-old rats [13].

Finally, our electrophysiological data indicate that dChAT fibroblasts exhibit constitutive release of ACh but also have the ability to package ACh in vesicles for quantal release mimicking synaptic release of ACh [43]. It is possible that the released ACh is presynaptically utilized by the aged, compromised septohippocampal and basal–neocortical fibers or stimulates postsynaptic ACh receptors directly. While the exact mechanisms by which ACh affects learning and memory are not known, working hypotheses have converged on an idea that, by reducing interference, ACh enhances the signal-to-noise ratio. Thus increased intrinsic cholinergic tone sets the network to a level appropriate for learning new information.

Exogenous and locally restricted supplementation of ACh has allowed us to demonstrate that ACh release from cellular implants can modulate aged cortical and hippocampal networks adequately to reverse cognitive deficits in learning and memory. In a very direct way, this study also tests the hypothesis that the ameliorative effects of NGF in the aged animal are in fact mediated through the cholinergic system. These findings allow us to conclude that ACh supplementation locally in the cortex and hippocampus is sufficient to partially ameliorate age-related cognitive deficits in rats.

Materials and Methods

Fibroblast Preparation

Fibroblasts expressing either *Drosophila* ChAT or *Escherichia coli* LacZ (β-Gal) were prepared as described previously [32]. Briefly, Fisher 344 rat skin fibroblasts were infected with a retroviral vector in which the dChAT or β-Gal transgene was expressed from the 5′ long terminal repeat promoter. The neomycin-resistance gene was expressed from an internal Rous sarcoma virus promoter. The neomycin analog G418 (200 μg/ml) was used to select stable transfectants.

Biochemical Assessment of ChAT

Expression of ChAT activity within transduced fibroblasts was confirmed prior to grafting by *in vitro* analysis of ChAT activity in cells obtained from sister flasks used for transplantation by the method of Fonnum [33] with the modification of Tucek [34]. ChAT activity is expressed as nanomoles of ACh formed per hour per milligram (mg) of protein. Protein content was determined by a Coomassie protein assay. [^{14}C]Acetyl-CoA (60.0 mCi/mmol) was purchased from New England Nuclear (Boston, MA). ACh chloride, acetyl-CoA, choline chloride, eserine salicylate, sodium tetraphenylborate, and 3-heptanone were from Sigma Chemical Co. (St. Louis, MO).

Electrophysiological Analysis of dChAT Cells to Release ACh

To examine the nature and quantity of ACh release from the dChAT cells, the transduced fibroblasts were incubated with 500 μM choline and 100 μM eserine for 24 h [32]. Eserine (physostigmine) was obtained from Sigma. After extensive washing, dChAT cells were detached from the culture dish using trypsin–EDTA solution (GIBCO, Gaithersburg, MD). The suspension of dChAT cells was transferred to the recording chamber containing cultured *Xenopus* myocytes [35]. Whole-cell patch-clamp recording methods were used as described previously [36]. Close contact of fibroblasts was made with the myocyte, which was voltage-clamped at −70 mV. The solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES buffer (pH 7.2). Recordings were made at room temperature in a solution containing 40 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.2). The membrane currents induced by spontaneous quantal release of ACh from fibroblasts were monitored using a patch-clamp amplifier (Axopatch-1D). Electrophysiological data were filtered at 1–5 kHz and stored on a videotape recorder for later playback onto a storage oscilloscope (Tektronix 5113) or an oscillographic recorder (Gould RS3200). The data were digitized and analyzed with the SCAN program kindly provided by Dr. J. Dempster (University of Strathclyde, UK). Similar results were obtained when fibroblasts on coverslips were transferred directly into the recording channel without trypsinization. Quantal release of ACh from fibroblasts can be detected with myocytes. d-Tubocurarine (0.5 mM) was used to block nicotinic ACh channels.

Animals

At the beginning of the experiment, subjects were 57 aged (24 months of age; NIH, Bethesda, MD) and 19 young (3 months of age; Harlan Sprague–Dawley, Indianapolis, IN) male Fischer 344 rats. Three aged animals were excluded from the experiment: 2 due to cataracts and 1 after histological evaluation showed nonviable grafts. Prior to surgery, 20 animals were sacrificed due to age-related somatic dysfunction (skin lesions and tumor formation) and 5 died postsurgery. The final groups consisted of young controls (n = 19), aged unimpaired β-Gal (n = 5), aged unimpaired dChAT (n = 6), aged impaired β-Gal (n = 9), and aged impaired dChAT animals (n = 9). Rats were housed in pairs in a temperature-controlled (22°C) colony room maintained on a 12-h light/12-h dark cycle with food and water available *ad libitum*. The animals were kept in the colony for at least 1 week prior to testing. Behavioral testing was performed during the daylight cycle. The temporal design of the present study is summarized in Fig. 1.

Behavioral Assessment

Pregrafting water maze testing: hidden platform. One week before surgery, rats were pretested in a black circular pool (120 cm diameter × 45 cm height) filled with clear tepid water (25–27°C) that had been turned opaque using black nontoxic tempura paint. The platform consisted of a black Plexiglas stand (14 × 10 cm) that was submerged 2 cm below the water surface. The four release points were equally spaced around the pool, and the starting position for the rats was chosen semirandomly for each trial to prevent the animal learning a simple motor pattern. All rats were given four training trials per day for 3 consecutive days. At the start of each trial, the rat was placed in the water facing the wall. Afterward, the rat was allowed to search for the submerged platform for up to 40 s. After the rat remained on the platform for 20 s, it was removed from the pool and placed in a holding box for 30 s. If the rat did not find the platform within 40 s, it was gently guided to the platform. Swim path lengths (cm), latencies (s), and swim speed (cm/s) were recorded. To assess spatial bias for the platform quadrant, a single, 40-s spatial probe trial without the platform present was conducted 4 h after the last block of training trials.

Pregrafting water maze testing: visible platform. After the hidden version of the water maze testing, all animals were tested for 2 days in a pool with

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a visible platform to assess age-related sensorimotor or motivational deficits. The visible platform is a square (9.5 × 9.5 cm) Plexiglas stand raised 3 cm above the water level with four attached wooden dowels (20 cm long) supporting a black cardboard square. To enhance visibility of the platform further, geometric shapes in contrasting white and black stripes were suspended between the dowels. The animals were tested for six trials on both days. Rats were placed in the pool at the same location for all trials and given 40 s to locate and to escape onto the platform. The platform was moved to a novel location for each trial. The rat was allowed to remain on the platform for 10 s and was then placed in a holding cage for approximately 20 s. Rats that showed no improvement in escape performance during the pregrafting visible water maze testing ($n = 2$), most likely due to impaired vision, were excluded from the study.

Group assignment. Based on their performance during the last acquisition day of the pregrafting water maze testing (hidden version; day 3), the aged rats were divided into two groups for surgery according to the criteria of Gage and Björklund [24]: aged impaired and aged unimpaired. The aged impaired group included rats that had mean escape distances (swim path length in cm) of greater than 2 SD from the mean of the young controls. The aged unimpaired group consisted of rats whose performance was within 2 SD of young animals.

Postgrafting water maze testing: hidden and visible platform. One week postgrafting, rats were retested on the hidden and visible portions of the water maze task. The procedures followed were identical to those of the pregrafting testing paradigm with two exceptions: (1) the escape platform was located in a novel quadrant and (2) the reacquisition phase lasted 1 day longer.

Motor activity and habituation. One day after completion of the water maze testing, locomotor activity was examined by placing each rat into a novel environment for 40 min and then returning the rat to the same environment 24 h later for another 40-min session. The novel environment consisted of a square (61 × 61 cm) chamber equipped with eight photoelectric sensors that automatically recorded rat movements throughout the enclosure during the test period (San Diego Instruments, San Diego, CA). Activity counts were sampled once every 15 s and subsequently collapsed into 165-min time bins for data analysis.

Cell Preparation and Transplantation

Fibroblasts were removed from tissue culture plates with 1 mM EDTA and trypsin (0.05%) in phosphate-buffered saline (PBS). Cells were washed twice in PBS and suspended in PBS supplemented with $MgCl_2$, $CaCl_2$, 0.1% glucose, and 2% rat serum at a density of 100,000 cells/μl for grafting.

All aged rats received grafts of either β-Gal or dChAT fibroblasts into the hippocampus and the neocortex (frontal and parietal). Based on pregrafting hidden platform water maze performances, aged rats were assigned to one of four graft groups: unimpaired β-Gal ($n = 5$), unimpaired dChAT ($n = 6$), impaired β-Gal ($n = 9$), and impaired dChAT ($n = 9$). Young controls ($n = 19$) were not grafted but were anesthetized to control for effects of anesthesia on behavior. Before grafting, rats were anesthetized with a mixture of acepromazine (5.6 mg/kg), ketamine (75 mg/kg), and xylazine (4.0 mg/kg). Transduced fibroblasts were implanted into both the dorsal hippocampi (three sites with 100,000 cells/site: AP −3.6, ML ±2.0, DV −3.0 and −2.7 and AP −4.3, ML ±2.4, DV −2.8) and the fronto-parietal neocortices (four sites with 75,000 cells/site: frontal, AP +2.5, ML ±2.5, DV −2.0 and −1.4 from dura; parietal, AP −2.3, ML ±5.8, DV −5.0 and −3.8). Five aged animals (unimpaired dChAT, $n = 2$, and from all other aged groups, $n = 1$) died postgrafting, most likely due to an increased age-related surgical stress. All coordinates are taken from bregma according to the atlas of Paxinos and Watson [37]. Cells were implanted using a 26-gauge Hamilton syringe over a period of 1 min/deposit. After each injection, the syringe was left in place for an additional minute to prevent cell migration up the needle track.

Quantitative RT-PCR of Grafts

For the *in vivo* analysis of gene expression postgrafting, three aged impaired rats with dChAT grafts were selected based on water maze performance acquisition. Two of the young control rats were used as controls. Animals were anesthetized and killed by decapitation 40 days postgrafting. Brains were rapidly removed under aseptic conditions, and grafted and nongrafted hippocampal and cortical areas were dissected, under microscopic guidance, on ice and processed for detection of dChAT mRNA by using standard guanidinum–thiocyanate–CsCl gradient purification methods as previously described [38]. Total RNA (200 ng) was reverse-transcribed in 1× PCR buffer (10 mM Tris–Cl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 1 mM dNTP (Boehringer, Mannheim, Germany), 100 pM random hexamers, 20 units RNasin, and 12.5 units AMV reverse transcriptase in a 20-μl volume for 75 min at 42°C followed by 10 min at 95°C. PCR amplification was performed by adding premixed 1× PCR buffer in water, additional $MgCl_2$ (2.5 mM final concentration), 2×10^{-3} mCi [^{32}P]dCTP, 2.5 units *Taq* polymerase, and 0.5 μg each primer. Of this mix, 80 μl was added to each RT reaction for a total of 100 μl. Samples were overlaid with mineral oil and amplified in a PEC thermocycler 480 as follows: 2 min at 95°C, 2 min at 60°C, and 2 min at 72°C, for 23 cycles as previously described [30]. A 40-μl sample of reaction product was analyzed on 6.5% polyacrylamide gel electrophoresis. Primer sequences were dChAT-forward (F) primer, 5′-CTGGAGAAGCGTGAGGCGAG; dChAT-reverse (R) primer, 5′-GCACGTCGTGGACGGTCTTG; ribosomal protein (RP) L27A F primer, 5′-ATCGGTAAGCACCGCAAGCA; RP L27A R primer, 5′-GGGAGCAACTCCATTCCTTGT. dChAT primers were designed and tested for non-cross-hybridization with endogenous rat ChAT (results not shown). RP L27A PCR product in the absence of RT presumably reflects the presence of small amounts of genomic DNA.

Histology

Four weeks postgrafting, the remaining rats were deeply anesthetized and perfused transcardially with 50 ml of 0.1 M phosphate-buffered saline (pH 7.3) followed by 250 ml of ice-cold 4% paraformaldehyde. Brains were removed, postfixed overnight, and then placed in 30% phosphate-buffered sucrose for several days at 4°C. Forty-micrometer-thick coronal sections were cut on a freezing microtome. Every sixth section was stained for Nissl substance using Thionin. Grafts were visualized by immunolabeling sections for β-Gal (Promega; 1:5000) [31]. Stained sections were mounted onto gelatin-coated slides, air dried, dehydrated through a series of alcohols, and coverslipped.

Data Analyses

A two-way (group × blocks) ANOVA with repeated measures was used to analyze changes in behavioral indices (swim path length and activity counts). A one-way (group) ANOVA was applied to analyze differences in speed and locomotor activity. When statistical differences were obtained at the $P < 0.05$ level between groups, post hoc comparisons were made using the Fisher least-square differences test. Within-group changes in performance were assessed using one-way ANOVA with repeated measures (across trials) and by paired t test (between days).

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REFERENCES

1. Bartus, R. T., Dean, R. L., Beer, B., and Lippa, A. S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217:** 408–417.
2. Collerton, D. (1986). Cholinergic function and intellectual decline in Alzheimer's Disease. *Neuroscience* **19:** 1–28.
3. Emre, M., Heckers, S., Mash, D. C., Geula, C., and Mesulam, M. M. (1993). Cholinergic innervation by the amygdaloid complex in the human brain and its alterations in old age and Alzheimer's disease. *J. Comp. Neurol.* **336:** 117–134.
4. Cummings, J. L., and Kaufer, D. (1996). Neuropsychiatric aspects of Alzheimer's disease: the cholinergic hypothesis revisited. *Neurology* **47:** 876–883.
5. Lehericy, S., *et al.* (1993). Heterogeneity and selectivity of the degeneration of cholinergic neurons in the basal forebrain of patients with Alzheimer's disease. *J. Comp. Neurol.* **330:** 15–31.

6. Davis, K. L., *et al.* (1999). Cholinergic markers in elderly patients with early signs of Alzheimer's disease. *JAMA* **281:** 1401–1434.
7. Geula, C., and Mesulam, M.-M. (1999). Cholinergic systems in Alzheimer disease. In *Alzheimer Disease,* pp. 269–292. Lippincott Williams & Wilkins, Philadelphia.
8. Mandel, R. J., Gage, F. H., and Thal, L. J. (1989). Spatial learning in rats: correlation with cortical choline acetyltransferase and improvement with NGF following NBM damage. *Exp. Neurol.* **104:** 208–217.
9. Luine, V., and Hearns, M (1990). Spatial memory deficits in aged rats: contributions of the cholinergic system assessed by ChAT. *Brain Res.* **523:** 321–324.
10. Dunbar, G. L., Rylett, R. J., Schmidt, B. M., Sinclair, R. C., and Williams, L. R. (1993). Hippocampal choline acetyltransferase activity correlates with spatial learning in aged rats. *Brain Res.* **604:** 266–272.
11. Smith, D. E., Roberts, J., Gage, F. H., and Tuszynski, M. H. (1999). Age-associated neuronal atrophy occurs in the primate brain and is reversible by growth factor gene therapy. *Proc. Natl. Acad. Sci. USA* **96:** 10893–10898.
12. Koh, S., and Loy, R. (1988). Age-related loss of nerve growth factor sensitivity in rat basal forebrain neurons. *Brain Res.* **440:** 396–401.
13. Fischer, W., Björklund, A., Chen, K., and Gage, F. H. (1991). NGF improves spatial memory in aged rats as a function of age. *J. Neurosci.* **11:** 1889–1906.
14. Thal, L. (1999). Clinical trials in Alzheimer's disease. In *Alzheimer's Disease,* 2nd ed., pp. 423–439. Lippincott Williams & Wilkins, Philadelphia.
15. Fischer, W., Wictorin, K., Björklund, A., Williams, L. R., Varon, S., and Gage, F. H. (1987). Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* **329:** 65–68.
16. Markowska, A. L., Koliatsos, V. E., Breckler, S. J., Price, D. L., and Olton, D. S. (1994). Human nerve growth factor improves spatial memory in aged but not in young rats. *J. Neurosci.* **14:** 4815–4824.
17. Markowska, A. L., Price, D., and Koliatsos, V. E. (1995). Selective effects of nerve growth factor on spatial recent memory as assessed by a delayed nonmatching-to-position task in the water maze. *J. Neurosci.* **16:** 3541–3548.
18. Chen, K. S., and Gage, F. H. (1995). Somatic gene transfer of NGF to the aged brain: behavioral and morphological amelioration. *J. Neurosci.* **15:** 2819–2825.
19. Frick, K. M., Price, D. L., Koliatsos, V. E., and Markowska, A. L. (1997). The effects of nerve growth factor on spatial recent memory in aged rats persist after discontinuation of treatment. *J. Neurosci.* **17:** 2543–2550.
20. Gustilo, M. C., Markowska, A. L., Breckler, S. J., Fleischman, C. A., Price, D. L., and Koliatsos, V. E. (1999). Evidence that nerve growth factor influences recent memory through structural changes in septohippocampal cholinergic neurons. *J. Comp. Neurol.* **405:** 491–507.
21. Gage, F. H., Björklund, A., Stenevi, U., Dunnett, S. B., and Kelly, P. A. (1984). Intrahippocampal septal grafts ameliorate learning impairments in aged rats. *Science* **225:** 533–536.
22. Dunnett, S. B., Toniolo, A., Fine, C. N., Ryan, A., Björklund, A., and Iversen, A. (1985). Transplantation of embryonic ventral forebrain neurons to the neocortex of rats with lesions of nucleus basalis magnocellularis - II. Sensorimotor and learning impairments. *Neuroscience* **16:** 787–797.
23. Clarke, D. J., Gage, F. H., and Björklund, A. (1986). Formation of cholinergic synapses by intrahippocampal septal grafts as revealed by choline acetyltransferase immunohistochemistry. *Brain Res* **369:** 151–162.
24. Gage, F. H., and Björklund, A. (1986). Cholinergic septal grafts into the hippocampal formation improve spatial learning and memory in aged rats by an atropine-sensitive mechanism. *J Neurosci.* **6:** 2837–2847.
25. Nilsson, O. G., Shapiro, M. L., Gage, F. H., Olton, D. S., and Björklund, A. (1987). Spatial learning and memory following fimbria-fornix transection and grafting of fetal septal neurons to the hippocampus. *Exp. Brain Res.* **67:** 195–215.
26. Gage, F. H., Kawaja, M. D., and Fisher, L. J. (1991). Genetically modified cells: applications for intracerebral grafting. *Trends Neurosci.* **14:** 328–333.
27. Fisher, L. J. (1995). Engineered cells: a promising therapeutic approach for neural disease. *Restor. Neurol. Neurosci.* **8:** 49–57.
28. Sinden, J. D., Hodges, H., and Gray, J. A. (1995). Neural transplantation and recovery of cognitive function. *Behav. Brain Sci.* **18:** 10–35.
29. Martinez-Serrano, A., Fischer, W., and Björklund, A. (1995). Reversal of age-dependent cognitive impairments and cholinergic neuron atrophy by NGF-secreting neural progenitors grafted to the basal forebrain. *Neuron* **15:** 473–484.
30. Winkler, J., Suhr, S. T., Gage, F. H., Thal, L. J., and Fisher, L. J. (1995). Essential role of neocortical acetylcholine in spatial memory. *Nature* **375:** 484–487.
31. Dickinson-Anson, H., Aubert, I., Gage, F. H., and Fisher, L. J. (1998). Hippocampal grafts of acetylcholine-producing cells are sufficient to improve behavioral performance following a unilateral fimbria–fornix lesion. *Neuroscience* **84:** 771–781.
32. Fisher, L. J., Schinstine, M., Salvaterra, P., Dekker, A. J., Thal, L. J., and Gage, F. H. (1993). *In vivo* production and release of acetylcholine from primary fibroblasts genetically modified to express choline acetyltransferase. *J. Neurochem.* **61:** 1323–1332.
33. Fonnum, F. (1969). Radiochemical microassays for the determination of choline acetyltransferase and cholinesterase activities. *Biochem. J.* **115:** 465–472.
34. Tucek, S. (1978). *Acetylcholine Synthesis in Neurons.* Chapman & Hall, London.
35. Tabti, N., Alder, S., and Poo, M.-M. (1998). Culturing spinal neurons and muscle cells from *Xenopus* embryos. In *Culturing Nerve Cells.* MIT Press, Cambridge, MA.
36. Song, H.-J., Ming, G.-l., Fon, E., Bellucchio, E., Edwards, R. H., and Poo, M.-M. (1997). Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. *Neuron* **18:** 815–826.
37. Paxinos, G., and Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates*. 4th ed. Academic Press, San Diego.
38. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. Poo, M.-M., Dan, Y., Song, H.-J., Morimoto, T., and Popov, S. (1996). Calcium dependent vesicular exocytosis: from constitutive to regulated secretion. *Cold Spring Harbor Symp. Quant. Biol.* **V60:** 349–359.
40. Girod, R., Popov, S., Alder, J., Zheng, J. Q., Lohof, A., and Poo, M.-M. (1996). Spontaneous quantal transmitter secretion from myocytes and fibroblasts: comparison with neuronal secretion. *J. Neurosci.* **15:** 2826–2838.
41. Gage, F. H., Björklund, A., Stenevi, U., Dunnett, S. B., and Kelly, P. A. T. (1984). Intrahippocampal septal grafts ameliorate learning impairments in aged rats. *Science* **225:** 533–536.