

# Gene-experience interaction alters the cholinergic septohippocampal pathway of mice

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**Spatial learning requires the septohippocampal pathway. The interaction of learning experience with gene products to modulate the function of a pathway may underlie use-dependent plasticity. The regulated release of nerve growth factor (NGF) from hippocampal cultures and hippocampus, as well as its actions on cholinergic septal neurons, suggest it as a candidate protein to interact with a learning experience. A method was used to evaluate NGF gene-experience interaction on the septohippocampal neural circuitry in mice. The method permits brain region-specific expression of a new gene by using a two-component approach: a virus vector directing expression of *cre* recombinase; and transgenic mice carrying genomic recombination substrates rendered transcriptionally inactive by a “floxed” stop cassette. Cre recombinase vector delivery into transgenic mouse hippocampus resulted in recombination in 30% of infected cells and the expression of a new gene in those cells. To examine the interaction of the NGF gene and experience, adult mice carrying a NGF transgene with a floxed stop cassette (NGFXAT) received a *cre* recombinase vector to produce localized unilateral hippocampal NGF gene expression, so-called “activated” mice. Activated and control nonactivated NGFXAT mice were subjected to different experiences: repeated spatial learning, repeated rote performance, or standard vivarium housing. Latency, the time to complete the learning task, declined in the repeated spatial learning groups. The measurement of interaction between NGF gene expression and experience on the septohippocampal circuitry was assessed by counting retrogradely labeled basal forebrain cholinergic neurons projecting to the hippocampal site of NGF gene activation. Comparison of all NGF activated groups revealed a graded effect of experience on the septohippocampal pathway, with the largest change occurring in activated mice provided with repeated learning experience. These data demonstrate that plasticity of the adult spatial learning circuitry can be robustly modulated by experience-dependent interactions with a specific hippocampal gene product.**

*cre/loxP* | NGF | learning

The capacity of individual experience to modify the anatomical substrate(s) underlying memory formation likely involves regional changes in the expression of specific genes (1, 2). Whereas many brain regions undergo both learning and experience-dependent changes in synaptic density (3–5), the molecular determinants underlying these phenomena are obscure. The elucidation of such mediators requires dissection of specific neural circuitry by using tools to manipulate molecules within discrete synaptic compartments. Our criteria for a candidate molecule are that its synthesis and release from postsynaptic compartments be regulated by activity and its action on the presynaptic compartment would alter signaling in that cell. Neural growth factor (NGF) fulfills these criteria given its activity-dependent regulation within hippocampal postsynaptic target fields and through its actions on projecting NGF-responsive basal forebrain cholinergic neurons (6–9). However, convincing evidence for its role as a mediator of experience-driven alteration of the septohippocampal circuitry is lacking. To examine this potential role of NGF, we developed a method whereby precise temporal and spatial regulation of gene expression is achieved in the brain of adult mice. This approach involves a

two-fold molecular genetic manipulation. A transgene construct placed into the germline is designed to be silent by inclusion of a *loxP* bounded or “floxed” transcriptional stop cassette. Expression of the transgene or its activation is achieved by intracerebral delivery of a virus vector transducing *cre* recombinase that produces regional recombination and excision of the transcriptional stop cassette. The process of recombination-mediated *de novo* expression of a gene in such mice is hereafter referred to as “activation.”

By using this approach we initiated an analysis of NGF function in the mouse septohippocampal pathway. We constructed NGFXAT mice, a transgenic line carrying a silent NGF gene engineered to be activated only in neurons in which *cre* recombinase is expressed. We had demonstrated that, in adult NGFXAT mice, the intracerebral delivery of herpes simplex virus (HSV) vectors that express *cre* recombinase resulted in temporal and spatial NGF gene activation. Hippocampal delivery of HSV*cre*, a vector expressing *cre* recombinase, produced approximately a 10-fold local increase in NGF (10, 11). Given that cholinergic basal forebrain neurons respond to target-derived NGF, we examined whether selective hippocampal NGF activation would elicit an alteration in the afferent septal cholinergic projection. Localized hippocampal NGF activation caused an increase in cholinergic neurons projecting to the specific region of gene activation (12). These data, while supporting a role for NGF in promoting modification of the adult septohippocampal circuitry, raised the question as to whether reorganization can facilitate learning or enhance memory function. Recently, we demonstrated that unilateral hippocampal NGF gene activation does indeed augment learning and memory as measured in two spatial learning paradigms, the water maze and the newly developed repeated acquisition and performance chamber (RAPC; unpublished results). Thus, a localized increase in NGF elicited alteration in afferent septohippocampal input and also enhanced spatial learning.

Hebb’s postulate regarding the role of activity or experience in the modification of neural circuits led us to posit that an appropriate behavioral experience mediated through use of a specific neural pathway would stimulate its alteration. More specifically, we postulated that the extent of NGF release (from engineered hippocampal sites) and subsequent septohippocampal pathway alteration are related to the type and magnitude of experience. The present study was designed to examine the interaction of activated NGF gene expression and experience on septohippocampal pathway plasticity.

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Abbreviations: NGF, nerve growth factor; HSV, herpes simplex virus; RAPC, repeated acquisition and performance chamber; eGFP, enhanced green fluorescent protein;  $\beta$ -gal,  $\beta$ -galactosidase; FG, Fluorogold; ChAT, choline acetyltransferase.

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## Methods

**GFPcre Fusion.** The gfpcre fusion was produced by amplification of enhanced green fluorescent protein (eGFP) with primers (5'-CCGGAATTCGGATGTCCAATTTACTGACC-3' and 5'-TTCCCCCGGGGGGACTAATCGCCATCTT-3'). This fragment, directionally cloned into pEGFP-C2 (CLONTECH) to generate a fusion protein (gfpcre), was excised with *NheI* and *BamHI* and directionally cloned into the *XbaI* and *BamHI* sites in amplicon vector HSVprpuc to generate HSVgfpcre.

**CAG CATZ Mice.** Mice from S. H. Schneider (The Johns Hopkins University School of Medicine, Baltimore, MD) (13) were screened by using previously described *lacZ* primers (14).

**NGFXAT Mice.** Line 30 NGFXAT heterozygote construction and characterization has been described previously (11).

**HSV Vectors.** HSVlac and HSVcrelac and amplicon packaging have been previously described (11, 15). The amplicon component of virus stocks was titered by an expression assay previously described (16). Throughout this study, multiplicity of infection refers to expressing amplicon particles. The titers of virus stocks used in this study were as follows: HSVlac,  $7.8 \times 10^4$  infectious particles/ $\mu\text{l}$  of amplicon; and HSVcrelac,  $1.9 \times 10^5$  infectious particles/ $\mu\text{l}$  of amplicon.

**$\beta$ -Galactosidase ( $\beta$ -gal) Histochemistry and Measurement of  $\beta$ -Gal Activity.**  $\beta$ -gal histochemistry and measurement of  $\beta$ -gal activity were performed as previously described (17).

**Hippocampal Cultures.** E16.5 hippocampal cultures were prepared from NGFXAT mice as previously described (18).

**Arecoline Treatment.** Mice received an i.p. injection of saline or drug solution every day for 3 consecutive days. The dose of arecoline hydrobromide is 17.5  $\mu\text{g}$ /mouse. All solutions were coded, and the animals' identities were blinded until completion of the study. Arecoline hydrobromide (Sigma) was diluted in 0.9% saline immediately before injection.

**NGF ELISA.** Tissue dissection and preparation in addition to the NGF ELISA were performed as previously described (11).

**Stereotactic Injections of Virus Vectors and Fluorogold.** All stereotaxic procedures were performed as previously described (11, 17).

**Immunocytochemistry and Fluorogold Visualization.** Choline acetyltransferase (ChAT) immunocytochemistry and Fluorogold (FG) (Fluorochrome, Englewood, CO) detection were completed as previously described (12).

**Morphological Analyses.** Cell counts and somal size were performed as previously described with the following modifications (12). All sections were 120  $\mu\text{m}$  apart, thereby decreasing the chance of counting the same cell twice because of the fact that this distance between sections is significantly larger than the diameter of a single neuronal cell body with nucleus. In addition, an Abercrombie correction was applied. This technique accounts for section thickness and nuclear size as a function of the crude number of cell nuclei per section (19). This difference is reflected in the Abercrombie corrected cell counts. Every section in the compartment that encompassed the anatomical boundaries stated above was analyzed. Double-labeled cells, FG+ (ultraviolet) and ChAT+ [tetramethylrhodamine B isothiocyanate (TRITC)], were counted in the same manner after image superimposition.

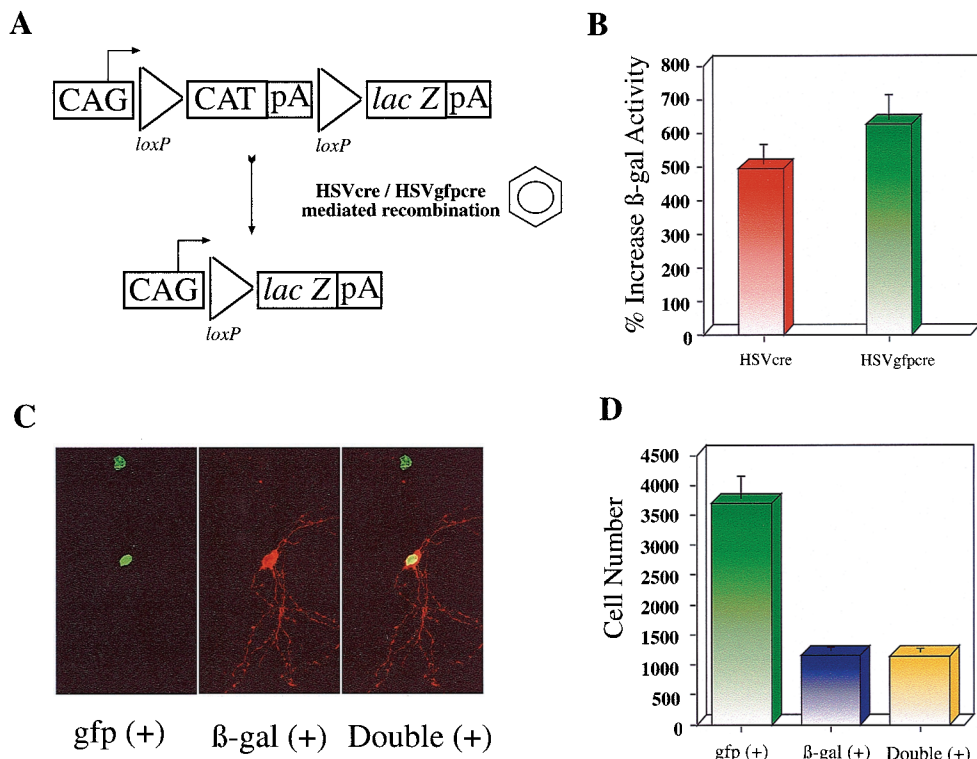
**Learning and Performance.** The RAPC is a hippocampal-dependent spatial learning task that allows for discrimination of spatial learning ability in mice (20). The RAPC, by affording the experimental measurement of both learning (learning component) and rote performance (performance component) at baseline and after gene transfer, provided a method for concurrent controlling for noncognitive influences in learning (20). The RAPC, a protocol for habituation and testing, has been described (20).

**Statistical Analyses.** Somal size differences between HSVcrelac and HSVlac groups were based on one-way ANOVAs. Cell count differences were determined by using 2-way ANOVAs with HSVcrelac vs. HSVlac and learning vs. performance as between-groups factors; individual group differences were then evaluated by least squares means analyses. Overall analyses for the behavioral data were based on 4-way RMANOVA (repeated measures ANOVA) with learning vs. performance and HSVcrelac vs. HSVlac as between-groups variables and both sessions and trials as within-group variables. These analyses were carried out separately for each week of the experiment. Subsequent analysis were carried out separately for each behavioral group (learning and performance) with a 3-way RMANOVA in which HSVcrelac vs. HSVlac served as a between-groups variable and both sessions and trials as within-group factors and finally by a 2-way RMANOVA carried out separately for each session based on HSVcrelac vs. HSVlac (between-groups factor) and trials (within-group factor). For the NGF ELISA data, one-way ANOVA was carried out. In all analyses,  $P$  values  $\leq 0.05$  were considered statistically significant.

## Results

**HSVcre Delivery to Hippocampus Produces Recombination and Activation of Gene Expression.** The efficiency of hippocampal recombination and gene activation was assessed in the CAG CATZ mice (13), a line expressing the  $\beta$ -gal gene after cre-catalyzed recombination (Fig. 1A). HSVcre and HSVgfpcre (contains a fusion of eGFP and cre) were delivered to the hippocampus, and, several days later, brains were removed and either dissected for homogenates or sectioned. In HSVcre- and HSVgfpcre-injected mice hippocampus, but not in uninjected regions or mock injected tissue (data not shown), there was a large and comparable amount of  $\beta$ -gal activity produced by both vectors (Fig. 1B). After sectioning, the GFP,  $\beta$ -gal, and double-positive cells were visualized, some in the hippocampus showing a characteristic neuronal appearance (Fig. 1C). Enumeration of cells expressing each of the labels indicated that approximately 30% of the GFP-positive cells had undergone recombination, evidenced by expression of  $\beta$ -gal (Fig. 1D). The numbers of double-positive (GFP- and  $\beta$ -gal-positive) and GFP-positive cells were equivalent.

**Hippocampal NGF Gene Activation Produces an Increase in the Amount of Releasable NGF.** A central postulate underlying this study is that NGF release from activated sites will be stimulated by afferent neurotransmission. In a prior study, HSVcre delivery to NGFXAT hippocampus caused a significant increase in local tissue levels of NGF (11). If increased levels of NGF are to produce the proposed activity-dependent action on the cholinergic septohippocampal pathway, NGF release should be regulated. To investigate this mechanism, we examined changes in NGF levels mediated by depolarization in dissociated hippocampal cultures or by cholinergic agonist treatment in the intact animal. Cultures from NGFXAT mice were transduced either with HSVcre to produce NGF activation or with control virus HSVlac. NGF release from cultures was measured under basal conditions and after depolarization with KCl. The data indicate that, in NGF-activated cultures, the amount of constitutive NGF release increased 50% ( $P = 0.007$ ) whereas the amount of activity-dependent NGF release was increased 300% ( $P < 0.0001$ ), compared with control (HSVlac-transduced) cultures.

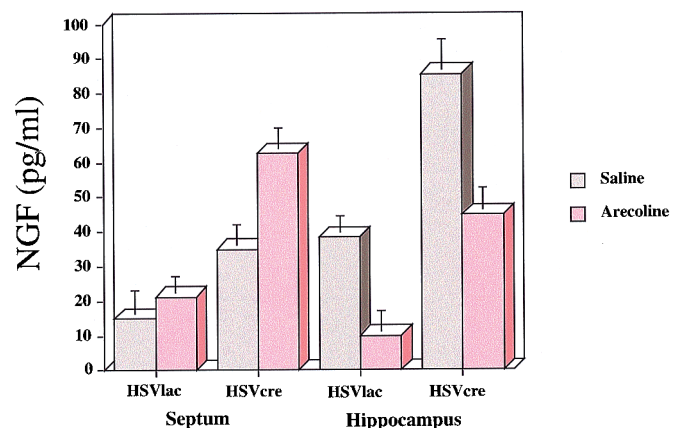


**Fig. 1.** HSVcre vectors mediate hippocampal recombination. (A) The CAG-CATZ reporter construct. (B) After delivery of HSVcre or HSVgfpcre, the hippocampus was excised and analyzed for β-gal. The data are the percentage increase of activity compared with non-transgenic animals infected with HSVcre or HSVgfpcre. (C) Confocal images of hippocampal neurons from CAG-CATZ mice infected with HSVgfpcre. The green image represents the eGFP whereas the red image represents β-gal. The combined channel illustrates β-gal in GFP+ cells. (D) Cell counts. β-gal, GFP+, and double-positive cells were enumerated.

Because the septohippocampal pathway releases acetylcholine, the likely regulator of NGF release, we asked whether a cholinergic agonist would stimulate NGF release from the NGF-activated hippocampus. Activated NGFXAT and control (HSVlac) mice were treated with the cholinergic agonist arecoline, and, after allowing time for NGF release and transport to cell bodies of afferent neurons, the hippocampus and medial septum were excised and homogenized and NGF was measured by ELISA. These data, presented in Fig. 2, reveal the following. First, and as expected, basal NGF levels are greater in activated (HSVcre) than nonactivated (HSVlac) mice in both hippocampus and septum. Second, basal hippocampal NGF levels are greater than corresponding septal levels in both groups of mice. Third, arecoline treatment significantly increased septal NGF content only in activated mice. Fourth, hippocampal NGF levels decline after arecoline administration in both activated and control mice. In aggregate, the ELISA data show that cholinergic neurotransmission augments NGF in the septum and reduction from the hippocampus.

**Behavioral Experience Modulates the Septohippocampal Projection in Activated NGFXAT Mice.** The hypothesis that NGF is a component of experiential modulation of the neural circuitry that subserves spatial learning leads to several predictions. First, an increase in the pool of activity-releasable NGF would serve to drive alteration of the septal cholinergic input to hippocampal sites with enhanced NGF availability. This alteration of the input was shown in our prior study in vivarium-housed mice: NGF gene activation within a hippocampal region elicited a time-dependent increase in the number of cholinergic septal neurons projecting to that region (12). A second prediction is that experience, by increasing activity of septohippocampal neurons, will further stimulate NGF release and consequently accelerate alteration of the afferent projection. To examine this postulate, we determined whether learning experience compared with other experiences would differentially modulate the extent of alteration of the septohippocampal pathway (supplementary Fig. 5, which is published as supplementary data on the PNAS web site, www.pnas.org). NGFXAT mice were transduced in the

hippocampus with HSVcrelac (activated) or HSVlac (control) and then assigned to one of three different behavioral experiences. These experiences were normal housing (Normal), repeated spatial learning in the RAPC (Learning), and repeated rote performance in the RAPC (Performance). The RAPC is a newly described reward-based spatial learning paradigm sensitive to both hippocampal lesioning and cholinergic agents. The RAPC paradigm incorporates two components, a repeated learning component and a repeated performance component, providing interventions that distinguish effects on learning *per se* from those alterations in motor



**Fig. 2.** Regulated NGF levels in arecoline-treated mice. Adult NGFXAT mice received HSVcre or HSVlac. Months later, animals received arecoline hydrobromide or saline before sacrifice. NGF in the medial septum and hippocampus was measured by ELISA. Septal HSVcre vs. HSVlac saline treated  $P = 0.013$ ; hippocampal HSVcre vs. HSVlac saline treated  $P < 0.0001$ ; HSVlac hippocampus vs. septum saline treated  $P = 0.0076$ ; HSVcre hippocampus vs. septum  $P = 0.0001$ ; HSVcre septum arecoline treated vs. HSVcre septum saline  $P = 0.0013$ ; HSVlac hippocampus arecoline vs. HSVlac saline  $P < 0.0001$ ; HSVcre hippocampus arecoline vs. HSVcre saline  $P < 0.0001$ .

**Table 1. Assessment of spatial learning in HSVcrelac (activated) and HSVlac (control) NGFXAT mice**

	Session 1, s			Session 2, s			Session 3, s		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
HSV crelac (activated)	375	300	250	275	150	125	175	120	75
HSVlac (control)	450	425	400	375	350	350	375	345	300

Latencies for three sessions each, including three trials, are shown. Shorter latencies were exhibited for HSVcrelac (learning) than HSVlac (learning) mice. Overall RMANOVA with HSVcrelac as a between-groups factor and component, trials and sessions as within-group factors: main effect of HSVcre/ac,  $F(1,27) = 9.03$ ,  $P = 0.006$ ; interaction of HSVcrelac by component,  $F(1,27) = 10.7$ ,  $P = 0.003$ ; interaction of trials by component by HSVcrelac,  $F(2,54) = 10.34$ ,  $P = 0.0002$ . Subsequent RMANOVA for each session for the learning component, all  $P$  values for HSVcrelac or interaction of HSVcrelac by treatment,  $P \leq 0.05$ .

function, sensory ability, and/or motivation (ref. 20 and unpublished observations). After assignment to Learning vs. Performance groups, latencies declined for both learning groups, HSVcrelac and HSVlac, confirming an acquisition phenomenon, but declined significantly more slowly in the HSVlac mice, as shown in Table 1. Latency data for performance groups showed no statistical trends and did not differ between groups (data not shown).

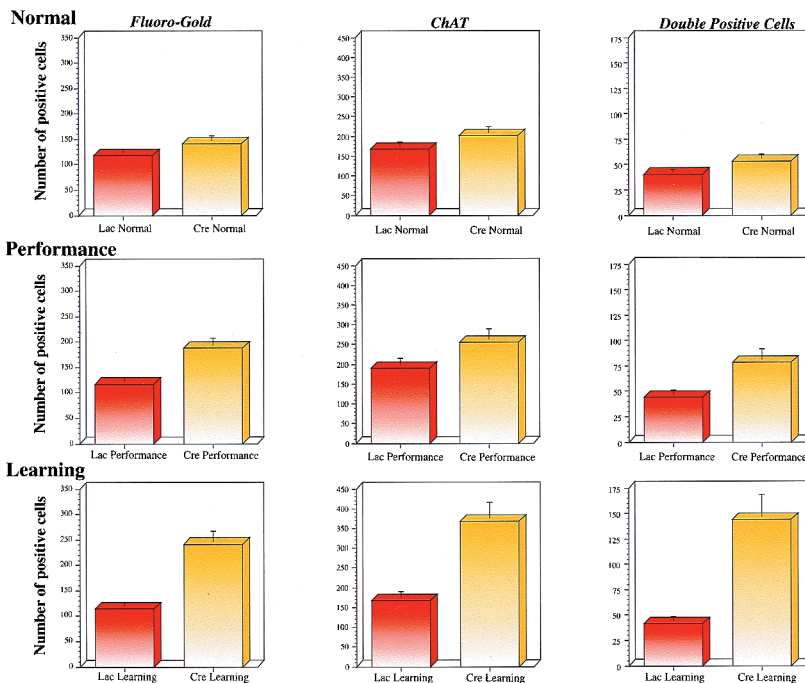
One week after the final behavioral sessions for the Learning and Performance groups, animals in all three groups received FG at the site of antecedent virus vector delivery to retrogradely label projecting basal forebrain cholinergic neurons. After tracer injection, animals were killed and brains were removed and processed for measurement of FG, ChAT, and double-positive cells in the medial septum and the diagonal band. Normally housed groups served as controls for the Performance and Learning groups.

In normally housed and activated mice (Cre Normal), FG-labeled cell number was increased 16% compared with normally housed control mice (Lac Normal) (Fig. 3 Normal, Column 1, Fluoro-Gold). To determine whether NGF gain of function stimulated cholinergic forebrain neurons, ChAT-labeled somata were counted. In normally housed and activated mice, there was a significant 21% increase in ChAT+ cell bodies compared with normally housed HSVlac-injected control mice (Fig. 3 Normal, Column 2, ChAT). The basal forebrain neurons most likely to be influenced by hippocampal NGF activation are those that directly project to the hippocampal site of NGF up-regulation (identified as FG+) and that express the NGF-responsive cho-

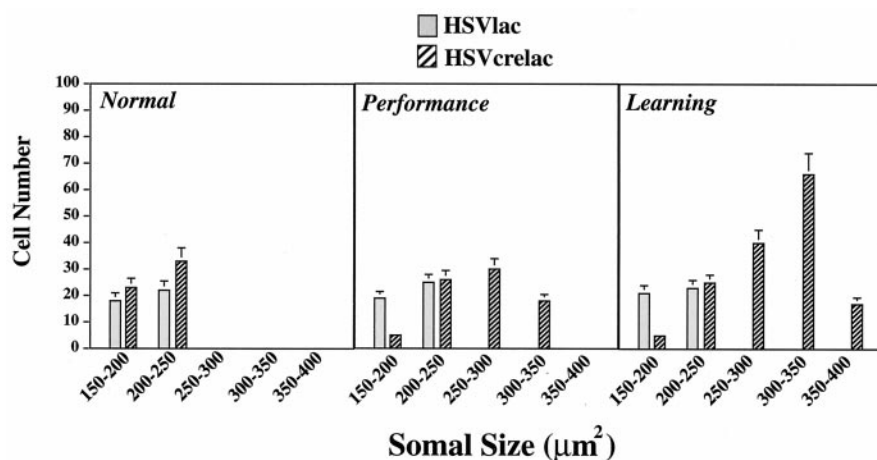
linergic marker ChAT, referred to as double-labeled cells. Double-labeled cells were increased 37% in normally housed and activated compared with control mice (Fig. 3, Column 3, Double Positive Cells). Thus, NGF gene activation alone is sufficient to cause a modest alteration of the septal projection to a hippocampal region engineered to produce additional NGF.

Corresponding data for the Performance group that repeatedly executed a rote response are depicted in Fig. 3 (Performance). Analyses disclosed significant differences. FG+ cells were increased 58% (Fig. 3, Performance, Column 1, Fluoro-Gold), ChAT+ cells were increased 37% (Fig. 3, Performance, Column 2, ChAT), and double-labeled cells increased 67% (Fig. 3, Performance, Column 3, Double Positive Cells) in activated (Cre Performance) as compared with control (Lac Performance) mice. Comparison of activated normally housed to activated Performance mice (*cf.* Fig. 3 Normal and Performance) revealed significant increases in all measured parameters in the Performance group. Thus, performance of a rote response further enhanced septohippocampal modification relative to NGF activation alone (normally housed activated group).

These parameters were most increased in the Learning groups. In HSVcrelac-injected mice (Cre Learning), FG+ cells were increased 108% (Fig. 3, Learning, Column 1, Fluoro-Gold); ChAT+ cells were increased by 115% (Fig. 3, Learning, Column 2, ChAT); and double-labeled cells were increased 235% (Fig. 3, Learning, Column 3, Double Positive Cells) relative to HSVlac-injected (Lac Learning) mice. Furthermore, activated groups



**Fig. 3.** Cell counts in activated and control mice. NGFXAT mice that received HSVcrelac (Cre) or HSVlac (Lac) virus were habituated and then maintained in the vivarium (Normal), exposed to rote performance (Performance), or exposed to a repeated learning paradigms (Learning). After retrograde tracing with FG, brains were removed, sectioned, and subjected to counting of cell bodies labeled with FG (FG+), ChAT (ChAT+), or both labels (double positive, FG+, and ChAT+). Histograms represent numbers of positive cells in each group. Column 1, Fluoro-Gold: total FG+ cells ipsilateral to virus injection and tracer deposition. Column 2, ChAT: total ChAT+ cells ipsilateral to virus injection and tracer deposition. Column 3, Double Positive Cells: total double-positive cells ipsilateral to virus injection and tracer deposition.



**Fig. 4.** Basal forebrain cell size in activated and control mice. NGFXAT mice received either HSVcrelac (activated) or HSVlac (control) virus and were assigned to one of three groups: Normal, Performance, or Learning. After retrograde tracing with FG, brains were removed, sectioned, and subjected to counting of fluorescent-labeled cell bodies (FG+), immunostained ChAT+ cell bodies, and double-positive (FG+ and ChAT+) cell soma. Double-positive cells have been binned according to somal size.

provided with a repeated learning experience exhibited significantly greater increases in FG+ cells, ChAT+ cells, and double-labeled cells as compared with HSVcrelac Performance group mice (*cf.* Fig. 3 Performance and Learning). Comparison of all activated groups showed a stratification for all measures of basal forebrain neuron numbers: Learning > Performance > Normal, consistent with a graded effect of experience on alteration of the septohippocampal pathway. Finally, comparison of control, non-activated animals in all three groups (Normal, Performance, and Learning) revealed no significant differences in FG+ cells, ChAT+, or double-positive cells. Experience did not modulate these parameters in nonactivated mice.

#### Septohippocampal Neurons Hypertrophy in Activated NGFXAT Mice.

One NGF response of cholinergic neurons is cell somal hypertrophy. Analyses of double-labeled somal sizes, represented as the number of cells in distributed size bins, in activated (HSVcrelac) vs. control (HSVlac) mice, disclosed differences among some but not all groups. Normally housed mice in either group were similar. Analysis of the distribution of the somal sizes indicated that an increasing number of double-labeled neurons in activated Performance and Learning mice had larger mean somal sizes. Activated Performance mice had a 21% increase in size whereas the activated Learning group displayed a 63% increase in mean somal size relative to their respective nonactivated groups ( $P < 0.05$ ; Fig. 4). This increase in cell size, reflected in a shift of the modal distribution of these activated groups, is not due to overestimation of cell number, given that the largest nuclear size, observed in activated Learning group, was increased only 15% (data not shown).

The somal size of ChAT+ cells not labeled by FG was also analyzed. A difference (18%) of ChAT+/FG- neurons between activated and control mice was observed only in the Learning groups (data not shown). Contralateral to gene activation and tracer injection, there were no differences in cell somal size among the groups.

#### Discussion

Experience is believed to modify the anatomic substrate that underlies the ability to learn, remember, and, in aggregate, shape cognitive functions. A long-held postulate is that new experience, via changes in neural activity, leads to the expression of specific sets of molecules that mediate learning and memory. The neuroanatomic structure often implicated in memory acquisition is the hippocampal formation; one of its inputs, the septohippocampal projection, is extensively involved in spatial learning (1, 21, 22).

Neurotrophins are candidates for these specific mediators of experience-dependent learning because they are localized

postsynaptically, are regulated by presynaptic activity, and modulate presynaptic function. Brain-derived neurotrophic factor (BDNF) and NGF secretion was shown to be regulated in hippocampal neurons or hippocampal slices, respectively, by depolarization (6, 23). A role for neurotrophins in learning and memory formation is inferred from analyses of mice harboring only a single copy of the gene. In +/- BDNF mice, electrophysiological studies of acute hippocampal slices disclose abnormalities in long term potentiation (LTP) (24). In +/- NGF mice, spatial learning is impaired, as is slice LTP (2). While provocative and supportive of the general thesis, these data must be interpreted cautiously, given that loss of neurotrophin function is likely to perturb nervous system development and, consequently, adversely impact mature functions apart from direct actions on those neural networks under study.

To evaluate NGF function in the adult, the *cre/loxP* recombination system was adapted for controlling spatial and temporal control of gene expression in the brain (10, 11). This approach uses a germline transgene (NGFXAT) that is a *cre* recombinase substrate that, once activated by recombination, will permanently express NGF in a neuron and brain region-specific manner (11). To activate the gene, HSV amplicon virus vectors were used to transiently express the *cre* recombinase gene (11). In this study, the frequency of recombination in hippocampal cells of the CAG CATZ reporter mouse was 32%. This recombination frequency is likely similar to that of HSVcre-transduced NGFXAT mice, given that the activity of the *gfpcre* chimera is comparable to that of the native gene product (Fig. 1).

Prior studies in NGFXAT mice showed that NGF gene activation in the dorsal hippocampus increased spontaneous motor activity, a phenotype suppressed by administration of a cholinergic antagonist (11). Modification of the septohippocampal cholinergic projection, assessed by retrograde tracing methods similar to those used in the current study, was observed 6 mo after gene activation (12). Thus, NGF activation in the hippocampus produced an alteration of the septohippocampal projection and a behavioral phenotype involving altered cholinergic neurotransmission.

We recently examined whether hippocampal activation of the NGFXAT transgene in adult mice would alter spatial learning ability (unpublished results). That study confirmed that gene-activation produced enhancement of learning. In the current study, the latencies of NGF-activated mice were shorter than those for control mice, an observation consistent with an effect of NGF activation on learning. We posit that this enhancement reflects, in part, the observed alteration of the septohippocampal projection.

We hypothesized that experience would affect the extent to which the cholinergic septohippocampal projection is altered in mice with hippocampal NGF activation. We reasoned that repeated spatial learning would provide the stimulus for modification of the septal input. To separate actual learning processes from the physical activity and sensory stimulation inherent in these procedures, another group was placed in the same environment (RAPC) to repeatedly perform a rote task rather than learn. A group not exposed to a new environment was maintained under normal conditions. Alteration of the septohippocampal projection was studied by FG retrograde labeling, ChAT immunocytochemistry and cell counting of single-labeled cells of both types, double-labeled cells, and measurement of somal size of double-labeled and ChAT+/FG- cells. All of the activated groups showed increases in FG+, ChAT+, and double-labeled cells compared with HSVlac-injected groups of the same type. The magnitude of differences in cell numbers between activated and control mice reflected the extent of experience; i.e., it was greatest in the Learning group, followed by the Performance and then normally housed groups. Likewise, increases in cell numbers were greater in the Learning-activated mice than in Performance-activated mice, with the least pronounced increases in Normal-activated mice. Measurement of cell numbers in activated mice could have been overestimated as a consequence of cellular hypertrophy in these groups. In the activated Learning group, where cellular hypertrophy was greatest, we measured a 15% increase in nuclear size, an increase insufficient to account for the increased cell numbers we determined. These data indicate that pathway alteration was directly related to type of experience.

The marked effect of learning, as compared with performance and normal experience on all parameters, suggests that spatial learning stimulates NGF release from activated hippocampal neurons. Regulated release is supported by other studies demonstrating activity-dependent neurotrophin release from hippocampal cells and slices (6, 23). Our results from cultured NGF-activated hippocampal neurons show that the magnitude of activity-releasable NGF is increased in hippocampal neurons. A similar cholinergically driven regulation of NGF levels was shown *in vivo*. The data are consistent with an activity-dependent NGF release model.

The differences in FG+ and ChAT+ double-labeled cells between the activated and control groups suggests that learning

stimulates an increase in the number of cholinergic septohippocampal neurons projecting to the NGF activation region. Such an increase could reflect sprouting from neurons under the influence of NGF. Although less likely, retraction/reprojection or up-regulation of FG transport can't be excluded. Regardless of the underlying mechanism, the experience-enhanced septohippocampal cholinergic modification reflects a NGF-initiated and an activity-requiring alteration in the neural circuitry facilitating learning and memory.

Hypertrophy of basal forebrain cholinergic neurons is a NGF response (25, 26). The cell somal size data are consistent with an experience enhancement effect. Within the Performance groups, a modest but significant increase in the size of double-labeled neurons was seen in activated animals. By contrast, in the activated Learning groups, a marked increase in somal size of double-labeled cells was observed. These data indicate that learning experience has a profound enhancing effect in the presence of NGF gene activation. The finding that, in activated Learning mice, ChAT+ cells that did not project to the gene activation site, i.e., FG- cells, also manifest increased somal size suggests other mechanisms could be contributory. For example, the effect of afferent activity within the hippocampus could stimulate a reciprocal pathway to release within the forebrain a neurotrophic factor. The known activity-dependent regulation of hippocampal brain-derived neurotrophic factor mRNA and the recent description of its anterograde transport make it an attractive candidate (27).

This study examined whether learning experience mediated by neural activity acts in concert with a specific molecule to modify neural circuits involved in spatial learning. By using a method to achieve spatial and temporal control of gene expression, we have demonstrated that a specific molecular mediator (NGF) in the hippocampus is sufficient to alter the cholinergic input to that region. More interestingly, experience and, most robustly, repeated learning specifically amplify the effect of expression of the molecular mediator on this cholinergic projection pathway. These data underscore the role of NGF in promoting the effects of experience on the neural circuitry subserving spatial learning. Moreover, they implicate septohippocampal activity-evoked NGF release as the molecular mechanism driving alteration of cholinergic basal forebrain neuronal plasticity.

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- Carew, T. J. (1996) *Neuron* **16**, 5–8.
- Chen, K. S. & Gage, F. H. (1995) *J. Neurosci.* **15**, 2819–2825.
- Rosenzweig, M. R., Krech, D., Bennett, E. L. & Diamond, M. C. (1962) *J. Comp. Physiol. Psychol.* **55**, 429–437.
- Wallace, C. S., Withers, G. S., Weiler, I. J., George, J. M., Clayton, D. F. & Greenough, W. T. (1995) *Mol. Brain Res.* **32**, 211–220.
- Greenough, W. T., Withers, G. S. & Wallace, C. S. (1990) in *The Biology of Memory*, eds. Squire, L. R. & Lindenlaub, E. (F. K. Schattauer Verlag, Stuttgart), pp. 159–185.
- Bloch, A. & Thoenen, H. (1995) *Eur. J. Neurosci.* **7**, 1220–1228.
- Thoenen, H. (1995) *Science* **270**, 593–597.
- Berzaghi, M. D., Cooper, J., Castren, E., Zafra, F., Sofroniew, M., Thoenen, H. & Lindholm, D. (1993) *J. Neurosci.* **13**, 3818–2826.
- Van der Zee, E. E. M., Lourenssen, S., Stanis, J. & Diamond, J. (1995) *Eur. J. Neurosci.* **7**, 160–168.
- Federoff, H. J., Brooks, A., Muhkerjee, B. & Corden, T. (1997) *J. Neurosci. Methods* **71**, 133–142.
- Brooks, A. I., Muhkerjee, B., Panahian, N., Cory-Slechta, D. & Federoff, H. J. (1997) *Nat. Biotechnol.* **15**, 57–62.
- Brooks, A. I., Halterman, M. W. & Federoff, H. J. (1999) *NeuroReport* **10**, 337–344.
- Agah, R., Frenkel, P. A., French, B. A., Michael, L. H., Overbeek, P. A. & Schneider, M. D. (1997) *J. Clin. Invest.* **100**, 169–179.
- Lu, B., Gupta, S. & Federoff, H. J. (1995) *Hepatology* **21**, 752–759.
- Geller, A. I. & Breakefield, X. O. (1988) *Science* **241**, 1667–1669.
- Geschwind, M. D., Lu, B. & Federoff, H. J. (1994) in *Providing Pharmacological Access to the Brain: A Volume of Methods in Neurosciences*, ed. Conn, P. (Academic, Orlando, FL), pp. 462–482.
- Brooks, A. I., Halterman, M. W., Chadwick, C. A., Davidson, B. L., Haak-Frendscho, M., Radcl, C. A., Porter, C. & Federoff, H. J. (1998) *J. Neurosci. Methods* **80**, 137–147.
- Bohn, M. C., Bloom, E., Goldstein, M. & Black, I. B. (1984) *Dev. Biol.* **105**, 130–136.
- Abercrombie, M. (1946) *Anat. Rec.* **94**, 274–329.
- Brooks, A. I., Cory-Slechta, D. A., Murg, S. M. & Federoff, H. J. (2000) *Neurobiol. Learn. Mem.* **74**, 241–258.
- Steckler, T., Keith, A. B., Wiley, R. G. & Sahgal, A. (1995) *Neuroscience* **66**, 101–114.
- Barnes, C. A. (1990) in *The Biology of Memory*, ed. Squire, L. & Lindenlaub, E. (F. K. Schattauer Verlag, Stuttgart), pp. 275–297.
- Goodman, L. J., Valverde, J., Lim, F., Geschwind, M. D., Federoff, H. J., Geller, A. I. & Hefti, F. (1996) *Mol. Cell. Neurosci.* **7**, 222–238.
- Patterson, S. L., Abel, T., Devel, A. S., Martin, K. C., Rose, J. C. & Kandel, E. R. (1996) *Neuron* **16**, 1137–1145.
- Higgins, G. A., Koh, S., Chen, K. S. & Gage, F. H. (1989) *Neuron* **3**, 247–256.
- Hefti, F. (1986) *J. Neurosci.* **6**, 2155–2162.
- Altar, C. A., Cai, N., Bliven, T., Juhasz, M., Conner, J. M., Acheson, A. L., Lindsay, R. M. & Wiegand, S. J. (1997) *Nature (London)* **389**, 856–860.