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Allosteric modulation of AMPA-type glutamate receptors increases activity of the promoter for the neural cell adhesion molecule, N-CAM

BRENT D. HOLST*, PETER W. VANDERKLISH*, LESLIE A. KRUSHEL*†, WEI ZHOU*, RONALD B. LANGDON†, JOHN R. MCWHIRTER*§, GERALD M. EDELMAN*†‡, AND KATHRYN L. CROSSIN*¶

*Department of Neurobiology and ‡The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037; and †The Neurosciences Institute, 10640 John Jay Hopkins Drive, San Diego, CA 92121

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ABSTRACT **To study regulation *in vivo* of the promoter for the neural cell adhesion molecule, N-CAM, we have used homologous recombination to insert the bacterial *lacZ* gene between the transcription and translation initiation sites of the N-CAM gene. This insertion disrupts the gene and places the expression of β-galactosidase under the control of the N-CAM promoter. Animals homozygous for the disrupted allele did not express N-CAM mRNA or protein, but the pattern of β-galactosidase expression in heterozygous and homozygous embryos was similar to that of N-CAM mRNA in wild-type animals. The homozygotes exhibited many of the morphological abnormalities observed in previously reported N-CAM knockout mice, with the exception that hippocampal long-term potentiation in the Schaffer collaterals was identical in homozygous, heterozygous, and wild-type animals. Heterozygous mice were used to examine the regulation of the N-CAM promoter in response to enhanced synaptic transmission. Treatment of the mice with an ampakine, an allosteric modulator of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that enhances normal glutamate-mediated synaptic transmission, increased the expression of β-galactosidase *in vivo* as well as in tissue slices *in vitro*. Similar treatments also increased the expression of N-CAM mRNA in the heterozygotes. The effects of ampakine in slices were strongly reduced in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor antagonist. Taken together, these results indicate that facilitation of AMPA receptor-mediated transmission leads to activation of the N-CAM promoter and provide support for the hypothesis that N-CAM synthesis is regulated in part by synaptic activity.**

Cell adhesion molecules (CAMs) mediate neuronal and glial adhesion during development of the nervous system and thereby affect processes such as neurite fasciculation, axonal pathfinding, and synaptogenesis (reviewed in refs. 1 and 2). It has been suggested that in the adult brain, regulation of adhesion alters the morphological and physiological properties of synapses through effects on membrane interactions (3–5). Changes in the levels of expression of CAMs may contribute to the maintenance of synaptic contacts and may be relevant to adult forms of activity-dependent synaptic plasticity (reviewed in refs. 3 and 4). The question therefore arises as to what extent CAM expression is regulated by synaptic activity.

The neural cell adhesion molecule (N-CAM) is a likely candidate for a role in synaptic plasticity. This molecule is expressed throughout the nervous system and has been detected in pre- and postsynaptic membranes (6, 7). The N-CAM protein can be glycosylated with polysialic acid, the presence of which diminishes N-CAM binding activity (8, 9). Perturbing N-CAM function with N-CAM antibodies or peptides or by enzymatic removal of polysialic acid interferes with long-term potentiation (LTP) and decreases performance in particular memory tasks (reviewed in refs. 4 and 10). These studies raise the possibility that alterations in N-CAM-mediated adhesion are important in adult plasticity and that dynamic modulation of N-CAM synthesis or breakdown by synaptic activity might be an integral part of this process.

To examine the potential relationships between N-CAM transcriptional regulation and altered synaptic activity in the adult brain, we produced mice in which the bacterial *lacZ* gene was inserted into the 3′ end of the first exon of the N-CAM gene, thereby disrupting N-CAM expression and placing β-galactosidase (β-gal) expression under the control of the N-CAM promoter. This design permitted studies in which N-CAM promoter activity could be assayed conveniently *in situ* by measuring β-gal activity in heterozygous animals and correlating the results with parallel measurements of N-CAM mRNA levels (see Fig. 1*A*). Mice homozygous for the *lacZ* insertion lacked N-CAM mRNA and protein and showed many of the phenotypic alterations seen in other N-CAM knockout animals (11, 12). However, unlike other N-CAM-deficient animals (6), they exhibited normal hippocampal LTP.

To assay the response of the N-CAM promoter to increased synaptic transmission *in vivo* and *in vitro* we used the ampakine CX547, a positive allosteric modulator of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (13). In contrast to glutamatergic agonists, ampakines do not open ligand-gated channels on their own (14, 15), but enhance synaptic transmission by increasing the time course and amplitude of receptor-mediated depolarization accompanying normal release events. Ampakines facilitate induction of LTP by patterned stimulation in slice preparations and have been shown to enhance learning in behavioral tests (16, 17).

After ampakine treatment in heterozygous mice, we observed an increase in N-CAM promoter activity as assessed by the β-gal reporter. N-CAM mRNA increased in ampakine-treated mice as shown by RNase protection assays. The response to ampakine was also observed in organotypic slice cultures of the hippocampus. In such slices, the response was similar to that found when glutamate receptors were stimulated with the agonist kainic acid, and it was prevented by 6-cyano-7-nitroquinoxaline-2.3-dione (CNQX), an AMPA receptor antagonist. Together these results

Abbreviations: N-CAM, neural cell adhesion molecule; β-gal, β-galactosidase; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; LTP, long-term potentiation; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; fEPSP, field excitatory postsynaptic potential; TBS, theta burst stimulation; E*n*, embryonic day *n*.

§Present address: Department of Biology, University of California, San Diego, CA 92037.
¶To whom reprint requests should be addressed at: Department of Neurobiology, SBR-14, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. e-mail: kcrossin@scripps.edu.

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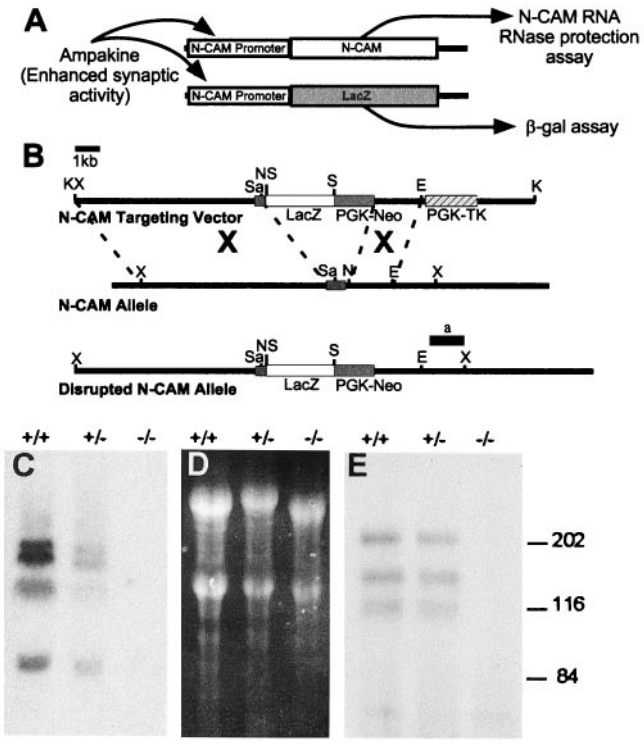

FIG. 1. Strategy for targeted replacement of the N-CAM allele with *lacZ* and analysis of N-CAM expression in knockout mice. (*A*) Diagram of the N-CAM locus in heterozygous animals. In heterozygous mice, N-CAM promoter activity can be assayed both by β-gal activity and by quantitating N-CAM mRNA. (*B*) Structures of the N-CAM targeting vector, wild-type N-CAM allele, and disrupted N-CAM allele. Restriction enzyme sites are abbreviated as follows: E, *Eco*RI; K, *Kpn*I; N, *Not*I; S, *Sal*I; Sa, *Sac*II; X, *Xho*I. (*C*) Northern blot. Total RNA isolated from the brains of wild-type (+/+), heterozygous N-CAM knockout (+/−), and N-CAM knockout (−/−) mice was hybridized to a 300-bp probe for N-CAM. (*D*) Ethidium bromide stain of the Northern gel shows approximately equal loading of the RNA. (*E*) Western blot of 30 μg of total protein isolated from the brains of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) N-CAM knockout mice with a polyclonal antibody to N-CAM.

suggest that facilitation of AMPA receptors leads to increased N-CAM promoter activity.

MATERIALS AND METHODS

Production and Analysis of N-CAM Knockout Mice. A targeted replacement vector was designed to disrupt the N-CAM allele and to insert a *lacZ* reporter gene under control of the endogenous N-CAM regulatory sequences (Fig. 1*B*). The vector contained the 8-kb *Xho*I–*Sac*II fragment of the N-CAM promoter and the 2-kb *Not*I–*Eco*RI fragment as 5′ and 3′ homologous recombinant arms, respectively. The region between the *Sac*II and *Not*I sites, which includes most of exon 1 and a small part of the first intron, was replaced by an *Escherichia coli lacZ* gene cassette containing an ATG codon followed by a nuclear localization signal and the neomycin-resistance gene driven by the phosphoglycerate kinase (PGK) promoter (18). To allow for selection against random insertion, a PGK-thymidine kinase (TK) gene was inserted at the 3′ end of the N-CAM homologous sequences. Of 16 transfected 129/SV ES (embryonic stem) cell clones (from ES cell clone D) selected for G418 resistance, five contained the recombined allele as assessed by Southern blot analysis. Cells were subjected to karyotype analysis to ensure a normal chromosome complement, and a single ES cell clone was used to generate chimeric founder mice from recombined ES cells and C57BL/6J blastocysts. The disrupted N-CAM allele was maintained by breeding in a background of both C57BL/J6 and CD-1 mice. In Northern blot analysis (Fig. 1*C*), a probe corresponding to nucleotides 183–505 of the N-CAM cDNA (according to numbering in GenBank accession no. X15049) hybridized to RNA from wild-type and heterozygous mouse brains but not to RNA from mice homozygous for the N-CAM gene disruption. Ethidium bromide staining indicated that similar amounts of RNA were loaded on the gel (Fig. 1*D*). In Western blot analysis of total brain proteins, a polyclonal antibody to N-CAM recognized three protein bands corresponding to the major 120-, 140-, and 180-kDa isoforms of N-CAM in the extracts from the wild-type and heterozygous mice (Fig. 1*E*). The N-CAM antibody did not bind to any proteins in the extracts from mice homozygous for the N-CAM gene disruption.

PCR Strategy for Analyzing Genotype. PCR analysis of tail DNA was performed as described for the analysis of transgenic N-CAM promoter/*lacZ* mice (19) to detect a product from the recombined allele of 305 base pairs. To detect the endogenous N-CAM allele, we sequenced into the first intron and prepared a 3′ primer (5′-ATG GCT CCC TTC TCA GCT CAG TG-3′) which produced a 425-bp product in combination with the 5′ primer used to amplify the mutant allele.

β-gal Histological Staining. Whole mount staining of embryos was performed as previously described (19, 20). Ampakine- or vehicle-treated animals were fixed by perfusion with 1% formaldehyde/0.25% glutaraldehyde in PBS. Brains were removed and postfixed for 1 h at 4°C and infiltrated with sucrose, and 50-μm cryosections were processed as described (19). By titrating the time of postfixation, we were able empirically to determine conditions that minimized activity in control animals and allowed observation of differences in β-gal staining.

β-gal Enzymatic Assay. Two- to 3-mm sagittal sections of the hippocampus from each hemisphere were isolated and prepared individually. The tissue was lysed by sonication in 100 mM Tris–acetate, pH 7.8/10 mM magnesium acetate/1 mM EDTA/1% Triton X-100/0.2% deoxycholate. Lysate was assayed for β-gal activity with the FluoReporter kit (Molecular Probes) according to manufacturer's protocols. β-gal values were normalized to DNA content of the samples as determined by the Picogreen assay (Molecular Probes). The values for β-gal from each hemisphere were averaged. Within an experimental group, β-gal activity for individual treatments was compared with the average β-gal levels for control animals. The percent increase was averaged over all experiments and analyzed statistically by using the Wilcoxon matched pairs test.

Ampakine Treatment. Heterozygous mice were injected i.p. with 80 mg/kg of the ampakine CX547 dissolved in 0.5× PBS with 20% (wt/vol) of the carrier cyclodextran. Control animals were injected with the vehicle alone.

Hippocampal Slice Culture. Hippocampal slices were prepared from heterozygous mice as described previously (21). After 7–10 days in culture, slices were incubated in 200 μM CX547 or carrier for 30 min, after which the medium was exchanged and the slices were incubated for an additional 7.5 h. Slices were harvested in a buffer composed of 0.32M sucrose, 2 mM EDTA, 2 mM EGTA, 25 mM Hepes, 50 μM leupeptin, pH 7.4, on ice, collected by centrifugation, and lysed by sonication in the β-gal assay buffer as described above.

RNase Protection Assay. RNase protection assays were performed on total brain RNA isolated by using RNAzol (Tel-Test, Friendswood, TX) extraction from ampakine- or vehicle-treated animals. A ^{32}P-labeled probe for the region of the N-CAM mRNA (nucleotides 183–505 according to GenBank no. X15049) and β-actin (Ambion) was used in the Ambion RNase protection assay according to manufacturer's protocols. Protected fragments were visualized and quantitated on a PhosphorImager (Molecular Dynamics). N-CAM values were normalized to levels of β-actin mRNA as determined by the protection assay. Values were analyzed statistically by the Wilcoxon matched pairs test.

Electrophysiology. Transverse slices of hippocampus (450 μm) were prepared from 12- to 20-week-old mice by parasag-

ittal section as previously described for rats (22); all operations were conducted in artificial cerebrospinal fluid (ACSF) composed of 114 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, 10 mM Hepes, 0.01 mM phenol red, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, bubbled with 95% O_2/5% CO_2 and titrated to pH 7.3 to 7.4 with NaOH. During recordings, slices were bathed on all sides by freely flowing ACSF at 32° to 33°C. Stimulation was provided via fine stainless steel wire electrodes (25 to 50 μm in diameter) placed approximately 300 μm to each side of a glass micropipette recording electrode (ACSF filled; 2–5 MΩ) in the stratum radiatum (Fig. 3*C*). Test stimuli were applied at 0.1 Hz, alternating between sites (i.e., 0.05 Hz per site). The stimulus-driven field excitatory postsynaptic potentials (fEPSPs) were amplified with an AxoClamp 2B amplifier to a final gain of 1000, low-pass filtered at 2 or 5 kHz, digitized at 5 or 10 kHz, analyzed on-line, and then stored on disk for further analysis.

Recording from each slice began with examination of its input–output relationship. In all cases, it was found that a fEPSP of 2–4 mV could be elicited with a stimulus current no greater than 80 μA for 200 μs. Subsequently, for the LTP experiments, the applied stimulation level was adjusted to evoke fEPSPs of 40–60% of maximal amplitude. After at least 15 min of baseline observation, theta burst stimulation (TBS) was applied to the orthodromic pathway. This consisted of nested trains of four stimuli at 100 Hz, repeated six times at 5 Hz, and this repeated twice at 0.1 Hz. The stimulus current amplitude and duration were the same during conditioning as during test cycling. The data from an LTP run were considered acceptable if responses to the nonconditioned input deviated in amplitude by less than 20% of initial value. The LTP group statistics were derived from all such runs, regardless of the magnitude of LTP observed. Group statistics were derived after calculating an averaged outcome for each animal; hence, the stated sample sizes are animal counts, rather than the actual number of slices tested (two or three per animal).

RESULTS

Insertion of the bacterial *lacZ* gene into the first exon and intron of the N-CAM gene disrupted expression of both N-CAM mRNA and protein (Fig. 1 *C* and *E*). Mice homozygous for the gene disruption expressed no gross phenotypic abnormalities other than a reduced body size and decreased breeding efficiency. As detailed below, the knockout mice that we report here have some of the morphological defects observed in other mutants (11, 12) although the various N-CAM knockout strains described here and by others represent different alleles due to mutation at widely distributed sites within the N-CAM gene.

β-gal Is Expressed in a Pattern Similar to That of Native N-CAM mRNA. Inserting the bacterial *lacZ* gene into the N-CAM gene placed β-gal expression under the regulation of the native N-CAM promoter. To establish that β-gal expression was regulated appropriately by the N-CAM promoter, we examined β-gal expression patterns by using enzymatic assays in heterozygous and homozygous embryos and compared them to N-CAM mRNA expression patterns as assessed by *in situ* hybridization. In embryonic day 9.5 (E9.5) and E13.5 embryos, β-gal staining was observed in a pattern similar to that observed for N-CAM mRNA (Fig. 2) and was seen only in tissues that express N-CAM mRNA. At E9.5, β-gal staining was expressed prominently throughout the spinal cord and in the dorsal root ganglia (Fig. 2 *A* and *B*). By E13.5, β-gal continued to be expressed throughout the spinal cord and in the dorsal root ganglia (Fig. 2 *C*, *D*, and *E*) and in heart and kidney (data not shown) and was similar to the pattern of *in situ* hybridization for N-CAM mRNA (Fig. 2*F*). At this stage, there was increased β-gal activity in the brain, particularly in the area of the hindbrain, midbrain, and the floor plate of the forebrain as well as in the trigeminal ganglia (Fig. 2*G*). In the head, β-gal expression was increased and was localized to postmitotic neurons of the midbrain and hindbrain and along the floor plate of the forebrain (Fig. 2*G*) comparable to the pattern of N-CAM mRNA expression (Fig. 2*H*). The β-gal staining patterns demonstrated that β-gal expression was regulated by the N-CAM promoter in both homozygous and heterozygous mice. These findings indicate that β-gal expression serves in these mice as a valid reporter of N-CAM expression.

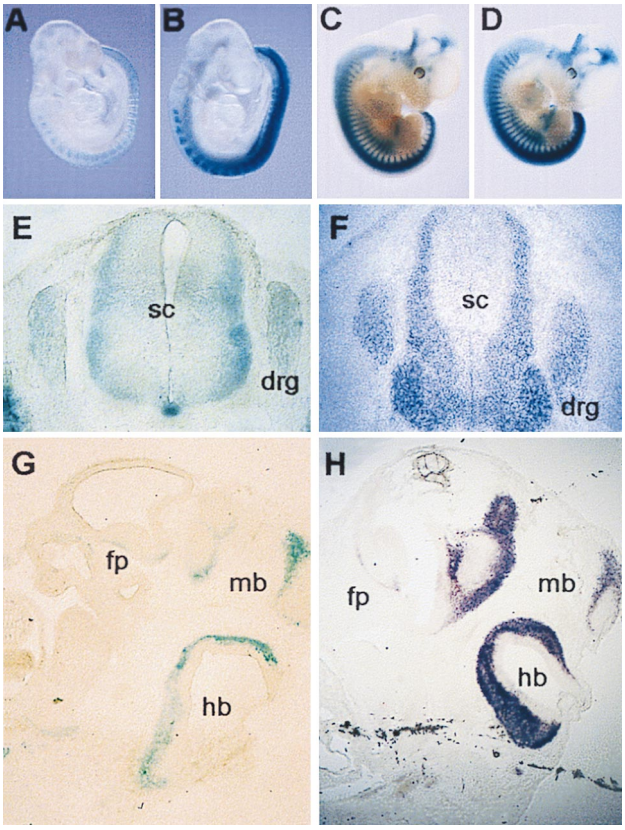

FIG. 2. β-gal expression in heterozygous and homozygous knockout mice compared with N-CAM mRNA localization. Whole mounts of heterozygous (*A* and *C*) and of homozygous (*B* and *D*) N-CAM knockout embryos, stained for β-gal expression at E9.5 (*A* and *B*) and E13.5 (*C* and *D*), expressed β-gal throughout the spinal cord and dorsal root ganglia. Homozygous E9.5 embryos appeared to express a greater amount of β-gal than heterozygotes (Fig. 3*B*) and β-gal activity quantitated by enzymatic assay was 2-fold higher in homozygotes, possibly reflecting a gene dosage effect. At E13.5 there was increased β-gal expression in the brain. (*E* and *G*) Sections of β-gal-stained E13.5 embryos. β-gal expression was localized throughout the spinal cord and in the dorsal root ganglia (*E*). In the brain, β-gal was expressed in the postmitotic neurons of the hindbrain and the midbrain as well as the in the floor plate (*G*). (*F* and *H*) *In situ* hybridization of E13.5 sections with N-CAM RNA probes. The expression of β-gal was similar to the pattern of N-CAM mRNA expression visualized by *in situ* hybridization. sc, spinal cord; drg, dorsal root ganglia; fp, floor plate; mb, midbrain; hb, hindbrain. (*A* and *B*, ×2.5; *C* and *D*, ×1.25; *E* and *F*, ×35; *G* and *H*, ×11.

Hippocampal Morphology and Physiology in N-CAM-Deficient Mice. Homozygous knockout mice had a bifurcation of the CA3 region of the hippocampus not present in wild-type animals (Fig. 3*A* and *B*) but similar to that reported in other N-CAM knockout mice (11, 23). Heterozygous animals showed an intermediate morphological phenotype with a less pronounced bifurcation. In spite of these defects, the projections from the dentate gyrus to the CA3 region were similar in mutant and wild-type animals as revealed by Timm's staining (not shown). It was also apparent in these sections that homozygous animals had an increase in the number of cells in the subventricular zone on the route of migration to the olfactory bulb. They also showed a reduction in the size of the olfactory bulb (data not shown).

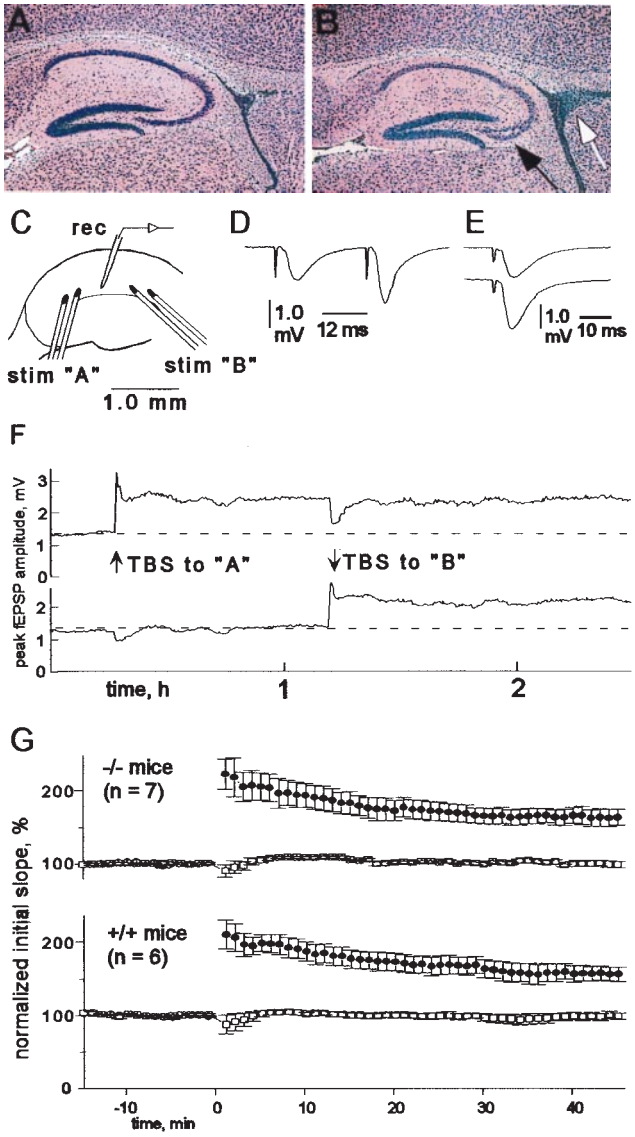

FIG. 3. Hippocampal morphology and physiology. (*A* and *B*) Morphology of adult hippocampus. Hematoxylin and eosin stain (×14) of wild-type (*A*) and homozygous (*B*) adult brain. Hippocampal physiology: (*C*) Typical stimulus (stim) and recording (rec) electrode positions. (*D*) Example of fEPSPs elicited with paired stimulation; interpulse interval equals 25 ms. Waveforms (*E*) are sample fEPSPs collected immediately before (upper trace) and 40 min after (lower trace) TBS. (*F*) Typical record of LTP induced in slices from knockout mice. The upper and lower traces are amplitudes of fEPSPs elicited alternately at 0.1 Hz along two independent pathways in the stratum radiatum of field CA1. Delivery of TBS to path A (orthodromic) resulted in a rapid enhancement of fEPSP amplitudes that decayed over a brief period to a stable plateau. Approximately 1 h later, application of the same high-frequency stimulus to pathway B (antidromic) resulted in a comparable enhancement, without affecting established LTP in path A. The initial period of enhancement after TBS is thought to reflect a mixture of post-tetanic potentiation and short-term potentiation, a more decremental form of *N*-methyl-D-aspartate (NMDA) receptor-dependent plasticity. The brief heterosynaptic depression observed after TBS is likely related to adenosine release accompanying high-frequency stimulation. (*G*) A plot of the average amount of LTP obtained in groups of slices from N-CAM knockout and wild-type mice. TBS-induced potentiation, expressed as a percentage of the preconditioning EPSP slope, was similar in the two groups of slices with regard to time course, magnitude, and input specificity (n = number of animals). Amplitudes of response to both the conditioned (●) and nonconditioned (□) pathways are shown.

We examined baseline synaptic physiology and LTP in hippocampal slices from heterozygous and homozygous N-CAM knockout mice in comparison with those of wild-type animals. Synaptic physiology was studied in the Schaffer collaterals (Fig. 3*C*) in hippocampal slices prepared from seven homozygous mutant mice (four from the CD1 background, three from the C57BL/6 background), two heterozygotes, and six wild-type animals. The fEPSPs measured in these groups were indistinguishable with respect to size and shape and were typical of those reported in the literature for slices from normal rodents. The fEPSP began after a latency of 2.5–3 ms, rose in 1.5–2.5 ms, and decayed with a half-width of about 8 ms (Table 1). Paired-pulse facilitation of the fEPSPs was compared in slices from the homozygous mutant and wild-type mice, by using an interstimulus interval of 25 ms (Table 1 and Fig. 3D). Mean facilitation ratios of 1.60 ± 0.06 and 1.47 ± 0.03 were observed, which did not differ significantly and were similar to those found in previous studies of these synapses (e.g., refs. 24 and 25). Half-maximal baseline fEPSPs used for LTP runs were 1–2 mV and were elicited by stimulus currents between 12 and 45 μA.

The slices were also examined with respect to TBS-induced LTP. Robust LTP, found in slices from all groups, was stable for the duration of the recordings regardless of whether N-CAM was expressed or not (Fig. 3 *F* and *G*). Our findings were essentially the same whether response amplitudes were determined at fEPSP peak or initial slope (Fig. 3 *F* and *G*). Typically, TBS in homozygous mutant and wild-type animals elicited a brief (<2 min) post-tetanic potentiation (PTP), a variable and also brief (<5 min) heterosynaptic depression, short-term potentiation, and LTP, the latter two phenomena being superimposed for the first 20–30 min after TBS. As determined from the initial slope of the fEPSP before applying TBS and 45 min after, the mean LTP magnitude (in percent increase) was 64.7 ± 10.9, and 56.9 ± 9.9 in slices from the homozygous mutant and wild-type animals, respectively. The means did not differ significantly when tested at the level of $P < 0.05$ (t test). Comparable short- and long-term plasticity was observed in slices prepared from heterozygous mutants (data not shown). In summary, we found that mice that lacked N-CAM exhibited normal LTP and baseline synaptic physiology.

Ampakine Administration Increases N-CAM Promoter Activity *in Vivo*. Heterozygous mice were used to examine regulation of the N-CAM promoter because β-gal expression from the *lacZ* gene insertion and N-CAM mRNA expression could both be used to monitor the activity of the N-CAM promoter. Synaptic activity was enhanced by treatment with ampakines, a class of drugs that are positive allosteric modulators of AMPA receptors that increase the amplitude and prolong the time course of synaptic responses to endogenous glutamate release (13–15). Mice were treated with ampakine, and after 8 h the brains were harvested, sectioned, and stained histologically for β-gal activity. Hippocampal tissue was also homogenized and assayed for β-gal activity. After homogenization of individual brains, N-CAM mRNA levels were measured by RNase protection.

Histological examination of the hippocampus revealed an increase in the level of β-gal expression driven by the N-CAM promoter (Fig. 4) 8 h after ampakine treatment in heterozygous mice. The most dramatic changes were in the CA1 and CA2 regions of the hippocampus, where a marked increase in the intensity of staining was observed relative to control mice treated with vehicle alone (Fig. 4). Increases in β-gal expression were also observed in areas outside of the hippocampus, most notably in the deep layers of the cortex (Fig. 4). At this level of analysis, however, β-gal staining did not appear to change in the superficial layers of the cortex.

To quantitate the percentage increase in N-CAM promoter activation in response to ampakine that was observed histologically, β-gal assays were performed on extracts of hippocampus isolated from mice 8 h after treatment with either CX547 or carrier. An average increase of 19% in β-gal activity was observed in the hippocampus as compared with that of vehicle-injected animals (Table 2).

Table 1. Conditions and fEPSP parameters in synaptic physiology experiments

Genotype	Stimulus current, μA × 0.2 ms	Presynaptic volley peak amplitude, mV	fEPSP peak amplitude, mV	fEPSP latency to 10% rise, ms	fEPSP risetime (10–90%), ms	fEPSP halfwidth, ms	Paired-pulse facilitation ratio	*n*
−/−	24.9 ± 3.1	0.13 ± 0.02	1.27 ± 0.09	2.97 ± 0.11	2.23 ± 0.09	7.90 ± 0.29	1.60 ± 0.06	7
−/+	24.7 ± 3.7	0.19 ± 0.01	1.08 ± 0.10	2.09 ± 0.20	1.68 ± 0.03	8.01 ± 0.01	ND	2
+/+	24.3 ± 3.4	0.17 ± 0.01	1.56 ± 0.14	2.87 ± 0.29	2.20 ± 0.15	7.72 ± 0.35	1.47 ± 0.03	6

Group means ± SEM are indicated; ND, not determined.

Ampakine Treatment in Heterozygotes Increases Expression of Endogenous N-CAM mRNA. To obtain an independent confirmation of the degree of ampakine stimulation of N-CAM promoter activity, we performed RNase protection assays on total RNA isolated from the brain tissue of the heterozygous mice that had been assayed for β-gal activity after ampakine treatment. Using a probe that hybridizes to all isoforms of N-CAM mRNA, we observed an average increase of 26% in N-CAM mRNA expression of in the ampakine-treated mice over control animals (Table 2). These data indicate that, in the heterozygous animals, ampakine treatment results in increases in levels of N-CAM mRNA the same magnitude as those of β-gal expression driven by the N-CAM promoter.

Ampakine Treatment Increases N-CAM Promoter Activity in Organotypic Slice Cultures *in Vitro*. To assay the effect of heightened glutamatergic transmission on N-CAM promoter activity in a system that is more amenable to experimental manipulation, hippocampal slices were cultured from mice heterozygous for the *lacZ* insertion into the N-CAM gene and the slices were then exposed to ampakines *in vitro*. After incubation of slices in 200 μM CX547 for 30 min, an average increase of 27% in β-gal activity was found in the hippocampal slices at 8 h posttreatment (Table 2). An increase of 20% was also observed in the presence of the AMPA receptor agonist kainic acid. The response to ampakine was strongly reduced in the presence of the AMPA receptor antagonist CNQX (average decrease = 89% ± 22%), indicating that activation of the N-CAM promoter was dependent on the ability of the ampakine to influence synaptic transmission through AMPA receptors (Table 2). This conclusion was further supported by the finding, in control experiments, that embryonic fibroblasts prepared from the heterozygous embryos showed no significant changes in N-CAM promoter activity as assessed by β-gal expression when treated with either ampakine or CNQX (data not shown).

DISCUSSION

We have found that mice expressing *lacZ* under the control of the N-CAM promoter showed increased β-gal reporter activity and N-CAM mRNA levels in response to ampakine treatment. By utilizing mice heterozygous for the *lacZ* insertion we were able to demonstrate in the same animal that there were similar increases in the levels of both N-CAM mRNA and β-gal activity. In control experiments, CX547 had no effect on fibroblasts cultured from heterozygous embryos and the effects of the drug on tissue slices were reduced by CNQX, a specific antagonist of AMPA receptors. These findings suggest that enhanced synaptic transmission leads to activation of the N-CAM promoter and that β-gal expression in these mice can be used in further studies as an indicator of patterns of neural activity in *in vivo*.

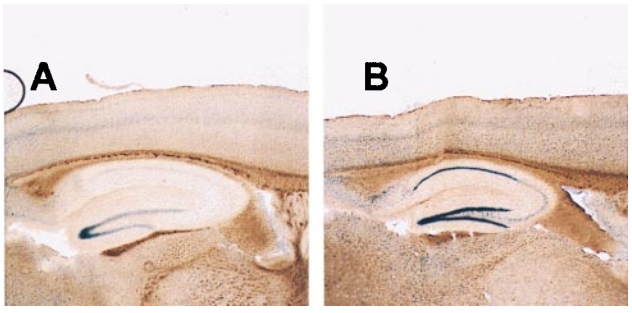

FIG. 4. Ampakine induction of β-gal expression resulting from N-CAM promoter activity in heterozygous N-CAM knockout mice. β-gal expression was examined in sagittal sections from vehicle-treated control (*A*) and ampakine-treated (*B*) mice. Ampakine injection increased β-gal expression in the CA1 and CA2 of the hippocampus and in the deep layers of the cortex. (×20.)

Ampakines have been reported to have efficacy as memory-enhancing agents in rodents and humans (17, 26, 27), presumably because of their ability to facilitate synaptic plasticity. As allosteric modulators of AMPA receptors, ampakines potentiate natural glutamate-mediated transmission. The approach used here therefore differs from studies that alter neural activity by using seizure-induction paradigms or chronic electrical stimuli, manipulations that can evoke abnormal physiology. Nevertheless, it should be noted that application to slice cultures of kainic acid, a potent agonist of AMPA-type and kainate receptors, gave results similar to those of ampakine treatment. This finding confirmed that depolarization mediated by non-NMDA-type glutamate receptors can lead to increased N-CAM transcription, although the indirect participation of multiple glutamate receptor subtypes in the induction of the response cannot be ruled out. Further analyses of slice cultures should allow pharmacological dissection of the neurotransmitter receptor selectivity of N-CAM promoter regulation.

Up-regulation of N-CAM promoter activity by pharmacological enhancement of endogenous transmission suggests that a coupling might exist between activity-dependent aspects of learning and memory and N-CAM expression (28). A role for N-CAM in synaptic modification in the adult has been suggested by studies in which perturbing N-CAM function by specific antibodies or removal of N-CAM polysialic acid interferes with LTP and decreases performance in particular memory tasks (reviewed in refs. 4 and 10). In addition, increases in polysialic acid immunoreactivity have been observed in rats after various learning paradigms (29–31). The present data complement these results in suggesting that N-CAM transcription may be subject to regulation by neural activity. Critical issues to be addressed in future studies are whether the increase in N-CAM promoter activity results in increased N-CAM protein levels, whether new N-CAM protein is preferentially targeted to the synapse, and whether such changes contribute to altered synaptic morphology.

It is noteworthy that LTP obtained in knockout and wild-type mice was of equal magnitude, did not decrease over the period tested, and was not accompanied by an attenuation of short-term plasticity that could indicate changes in signal transduction during burst stimulation. The apparent discrepancies between these data and those of a previous report that measured LTP in another strain of N-CAM null mice (6) may reflect differences in the efficacy of LTP induction mechanisms, given that both LTP and short-term potentiation were reduced in that study. They may also relate to strain differences, which are known to be responsible for a number of physiological and behavioral differences (32). It should be noted, however, that we observed normal LTP both in inbred crosses (C57BL/6) and in outbred animals (CD1). More detailed electrophysiological tests, such as a comparison of the relationships between number of bursts and LTP magnitude and longer recording periods, will be needed to rule out an influence of the loss of N-CAM on the induction or stabilization of LTP.

Table 2. Changes in β-gal activity and N-CAM mRNA after ampakine treatment

Treatments		Experiment							Mean % increase
		1	2	3	4	5	6	7	
In vivo	β-gal (hippocampus), % increase	25	38	13, 2	33	1, 29, 9	17, 38, 10	20, 12, 22	19 ± 3*
	N-CAM mRNA (total brain RNA), % increase	ND	ND	28	63	21, 32, 47	28, 46, 3	8, −13, 22	26 ± 6†
In vitro	β-gal (hippocampal slices), % increase	58, 31, 24, 26	59, 3, 0	−40, 31, 54, −2	77, 41, 16	22, 8			27 ± 8‡
	β-gal, % suppression by CNQX of ampakine induction	ND	ND	ND	42, 35, 118	100, 150			Mean % suppression 89 ± 22§

Changes in β-gal activity and mRNA are expressed as percentages over or under control values. For average percent increase, values are group means ± SEM. Statistics in Wilcoxon matched pairs test: *, $P < 0.001$; †, $P < 0.02$; ‡, $P < 0.003$; §, $P < 0.004$ by Student's *t* test. Animals were killed 8 h after i.p. injection of 80 mg/kg of ampakine. β-gal assays were performed on isolated hippocampi and RNase protection assays for N-CAM mRNA were performed on total brain RNA. ND, not done.

The N-CAM gene is one among a wide range of genes that are transcribed or translated in response to heightened neural activity and after certain learning paradigms (reviewed in ref. 33). The mechanism of activation of many of these genes has been linked to phosphorylation of the transcription factor CREB that binds to a consensus DNA element called a cAMP response element (CRE). A CRE-regulated promoter-reporter construct has been shown to be activated in slice cultures by LTP-inducing stimulation (34). Although no CRE has been found in the proximal 1 kb of the N-CAM promoter, the response of the entire promoter to cAMP-elevating agents has not yet been explored. Other genes activated by enhanced synaptic transmission include GAP-43 (35) and several transcription factors such as *zif*268 and NFκB (36–38). It will be important in future studies to examine and compare the time course of increased N-CAM transcription with respect to that of these other genes and to determine whether any of the transcription factors can activate the N-CAM promoter.

Our preliminary studies have indicated that animals expressing β-gal under the control of the N-CAM promoter sequence comprising 6.5 kb upstream of the transcriptional start site (19, 20) can also respond to ampakine treatment (unpublished observations). It should thus be possible to use transfection of promoter constructs into slice cultures to identify the cis sequences within the promoter that respond to membrane depolarization via activity-regulated transcription factors. Combined with pharmacological approaches to identify signaling intermediates (39), the slice culture paradigm should allow an identification of potential cis- and trans-acting factors that are important for the regulation of N-CAM expression by alterations in synaptic transmission. Potentially of equal importance, the availability of a construct that regulates the levels of a reporter gene product in response to neural activity *in vivo* may provide a tool of general utility for neurobiological investigations.

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