Synaptic Strength as a Function of Post- versus Presynaptic Expression of the Neural Cell Adhesion Molecule NCAM

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Summary

To evaluate the contributions of the pre- versus post-synaptic expression of NCAM in regulation of synaptic efficacy, we cultured dissociated hippocampal cells from NCAM-deficient and wild-type mice in homo- and heterogenotypic combinations. Double recordings from synaptically coupled neurons maintained in hetero-genotypic cocultures showed that synaptic strength of excitatory but not inhibitory synapses depended on expression of NCAM post- but not presynaptically. This correlated with higher levels of potentiation and synaptic coverage of NCAM-expressing neurons compared to NCAM-deficient neurons in heterogenotypic cocultures. Synaptic density was the same in homogenotypic cultures of NCAM-deficient and wild-type neurons as well as in heterogenotypic cocultures in which glutamate receptors were blocked. These observations indicate that the relative levels of postsynaptic NCAM expression control synaptic strength in an activity-dependent manner by regulating the number of synapses.

Introduction

Formation of synaptic connections is based on molecular recognition cues guiding interaction between growth cone and target followed by activity-dependent stabilization or retraction of formed connections. Cell adhesion molecules of the immunoglobulin (Ig) superfamily belong to such cues, mediating Ca^{2+}-independent homo- and heterophilic cell–cell and cell–matrix interactions. Particularly, the neural cell adhesion molecule (NCAM) is implicated in axonal growth and fasciculation and synaptogenesis in the hippocampus (Cremer et al., 1997; Seki and Rutishauser, 1998). In addition, NCAM has been shown to be important for synaptic plasticity. Polyclonal antibodies against NCAM and L1, another member of Ig superfamily, and synthetic peptides from the fourth Ig-like domain of NCAM, mediating interaction of NCAM with L1, significantly reduced long-term potentiation (LTP) in the CA1 area of the hippocampus (Lüthi et al., 1994; Rønn et al., 1995) and impaired spatial memory of rats in the Morris water maze (Arami et al., 1996). In agreement with these results, mice lacking the *NCAM* gene exhibited impaired spatial memory (Cremer et al., 1994) as well as reduced LTP in the CA1 and CA3 regions

of the hippocampus in vitro (Muller et al., 1996; Cremer et al., 1998; Artola, personal communication; but see Holst et al., 1998). Treatment with endoneuraminidase, an enzyme that specifically cleaves α-2,8-linked polysialic acid (PSA) from NCAM, impaired LTP and long-term depression (LTD) as well as acquisition and retention of spatial memory in rats (Becker et al., 1996; Muller et al., 1996). Available data suggest that NCAM is involved in the early stabilization phase of LTP rather than in the NMDA receptor–dependent induction of LTP in CA1 (Lüthi et al., 1994; Stäubli et al., 1998). Not only in rodents but also in invertebrates such as *Aplysia* and *Drosophila*, NCAM-related molecules have been implicated in control of synaptic strength (Zhu et al., 1995; Schuster et al., 1996a, 1996b; Bailey et al., 1997; Davis et al., 1997; Davis and Goodman, 1998).

This extensive involvement of NCAM in synaptic plasticity reflects its widespread expression in the central nervous system of adult mammals. In the hippocampal formation, NCAM immunostaining is intense in the hilus, inner molecular layer of the dentate gyrus, the mossy fiber tract, and the stratum radiatum and stratum oriens of CA1 (Miller et al., 1993). Of the three major isoforms of NCAM, NCAM120 is not detectable in synaptosomal membranes, whereas NCAM140 is expressed on both pre- and postsynaptic membranes, and NCAM180 is restricted mostly to postsynaptic sites (Persohn et al., 1989; Persohn and Schachner, 1990). Expression of NCAM/PSA in the hippocampus is abnormal in schizophrenic and epileptic brains as well as after lesioning (Wang et al., 1992; Barbeau et al., 1995; Jucker et al., 1995; Aubert et al., 1998; Mikkonen et al., 1998). Upon induction of LTP in the molecular layer of the dentate gyrus of adult rats, the expression of the major isoform of NCAM with the longest cytoplasmic domain, NCAM180, is upregulated in postsynaptic sites (Schuster et al., 1998). However, this link between LTP and expression of postsynaptic NCAM has remained correlative. To directly address the question of whether pre- or postsynaptically localized NCAM is necessary for synapse formation and/or stabilization, we compared the efficacies of synaptic connections between cultured murine hippocampal neurons in which NCAM was present or absent either pre- or postsynaptically. Our data show that postsynaptic expression of NCAM in a choice situation increases synaptic strength of excitatory but not inhibitory connections. This effect is related to the increased synaptic coverage of NCAM-expressing neurites.

Results

Identification of Neurons in Heterogenotypic Cocultures
To identify cells with a particular genotype, one population of cells was prelabeled with the fluorescent dye PKH-26 (Rivas and Hatten, 1995). After one week in culture, the dye was most strongly accumulated in astrocytes. In neurons, it was concentrated on the somata and proximal dendrites giving a weak punctate

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appearance that was sufficient, however, to provide reliable identification of labeled cell bodies (Figure 3A). Two experiments ensured reliability of identification. First, prelabeled neurons were plated on coverslips, and the percentage of neurons showing staining with PKH-26 was determined every 5 days for 100 large neurons, such as those used for electrophysiological recordings. After 1, 5, 10, 15, and 20 days in vitro, 96%–98% of all somata in the prelabeled cultures exhibited punctate staining with the dye. Second, *NCAM +/+* neurons were prelabeled and mixed with unlabeled *NCAM −/−* neurons in heterogenotypic cocultures that were then stained with NCAM antibodies. The percentage of neurons showing only single staining with either NCAM antibodies or PKH-26 was determined. Neurons on intensely PKH-26 stained astrocytes were excluded from the evaluation. At most, 6% of all neurons were labeled with only one fluorochrome, indicating that prelabeling of neurons with PKH-26 was a valuable tool for identification of the neuronal *NCAM* genotype before electrophysiological recording.

We next verified that dye loading per se does not change the electrophysiological properties of neurons. When a comparison of cell resistance, capacitance, inward Na$^+$, and outward transient and sustained K$^+$ currents in PKH-26-labeled and -unlabeled wild-type neurons was performed, no significant difference was found (10 cells in each group, 12–14 days in vitro, P > 0.1, unpaired t test, data not shown). To examine whether synaptic transmission to PKH-26-prelabeled neurons was normal, we compared spontaneous synaptic activity in labeled and unlabeled neurons. Patterns of synaptic activity in cultured hippocampal neurons were highly variable from culture to culture (compare upper traces in Figures 1A and 1B). Nevertheless, spontaneous postsynaptic currents simultaneously recorded in neighboring cells separated by a distance of 30–150 μm often exhibited striking similarities (Figures 1A, 1C, and 1E), most likely due to a similar local environment and set of contacting neurons. These data justified the use of double recordings to compare synaptic inputs received by neighboring neurons. There was no effect of PKH-26 on spontaneous synaptic activity: the mean difference between amplitudes recorded simultaneously in labeled and unlabeled wild-type neurons was −6.5% ± 19.1% (n = 5, P > 0.9, paired t test), with the activity in the unlabeled neurons being taken as 100%.

Spontaneous Activity Is Higher and Unitary Evoked EPSCs Are Larger in *NCAM +/+* Than in *NCAM −/−* Neurons in Heterogenotypic Cocultures

Spontaneous synaptic activity recorded in *NCAM −/−* neurons turned out to be lower than in neighboring *NCAM +/+* neurons in 8 of 10 pairs (Figures 1B and 1D). On average, the activity in *NCAM −/−* neurons was reduced by 36% ± 10.8%, with the activity in *NCAM +/+* neurons being taken as 100% (n = 10, P < 0.01, paired t test; Figure 1F). Thus, even in the absence of knowledge of the presynaptic *NCAM* genotype, we can state that postsynaptic NCAM is an important contributor to synaptic activity. Interestingly, slow spontaneous events, most likely inhibitory currents, appeared almost simultaneously in neighboring *NCAM +/+* and −/− neurons,
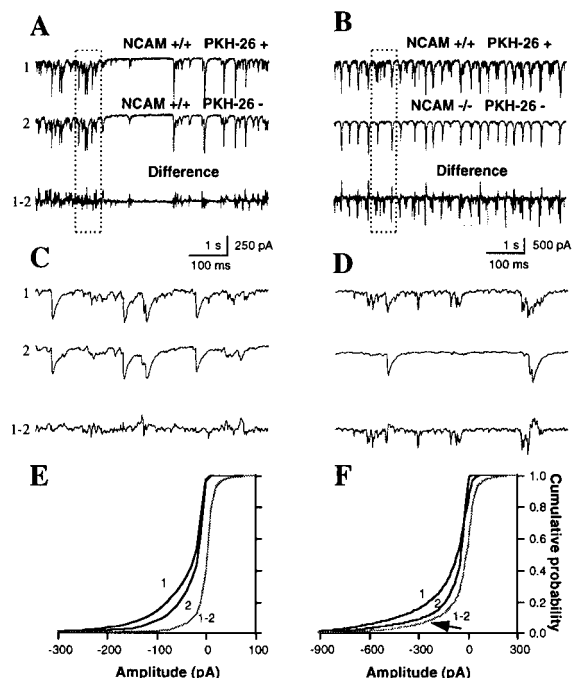

Figure 1. *NCAM −/−* Neurons Show Less Spontaneous Synaptic Activity Than *NCAM +/+* Neurons

(A–D) Low and high temporal resolution double recordings of spontaneous currents in neighboring neurons in homogenotypic cultures from *NCAM +/+* mice (A and C) or in heterogenotypic cocultures (B and D). In (A) and (C), recordings were performed in *NCAM +/+* neurons prelabeled or not prelabeled with PKH-26. In (B) and (D), *NCAM +/+* cells were identified by prelabeling with PKH-26 before mixing them with *NCAM −/−* cells. Genotypes are indicated above the traces. (C) and (D) show the regions selected in (A) and (B) (see stippled boxes in [A] and [B]). Difference refers to the difference between traces recorded in neurons 1 and 2.

(E and F) Cumulative distributions of current amplitudes recorded in neurons 1 and 2 and amplitude difference (1–2) for (A) and (B), respectively. In (E), the difference is distributed around 0 (~50% of the values are less or more than 0), i.e., there is no systematic difference between spontaneous activity recorded in PKH-26-labeled and -unlabeled *NCAM +/+* neurons. In (F), the distribution is skewed to the left (arrow), which reflects an excess of spontaneous activity in *NCAM +/+* neuron. In (A)–(F), the cultures were maintained for 15 days.

but fast EPSCs were present more often or with higher amplitude in *NCAM +/+* neurons (Figure 1D).
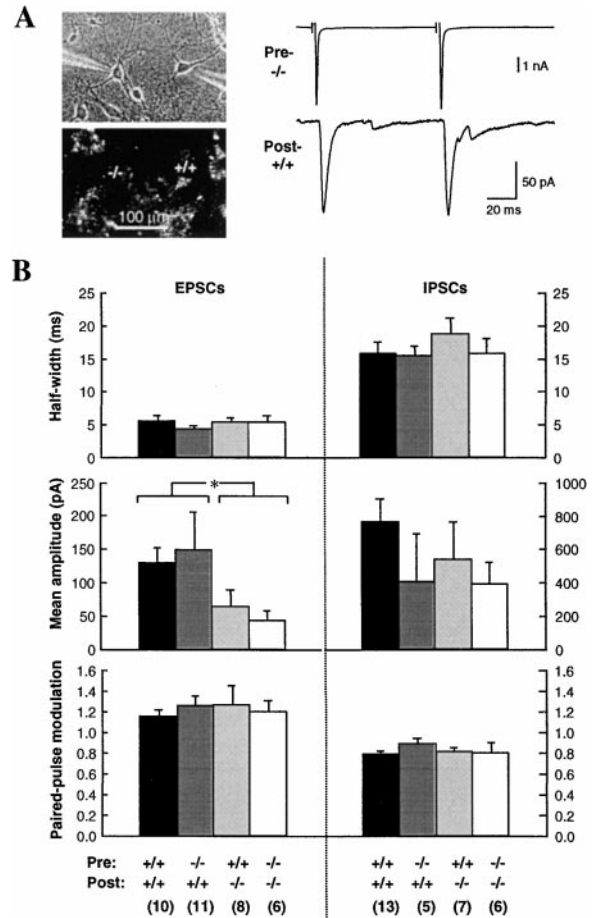
To determine whether inhibitory or excitatory transmission to *NCAM −/−* neurons was reduced, unitary EPSCs and IPSCs were first recorded in pairs of neurons with *NCAM +/+*, *NCAM +/−*, and *NCAM −/−* genotypes maintained in homogenotypic cultures (Figure 2A). EPSCs could be unambiguously distinguished from IPSCs by their fast decay time. The half-time of EPSCs was always <10 ms and on average ~5 ms for the three different genotypes. EPSCs could be blocked by 20 μM CNQX. IPSCs displayed a decay time longer than EPSCs. IPSCs had a half-time of more than 10 ms and on average ~15 ms. They could be blocked by 50 μM picrotoxin. The mean amplitude of unitary IPSCs was several fold higher than that of EPSCs (Figure 2, left versus right panels). Comparison of half-times and mean amplitudes of EPSCs and IPSCs in *NCAM +/+*, +/−,
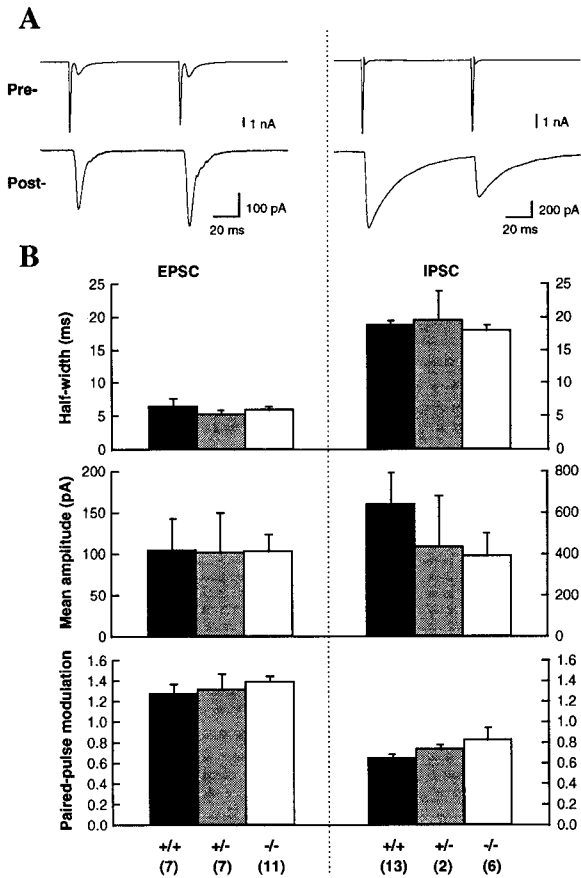
Figure 2. Evoked Unitary Postsynaptic Currents Are Similar in Neurons with Different *NCAM* Genotypes in Homogenotypic Cultures

(A) Intracellular depolarization (2 ms from −60 mV to −20 mV) of presynaptic neurons produced inward Na^+ currents (upper traces) that evoked fast EPSCs (left) or slow IPSCs (right) in the postsynaptic neurons. IPSCs are seen downward due to the fact that the reversal potential was −49 mV with the extracellular/intracellular Cl^- concentrations used in our experiments. Each sweep is an average of 30 individual currents recorded in cultures of *NCAM* +/+ neurons. Note the large autaptic EPSC produced in the presynaptic neuron (left upper trace).
(B) Cumulative values of half-time, mean amplitude, and paired-pulse modulation of unitary EPSCs and IPSCs recorded in homogenotypic cultures of neurons with the genotypes *NCAM* +/+, *NCAM* +/−, and *NCAM* −/−. Note the different scales for the amplitudes of EPSCs and IPSCs. Paired-pulse stimulation was performed with an interstimulus interval of 80 ms. Labels below graphs indicate the genotypes of neurons, and the numbers in parentheses provide the number of the synapses studied. The cultures were maintained for 10–20 days.

Figure 3. Mean Amplitude of Evoked Unitary Excitatory Postsynaptic Currents in Synapses Lacking Postsynaptic NCAM Is Reduced in Heterogenotypic Cocultures of *NCAM* +/+ and −/− Neurons

(A) Phase contrast image, PKH-26 fluorescence, and double whole-cell recordings in pairs of *NCAM* +/+ and −/− neurons. *NCAM* +/+ neurons were prelabeled with PKH-26 in this experiment. Glass pipettes evident in the phase contrast image point to the selected pyramidal-shaped neurons. Paired-pulse stimulation of *NCAM* −/− neurons evoked in this case nonfacilitating EPSCs (lower trace). Note that the spontaneous IPSCs remain detectable even after averaging of 20 sweeps.
(B) Cumulative values of half-times, mean amplitudes, and paired-pulse modulations of unitary EPSCs and IPSCs recorded in cocultures of *NCAM* +/+ and −/− neurons. Note different scales for the amplitudes of EPSCs and IPSCs. Labels below graphs indicate genotypes of pre- and postsynaptic partners, and the numbers in parentheses provide the numbers of the synapses studied. Asterisk, $P < 0.05$, two-way ANOVA. The cocultures were maintained for 10–20 days.

and −/− neurons maintained in homogenotypic cultures revealed no difference between genotypes (one-way ANOVA, $P > 0.1$, Figure 2B).

As a next step, heterogenotypic cocultures were used to record excitatory and inhibitory unitary responses in pairs with the four different combinations of *NCAM* +/+ and *NCAM* −/− neurons as pre- and postsynaptic partners (Figure 3A): presynaptic *NCAM* −/− with postsynaptic *NCAM* −/−, presynaptic *NCAM* −/− with postsynaptic *NCAM* +/+, presynaptic *NCAM* +/+ with postsynaptic *NCAM* −/−, and presynaptic *NCAM* +/+ with postsynaptic *NCAM* +/+. No significant differences

between these four types of connections with regard to half-width of postsynaptic currents were found (two-way ANOVA, $P > 0.1$, Figure 3B). The mean amplitudes of EPSCs in connections between *NCAM* +/+ or *NCAM* −/− presynaptic neurons and *NCAM* +/+ postsynaptic neurons were not different (Figure 3B). Also, the mean amplitudes of EPSCs in connections between *NCAM* +/+ or *NCAM* −/− presynaptic neurons on *NCAM* −/− postsynaptic neurons were similar to each other but different from the amplitudes found in connections established with *NCAM* +/+ postsynaptic neurons. Thus,

amplitudes of EPSCs depended on the genotypes of the post- but not the presynaptic neurons (two-way ANOVA, P < 0.05, Figure 3, asterisk). The difference found was not due to prelabeling of neurons with PKH-26 as verified by comparison of parameters of excitatory connections in which either NCAM −/− or NCAM +/+ neurons were prelabeled with the dye (three-way ANOVA, P > 0.8, data not shown). The mean amplitude of unitary EPSCs recorded in NCAM −/− postsynaptic neurons in heterogenotypic cocultures was lower than that in NCAM −/− homogenotypic cultures (43 ± 15 pA versus 102 ± 22 pA; P < 0.1, unpaired t test; P < 0.05, Mann-Whitney U test; Figures 2 and 3). The mean amplitude of EPSCs recorded in NCAM +/+ postsynaptic neurons in heterogenotypic cocultures was slightly higher than that in NCAM +/+ homogenotypic cultures (129 ± 23 versus 104 ± 39 pA, P > 0.6; Figures 2 and 3).

Analysis of inhibitory connections in cocultures did not reveal any dependence of amplitudes of IPSCs on the presence of NCAM on either pre- or postsynaptic neurons (two-way ANOVA, P > 0.1, Figure 3B).

Paired-Pulse Modulation and Quantal Size Are Similar in NCAM +/+ and NCAM −/− Neurons Maintained in Heterogenotypic Cocultures

Several factors could contribute to the difference found between NCAM genotypes in terms of mean EPSC amplitude. The number of synapses or properties of individual synapses could differ: synapses on NCAM −/− neurons could either be less effective in transmitter release than those on NCAM +/+ neurons or they could have a reduced postsynaptic sensitivity or, in other words, smaller quantal size.

Facilitation of EPSCs evoked by paired-pulse stimulation with a short interstimulus interval is believed to be a function of probability of release (Schulz, 1997). The values of the paired-pulse facilitation of EPSCs were remarkably similar for pairs of NCAM +/+, +/−, and −/− neurons in homogenotypic cultures (one-way ANOVA, P > 0.1; Figure 2B, lower panel) as well as for four types of pre- and postsynaptic combinations between NCAM +/+ and −/− neurons maintained in heterogenotypic cocultures (two-way ANOVA, P > 0.1; Figure 3B, lower panel). IPSCs exhibited paired-pulse depression rather than facilitation as seen for EPSCs. The magnitude of depression was independent of the genotypes of pre- and postsynaptic neurons (Figures 2B and 3B).

To assess the magnitude of quantal size in NCAM +/+ and −/− neurons, we recorded miniature PSCs that are supposed to reflect postsynaptic responses produced by spontaneous single vesicle release. Excitatory and inhibitory miniature postsynaptic currents (mEPSCs and mIPSCs) were pharmacologically isolated (Figure 4). To increase detectability of mIPSCs, intracellular concentrations of Cl− were raised. There was no significant difference between amplitudes of mEPSCs or mIPSCs recorded in NCAM +/+ and −/− neurons maintained in heterogenotypic cocultures (P > 0.1, unpaired t test; Figure 4).

Neurite Outgrowth and Morphology of NCAM −/− Neurons Are Normal but Synaptic Coverage Is Reduced in Heterogenotypic Cocultures

Similarities between genotypes in terms of paired-pulse modulation and amplitude of miniature PSCs imply that
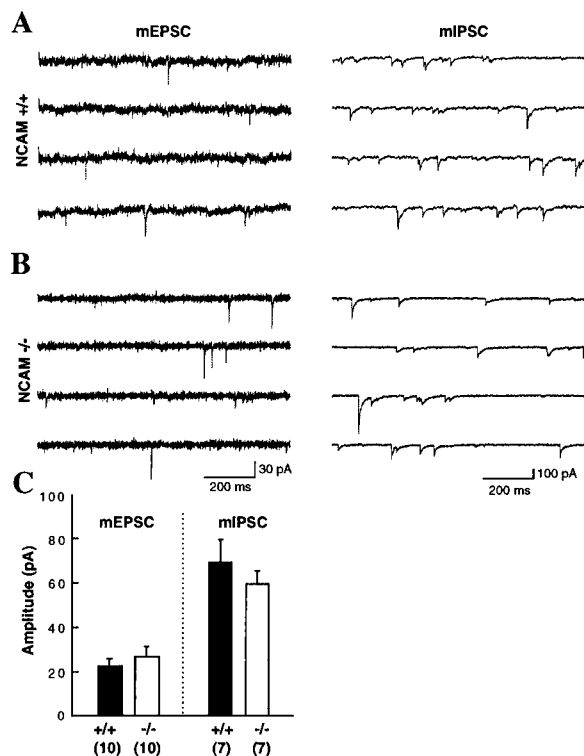

Figure 4. Quantal Size, Measured as the Amplitude of Miniature Postsynaptic Currents, Is Similar in NCAM +/+ and −/− Neurons in Heterogenotypic Cocultures

(A and B) Sample traces of miniature EPSCs (mEPSCs) and IPSCs (mIPSCs) recorded in NCAM +/+ (A) and −/− (B) neurons maintained in cocultures.

(C) Cumulative values of mean amplitudes of miniature EPSCs and IPSCs recorded in cocultures of neurons with different NCAM genotypes. Labels below graphs indicate genotypes of neurons and numbers in parentheses provide the numbers of neurons studied. The cocultures were maintained for 14–15 days.

the reduced amplitude of evoked EPSCs observed in NCAM −/− postsynaptic neurons in heterogenotypic cocultures may be due to the possibility that NCAM −/− neurons receive less synaptic contacts. This could either be due to a smaller total surface of NCAM −/− neurons or to a lesser density of synaptic contacts formed on them. Two approaches were used to study the first possibility. First, the length of the longest neurite per cell was measured one day after plating of cells. Both NCAM +/+ and −/− neurons maintained on a poly-L-lysine/laminin substrate had mostly one prominent neurite of ∼30 μm length (Figure 5D) distinguishing it from the shorter neurites. Second, more mature pyramidal-like neurons were visualized by DiI and analyzed by laser confocal microscopy. The shape of pyramidal-like neurons maintained in cocultures of NCAM +/+ and −/− cells were reminiscent of pyramidal neurons in vivo. In addition to the total length of neurites, the cell total surface area, a parameter representing the surface available for synapse formation, was measured by confocal microscopy. Comparison of the total surface areas and total lengths of NCAM +/+ and −/− neurons did not reveal any difference between the two genotypes in cocultures (P > 0.9, unpaired t test; Figures 5E and 5F).
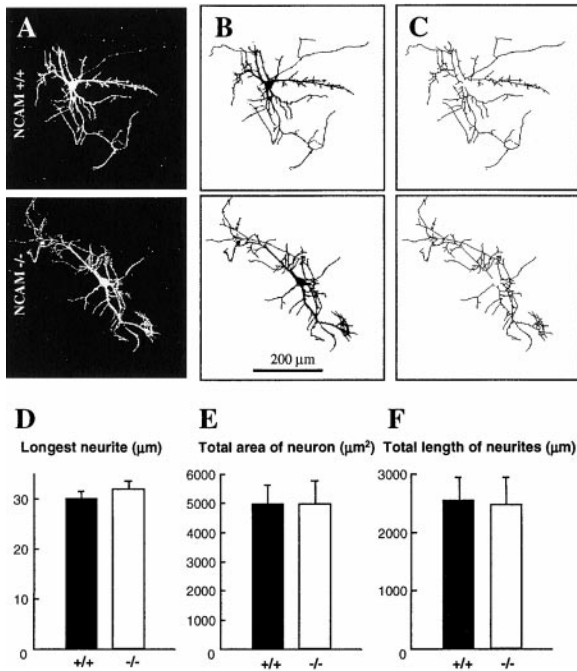
To analyze synaptic coverage of neurites in homo-

Figure 5. Morphological Features of *NCAM* +/+ and −/− Neurons Are Similar

(A) Confocal images of *NCAM* +/+ and −/− neurons stained in cocultures with DiI.

(B) Shape of neurons extracted from the background by setting a threshold of fluorescence. Magnification bar in lower (B) is representative for all panels.

(C) Results of skeletalization that was used to estimate the total length of neurites.

(D) Cumulative values of neurite outgrowth measurements in cultures of neurons with different genotypes (300 cells from two mice each of *NCAM* +/+ and −/− genotypes). Cells were maintained in homogenotypic cultures for 24 hr.

(E–F) Cumulative values of total neuron area and total neurite length measurements for *NCAM* +/+ (n = 6) and *NCAM* −/− (n = 8) pyramidal-like neurons maintained 8 days in heterogenotypic cocultures. No significant differences were found.
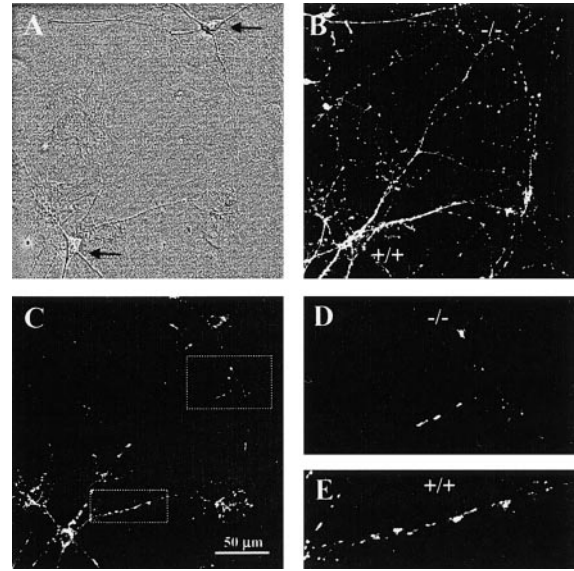

Figure 6. *NCAM* +/+ Neurons Have More Synaptic Contacts Than *NCAM* −/− Ones in Heterogenotypic Cocultures

(A and B) Two pyramidal-like cells (A, arrows) were identified by indirect immunofluorescence with NCAM antibodies as *NCAM* +/+ (lower) and −/− (upper) neurons.

(C) Synaptic boutons formed on two cells shown in (A) and (B) were visualized by indirect double immunofluorescence with synaptophysin antibodies.

(D and E) Two regions with synaptophysin-rich spots outlined by stippled boxes in (C). The NCAM-immunopositive cell and its neurites are covered more densely by the spots than the NCAM-immunonegative cell. Neurons were maintained in coculture for 10 days.

and heterogenotypic cultures, we immunostained for synaptophysin, a vesicular protein known to be enriched in synaptic boutons. The genotype of neurons in heterogenotypic cocultures was determined by labeling with NCAM antibodies (Figure 6), since PKH-26 stained mostly the neuronal somata, and this staining was strongly disturbed by permeabilization of cells required for penetration of synaptophysin antibodies. Synaptophysin immunostaining appeared as bright spots and was used for quantitation of the number of synapses. As shown in Figures 7A and 7B, the neurons selected for analysis had a similar local environment (as measured by the number of cells per frame) and similar mean diameters of neurites as measured by confocal microscopy with a resolution of 0.22 μm. The mean intensity of synaptophysin immunofluorescence along traced neurites from *NCAM* +/+ and −/− neurons was similar when neurons were maintained in homogenotypic cultures but differed in heterogenotypic cocultures (Figure 7C). The number of synaptophysin-rich spots per 10 μm of neurite length was taken as another measure of synaptic density and was also smaller in *NCAM* −/− neurons maintained in cocultures (Figure 7D). Similar to

the difference found between mean amplitudes of unitary EPSCs recorded in *NCAM* −/− neurons in homo- versus heterogenotypic cultures, synaptic density of *NCAM* −/− neurons in heterogenotypic cocultures was lower than that in *NCAM* −/− homogenotypic cultures (2.44 ± 0.40 versus 3.31 ± 0.31, P < 0.05, unpaired t test). Coverage of *NCAM* +/+ neurons with synaptophysin-immunoreactive spots in heterogenotypic cocultures was higher than the coverage of *NCAM* +/+ neurons in homogenotypic cultures (4.72 ± 0.38 versus 3.78 ± 0.29, P < 0.05, unpaired t test). Mean synaptic coverage of *NCAM* +/+ and −/− neurons (accumulated measurements for all neurites) in heterogenotypic cocultures was very close to that in homogenotypic cultures (3.58 versus 3.54, respectively). This implies that the same number of synapses is formed in choice and no-choice situations, but in a choice situation the synapse formation occurs preferentially on *NCAM* +/+ neurons.

Postsynaptic NCAM Is Involved in Activity-Dependent Strengthening of Synaptic Connections

In the next series of experiments we tried to distinguish over which time in culture the difference in synaptic coverage of *NCAM* +/+ and −/− neurons develops and whether it depends on synaptic activity. After three days in vitro (DIV), there was no difference in coverage of neurites by synaptophysin-rich spots between *NCAM* +/+ and −/− neurons (Figure 7D). However, between 3 and 5 DIV the synaptic coverage of *NCAM* +/+ neurites was doubled, whereas there was no further increase in
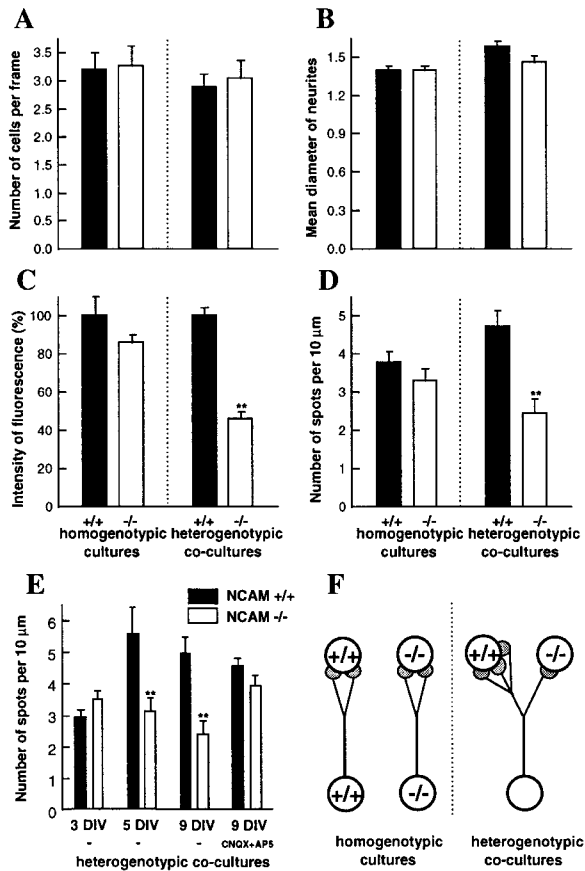
Figure 7. Cumulative Values of Synaptic Density Measurements in *NCAM* +/+ and −/− Neurons in Homo- and Heterogenotypic Cultures

(A) On average, three somata per frame were counted in all groups of neurons in homo- or heterogenotypic cultures.
(B) Mean diameters of traced neurites were similar for *NCAM* −/− and *NCAM* +/+ neurons in homo- or heterogenotypic cultures.
(C) Mean intensity of synaptophysin immunofluorescence within traced neurites was higher for *NCAM* +/+ neurons than for *NCAM* −/− neurons when they were cocultured together. Mean intensity of fluorescence in *NCAM* +/+ neurites was used to normalize the intensities in the different experiments.
(D) The number of synaptophysin-rich spots was counted for the same set of neurites taken for (B) and (C). *NCAM* +/+ neurites in cocultures were more densely covered by spots than *NCAM* −/− neurons and than neurites in homogenotypic *NCAM* +/+ and −/− cultures. Double asterisk, difference between genotypes P < 0.01, one-way ANOVA.
(A–D) The cells were maintained in vitro for 9–10 days. Data are from three independent experiments (three pairs of *NCAM* +/+ and −/− mice), 120–150 neurites in each group.
(E) The number of synaptophysin-rich spots as a function of time and synaptic activity. The difference between *NCAM* +/+ and −/− neurons maintained 9 days in vitro (DIV) in this experiment was similar to that found in three previous experiments summarized in (D). However, there was no significant difference between genotypes at 3 DIV. Block of excitatory synaptic transmission with antagonists to glutamate receptors (CNQX + AP5) led to equal synaptic coverage of *NCAM* +/+ and −/− neurites.
(F) A scheme showing preferential formation and/or stabilization of synapses on *NCAM* +/+ neurites exclusively in a choice situation, i.e., in heterogenotypic cocultures.
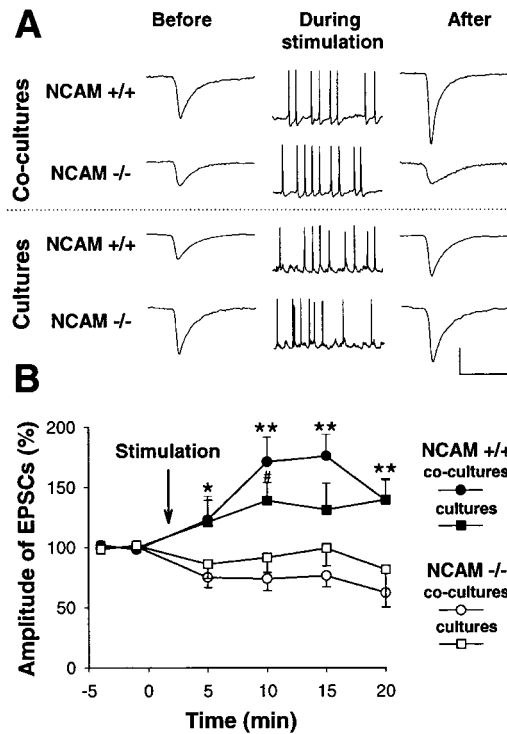

Figure 8. Glutamate-Induced Potentiation of Amplitudes of Spontaneous Excitatory Postsynaptic Currents Is Impaired in *NCAM* −/− Neurons in Homo- and Heterogenotypic Cultures

(A) Averaged aligned spontaneous EPSCs collected 1 min before and 20 min after the stimulation given by application of culture medium (1 min, 3 ml) containing 50 μM glutamate, 3 mM CaCl$_2$ (but 0 mM MgCl$_2$), 10 μM picrotoxin, and 1 μM glycine. Middle panel illustrates firing of neurons during application of the medium. Double whole-cell recordings were performed in synaptically noncoupled *NCAM* +/+ and −/− neurons in heterogenotypic cocultures (six pairs). Single whole-cell recordings were performed in homogenotypic cultures (six *NCAM* +/+ and seven *NCAM* −/− neurons).
(B) Significant differences in the level of potentiation between *NCAM* +/+ and −/− neurons were found: asterisk, P < 0.05 and double asterisk, P < 0.01, paired t test for neurons in heterogenotypic cocultures; pound sign (#), P < 0.05, unpaired t test for neurons in homogenotypic cultures. Bars: 20 pA and 10 ms for EPSCs; 30 mV and 500 ms for action potentials. Neurons were maintained 12–15 days in vitro.

the number of spots on *NCAM* −/− neurites. Block of glutamatergic transmission with CNQX and AP5 applied starting from 3 DIV completely abolished the difference seen between genotypes on 9 DIV, implying that the molecular mechanisms underlying the preferential coverage of NCAM-positive targets are activity-dependent.

Short-term application of glutamate was reported to induce NMDA receptor-dependent long-term potentiation of synaptic currents in cultured hippocampal neurons (Malgaroli and Tsien, 1992). Here, we used this protocol to compare the levels of potentiation simultaneously recorded in *NCAM* +/+ and *NCAM* −/− pyramidal-like neurons in heterogenotypic cocultures. Application of 50 μM glutamate in Mg^{2+}-free solution produced a strong depolarization of neurons and evoked their firing (Figure 8A). Several parameters of spontaneous activity were analyzed before and after such stimulation. The most significant difference between genotypes was

seen for the amplitudes of spontaneous EPSCs that could be reliably measured in time intervals between bursts of activity. In wild-type neurons, a slow increase in the amplitude was observed that reached the level of 171% $\pm$ 20% (n = 6) 10 min after stimulation (Figure 8B). The amplitude of EPSCs simultaneously recorded in NCAM $-/-$ neurons was decreased to 74% $\pm$ 10% (n = 6) from baseline levels. The profiles of changes in the amplitude of EPSCs seen in NCAM $+/+$ and $-/-$ neurons were significantly different from each other (Figure 8B; P < 0.001 for difference between genotypes, two-way ANOVA). Similar differences were observed for the total spontaneous activity (including bursts of events) measured as standard deviation of current (P < 0.01, two-way ANOVA). The latter measure was 161% $\pm$ 10% and 90% $\pm$ 19% 10 min after the stimulation in NCAM $+/+$ and $-/-$ neurons, respectively (P < 0.01, n = 6, paired t test). Frequencies of spontaneous events were also more potentiated in wild-type neurons, but differences did not reach significance levels (236% $\pm$ 50% versus 171% $\pm$ 21%, P > 0.1, n = 6, paired t test). When glutamate was applied to the homogenotypic cultures, potentiation of amplitudes of EPSCs was evoked in NCAM $+/+$ but not in NCAM $-/-$ neurons (Figure 8B). However, the level of potentiation in NCAM $+/+$ neurons relative to NCAM $-/-$ neurons in homogenotypic cultures was 2-fold smaller than that in heterogenotypic cultures (Figure 8B; P < 0.01, 10–15 min after the stimulation, unpaired t test). Also, the standard deviation of current and frequency of spontaneous events were significantly potentiated after stimulation to the same extent in NCAM $+/+$ and $-/-$ neurons in homogenotypic cultures (P < 0.05 for potentiation above baseline levels and P > 0.3 for difference between genotypes, two-way ANOVA).

Discussion

We have used heterogenotypic cocultures of NCAM $-/-$ and NCAM $+/+$ hippocampal cells to study the contribution of pre- and/or postsynaptically expressed NCAM on formation and/or stabilization of synapses. This in vitro system has several advantages over other available methods to study this problem in vivo. Precise manipulation of individual cell types in a living animal is presently only possible by the very restricted conditional knockout technology based on the cre/loxP recombination system, where the cre recombinase is expressed only in certain parts and cell types of a particular brain region (Tsien et al., 1996; Mayford et al., 1997). And even with this refined technology, there is no assurance that all cells express sufficient cre recombinase and that the enzyme ablates the gene at the exact desired time point. Cocultures prepared from wild-type and mutant animals (Shine and Sidman, 1984; Ueda et al., 1994) provide a model where cells of different genotypes are maintained under identical conditions during neurite outgrowth and synapse formation and stabilization. Prelabeling of one cell population by fluorochrome tagging or identification of the different populations by immunostaining for NCAM has allowed an unequivocal identification of the genotype at the single-cell level. Furthermore, identification of the genotypes of the cells before electrophysiological recordings and morphometric analysis of single cells in two rather than three dimensions as in vivo have allowed a precise and efficient characterization of the cells under study.

Postsynaptically Expressed NCAM Determines Synaptic Strength in a Choice Situation

We have made use of the possibility to study contacts between heterogenotypic hippocampal cells in culture in four possible combinations: NCAM $+/+$ and NCAM $+/+$, NCAM $-/-$ and NCAM $+/+$, NCAM $+/+$ and NCAM $-/-$, and NCAM $-/-$ and NCAM $-/-$, respectively. These combinations permit an unequivocal determination of the contribution of the pre- and postsynaptically localized NCAM on synapse formation and/or stabilization. Thus, we have determined the electrophysiological properties of unitary postsynaptic currents evoked by excitation of single neurons and morphometrically evaluated the numbers of synaptic contacts, making it possible to correlate synaptic strength or efficacy, as determined by the mean amplitude of postsynaptic currents, and synaptic coverage of the postsynaptic neuron, as estimated by the number of synaptophysin-immunoreactive spots along neurites of a particular neuron. Synaptophysin immunofluorescence has been shown to be highly correlated with activity-dependent uptake of the fluorescent dye FM1-43 that is a measure of exocytotic/endocytotic activity in presynaptic boutons (Staple et al., 1997). Cells with pyramidal cell–like morphology were chosen for these studies.

We have found that, in order for synapse formation and/or stabilization to occur maximally under the conditions of heterogenotypic coculture, NCAM needs to be present postsynaptically (for a scheme, see Figure 7F). Elimination of NCAM presynaptically influences neither synaptic strength in terms of mean amplitude of excitatory postsynaptic currents nor synaptic coverage as measured by immunostaining with synaptophysin. It is noteworthy that the electrophysiological data are in agreement with the morphometric observations, indicating a direct correlation between synaptic strength and number of synapses. Other parameters related to the probability of release (paired-pulse modulation), quantal size (amplitude of miniature PSCs), and the kinetics of PSCs (half-width) are not affected by the expression of NCAM either pre- or postsynaptically. Interestingly, only excitatory postsynaptic currents are significantly affected by postsynaptic expression of NCAM, while inhibitory postsynaptic currents are affected by neither the pre- nor the postsynaptic presence or absence of NCAM, probably reflecting differences in molecular organization of glutamatergic and GABAergic synapses (Kirsch, 1999). Thus, in a choice situation, NCAM expression postsynaptically is a crucial determinator of the efficiency of excitatory but not inhibitory synapse formation and/or stabilization. Analysis of developmental time course of synaptophysin immunoreactivity showed that during the first three days in heterogenotypic coculture the formation and stabilization occurs effectively on NCAM $+/+$ and $-/-$ neurons equally but that axons later start to prefer NCAM $+/+$ to NCAM $-/-$ cells.

These results are interesting to review in the context of the functions of pre- and postsynaptically expressed NCAM-like molecules in Drosophila and Aplysia. At the

neuromuscular junction in *Drosophila*, a motor axon prefers to make contact with a Fasciclin II–overexpressing muscle cell rather than a muscle cell that expresses wild-type Fasciclin II in a choice situation (Davis et al., 1997). This bias is enhanced when one target expresses increased levels of Fasciclin II and another target expresses reduced levels of Fasciclin II, indicating that the relative difference between targets directs synapse formation in *Drosophila*. Our data are consistent with this principle. Another parallel between Fasciclin II and NCAM is that the total number of synaptic contacts in heterogenotypic cocultures does not change significantly when synapse formation is biased toward a target expressing relatively high levels of an adhesion molecule, suggesting that there are some limiting factors conserving the total number of contacts formed by a single axon. In contrast to our data in hippocampal cultures, however, Fasciclin II has to be present both pre- and postsynaptically at the *Drosophila* neuromuscular junction for formation of stable synapses (Schuster et al., 1996a).

As for the peripheral nervous system synapses of *Drosophila*, observations on the NCAM-like molecule in *Aplysia*, called apCAM, also indicate its important role in synaptogenesis. Activation of the sensory neuron serving the gill withdrawal reflex leads to formation of new synapses between the sensory neuron and the motoneuron (Zhu et al., 1995). The formation of new presynaptic boutons by sensory neurons is directed by the presence of preexisting zones on the postsynaptic motoneuron that are enriched for apCAM, reminiscent of the role of postsynaptic NCAM revealed in our study. Electrical stimulation or release of serotonin from the inhibitory interneuron impinging on the sensory neuron leads to an overall downregulation of apCAM on the sensory neuron, concomitant with an increase of apCAM at synaptic contacts between the sensory neuron and motoneuron. This redistribution of apCAM involving downregulation of extrasynaptic apCAM by internalization and accumulation at sites of contact between pre- and postsynaptic neurons appears to be a salient feature of synaptic plasticity in the central nervous system of *Aplysia* (Zhu et al., 1995; Bailey et al., 1997). In vertebrates, local changes in cell adhesion that may be required for synaptic plasticity could be achieved by activity-dependent proteolytic cleavage of NCAM (Fazeli et al., 1994; Endo et al., 1998) or by cell surface expression of the less adhesive, polysialylated form of NCAM that promotes neurite outgrowth (Becker et al., 1996; Muller et al., 1996, and references therein).

NCAM-Independent Synapse Formation and/or Stabilization in No-Choice Situations

In homogenotypic cultures two combinations are possible, namely the culture of *NCAM* −/− and *NCAM* +/+ cells. In such no-choice situations, synaptic strength as well as the number of synapses were the same in *NCAM* −/− and *NCAM* +/+ cultures. If expression of Fasciclin II is necessary for synaptic maintenance and changes in Fasciclin II levels are sufficient for altering synaptic bouton formation, NCAM appears to be sufficient but not necessary for formation and stabilization of synapses. A similar situation appears to hold true for the formation

of synapses in *NCAM* knockout mice in vivo, where endplates at the neuromuscular junction were not significantly smaller in NCAM-deficient mice than those in wild-type controls (Moscoso et al., 1998). Similarly, basal synaptic transmission in Schaffer collateral-CA1 synapses is normal in acute slices and organotypic cultures prepared from *NCAM* knockout animals (Muller et al., 1996). Thus, the mechanisms underlying synapse stabilization appear to be different at neuromuscular junction of *Drosophila*, which can not be stabilized in the absence of Fasciclin II, reflecting differences in types of synapses or species. In *Drosophila*, the homophilic *trans*-interaction between pre- and postsynaptic Fasciclin II molecules appears to be dominant. In our case, NCAM-mediated homophilic *trans*-interaction is not predominant, but postsynaptic NCAM must evoke retrograde signaling, either via heterophilic interactions or via diffusible factors to promote synapse formation and/or stabilization. Since *NCAM* −/− neurons in homogenotypic situations receive normal numbers of contacts, NCAM may be just one of several signals that are integrated by the developing axon to confer preference for contact formation at sites where the integrated signal is optimal. It appears, therefore, that the simple absence, presence, or overexpression of NCAM is not the decisive determinant but that the relative differences in postsynaptic target molecules guide the formation of synapses.

An example of NCAM-enhanced signals is given by studies showing that in vertebrates *trans*-interactions between neighboring cell surfaces can be enhanced by helper molecules that act in concert with a "lead" molecule. For instance, L1 at the surface of one cell interacts more avidly with L1 exposed at the cell surface of another cell when L1 on one cell forms a molecular complex with NCAM (Kadmon et al., 1990a, 1990b). Similarly, the immunoglobulin superfamily molecule TAG-1 activates L1 by a *cis*-interaction to enhance homophilic cell adhesion by recruiting the cytoskeleton protein ankyrin (Malhotra et al., 1998). Furthermore, integrins can interact with L1 in *cis*- and *trans*-interaction in a complex network, also involving extracellular matrix molecules (Schmidt et al., 1995; for review see Brummendorf and Rathjen, 1994). Notably, integrins and cadherins have been found to be important for LTP in the CA1 region of the hippocampus (Bahr et al., 1997; Tang et al., 1998). Moreover, an interaction between NCAM and L1 has been implicated in this form of plasticity (Lüthi et al., 1994).

Postsynaptic NCAM Is Involved in Activity-Dependent Strengthening of Synaptic Connections

Our data demonstrate that the preference of NCAM-expressing cells as postsynaptic targets is abolished when glutamate receptors are inhibited in the heterogenotypic cocultures, suggesting that activation of glutamate receptors is involved in target selection at least at certain developmental stages. These results are interesting in view of studies demonstrating that activation of AMPA receptors increases the activity of the NCAM promoter (Holst et al., 1998), and postsynaptic expression of the largest NCAM isoform, NCAM180, is increased following long-term potentiation of synapses in the dentate gyrus (Schuster et al., 1998). Thus, activation

of glutamate receptors can upregulate expression of NCAM and other adhesion molecules and enhance the relative difference in attractiveness of postsynaptic targets. This likely happens at a relatively slow time scale of hours and days. Our study also shows that postsynaptic NCAM is important for rapid activity-dependent synaptic modifications such as LTP, since no significant potentiation of amplitude of spontaneous EPSCs was induced in response to glutamate stimulation in NCAM-deficient neurons in homo- or heterogenotypic situations. Interestingly, the levels of potentiation in NCAM +/+ neurons relative to NCAM −/− neurons were higher in heterogenotypic cocultures than in homogenotypic cultures, again highlighting the role of a differential expression of NCAM in regulation of synaptic functions. These experiments link our work with previous studies that have made use of NCAM antibodies, NCAM-derived peptides, and knockout mice (Lüthi et al., 1994; Rønn et al., 1995; Muller et al., 1996; Cremer et al., 1998) to demonstrate an involvement of NCAM in LTP. Our study adds a novel point to these data, implicating postsynaptically expressed NCAM in enhancement of synaptic strength. The importance of postsynaptic NCAM for LTP is in agreement with our recent data obtained in hippocampal slices, in which the largest postsynaptic major isoform of NCAM, NCAM180, appears to be an essential determinant in synaptic plasticity, since transgenic reintroduction of NCAM180 into NCAM knockout animals normalizes long-term potentiation in comparison to wild-type animals, with NCAM knockout mice being deficient in long-term potentiation (Dityatev et al., 1999). The observation that NCAM is an important molecule in enhancing synaptic strength by a postsynaptic mechanism will be instrumental in defining the molecular mechanisms that underlie the positioning of adhesion molecules via the cytoskeleton and via signal transduction mechanisms in activity-dependent processes.

Experimental Procedures

Cultures of Hippocampal Neurons

Preparation of cultures was essentially based on the method used for rats and published elsewhere (Malgaroli and Tsien, 1992; Reuter, 1995). The hippocampi of 1- to 3-day old mice were isolated, cut into small pieces, and treated with trypsin (6 mg/1.8 ml) and DNase I (1.5 mg/1.8 ml) in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution for 10 min at room temperature. The cells were dissociated by pipetting up and down with three fire-polished Pasteur pipettes with sequentially smaller diameters. After dissociation, the cells were centrifuged twice (100 × g, 15 min, 4°C) to remove cellular debris and plated at a density of 700 cells/mm^2 on glass (Assistent, Sondheim, Germany) or plastic coverslips of CELLocate type (Eppendorf, Hamburg, Germany) coated with poly-L-lysine (100 μg/ml) and laminin (20 μg/ml). For the first 2 days the neurons were maintained in culture medium (Eagle's MEM containing glucose [5 g/l], glutamax I [2 mM], bovine transferrin [100 μg/ml], insulin [100 μg/ml], gentamycin [5 μg/ml], and specifically tested horse serum [10%]). Starting from day 3, gentamycin was omitted, and the concentration of serum was reduced to 5%. The culture medium was then supplemented with 5 μM cytosine-β-arabinofuranoside and 2% B-27 supplement (Life Technologies).

Heterogenotypic Cocultures of Hippocampal Neurons

Before decapitation, the tails of animals were collected for genotyping by PCR (Cremer et al., 1994). NCAM −/− mice were obtained from H. Cremer (Cremer et al., 1994) and backcrossed to C57BL/6J mice for eight generations. Age-matched C57BL/6J mice served as controls. The red-fluorescent dye PKH-26 (Sigma) was used to label cells derived from one mouse before mixing them in cocultures with neurons derived from another mouse. To stain cells from one hippocampus, 0.25 ml of cell suspension (6 × 10^6/ml) was added to 1 μl PKH-26 dissolved in 0.25 ml diluent supplied with the dye and incubated for 1 min at room temperature. The reaction was stopped by addition of 1 ml of horse serum. Finally, cells were centrifuged three times (200 × g, 5 min, 4°C) in 6 ml of Hanks' solution.

Measurements of Neurite Outgrowth and Size of Neurons

For morphometric analysis of neurite outgrowth, cells were fixed after 24 hr in culture by the addition of 4% paraformaldehyde in PBS and stained with toluidine blue. Kontron software was used for image acquisition and measurement of neurite lengths. To stain single neurons in cocultures, a drop of DiIC$_{18}$(3) (Molecular Probes, Eugene, USA) was dissolved in sesame oil (Sigma) at saturating concentration and applied under visual control onto the somata of pyramidal-like neurons that had been fixed for 15 min at room temperature in 4% paraformaldehyde. To allow DiI diffuse along neurites, cocultures with labeled neurons were kept in darkness for 24 hr in PBS at room temperature before images were acquired. Stacks of images of DiI-stained neurons and synaptophysin-immunolabeled synapses were collected using a confocal laser scanning microscope (LSM 510, Zeiss) with Z-step of 0.5 μm, projected into XY-plane, and examined using Scion Image software. The shape of neurons stained with DiI was extracted from the confocal images by setting a threshold of fluorescence. Then, the total surface area of projected neurons was estimated as the product of pixel area (0.45 μm × 0.45 μm) and the number of black pixels in the resultant binary images (Figure 5B). The total length of neurites was estimated as a product of pixel size (0.45 μm) and the number of black pixels after skeletalization of images (automatically performed by Scion Image software) that compress neurites into linear structures with the thickness of 1 pixel (Figure 5C).

Double Immunostaining for NCAM and Synaptophysin and Measurement of Synaptic Density

The cultures were briefly washed with phosphate-buffered saline (PBS), pH 7.3, fixed with 4% paraformaldehyde in PBS, and treated for 20 min with blocking solution (PBS containing 0.3% BSA and 10% horse serum). NCAM rabbit polyclonal antibodies (pAb, 30 μg/ml; Lüthi et al., 1994) and secondary anti-rabbit IgG antibodies coupled to Cy2 (Dianova, Hamburg, Germany, diluted 1:100) were consecutively applied for 30 min at room temperature. Washing (4 times for 5 min) followed each application of antibodies. After staining with secondary antibodies, the cultures were fixed with 2% paraformaldehyde in PBS for 10 min and permeabilized by PBS containing 0.2% Triton X-100, 0.3% BSA, and 10% horse serum. Monoclonal mouse synaptophysin antibodies (Camon, Wiesbaden, Germany, 1:2) and secondary anti-mouse IgG antibodies coupled to Cy3 (1:100) were consecutively applied for 90 and 45 min, respectively. Finally, the cultures were embedded in Aqua-Poly/Mount (Polyscience, Eppelheim, Germany). Well-isolated cells with distinguishable, nonfasciculating neurites were selected for analysis without knowing the cell genotype and the distribution of synaptophysin immunostaining. The thick tapering neurites, very likely dendrites, were traced starting from the origin to 2–3 order of branching using high-resolution (pixel size of 0.22 μm) images. The synapses on somata were not analyzed. Synaptophysin immunofluorescence was quantified using confocal images (as above). Two measures were used to assess synaptic density: (1) mean intensity of synaptophysin staining within traced area of neurites after subtraction of background fluorescence and (2) the number of spots (counted by the operator) normalized for the measured length of traced neurites.

Electrophysiological Recordings

All recordings were performed in the whole-cell mode at room temperature in extracellular solution containing (in mM) 140 NaCl, 4 KCl, 10 HEPES, 2 CaCl$_2$, 1.5 MgCl$_2$, 30 glucose, and 4 g/l sucrose. pH was adjusted with NaOH to 7.25. Patch pipettes were filled with 125 mM K-gluconate, 20 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM Mg-ATP, 0.2 mM Na-GTP, and 10 mM glucose. The pH was adjusted

with KOH to 7.2 and the osmolarity was 310–315 mOsm. The hippo-campal neurons used for recordings were maintained in vitro for 10–20 days. Double whole-cell voltage clamp recordings were per-formed using EPC-9 and EPC-8 amplifiers (HEKA, Lambrecht, Ger-many). Serial resistance as well as cell resistance and capacitance were routinely measured during the experiments. There was no systematic difference between neurons with different genotypes in these three parameters. Recordings were performed only in configu-rations where at least three cells per visual field (0.06 mm^2) were found but their somata were not clustered touching each other. In two experiments, large autaptic EPSCs with mean amplitude greater than 1 nA were recorded exclusively in *NCAM* +/+ neurons, which were not included in the analysis. Recordings of miniature PSCs were performed in the presence of tetrodotoxin (TTX, 1 μM, Calbio-chem, Bad Soden, Germany). Non-NMDA receptor–mediated mEPSCs were isolated by addition of 50 μM picrotoxin (Sigma) and 50 μM D(-)-2-amino-5-phosphonopentanoic acid (AP5, Tocris, Bristol, UK). mIPSCs were collected at the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris) and 50 μM AP5 in the perfusion medium and after substitution of K$^+$ salts in the intracellular solution by CsCl (140 mM). Miniature EPSCs and IPSCs were detected using the template method implemented in the AxoGraph 4.0 software. Statistical analysis of all data was per-formed by the StatView 4.02 program. In the case of three-way variance analysis (ANOVA), the genotypes of pre- and postsynaptic neurons as well as the kind of prelabeling by PKH-26 (+/+ or −/− neurons) were considered as factors contributing to the variability of data.

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