

A Role for the Cadherin Family of Cell Adhesion Molecules in Hippocampal Long-Term Potentiation

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Summary

The cadherins are a family of cell–cell adhesion molecules that mediate Ca^{2+} -dependent homophilic interactions between cells and transduce signals by interacting with cytoplasmic proteins. In the hippocampus, immunostaining combined with confocal microscopy revealed that both neural- (N-) and epithelial- (E-) cadherin are present at synaptic sites, implying a role in synaptic function. Pretreatment of hippocampal slices with antibodies (Abs) raised against the extracellular domain of either N-cad or E-cad had no effect on basal synaptic properties but significantly reduced long-term potentiation (LTP). Infusion of antagonistic peptides containing the His-Ala-Val (HAV) consensus sequence for cadherin dimerization also attenuated LTP induction without affecting previously established LTP. Because the intense synaptic stimulation associated with LTP induction might transiently deplete extracellular Ca^{2+} and hence potentially destabilize cadherin–cadherin interactions, we examined whether slices could be protected from inhibition by N-cad Abs or HAV peptides by raising the extracellular Ca^{2+} concentration. Indeed, we found that high extracellular Ca^{2+} prevented the block of LTP by these agents. Taken together, these results indicate that cadherins are involved in synaptic plasticity, and the stability of cadherin–cadherin bonds may be regulated by synaptic stimulation.

Introduction

The idea that cell adhesion molecules may regulate synaptic function has gained support from recent demonstrations that some families of adhesion proteins are required for some forms of long-term synaptic plasticity (Staubli et al., 1990; Xiao et al., 1991; Bailey et al., 1992; Mayford et al., 1992; Luthi et al., 1995; Muller et al., 1996; Nosten-Bertrand et al., 1996; Bahr et al., 1997). Antibodies (Abs) to neural cell adhesion molecule (NCAM) or L1 can prevent long-term potentiation (LTP) in the hippocampus if applied prior to the inducing stimulus (Luthi et al., 1995), whereas peptide inhibitors of integrins can be applied immediately after the inducing stimulus and still prevent LTP (Bahr et al., 1997). Mice that lack the Thy-1 protein exhibit diminished LTP at synapses in the dentate gyrus, whereas LTP in area CA1 of the hippocampus and spatial learning are intact (Nosten-Bertrand et al., 1996). In addition, synaptic activity can

differentially regulate the expression of cell adhesion molecules (Bailey et al., 1992; Mayford et al., 1992; Itoh et al., 1995, 1997).

The classic cadherins are a family of adhesion molecules that span many different intercellular junctions by forming Ca^{2+} -dependent homophilic bonds (Hyafil et al., 1981; Buxton and Magee, 1992; Takeichi, 1995; Vaughn and Bjorkman, 1996). The highly conserved cytoplasmic C termini of cadherins interact with the cytoplasmically localized catenins (Aberle et al., 1994; Jou et al., 1995), which are in turn associated with the cytoskeleton. The extracellular domain of the vertebrate classic cadherins is made up of five repeats (EC1–EC5), which are joined together in a Ca^{2+} -dependent manner (Pokutta et al., 1994; Koch et al., 1997). The most distal EC repeat is important for homophilic binding (Blaschuk et al., 1990; Nose et al., 1990); this region also contains a relatively conserved tripeptide sequence (His-Ala-Val [HAV]). X-ray crystallographic analysis of the neural-cadherin (N-cad) structure (Shapiro et al., 1995) suggests that the two adjacent extracellular domains arranged in parallel within a membrane form a dimer, the so-called “strand dimer.” Strand dimers can exist on each side of juxtaposed membranes and each cadherin monomer can interact across the intercellular junction to form “adhesion dimers.” Together, the strand and adhesion dimers could form a zipper-like structure across the junction (Shapiro et al., 1995).

Although the cadherins are best known for their roles in cell recognition and adhesion during development (Buxton and Magee, 1992; Takeichi, 1995), they continue to be expressed at high levels in many tissues during adulthood. Biochemical and immunohistochemical studies have indicated the presence of cadherins in the adult hippocampus and forebrain (Beesley et al., 1995) and demonstrated their localization at synaptic sites (Yamagata et al., 1995; Fannon and Colman, 1996; Uchida et al., 1996). Moreover, several recent developmental studies have demonstrated directly cadherin involvement in neurite outgrowth and the formation and/or maintenance of synapses (Yamagata et al., 1995; Riehl et al., 1996; Inoue and Sanes, 1997), raising the possibility that cadherins present in the adult CNS may play an analogous role in the activity-dependent rearrangement of synaptic structures.

The established signaling capabilities of the cadherins coupled with their synaptic localization suggests that they may regulate synaptic transmission and plasticity. To begin to address these issues in the hippocampus, we have determined whether the cadherins are present at synapses in the CA1 area using immunostaining combined with confocal microscopy. We have then asked whether disrupting cadherin function with either function-blocking cadherin Abs (Volk et al., 1990; Meyer et al., 1992) or HAV peptides (Chuah et al., 1991; Doherty et al., 1991; Mege et al., 1992; Mbalaviele et al., 1995; Willems et al., 1995) affects synaptic transmission and LTP in adult hippocampal slices.

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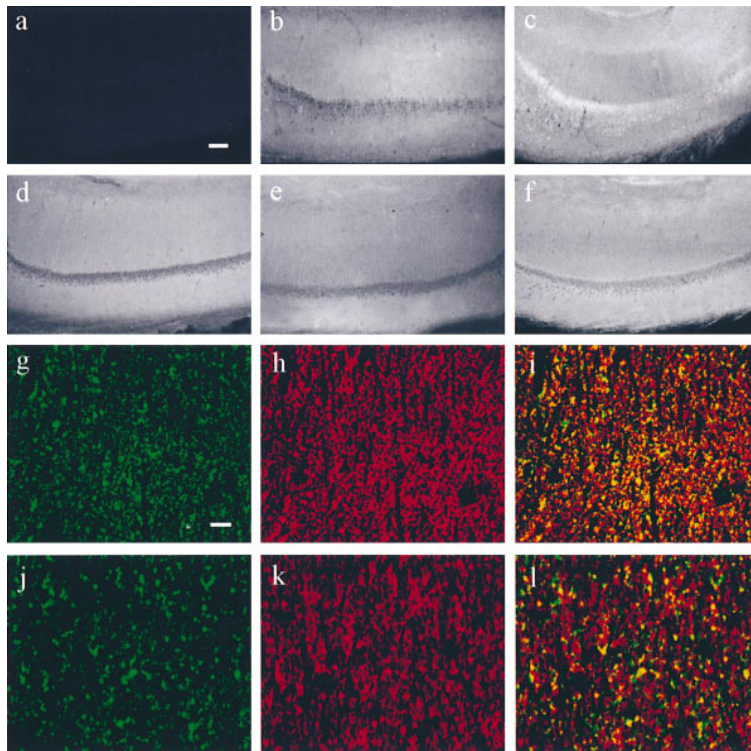


Figure 1. Immunohistochemical Localization of Cadherins in the CA1 Region of the Adult Hippocampus

(a–f) Low power (10 \times) confocal images of the CA1 region of a hippocampal slice, showing portions of stratum oriens (bottom), pyramdale (middle), and radiatum (top). Immunopositive tissue is indicated in white. Slices were treated with the following Abs: no primary Ab (control) (a), anti-N-cad (b), anti-E-cad (c), anti-Pan-cad (d), anti- α -catenin (e), and anti- β -catenin (f). Scale bar, 100 μ m.

(g–l) High power (63 \times) confocal images of dual immunolabeled slice sections from stratum radiatum in CA1. Immunopositive tissue is indicated in either green (FITC) or red (Cy3). Immunolabeling was done for the following Abs: anti-N-cad (g), anti-synapsin I (h), overlapping images of (g) and (h) showing coincidence of N-cad and synapsin I staining (i), anti-E-cad (j), anti-synapsin I (k), and overlapping images of (j) and (k) showing coincidence of E-cad and synapsin I staining (l). Round structures in stratum radiatum, which appear negative, likely represent the somas of interneurons. Scale bar, 10 μ m.

Results

Cadherins Colocalize with Synaptic Markers in the CA1 Area of the Hippocampus

To examine first whether cadherins are localized to specific cellular and/or synaptic regions in the CA1 region of the hippocampus, we combined immunofluorescent labeling with confocal microscopy and visualized labeled sites in mouse (E-cad) and rat (N-cad, Pan-cad, α/β -catenin). At low power, strong cadherin-positive staining was evident throughout the stratum radiatum and oriens; three different Abs, anti-neural- (N-), anti-epithelial- (E-), and anti-Pan-cad, yielded similar patterns of labeling (Figures 1A–1D). The catenins have also recently been shown to be associated with synaptic structures (Uchida et al., 1996). Like the cadherins, immunostaining for both α - and β -catenin was present throughout the synaptic neuropil in stratum radiatum and stratum oriens (Figures 1E and 1F). High power imaging of hippocampal sections immunolabeled for N-cad (Figure 1G), E-cad (Figure 1J), Pan-cad, and the catenins (data not shown) revealed punctate staining throughout the synaptic neuropil, suggesting localization of these proteins at or near synaptic sites.

To examine further the localization of the cadherins, we compared the labeling for N- or E-cad with that of presynaptic protein synapsin I in the same hippocampal section, using either FITC-conjugated (green; cadherin) or Cy3-conjugated (red; synapsin) secondary Abs. Synapsin I labeling was punctate and abundant throughout the slice (Figure 1H) and the majority of the N-cad signal colocalized with the synapsin I signal (Figure 1I). A similar pattern was observed for E-cad staining, although the labeling for E-cad was less abundant, and the synapsin I

staining was more diffuse in mouse slices. There were also a number of synapsin I-positive sites that were not colabeled with a cadherin Ab, as has been observed by others (Fannon and Colman, 1996). Similar experiments conducted with synapsin I and a Pan-cad Ab still indicate the presence of sites that are only recognized by the synapsin I Ab (data not shown), suggesting that there exists a population of synapses that contain cadherins not recognized by this Ab or, alternatively, other types of adhesion molecules.

Function-Blocking Antibodies to N- or E-cad Do Not Affect Basal Synaptic Parameters

To perturb cadherin function, we used the above Abs raised against the extracellular region of two different cadherin family members, N- or E-cad, which have been shown to interfere with cadherin-mediated processes (Volk et al., 1990; Meyer et al., 1992). In paired experiments, adjacent hippocampal slices were incubated in either N- or E-cad Ab or a control solution containing either normal artificial cerebrospinal fluid (ACSF), preimmune rat IgG, a non-function-blocking cadherin Ab, or an Ab to glypican, a cell-surface glycosylphosphatidylinositol-anchored (GPI-anchored) heparan sulfate proteoglycan, which is present in the adult hippocampus (Karthikeyan et al., 1994; Litwack et al., 1994). To confirm that our incubations resulted in successful Ab penetration into the depth of the slice, we analyzed the extent of Ab labeling in incubated slices. We found that 2–3 hr Ab incubations were sufficient to penetrate the interior of the slice and that Abs remained in the slice following perfusion with ACSF for at least 1 hr (Figure 2).

We determined whether the cadherin Abs affect basal

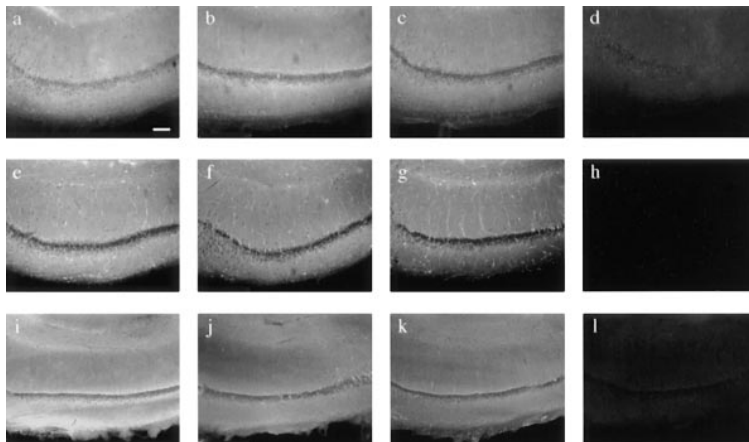


Figure 2. Cadherin Antibodies Remain in Hippocampal Slices for at Least 60 Minutes Following Perfusion with Normal ACSF

Each row shows sections taken from the middle 50 μm of Ab-treated slices perfused with normal ACSF for 0, 30, or 60 min as well as a no primary Ab control. Abs are anti-N-cad (a-c), anti-E-cad (e-g), anti-glypican (i-k). Scale bar, 100 μm .

synaptic transmission by examining several different parameters that reflect normal synaptic function. We found that the cadherin Abs had no effect on the relationship between stimulus strength and the size of the post-synaptic response (Figures 3A and 3B), paired-pulse facilitation (PPF) (Figure 3C and D), or posttetanic potentiation (PTP) (see Figure 4). Taken together, these observations indicate that the cadherin Abs do not affect basal synaptic transmission, nor do the Abs appear to disturb the structural integrity of the slice.

Function-Blocking Antibodies to N- or E-cad Inhibit LTP

Adjacent slices from the same hippocampus were treated with either a function-blocking cadherin Ab or a control Ab (or solution) prior to LTP induction by high frequency stimulation of the Schaffer collateral axons. Incubation of slices in an N-cad function-blocking Ab significantly attenuated the magnitude of LTP relative to control slices treated with an equal dilution of a non-

function-blocking cadherin Ab (Figures 4A and 4C) (mean percent of baseline 1 hr after LTP induction: N-cad, $109.1\% \pm 7.1\%$; control, $147.6\% \pm 9.5\%$ [$n = 8$]). Similarly, slices treated with a function-blocking E-cad Ab also exhibited significantly less LTP than adjacent control slices incubated in ACSF (Figures 4B and 4C) (mean percent of baseline 1 hr after LTP induction: E-cad, $122.9\% \pm 7.9\%$; control, $150.7\% \pm 11.5\%$ [$n = 9$]). In contrast, paired experiments examining LTP in slices exposed to normal ACSF versus slices exposed to non-function-blocking Abs raised against the cytoplasmic domain of N- or E-cad did not reduce LTP (Figure 4C) (mean percent of baseline: cad_{cyto}, $148.7\% \pm 6.7\%$; control, $148.6\% \pm 4.9\%$ [$n = 16$]). In addition, rat hippocampal slices pretreated with an additional control Ab, to glypican, exhibited robust and significant potentiation of synaptic transmission that did not differ significantly from that observed in control ACSF slices (Figure 4C) (mean percent of baseline: glypican, $146.2\% \pm 7.5\%$; control, $152.8\% \pm 5.5\%$ [$n = 12$]).

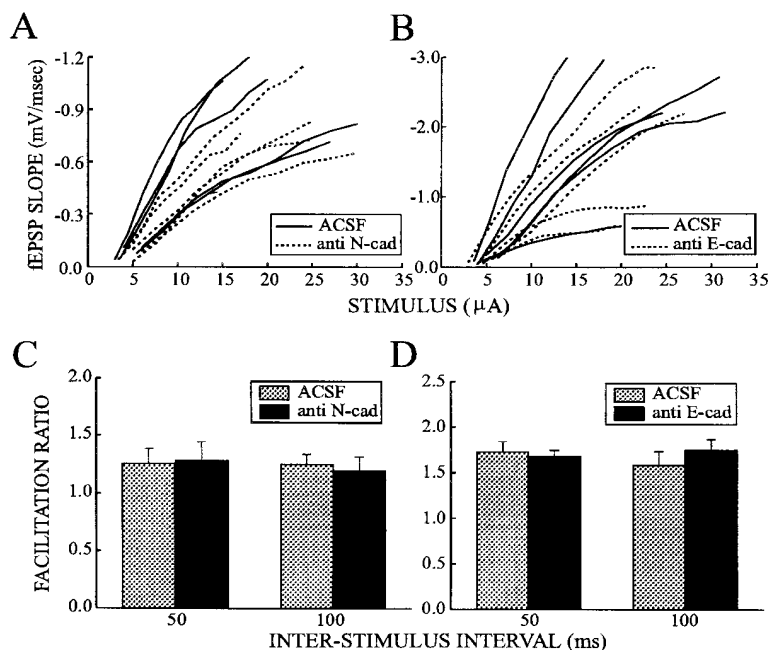


Figure 3. Cadherin Antibodies Do Not Alter Basal Properties of Synaptic Transmission in Hippocampal Slices

(A and B) Input-output curves depicting the relationship between stimulus current intensity and the size of the field EPSP slope. Slices treated with either an N-cad (A) or an E-cad (B) Ab were not significantly different from ACSF controls in their input-output curves.

(C and D) Facilitation ratios for N-cad-treated and E-cad-treated slices and controls. The facilitation ratio represents the slope of the second field EPSP divided by the slope of the first field EPSP for the interstimulus intervals shown.

HAV Peptides Inhibit LTP without Disrupting Normal Synaptic Function

Peptides containing the HAV sequence have been shown to inhibit cadherin-dependent processes, including neurite outgrowth and embryo compaction (Blaschuk et al., 1990; Nose et al., 1990; Willems et al., 1995). We have used different HAV-containing peptides as inhibitors to disrupt specifically cadherin dimerization (Blaschuk et al., 1990; Nose et al., 1990; Willems et al., 1995).

In the first set of experiments, we incubated slices in ACSF containing long (17-mer) HAV peptides, attempting to maximize the inhibition and specificity of the peptide interaction with endogenous cadherins (Doherty et al., 1991; Akli et al., 1993; Mbalaviele et al., 1995). As was the case for the function-blocking Ab, incubation with the 17-mer peptides had no apparent effect on either the input-output relations of synaptic transmission (Figures 5A–5C) or PPF (Figures 5D–5F). In addition, slices incubated with either an N-cad-derived or an E-cad-derived 17-mer peptide (1 mM) exhibited significantly less LTP than adjacent control slices incubated with a scrambled version of the peptide (Figures 6A and 6C) (mean percent of baseline: N-cad 17-mer, 109.7% ± 9.0%; scrambled 17-mer, 144.3% ± 9.6% [n = 6]; E-cad 17-mer, 119.7% ± 8.3%; scrambled 17-mer, 177.7% ± 20.2% [n = 7]). The combined pretreatment of slices with both N- and E-cad-derived peptides (1 mM for each peptide) did not result in greater inhibition than that observed with a single peptide (Figures 6B and 6C) (mean percent of baseline: N-cad [1 mM] + E-cad [1 mM] 17-mer, 113.3% ± 5.2% [n = 6]; scrambled 17-mer [2 mM], 146.0% ± 11.2% [n = 6]), suggesting that each peptide may be capable of inhibiting both N- and E-cad function.

HAV Peptides Block Only When Applied during LTP Induction

Because relatively short peptides can be introduced into slices in the perfusion media, we were able to address specifically whether peptides containing the HAV sequence alter the initiation and/or maintenance of LTP. Two independent pathways were stimulated in a single slice (Figure 7A). Thirty minutes following LTP induction in the first pathway, the HAV peptide (200 μM) was introduced into the ACSF. High frequency stimulation was then delivered to the second pathway in the continued presence of the peptide. The introduction of two different HAV peptides, in which flanking amino acids were derived from either N- (AHAVD) or E-cad (SHAVS) sequences, attenuated LTP induction in the second pathway (Figures 7B and 7D) (mean percent of baseline: AHAVD, 114.8% ± 4.3% [n = 6]; control pathway, 143.2% ± 8.7% [n = 6]). The same peptide applied 30 min after LTP induction, however, had no effect on previously established LTP (Figure 7B). The combined application of both the N- and E-cad-derived peptides (AHAVD and SHAVS; 200 μM each) did not produce greater inhibition of LTP than either peptide alone (Figure 7D) (mean percent of baseline: AHAVD + SHAVS, 132.7% ± 6.9% [n = 5]; control pathway, 175.2% ± 13.0% [n = 5]), suggesting that there may be a cadherin-independent aspect to potentiation that cannot be

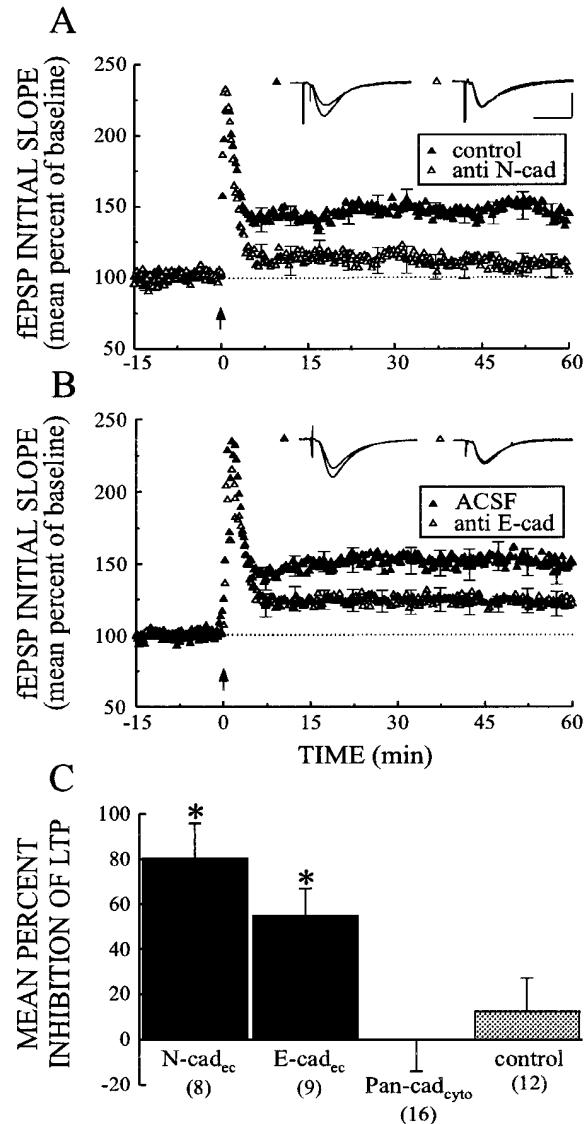


Figure 4. Adhesion-Blocking Antibodies Raised against the Extracellular Domains of Either N- or E-cad Attenuate LTP

(A) Ensemble average of all experiments (n = 8) in which slices were pretreated with an N-cad Ab or a non-function-blocking E-cad Ab. Superimposed representative field EPSPs taken before and 50–60 min after tetanus for each group are shown in (A) and (B).

(B) Ensemble average of all experiments (n = 9) in which slices were pretreated with an Ab to E-cad or normal ACSF. Scale bar, 0.5 mV and 20 ms.

(C) Summary of all Ab experiments. Mean percent inhibition of LTP is expressed relative to the amount of LTP observed in the control pathway for each set of experiments. Shown are the summary data for the N-cad-treated (A) and E-cad-treated (B) slices as well as slices treated with cytoplasmic cadherin Ab or an Ab to the cell surface GPI-anchored molecule glypican. Numbers in parentheses indicate the n for each set of experiments. Asterisks indicate significant inhibition (p < 0.05) relative to control.

blocked by the peptides in these experiments. In several sets of control experiments, introduction of either scrambled (AADHV; VSHSA) or single amino acid mutation (AEAVD) peptides had no apparent effect on either LTP induction (Figures 7C and 7D) or maintenance in the

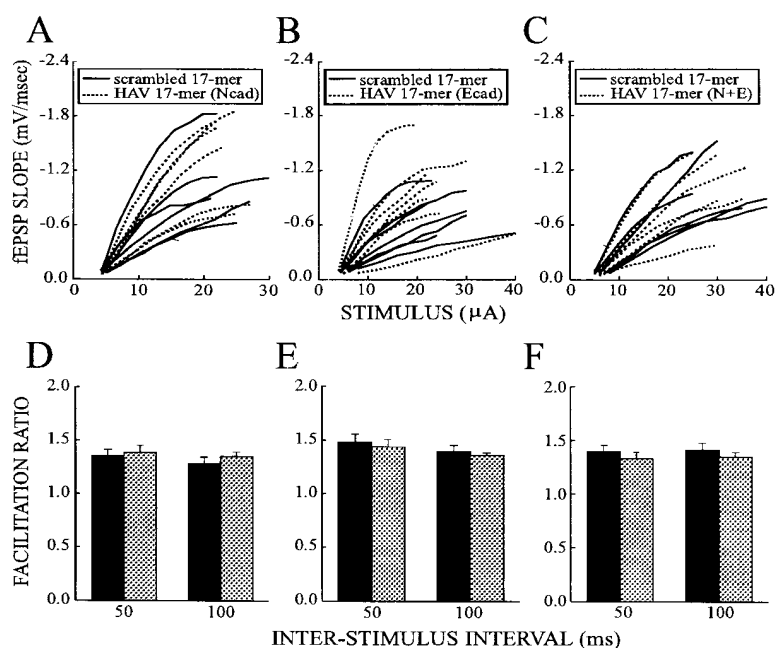


Figure 5. HAV Peptides Do Not Alter Basal Properties of Synaptic Transmission in Hippocampal Slices

(A–C) Input–output curves depicting the relationship between stimulus current intensity and the size of the field EPSP slope. Slices were treated with either an N-cad-derived HAV peptide (17-mer) (A), an E-cad-derived HAV peptide (17-mer) (B), or a combination of the two (C). These slices did not differ significantly from scrambled HAV peptide or ACSF (data not shown) controls in their input–output curves.

(D–F) Facilitation ratios for HAV peptide-treated slices and controls. The facilitation ratio represents the slope of the second field EPSP divided by the slope of the first field EPSP for the interstimulus intervals shown.

second pathway (Figure 7C). These results indicate that HAV-containing peptides can block LTP when present at the time of induction but cannot reduce previously established LTP when applied after induction.

HAV Peptides Also Block Theta Burst Stimulation- and Pairing-Induced LTP

Several studies have shown that different LTP induction protocols can utilize distinct biochemical signaling pathways (e.g., Kang et al., 1997). We therefore examined the sensitivity of LTP induced by two additional protocols, theta burst stimulation (TBS) (Bliss and Lomo, 1973; Larson and Lynch, 1986) and pairing postsynaptic depolarization with low frequency stimulation (Gustafsson et al., 1987) to the HAV peptides. In two-pathway experiments, we found that pathways exposed to the HAV peptide (200 μ M) exhibited significantly reduced TBS-induced LTP relative to the control pathway (Figure 8A) (mean percent of baseline: AHAVD pathway, 112.4% \pm 8.7%; control pathway, 139.0% \pm 10.8% [n = 5]). In addition, slices treated with the HAV peptide (200 μ M) exhibited significantly less pairing-induced LTP than control slices treated with a scrambled peptide (Figure 8B) (mean percent of baseline: AHAVD slices, 111.4% \pm 11.9%; AADHV slices, 183.6% \pm 23.9% [n = 5]). Taken together, these data indicate that the HAV peptides are capable of inhibiting LTP induced by several different protocols, suggesting a fundamental role of cadherins in synaptic plasticity.

HAV Peptides Do Not Inhibit the Postsynaptic Neuron's Ability to Depolarize

Like the cadherin Abs and the long HAV peptides, the 5-mer HAV-containing peptides had no detectable effect on basal synaptic responses, PTP (see Figure 7), or PPF (Figure 9A). Another potential mechanism for interfering with LTP could be the alteration of the postsynaptic

response during high frequency stimulation or by altering inhibitory or NMDA receptor-mediated responses. Application of an HAV (5-mer) peptide, however, had no effect on the magnitude of the inhibitory postsynaptic potential (IPSP) recorded intracellularly in pyramidal neurons (mean percent of baseline after 30 min peptide exposure: 97.4% \pm 15.4% [n = 2]). The peptides were also without effect on the pharmacologically isolated NMDA receptor field excitatory postsynaptic potentials (EPSPs) (Figure 9B) (mean percent of baseline value after 30 min: fEPSP, 111.6% \pm 11.5% [n = 5]) or the magnitude of excitatory postsynaptic currents (EPSCs) recorded at a range of holding potentials (mean percent of control value: at –60 mV, 102.0% \pm 12.0%; –50 mV, 105.0% \pm 5.0%; –40 mV, 108.0% \pm 2.0%; –30 mV, 94.0% \pm 16.0%; –20 mV, 94.0% \pm 10.0% [n = 2]). In addition, the application of an HAV-containing peptide did not significantly alter the postsynaptic neuron's ability to depolarize during high frequency stimulation (Figures 9C and 9D). These results rule out any obvious effect of the HAV peptide on the first few events (e.g., postsynaptic depolarization and NMDA receptor activation) associated with LTP induction.

The Block of LTP by HAV Peptides and N-cad Antibody: Dependence on $[Ca^{2+}]_{ext}$

The two-pathway experiments shown in Figures 7 and 8A indicate that HAV peptides are only effective inhibitors of LTP when applied around the time of LTP induction. There are two general possibilities to account for this exclusive effect of cadherin manipulations on the induction of plasticity, rather than its maintenance. LTP induction might render the adhesive junctions formed by existing cadherins susceptible to inhibition by the peptide inhibitors. For example, reductions in Ca^{2+} in the synaptic cleft during high frequency stimulation (Nicholson et al., 1978; Krnjevic et al., 1982; Smith, 1992;

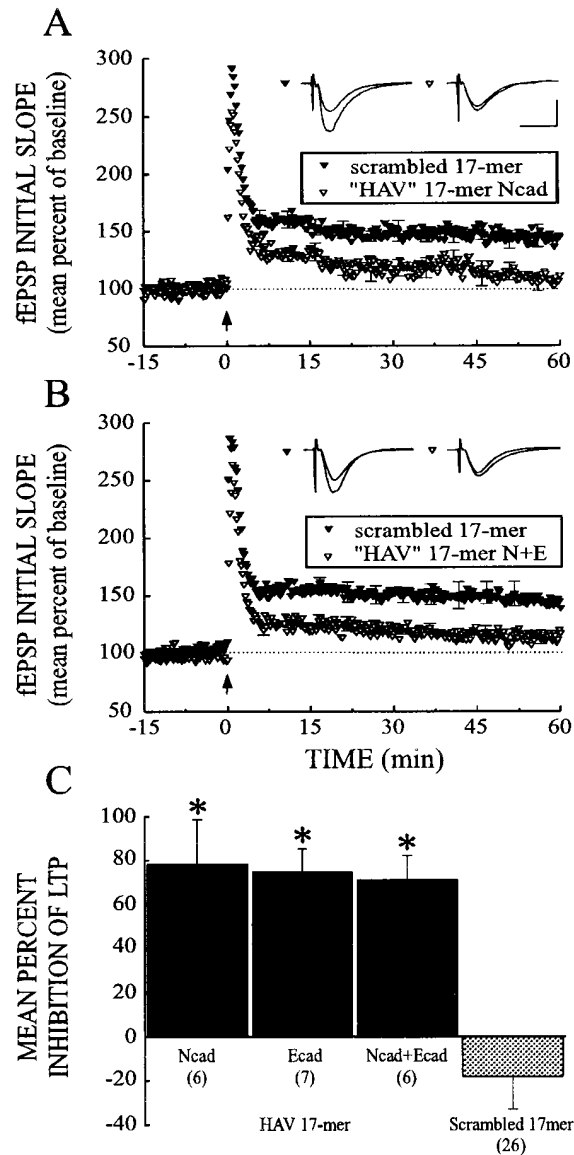


Figure 6. Slices Incubated in HAV Peptides Also Exhibit Diminished LTP

Superimposed representative field EPSPs taken before and 50–60 min after tetanus for each group are shown in (A) and (B). Scale bar, 0.5 mV and 20 ms.

(A) Slices pretreated with an N-cad-derived HAV 17-mer showed significantly reduced LTP relative to adjacent slices pretreated with a scrambled 17-mer.

(B) Slices pretreated with a combination of N-cad-derived (1 mM) and E-cad-derived (1 mM) HAV 17-mer showed significantly reduced LTP relative to slices pretreated with a scrambled 17-mer (2 mM).

(C) Summary graph for all 17-mer HAV peptide experiments. Mean percent inhibition of LTP is expressed relative to the amount of LTP observed in the control pathway for each set of experiments. Numbers in parentheses indicate the "n" for each set of experiments. The controls for the scrambled peptide experiments were adjacent ACSF-treated slices.

Egelman and Montague, personal communication) might transiently destabilize the Ca^{2+} -dependent cadherin-cadherin interaction (Hyafil et al., 1981). Alternatively,

new cadherin bonds may be formed during LTP induction; as such, the peptide could block the formation of these new junctions. We reasoned that if the former possibility were true, we should be able to prevent the inhibition by HAV peptides by raising the extracellular Ca^{2+} concentration. We thus elevated the Ca^{2+} concentration to 5 mM and reexamined the inhibitory efficacy of the HAV peptides in two-pathway experiments. As would be expected, the increased concentration of Ca^{2+} in the extracellular solution had a modest (15.5%) enhancing effect on basal synaptic transmission. The ability of the HAV peptide to inhibit LTP, however, was completely prevented by the elevated Ca^{2+} in the ACSF (Figure 10A) (mean percent of baseline: AHAVD, $138.3\% \pm 7.4\%$; control pathway, $142.4\% \pm 9.2\%$ [n = 9]). Similarly, when slices were pretreated with N-cad Ab in the presence of elevated Ca^{2+} , the Ab no longer attenuated LTP (Figure 10B; compare with Figure 4A) (mean percent of baseline: N-cad/high Ca^{2+} , $141.7\% \pm 13.5\%$ [n = 6]; control Ab/high Ca^{2+} , $138.8\% \pm 4.6\%$ [n = 6]). These results suggest that elevated extracellular Ca^{2+} protects synaptic cadherins from inhibition by the HAV peptide and function-blocking Ab.

Discussion

Taken together, our data indicate that cadherins participate in modulating activity-dependent changes in synaptic strength. Pretreatment of hippocampal slices with function-blocking Ab to either N- or E-cad attenuated LTP. Similar inhibitory effects on LTP were observed when peptides containing the HAV consensus sequence were applied to slices. The peptide- or Ab-induced inhibition of LTP was not accompanied by any changes in basal synaptic transmission, posttetanic potentiation, paired-pulse facilitation, NMDA receptor-mediated currents, or postsynaptic depolarization mechanisms. The lack of effect of the function-blocking Abs or HAV peptides on these basal synaptic properties strongly suggests that these blockers work by perturbing mechanisms involved in synaptic plasticity, rather than imparting a general anti-adhesive and disruptive effect on the slice. The small amount of residual enhancement consistently observed in the presence of the function-blocking Abs and HAV peptides may reflect the involvement of other types of cadherin molecules, as has been suggested by studies of localization (Fannon and Colman, 1996), or a cadherin-independent portion of plasticity, perhaps mediated by other adhesion molecules implicated in area CA1 LTP (Luthi et al., 1995; Muller et al., 1996; Nosten-Bertrand et al., 1996; Bahr et al., 1997).

Previous studies have shown that HAV peptides can interfere with several cadherin-dependent processes, including neurite outgrowth (Chuah et al., 1991; Doherty et al., 1991), osteoclast formation (Mbalaviele et al., 1995), and myoblast fusion (Mege et al., 1992). The HAV motif may also mediate heterophilic interactions of cadherins with another HAV-containing molecule, the fibroblast growth factor (FGF) receptor (Williams et al., 1994). Given that FGF does not appear to modulate LTP (Hisajima et al., 1992), perturbation of an FGFR-cadherin interaction is an unlikely explanation for our results. In

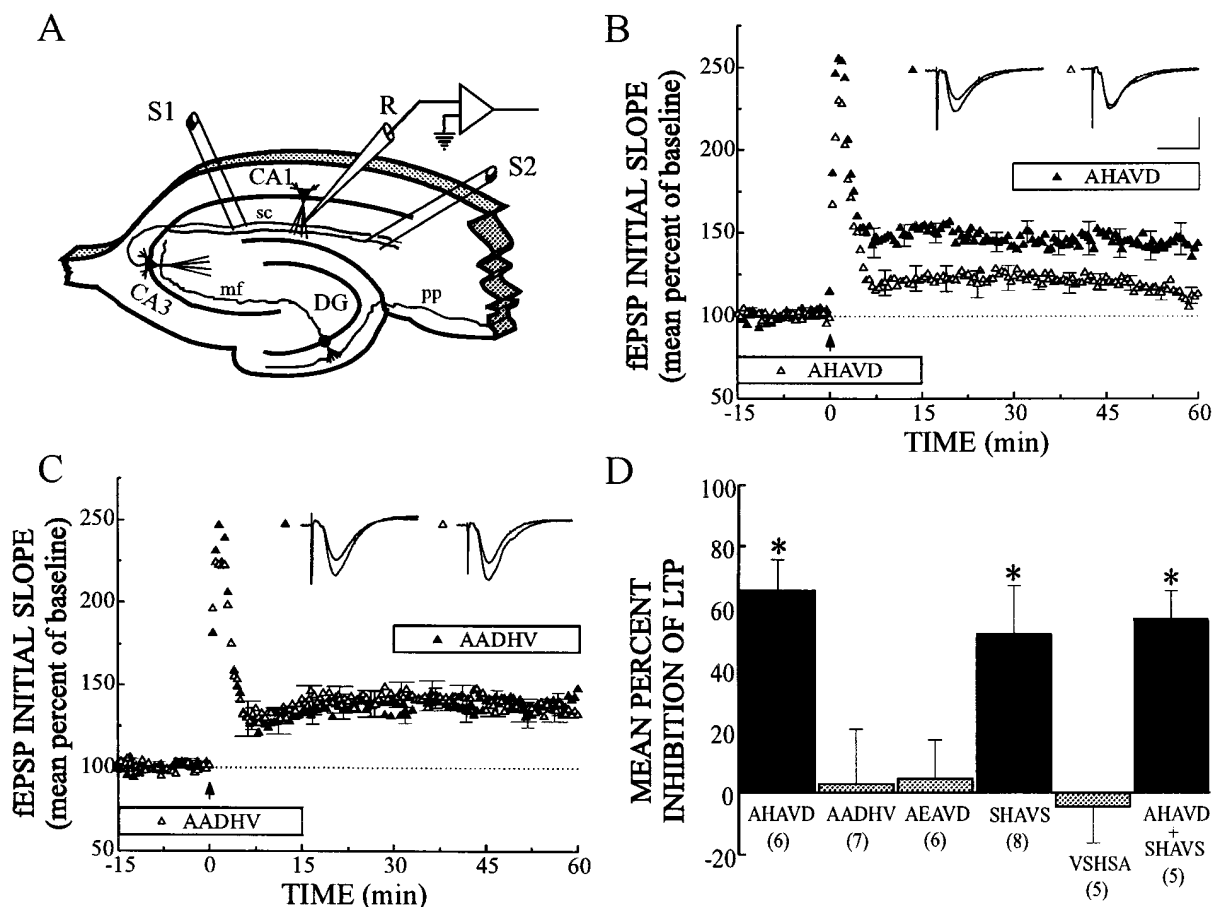


Figure 7. Peptides Containing the Sequence HAV Perturb the Induction but Not the Maintenance of LTP

(A) Diagram of two-pathway experiment design indicating the approximate position of the recording electrode (R) and two stimulating electrodes (S1 and S2).

(B and C) Superimposed ensemble averages from two-pathway experiments conducted in the same slices. Individual superimposed representative electrophysiological traces are shown for each experimental group 10 min before and 50–60 min after LTP induction.

(B) Bath application of an AHAVD peptide for 30 min prior to tetanus significantly reduces LTP in pathway 2 (open triangles), whereas application of the same peptide 30 min after LTP induction in pathway 1 has no significant effect on established potentiation (closed triangles). Scale bar, 0.5 mV and 20 ms.

(C) Bath application of a control AADHV peptide for either 30 min prior to tetanus (open triangles) or 30 min after tetanus (closed triangles) had no significant effect on the initiation or maintenance of LTP.

(D) Summary of all two-pathway experiments conducted with 5-mer peptides; only peptides containing the HAV motif in the correct orientation showed inhibitory activity. Mean percent inhibition of LTP is expressed relative to the amount of LTP observed in the control pathway for each set of experiments. The numbers in parentheses indicate the n for each experimental group. Asterisks indicate significant inhibition ($p < 0.05$) relative to control slice LTP.

cadherin-cadherin interactions, the HAV peptides likely interfere with the between-cell, rather than the within-dimer formation (Shapiro et al., 1995; Vaughn and Bjorkman, 1996). The inhibition by the HAV peptides thus suggests that intercellular interactions between cadherins are essential for LTP formation. Cadherin-mediated interactions could be required for intracellular signaling on either side of the synapse, or to increase the area of contact between pre- and postsynaptic elements, or even to transmit mechanical signals across the synaptic cleft.

Our studies further indicate a time- and Ca^{2+} -dependent vulnerability to inhibition by the peptides. HAV peptides applied after LTP had been successfully established were ineffective in returning transmission to baseline values; only peptides present around the time of

induction prevented LTP. Similar observations have been made for integrins, L1, and NCAM (Luthi et al., 1995; Muller et al., 1996; Bahr et al., 1997), suggesting that adhesion molecules are involved in the very early steps associated with synaptic modification. Another interesting feature of the block of LTP by the HAV peptides and the N-cad Ab is the Ca^{2+} concentration dependence: these agents do not inhibit LTP when extracellular Ca^{2+} is elevated to 5 mM. Studies using ion-sensitive microelectrodes have shown that repetitive stimulation in the hippocampus (Krnjevic et al., 1982) and cerebellum (Nicholson et al., 1978) can cause dramatic reductions in extracellular Ca^{2+} . Moreover, models of Ca^{2+} dynamics in the synaptic cleft and extrasynaptic space predict that action potential activity can transiently deplete Ca^{2+} in the cleft due to flux through voltage-gated

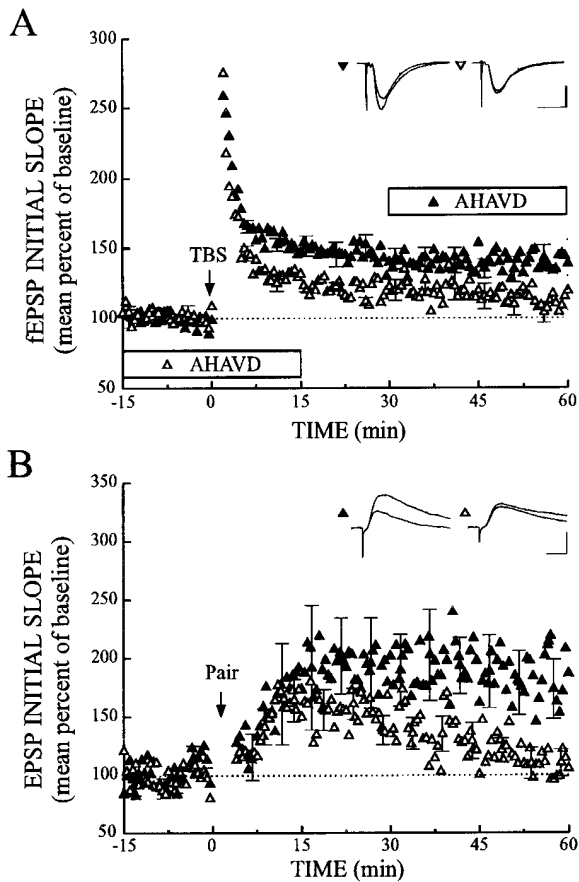


Figure 8. HAV Peptides Also Inhibit LTP Induced by Theta Burst Stimulation or Pairing Postsynaptic Depolarization with Low Frequency Stimulation

Individual superimposed representative electrophysiological traces are shown for each experimental group 10 min before and 50–60 min after LTP induction.

(A) Ensemble average for a series of two-pathway experiments in which the HAV peptide was applied either 30 min before (open triangles) or after (closed triangles) LTP induction by TBS. Scale bar, 0.5 mV and 20 ms.

(B) Ensemble averages for experiments in which LTP was induced by pairing in the presence of the correct orientation HAV peptide (open triangles) or the scrambled (closed triangles) peptide. Scale bar, 5.0 mV and 20 ms.

Ca²⁺ channels and NMDA channels (Smith, 1992; Egelman and Montague, personal communication). Cadherin–cadherin interactions exhibit a strong Ca²⁺ dependence: removal of Ca²⁺ from the medium results in a loss of adhesion (Hyafil et al., 1981) and a change in the structure of the cadherin extracellular domains from their native rod-like structure to a globular structure (Pokutta et al., 1994; Koch et al., 1997). Thus, bouts of intense synaptic activity, such as those used to induce LTP, may transiently destabilize existing cadherin bonds, rendering them susceptible to inhibition by the HAV peptides. Nascent cadherin–cadherin bonds might also be vulnerable at this time, due to reduced extracellular Ca²⁺. The inability of the HAV peptides to block at elevated Ca²⁺ concentrations may thus reflect a relative protection of the cadherin bonds owing to a less dramatic reduction in cleft Ca²⁺ during stimulation. In the

case of the N-cad Ab, our results do not distinguish between a protective effect of elevated Ca²⁺ associated with LTP induction versus protection associated with the Ab preincubation period (2–3 hr), during which time the Ab may gain access to synaptic sites.

Taken together with previous studies (Bailey et al., 1992; Mayford et al., 1992; Chen and Grinnell, 1995; Luthi et al., 1995; Muller et al., 1996; Nosten-Bertrand et al., 1996; Bahr et al., 1997), these data indicate that local, mechanical, or structural signals must also be considered as important regulators of synaptic plasticity. It is presently unclear to what extent the various adhesion molecules implicated in LTP play distinct or redundant roles. It is possible that some molecules may be permissive for plasticity, whereas others may play a more direct, instructive role. The use of recombinant DNA and transgenic animals to manipulate specific domains of adhesion molecules may help to elucidate their particular functions. For example, a recent study of NCAM mutant mice has revealed that LTP is intact in mutant animals, indicating that under some circumstances NCAM is not required for LTP (Holst et al., 1998).

Cadherins are involved in the dynamic rearrangements of cells during morphogenesis (Takeichi, 1995) and neurite outgrowth (Riehl et al., 1996); they may play a similar role in the adult nervous system, directing the growth of synaptic connections (Sargent Jones, 1996; Fannon and Colman, 1996; Uchida et al., 1996; Colman, 1997; Serafini, 1997). Indeed, the attractive zipper motif of some cadherin–cadherin homodimers (Shapiro et al., 1995) raises the possibility that cadherins might modulate synaptic transmission by increasing or decreasing the area of contact between pre- and postsynaptic elements (e.g., Lisman and Harris, 1993). Finally, the suggestion that cadherin bonds are sensitive to dynamical changes in extracellular Ca²⁺ might provide a mechanism for the direct coupling of cadherin-mediated adhesive or signaling events to synaptic activity.

Experimental Procedures

Immunohistochemistry

500 μ m slices were fixed on ice with 4% paraformaldehyde and 0.2% glutaraldehyde for 4 hr and transferred to phosphate buffered saline (PBS). Sections (25 or 50 μ m) were then cut using a vibratome and a sapphire knife. The sections were then put on a shaker and treated sequentially with 0.7% Triton X-100 in PBS, PBS, 0.1 M glycine in PBS, dH₂O, 1% Na borohydride, dH₂O, preblock buffer (0.05% Triton X-100, 5% goat serum in PBS), primary Ab in preblock at 4°C overnight, preblock buffer, FITC- or Cy3-conjugated secondary Ab in preblock, preblock, and PBS. Sections were mounted in the mounting medium (80% glycerol and p-phenylenediamine in 0.1 M Na carbonate buffer [pH 9.0]). Immunostained specimens were viewed with a Zeiss LSM 310 laser-scan confocal microscope through either a 10 \times or a 63 \times oil-immersion lens. Cy3 was excited at 543 nm and fluorescein at 488 nm. Images were recorded through standard emission filters at contrast settings for which the crossover between the two channels was negligible.

Antibodies

The following Abs were used for immunostaining and/or electrophysiology, as indicated in the text: (1) a mouse anti-N-cad Ab (GC-4, Sigma) raised against purified chicken N-cad that reacts with the N-terminal half of the extracellular domain of N-cad; (2) a monoclonal mouse anti-Pan-cad Ab (CH-19, Sigma) raised against a synthetic peptide corresponding to the C-terminal 24 amino acids

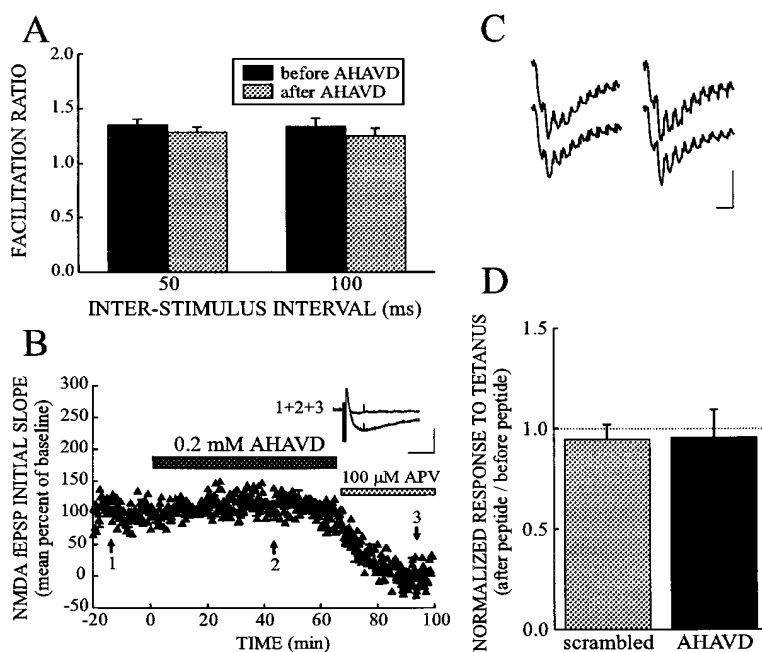


Figure 9. HAV Peptides Do Not Inhibit Paired-Pulse Facilitation, NMDA Receptor-Mediated EPSPs, or Postsynaptic Depolarization Mechanisms

(A) Facilitation ratios for HAV peptide-treated slices and controls. The facilitation ratio represents the slope of the second field EPSP divided by the slope of the first field EPSP for the interstimulus intervals shown.

(B) Ensemble average of experiments ($n = 5$) examining field EPSPs mediated by NMDA receptors before and after the addition of the AHAVD peptide. Similar data ($n = 2$) were also obtained for NMDA receptor-mediated EPSCs. Representative traces taken from the times indicated. Scale bar, 0.25 mV and 30 ms.

(C) Representative field potential recordings obtained during a tetanus before (top) and after (bottom) addition of either a scrambled (left) or correct (right) orientation HAV peptide. Scale bar, 0.5 mV and 20 ms.

(D) Summary of the normalized response to tetanus for either the scrambled or the correct orientation HAV peptide.

of chicken N-cad and which reacts with many members of the cadherin family; (3) monoclonal rat anti-E-cad (Clone DECMA-1, Sigma) raised against a mouse embryonal carcinoma cell line; (4) polyclonal rabbit anti-synapsin I Ab (a gift from Mary Kennedy); (5) a polyclonal rabbit antiserum to a full-length recombinant rat glypican-GST fusion protein (a gift from R. U. Margolis); (6) a polyclonal rabbit antiserum (E2) to a recombinant E-cad cytoplasmic domain GST fusion protein (a gift from James Nelson); (7) rabbit α - and β -catenin antiserum (Sigma); and (8) purified rat or mouse IgG (Sigma). FITC- or Cy3-conjugated goat IgGs to rabbit, mouse, or rat IgG (Jackson Labs) were used as secondary Abs.

Slice Preparation and Electrophysiology

Hippocampal slices were prepared using a Stoelting tissue chopper from young (6- to 8-week-old) adult male Sprague-Dawley rats or young (5- to 7-week-old) adult male BALB/C57I mice. Prior to electrophysiological recording, slices were stored for at least 1.5 hr on a Millipore membrane placed over a tissue culture dish containing oxygenated artificial cerebral spinal fluid (ACSF) solution. The slice was exposed to 95% O_2 /5% CO_2 circulating in an enclosed chamber. For electrophysiological recordings, slices were submerged in a stream of ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4 , 26.2 mM $NaHCO_3$, and 11.0 mM glucose) maintained at room temperature (22°C–25°C) and gassed with 95% O_2 /5% CO_2 . Field, intracellular, or whole-cell EPSP/Cs measured in stratum radiatum or in CA1 pyramidal cells, respectively, were evoked by stimulation of the Schaffer collateral-commissural afferents (1 stimulation every 15 s). In order to be included for further analysis, baseline field EPSPs of slope 0.1–0.2 mV/ms had to be elicited with stimulation currents of $\leq 20 \mu A$ and have fiber volley amplitudes of ≤ 0.15 mV. Extracellular recording electrodes were filled with 3 M NaCl; intracellular recording electrodes were filled with 2 M cesium acetate. In experiments measuring NMDA receptor-mediated EPSP/Cs, whole-cell pipettes were filled with 100 mM cesium gluconate, 0.6 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, 0.3 mM GTP, and 40 mM HEPES (pH 7.3). Recordings were made in the single-electrode voltage-clamp mode with 75%–90% of series resistance compensation. NMDA receptor-mediated EPSP/Cs were pharmacologically isolated by including CNQX (10 μM), glycine (10 μM), and picrotoxin (50 μM). In field recordings, a modified ACSF containing 0.1 mM Mg^{2+} was used. The NMDA receptor antagonist APV (50 mM) was applied at the conclusion of each experiment to confirm that the recorded EPSP/C was mediated by NMDA receptor activation. Tetanic stimulation was delivered at the test intensity in

1 s trains at 100 Hz, with one to four trains delivered 30 s apart. (Control and experimental slices always received the same number of trains.) TBS consisted of three applications (30 s apart) of the following: four bursts of stimuli, each of five pulses at 100 Hz with an interburst interval of 200 ms. Pairing was accomplished by sustained depolarization (to 0 mV) of the intracellularly recorded neuron by DC injection in conjunction with low frequency (1 Hz) stimulation of the test pathway for 60 s.

Antibody and Peptide Incubation

In Ab incubation experiments, slices were placed in individual wells containing 200 μl of ACSF and 1:100 dilutions of either preimmune rat IgG, N-cad (GC4), E-cad (DECMA-1 or E2), Pan-cad (Sigma), or glypican Abs. In the anti-E-cad experiments, in which the Ab (DECMA-1) was raised in rat against a mouse antigen, we used slices from mouse, since the Ab does not recognize the rat E-cad protein. Following incubation for 2–3 hr, slices were transferred to a recording chamber and perfused with normal ACSF for the duration of the experiment. LTP was induced within 45 min of transfer to normal ACSF. In the 17-mer (0.5, 1, or 2 mM) peptide experiments, slices were incubated as above for 2–3 hr. The 17-mer peptide sequences used, derived from mouse N- and E-cad and previously shown by others (Blaschuk et al., 1990; Doherty et al., 1991) to interfere with cadherin-dependent processes in rat or mouse tissue, were as follows: E-cad, AKYILYSHAVSSNGEAV; N-cad, ARFHLRA HAVDINGNQV; and scrambled (derived from E-cad sequence), VAV LYEKSGIAYHNSAS.

Analysis

We analyzed the initial slope of the field EPSP and the slope and amplitude of the intracellular EPSP. Ensemble averages were constructed using all data points, aligned with respect to the time of LTP induction. Two statistical comparisons were performed for each set of experiments: (1) a within-experiment comparison examining whether the magnitude of synaptic transmission measured 50–60 min following LTP induction was significantly different from that measured before tetanus and (2) a between-experiment comparison examining whether the HAV peptide or Ab-treated slice differed significantly from its associated control experiments in the magnitude of potentiation measured 50–60 min after tetanus. Mean percent inhibition values indicate the inhibition of LTP in peptide or Ab-treated slices relative to same day, adjacent control slices. Paired-pulse facilitation was measured as the ratio of the slope of the second response to that of the first response at a 50 or 100 ms

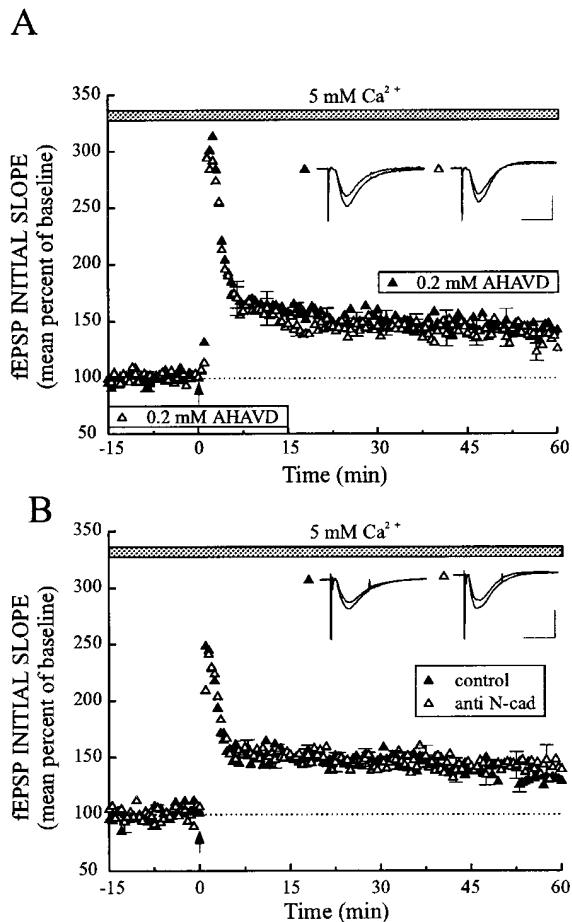


Figure 10. Elevated Extracellular Ca^{2+} Prevents the Block of LTP by the AHAVD Peptide or the Function-Blocking N-cad Antibody (A) Ensemble averages for two-pathway experiments in which the HAV peptide was applied either 30 min before (open triangles) or after (closed triangles) LTP induction by tetanus in an altered ACSF containing 5.0 mM Ca^{2+} . (B) Ensemble average of all experiments ($n = 6$) in which slices were pretreated with either a function-blocking N-cad Ab or a non-function-blocking E-cad Ab in the presence of an ACSF containing 5.0 mM Ca^{2+} . Individual superimposed representative electrophysiological traces are shown for each experimental group 10 min before and 50–60 min after LTP induction. Scale bar, 0.5 mV and 20 ms.

interstimulus interval. The magnitude of PPF in Ab or peptide-treated slices was compared to their associated controls. In depolarization measurements, the integrated area below baseline during high frequency stimulation was calculated.

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