Reg1ulatory Role and Molecular Interactions of a Cell-Surface Heparan Sulfate Proteoglycan (*N*-syndecan) in Hippocampal Long-Term Potentiation

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The cellular mechanisms responsible for synaptic plasticity involve interactions between neurons and the extracellular matrix. Heparan sulfates (HSs) constitute a group of glycosaminoglycans that accumulate in the β-amyloid deposits in Alzheimer's disease and influence the development of neuron–target contacts by interacting with other cell surface and matrix molecules. However, the contribution of HSs to brain function is unknown. We found that HSs play a crucial role in long-term potentiation (LTP), a finding that is consistent with the idea that converging molecular mechanisms are used in the development of neuron–target contacts and in activity-induced synaptic plasticity in adults. Enzymatic cleavage of HS by heparitinase as well as addition of soluble heparin-type carbohydrates prevented expression of LTP in response to 100 Hz/1 sec stimulation of Schaffer collaterals in rat hippocampal slices. A prominent carrier protein for the type of glycans implicated in

LTP regulation in the adult hippocampus was identified as *N*-syndecan (syndecan-3), a transmembrane proteoglycan that was expressed at the processes of the CA1 pyramidal neurons in an activity-dependent manner. Addition of soluble *N*-syndecan into the CA1 dendritic area prevented tetanus-induced LTP. A major substrate of src-type kinases, cortactin (p80/85), and the tyrosine kinase fyn copurified with *N*-syndecan from hippocampus. Moreover, association of both cortactin and fyn to *N*-syndecan was rapidly increased after induction of LTP. *N*-syndecan may thus act as an important regulator in the activity-dependent modulation of neuronal connectivity by transmitting signals between extracellular heparin-binding factors and the fyn signaling pathway.

Key words: long-term potentiation; synaptic plasticity; extracellular matrix; heparan sulfate proteoglycans; hippocampus; src family tyrosine kinases; cortactin

Long-term potentiation (LTP) is a long-lasting augmentation of synaptic strength that has been suggested as a cellular mechanism underlying learning and memory (Bliss and Collingridge, 1993; Larkman and Jack, 1995; Nicoll and Malenka, 1995). Considerable evidence suggests that the maintenance of LTP involves structural rearrangements within neuronal connections (Lee et al., 1980; Chang and Greenough, 1984; Edwards, 1995) (but see also Sorra and Harris, 1998), a process that is critically dependent on cell–matrix interactions. Antibodies or peptides inhibiting cell adhesion molecules disrupt expression of LTP already at the early stages (Lüthi et al., 1994; Bahr et al., 1997; Tang et al., 1998), which are dependent on the activity of protein kinases and phosphatases. Changes in kinase activity can directly influence synaptic transmission by phosphorylation of postsynaptic neurotransmitter receptors or components of the presynaptic releasing machinery (Walaas and Greengard, 1991; Smart, 1997). In addition, protein kinases, in particular the src family tyrosine kinases, associate to and control the organization of the cytoskeleton, and may thereby regulate cellular morphology and biological func-

tions dependent on it (Thomas et al., 1995; Lowell and Soriano, 1996).

Heparan sulfates (HSs) are glycosaminoglycans that influence cell–environment interactions by binding to a heterogeneous group of growth factors, matrix ligands, and cell surface molecules (Gallagher, 1989; Rapraeger, 1993; Lindahl et al., 1994). In the nervous system, proteoglycans and factors binding to them have an important role in axonal guidance and synaptogenesis during prenatal and early postnatal periods (Lander, 1993; Margolis et al., 1996; Rauvala and Peng, 1997). The majority of cell surface heparan sulfate proteoglycans belong to the family of syndecans, whose expression in rat brain is developmentally regulated (Bernfield et al., 1992; Nolo et al., 1995; Carey, 1997). In addition, HSs and heparin-binding factors have been found to accumulate in the β-amyloid deposits in Alzheimer's disease (Snow et al., 1988, 1994; Wisniewski et al., 1996) and are proposed to be important for the promotion, deposition, and persistence of the senile plaques.

Molecules whose cellular effects on the development of neuronal connections are modulated by or dependent on HS include at least the neural cell adhesion molecule (NCAM) (Cole et al., 1986), fibroblast growth factors (FGFs) (Lander, 1993; Nurcombe et al., 1993), and heparin-binding growth-associated molecule (HB-GAM; Rauvala and Peng, 1997). Interestingly, all these three molecules have been found to influence LTP (Ishiyama et al., 1991; Lüthi et al., 1994; Lauri et al., 1998). Binding of HSs to several molecules implicated in synaptic plasticity suggests that

HSs might play a key role in the regulation of brain function. Nevertheless, the possible role of HSs in synaptic transmission has not been previously examined.

Here we show that hippocampal LTP is critically dependent on heparan sulfates, and identify *N*-syndecan as a major heparan sulfate proteoglycan expressed in the pyramidal neurons of hippocampus. The expression of *N*-syndecan as well as its interaction with the intracellular, cytoskeleton-regulating molecules cortactin and fyn-kinase was increased after LTP induction. Thus, we suggest that *N*-syndecan is one of the molecules that modulate cell–matrix interactions associated with synaptic plasticity.

MATERIALS AND METHODS

In vitro *electrophysiological recordings and protein injections.* Transverse slices (300–400 μm) were cut by means of a vibratome from the hippocampi of Wistar rats (100–200 gm), which were decapitated under deep pentobarbital anesthesia (30–40 mg/kg, i.p.). The slices were allowed to recover at room temperature for at least 60 min before any experiments were started. All the recordings were made at +32°C in an interface-type chamber, which was perfused with a solution containing (in mM) NaCl 124, KCl 3, CaCl$_2$ 2, NaHCO$_3$ 25, NaH$_2$PO$_4$ 1.1, MgSO$_4$ 2, and glucose 10, and gassed with 5% CO$_2$ and 95% O$_2$. A constant perfusion at a rate of ~1 ml/min was applied, except for the experiments with desulfated glycans, which were done in a static perfusion (chamber volume, 1 ml).

Extracellular recordings from CA1 stratum radiatum were made by using glass capillary microelectrodes filled with 150 mM NaCl. Intracellular current-clamp recordings were made from CA1 pyramidal neurons by using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) in active bridge mode. The intracellular electrodes were filled with electrolyte solution that contained 1 M K$^+$ methyl sulfate, 0.5 M K$^+$ acetate, and 10 mM KCl, and their resistance was 70–120 MΩ. A bipolar electrode was used for Schaffer collateral stimulation (pulse length, 100 μsec), and the stimulus intensity was adjusted to give a half-maximal field EPSP (fEPSP) amplitude. After at least 10 min of stable baseline recording (0.05 Hz), LTP was induced by high-frequency stimuli (100 Hz/1 sec), during which the pulse length was doubled. In some of the experiments (protein injections), induction of LTP in the slice was controlled by recording simultaneously an independent pathway. Asystant software package (MacMillan) was used for all data acquisition and analysis. The slope of fEPSP was used as an indicator of synaptic efficacy and was calculated between 20 and 80% of the maximal amplitude.

Organotypic hippocampal cultures were prepared from 9- to 11-d-old rats by the method of Stoppini et al. (1991). After 10–12 d in culture, the slices were incubated for 15 hr with heparitinase (heparinase III, Sigma, St. Louis, MO) (1 U/ml) or bovine serum albumin (BSA) (0.01%), and transferred to the recording chamber for the measurement of LTP.

N-syndecan was purified from rat brain as described (Raulo et al., 1994), and dialyzed against PBS before use. The protein was pressure-injected (Perfusor 1–300; B. Braun Melsungen AG) with a micropipette into the dendritic area of CA1 between stimulation and recording electrodes within 0.3 mm distance from the recording site. The protein ($\approx$3 $\times$ 0.3 μl) was applied by three brief injections within 5 min. According to previous data, pressure-injected proteins of sizes of 150 kDa (IgG; total amount, 0.5 pmol) and 18 kDa (HB-GAM; total amount, 1 pmol) can be detected by immunostaining in a 400-μm-thick hippocampal slice in a round-shaped area of a diameter of ~200 μm and 1 mm, respectively (Ronn et al., 1995; Lauri et al., 1998). Based on the data above and the Stokes–Einstein relation, injected *N*-syndecan (210 kDa, 0.1 pmol) is expected to spread into an area with a diameter of ~50 μm.

In vivo *electrophysiology.* Male Wistar rats (250–300 gm) were anesthetized with urethane (1.3 gm/kg) and placed in a stereotaxic apparatus. The scalp was removed, and a small (1.5 $\times$ 1.5 mm) bone window was drilled above the hippocampus (the anteromedial edge at anteroposterior = 3.3 and lateral = 2.2 mm from bregma). A pair of stimulating electrodes (60 μm wire) was lowered simultaneously with the recording electrode. The positioning of the recording electrode to CA1 stratum radiatum was based on stereotaxic coordinates, evoked responses, and on CA1 pyramidal cell unit monitoring. The pair of stimulating electrodes was positioned into the same layer 0.5 mm laterally. The signals were amplified by a Brownlee Model 440 instrumentation amplifier and stored on a hard disk for off-line analysis. After a stable baseline recording, LTP was induced with the same stimulation patterns as *in vitro* (100 Hz/1 sec)

using a stimulation intensity that yields a half-maximal fEPSP amplitude. During the recordings, the electrical activity of the hippocampus was followed through oscilloscope and an audiomonitor, and no epileptiform discharges were observed. If potentiation of the fEPSP slope was <30% 1 hr after the high-frequency stimulation (HFS), the experiment was rejected. Control rats received the same number of pulses at 0.05 Hz. The rats were perfusion-fixed for immunohistochemistry and *in situ* hybridization 3–4 hr after the stimulation, a time period known to result in changes in the expression of activity-regulated genes (Armstrong and Montiminy, 1993; Silva and Giese, 1994).

In situ *hybridization and immunohistochemistry.* Anesthetized Wistar rats were perfused transcardially with 0.9% saline followed by 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4; 5 min). A part of brain containing the hippocampi was removed and post-fixed overnight with 4% paraformaldehyde. Alternatively, hippocampal slices (400 μm) were fixed after electrophysiological recordings with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for at least 4 hr. Fixed tissue was cryoprotected in PBS containing 15% sucrose for several days, and 40 μm cryosections were cut transversally and treated in a free-floating state through the *in situ* or immuno-labeling procedures.

Antibodies against heparan sulfate 10E4 epitope were from Seikagaku Corporation (Tokyo, Japan) and were used for immunohistochemistry at a concentration of 10 μg/ml. Affinity-purified antibodies against an N-terminal peptide of *N*-syndecan (described in Nolo et al., 1995) were used for immunohistochemistry at a concentration of 0.1 μg/ml. The immunostain was visualized with biotinylated secondary antibodies and the Vector Laboratories (Burlingame, CA) immunoperoxidase reagents. For electron microscopy, the stained sections were post-fixed with 1% osmium tetroxide (1 hr), dehydrated in ethanol series, and embedded in Epon. Ultrathin sections (60 nm) were cut, stained with uranyl acetate and lead citrate, and examined by a transmission electron microscope (Jeol 1200; Jeol).

Sense and antisense digoxigenin-labeled RNA probes were synthesized in the presence of digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, IN) with T7 and T3 RNA polymerases from a linearized pBluescript vector containing the full-length *N*-syndecan sequence, and were used at the concentration of 20 ng/ml. The hybridization was performed as described (Lauri et al., 1996), and the hybridization signal was detected by alkaline phosphatase-conjugated anti-digoxigenin–Fab fragments. The stained sections were digitized with an Olympus AX70 Provis microscope and a Photometrics Sensys CCD camera. Image-Pro Plus 3.0 software was used for quantification of the intensity of the *in situ* and immunostain from the digitized images of the sections.

HB-GAM affinity chromatography and immunoblotting. HB-GAM binding proteins were purified from crude extracts of adult rat hippocampi by HB-GAM affinity chromatography as described (Raulo et al., 1994). Bound proteins were eluted with a linear NaCl gradient, and the fractions were analyzed on 3–12% gradient SDS-PAGE. To visualize both proteins and proteoglycans, the gels were stained with Alcian blue–silver nitrate (Møller et al., 1993). Aliquots of the fractions were digested with nitrous acid or heparitinase (10 U/ml, 15 hr) to remove carbohydrate side chains or incubated with 50 μM herbimycin A (Calbiochem, La Jolla, CA). Kinase activity was assayed as described (Kinnunen et al., 1998).

For analysis of *N*-syndecan-binding components after induction of LTP, the CA1 region of a hippocampal slice was rapidly dissected and frozen in liquid nitrogen 10 or 20 min after high-frequency Schaffer collateral stimulation. CA1 regions from nontreated slices of the same animals were prepared for controls. The tissue was homogenized in ice-cold PBS containing 1% Nonidet P-40, 2 μg/ml aprotinin, 1 mM Na$_3$VO$_4$, 1 mM NaF, 0.7 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 1 mM phenylmethylsulfonylfluoride. Lysates from 5–10 slices were combined, and samples of equal protein concentration were submitted to precipitation with HB-GAM–Sepharose (15 hr, +4°C). After washing with PBS containing 0.3 M NaCl, *N*-syndecan and associated components were eluted from the precipitates with heparin (10 μg/ml) in the presence of 0.3 M NaCl or with 0.6 M NaCl.

N-syndecan was immunoblotted using affinity-purified polyclonal antibodies against the N-terminal or C-terminal peptide of *N*-syndecan as described (Raulo et al., 1994). The C-terminal and N-terminal antibodies detected *N*-syndecan in a similar manner in hippocampal fractions. The C-terminal antibody was produced in rabbits using a synthetic peptide corresponding to the full-length cytoplasmic moiety of rat *N*-syndecan as an immunogen. Production of immunosera and affinity purification of the antibodies on peptide–Sepharose column as well as immunoblotting of
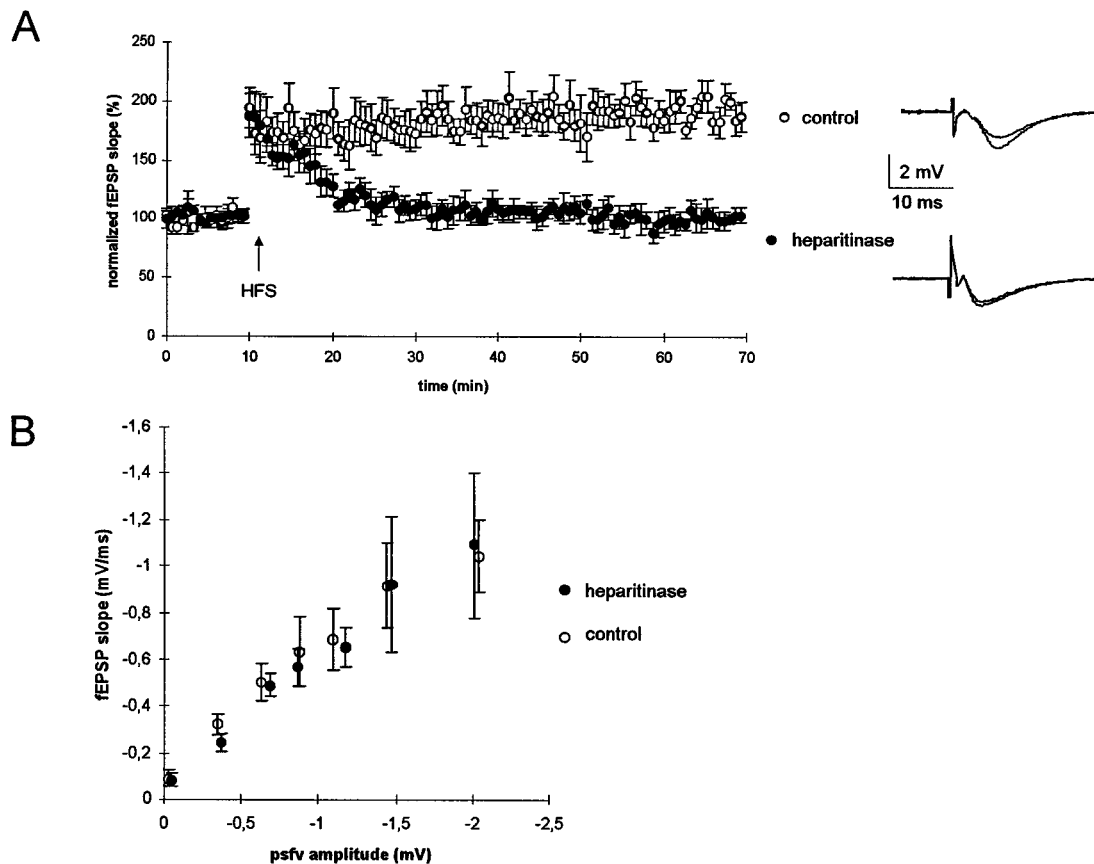
Figure 1. Enzymatic cleavage of heparan sulfate prevents LTP but has no effect on single pulse-evoked synaptic responses in the area CA1 of hippocampal slices. *A*, Effect of HFS on the fEPSP slope in rat hippocampal slices (300 μm) preincubated with heparitinase–0.2% BSA (20 U/ml; volume, 500 μl; 3 hr; +24°C) (●) or 0.2% BSA only (○) (average ± SEM; n = 7 on both groups; p < 0.01; Student's *t* test). fEPSP traces before and 30 min after the HFS are shown superimposed on the *right*. *B*, Slopes of fEPSPs plotted as a function of presynaptic fiber volley (*psfv*) amplitude show the lack of effect of heparitinase on baseline synaptic responses. Data were obtained from five heparitinase-treated (●) and five control (○) slices (average ± SEM shown).

tyrosine kinases, HS, and cortactin were performed as described (Rauvala, 1989). Polyclonal anti-src (SRC-2), anti-pp60[c-src], anti-yes, and anti-fyn antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-cortactin (p80/85) antibody was from Upstate Biotechnology (Lake Placid, NY). Heparan sulfate-containing proteins were immunoblotted with antibodies against heparan sulfate 10E4 epitope (Seikagaku Corporation) from hippocampal slices dissolved in SDS-PAGE buffer (40 μl/slice). All Western blots were developed using enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, England), and quantified according to the optical density of the protein bands.

RESULTS

Heparitinase treatment prevents LTP in area CA1 of acute and cultured hippocampal slices

To investigate the role of HS-type carbohydrates in synaptic transmission and plasticity, hippocampal slices (300 μm) were incubated for 3 hr with heparitinase (heparinase 3) or in similar conditions without the enzyme (control slices). This treatment reduced the HS content of the slices ~50% (total area of histologically detected HS immunoreactivity was 54 ± 6% of control, and the optical density of anti-HS-reactive bands in Western blots was 56 ± 8% of control). After heparitinase treatment, no LTP was observed after a 100 Hz/1 sec HFS, whereas in control slices a long-lasting synaptic potentiation was induced (Fig. 1*A*). Removal of HS did not affect single stimulus-evoked (0.05 Hz) synaptic responses at the Schaffer collateral–CA1 synapses (Fig.

1*B*). Also, the initial post-tetanic potentiation 1–2 min after the HFS was not affected by heparitinase (heparitinase 217 ± 20%, control 205 ± 18%), indicating that intact HS was needed for expression of early LTP, but not for baseline synaptic transmission or induction of short-term potentiation in the time scale <5 min.

In acute slices, a rather high heparitinase concentration (20 U/ml) was needed for removal of HS and inhibition of LTP. Therefore, the experiment was repeated in hippocampal slice cultures (Stoppini et al., 1991), which allowed a prolonged exposure (15 hr) to the enzyme. A similar inhibition of HFS-induced synaptic potentiation was seen in slice cultures treated with a low concentration (1 U/ml) of heparitinase (potentiation of the fEPSP slope 15 min after the HFS 178 ± 16% in controls and 95 ± 15% in heparitinase-treated cultures; n = 4).

Sulfation pattern of heparin-type glycans has a critical effect on their capability to influence LTP

As an alternative approach to study the effect of HS on synaptic plasticity, soluble heparin-type glycans were added to the perfusion solution of hippocampal slices while recording synaptic responses in the area CA1. Purification of HS as well as the selective desulfation of the glycans was done as described previously (Maccarana et al., 1993). Synaptic potentiation induced by HFS was markedly inhibited in the presence of heparin at a low concen-
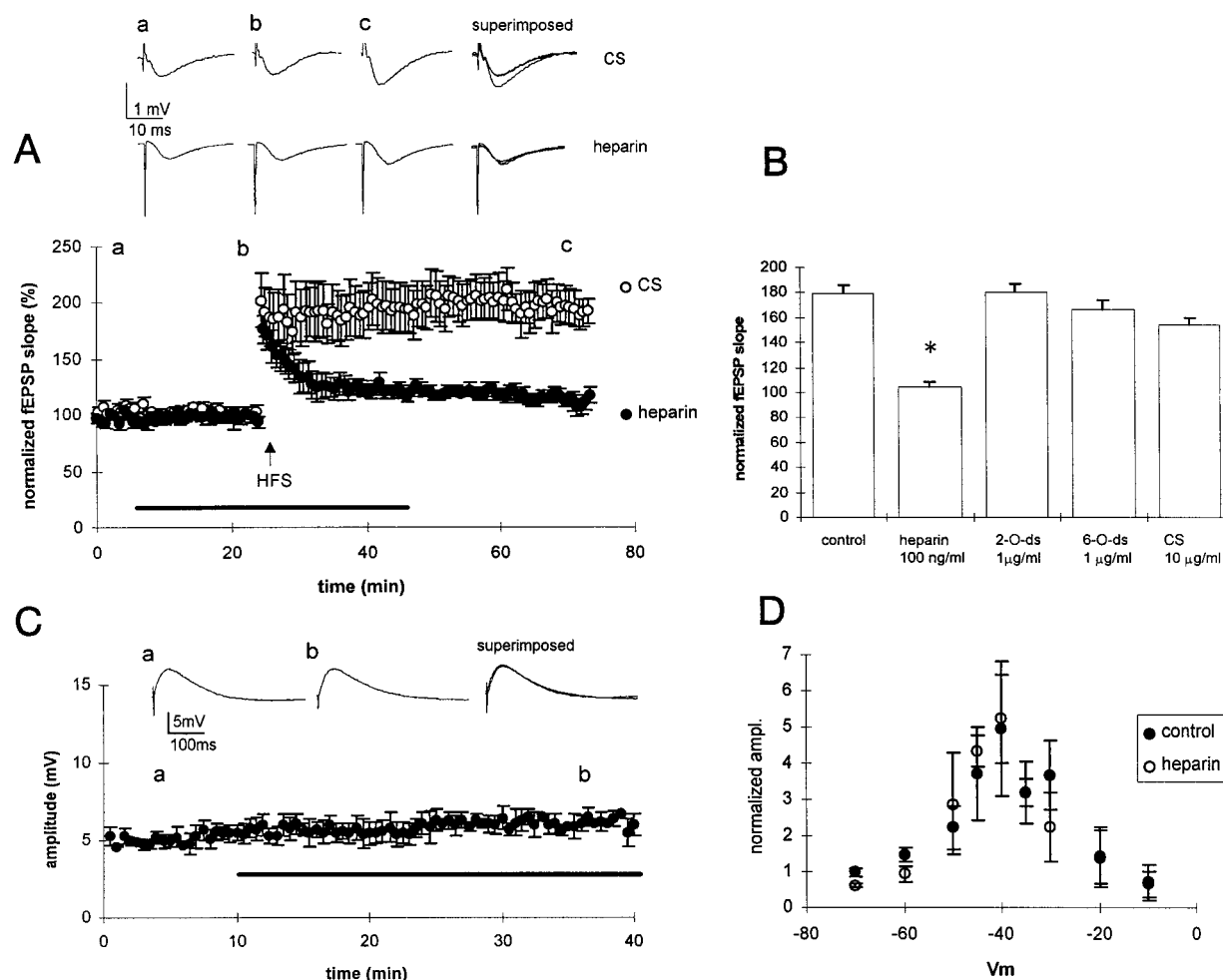
Figure 2. Heparin prevents LTP in a manner dependent on the sulfation pattern of the glycan, but has no effect on pharmacologically isolated NMDA receptor-mediated responses. *A*, Effect of heparin (*n* = 8) and chondroitin sulfate (*CS*) (*n* = 7) on LTP. Both glycans were bath-applied at the concentration of 100 ng/ml as shown by the *bar*. *Top traces* show sample fEPSPs before (*a*) and after (*b*) application of the glycan, and 60 min after HFS (*c*). *B*, Pooled data showing the effect of selectively 2-O-desulfated (*2-O-ds*) and 6-O-desulfated (*6-O-ds*) heparins on LTP. Because the desulfated glycans were available in low amounts, these experiments were performed in static perfusion (volume, 1 ml). The values represent fEPSP slope (% from control ± SEM; *n* = 4) 30 min after the HFS (**p* < 0.01; one-way ANOVA with Tukey *post hoc* comparison). *C*, Averaged amplitude of NMDA receptor-mediated responses recorded under current clamp from CA1 pyramidal neurones in the presence of 10 μM NBQX and 100 μM PiTX. Application of heparin (0.5 μg/ml) (shown by the *bar*) did not affect the amplitude of these responses (*n* = 3) at the resting membrane potential. The lack of effect of heparin on the voltage dependence of NMDA responses is shown in *D*.

tration (100 ng/ml), but not by another polyanionic glycosamino-glycan, chondroitin sulfate (Fig. 2*A*). Heparin did not influence post-tetanic potentiation or baseline synaptic responses evoked by low-frequency stimulation (0.05 Hz) at the concentration used. Interestingly, selectively 2-O- or 6-O-desulfated heparin did not influence LTP (Fig. 2*B*), indicating that the 2-O-sulfated iduronic acid units as well as glucosamine 6-O sulfate groups of heparin are essential for the inhibition of LTP.

Because induction of LTP in area CA1 is dependent on the activation of the NMDA receptors (Collingridge et al., 1983), we tested the possibility that heparin might have a direct influence on the NMDA receptor activation. NMDA receptor-mediated responses were recorded from CA1 pyramidal neurons with intra-cellular microelectrodes under current clamp in the presence of 10 μM 6-nitro-7-sulfamoylbenzoquinoxaline (NBQX; Tocris Cookson) and 100 μM picrotoxin (Sigma). Heparin had no apparent effect on pharmacologically isolated, NMDA receptor-mediated responses in terms of their voltage dependence or

the response amplitude at the resting membrane potential (Fig. 2*C,D*).

N-syndecan is isolated from hippocampus as a plasticity-associated heparan sulfate proteoglycan

Because the above data pointed to an important role of heparin-type glycans in LTP, we next pursued to identify the HS carrier proteins involved. We chose to use HB-GAM affinity chromatog-raphy to purify plasticity-associated heparan sulfate proteogly-cans (HSPGs) from crude extracts of adult rat hippocampi, be-cause (1) a similar sulfation pattern of heparin is needed for both its binding to HB-GAM (Kinnunen et al., 1996) and for inhibition of LTP, and (2) application of HB-GAM inhibits LTP in the area CA1 (Lauri et al., 1998). In a salt gradient elution, two compo-nents (400 and 200 kDa) with a broad electrophoretic mobility were eluted from the HB-GAM affinity columns at a reasonably high (0.4–0.6 M) NaCl concentration. After nitrous acid cleavage of the glycan side chains, the 200 kDa component was reduced to
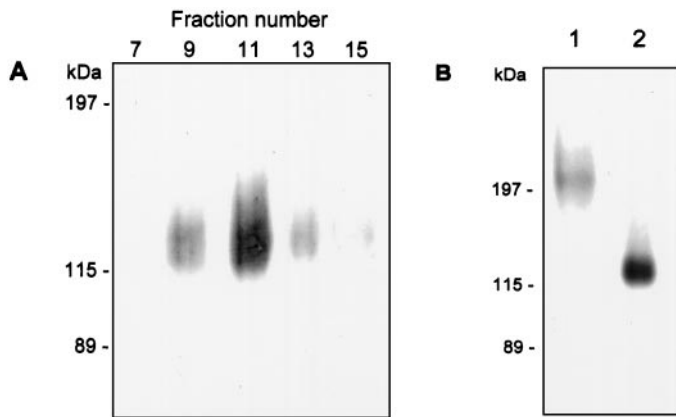
Figure 3. *N*-syndecan is isolated as the major HB-GAM-binding HSPG from adult hippocampus. *A*, Adult rat hippocampi (10 gm wet tissue) were solubilized in octyl glucoside and fractionated by salt gradient elution on HB-GAM–Sepharose. Alcian blue–silver staining was used to detect both proteins and proteoglycans, and it revealed a proteoglycan-type smear in fractions eluting at 0.4–0.6 M NaCl (fractions 9–13). The figure shows a Western blot of fractions deglycosylated by nitrous acid. The immunoblot was stained with affinity-purified antibodies against *N*-syndecan. *B*, Western blot of the hippocampal fractions with anti-*N*-syndecan antibodies showing that heparitinase digestion reduces the 200 kDa proteoglycan (*lane 1*) to a 120 kDa core protein (*lane 2*).

a 120 kDa band and was identified by Western blotting as the core protein of *N*-syndecan (syndecan-3) (Fig. 3*A*), a transmembrane HSPG enriched in neuronal cells (Carey et al., 1992). Significantly, the carbohydrates of hippocampal *N*-syndecan were quantitatively cleaved by the same enzyme (heparitinase) that was used to inhibit LTP (Fig. 3*B*). In contrast, the 400 kDa component did not contain HS but was digested by chondroitinase (data not shown).

To test directly whether *N*-syndecan actually influences synaptic plasticity, its effect on LTP was examined in a set of experiments in which *N*-syndecan was applied into the hippocampal slice. *N*-syndecan was purified from postnatal rat brain (Raulo et al., 1994) and pressure-injected into the CA1 dendritic area close to the recording site. An independent, noninjected pathway was recorded in the same slice to control induction of LTP. Injection of *N*-syndecan blocked HFS-induced LTP without affecting post-tetanic potentiation or baseline synaptic responses (Fig. 4). A number of control injections with physiological saline did not influence LTP (Fig. 4). Given that soluble *N*-syndecan inhibits ligand binding to the endogenous transmembrane proteoglycan at the concentration used (20 μg/ml) (Kinnunen et al., 1996), the above results suggest that *N*-syndecan is an essential part of the machinery supporting activity-evoked neuronal plasticity in the area CA1 of adult hippocampus.

N-syndecan is expressed in the processes of CA1 pyramidal neurons in an activity-dependent manner

In situ hybridization with antisense-*N*-syndecan RNA probes in hippocampal cryosections showed that *N*-syndecan mRNA is expressed in all the pyramidal neurons of the hippocampus proper as well as in the granule cells of the dentate gyrus (Fig. 5*A*). The sense probe used as a control showed no reactivity (Fig. 5*B*). Immunostaining against *N*-syndecan showed that expression of the protein product was strongest in the alveus, in the hilar region of the dentate gyrus, and in the CA3 stratum radiatum. *N*-syndecan immunoreactivity was localized to fiber-like struc-

tures (Fig. 5*C*), which were identified as nonmyelinated neuronal processes by immunoelectron microscopy (Fig. 5*D*).

Several molecules that are involved in activity-induced plasticity show changes in the level or pattern of expression as a consequence of an LTP-inducing high-frequency stimulation (Armstrong and Montiminy, 1993; Silva and Giese, 1994; Ghosh and Greenberg, 1995). Therefore, we tested the effect of LTP induction on expression of *N*-syndecan in hippocampus. High-frequency stimulation (100 Hz/1 sec) of Schaffer collaterals resulted in a long-lasting potentiation of the fEPSP slope in urethane-anesthetized rats (173 $\pm$ 7%; $n = 3$; Fig. 6*A*). Control rats received the same amount of stimulus pulses at low frequency (0.05 Hz). *In situ* hybridization as well as the immunostain against *N*-syndecan was quantified from areas CA1, CA3, and dentate gyrus of 10 sections from control and 20 sections from high-frequency-stimulated hippocampi. The quantified sections were from the same batch of color development reactions, thus comparable with each other. Sample sections of these stainings are shown on Figure 6. Expression of *N*-syndecan mRNA was clearly increased in the stimulated area CA1 4 hr after induction of LTP (stain intensity 261 $\pm$ 19% from control; $p < 0.01$, Student's t test) (Fig. 6*B,C*). *N*-syndecan mRNA expression was also slightly increased in the area CA3 and in the granule cells of dentate gyrus (154 $\pm$ 19% and 159 $\pm$ 8%, respectively), suggesting that neuronal activity in response to electrical stimulation is rather widely distributed in an intact hippocampus. At the protein level, immunoreactivity against *N*-syndecan was most clearly increased in the CA1 stratum radiatum, where neuronal processes were stained with *N*-syndecan antibodies in stimulated animals, but hardly at all in nonstimulated animals (area of *N*-syndecan immunoreactivity 220 $\pm$ 15% from control in area CA1, $p < 0.01$; 146 $\pm$ 16% in area CA3, and 156 $\pm$ 22% in the hilus of dentate gyrus) (Fig. 6*D,E*). In addition to the immunostaining of neuronal fibers, *N*-syndecan immunoreactivity was often found at the periphery of synaptic terminals after induction of LTP (Fig. 6*F*). This might reflect a change in the localization of *N*-syndecan after an HFS. Alternatively, low levels of *N*-syndecan expressed in the synaptic structures of control animals might not be detected by the antibodies. A similar induction of *N*-syndecan expression was also observed *in vitro* in the area CA1 of hippocampal slices 2–4 hr after an LTP-inducing HFS (data not shown).

Hippocampal *N*-syndecan copurifies with fyn-kinase/cortactin in a manner that is increased by an LTP-inducing high-frequency stimulation

Recent biochemical and cell biological studies have shown that the cytosolic tail of *N*-syndecan binds to a tyrosine kinase-active protein complex containing pp60-src, fyn, and the F-actin-binding src substrate cortactin (p80/85) (Kinnunen et al., 1998). Because tyrosine kinase activity is required for synaptic plasticity (O'Dell et al., 1991; Abe and Saito, 1993; Boxall et al., 1996), the presence of src family kinases and their substrates in the affinity-purified hippocampal fractions containing *N*-syndecan was investigated. A strong, herbimycin-sensitive kinase activity copurified with *N*-syndecan from hippocampus (data not shown). Immunoblotting of the *N*-syndecan-containing fractions from two different isolations indicated the presence of an src family tyrosine kinase and cortactin (Fig. 7*A*). Unlike in the developing brain (c.f. Kinnunen et al., 1998), only fyn of the src-type kinases copurified with *N*-syndecan from adult hippocampus (Fig. 7*B*).

Because of the rapid inhibition of LTP by soluble *N*-syndecan and heparin-type glycans, we presumed that fast alterations in the
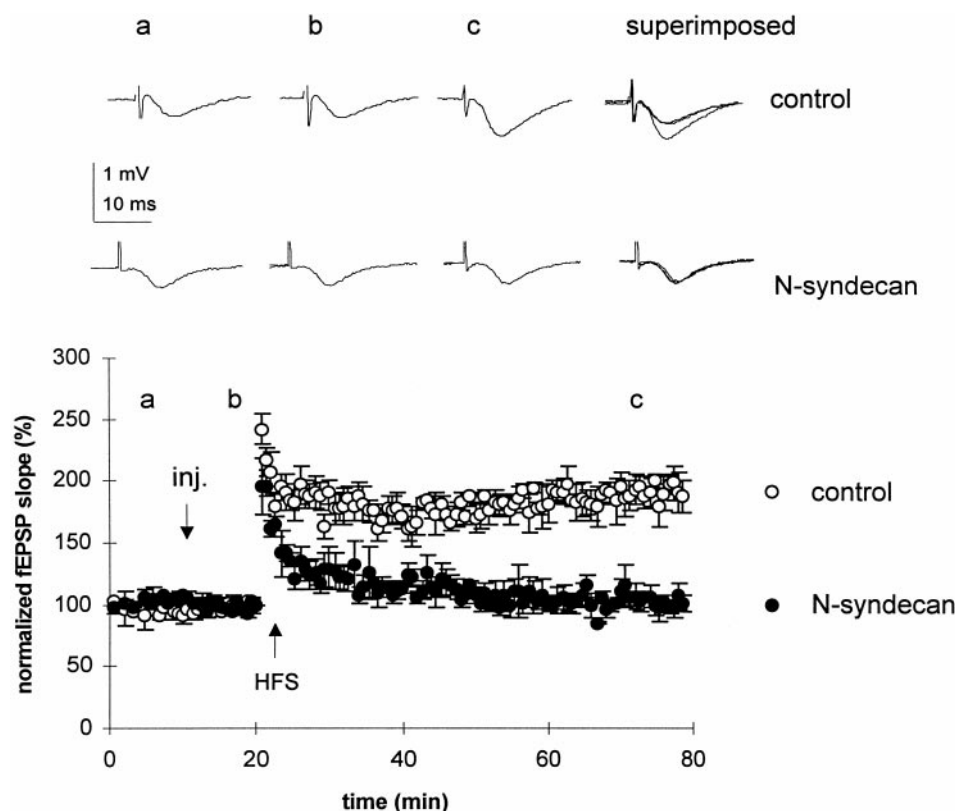
Figure 4. Effect of soluble *N*-syndecan on LTP. After a stable baseline recording, *N*-syndecan (20 μg/ml) (●) was pressure-injected into the CA1 dendritic area close to the recording site (*arrow*). Control injections were performed with saline (○). LTP was induced by HFS 10 min after the injection. The data represents the average ± SEM of six experiments ($p < 0.05$; Student's *t* test). Sample responses before (*a*) and after (*b*) injection, and 30 min after high-frequency stimulation (*c*) are shown.
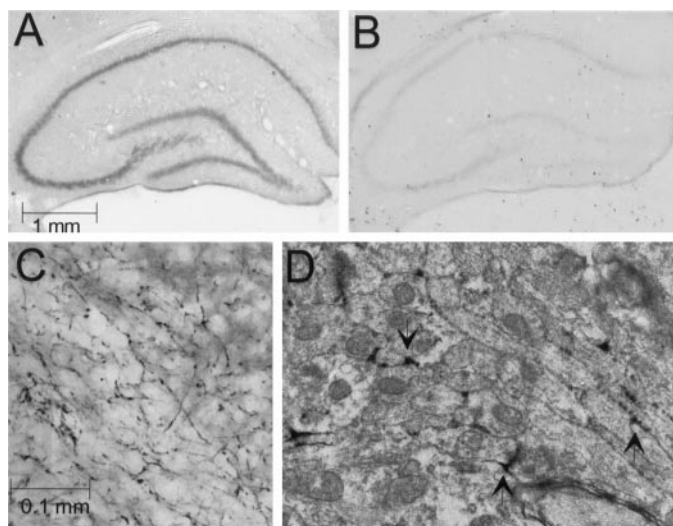

Figure 5. Expression of *N*-syndecan in adult rat hippocampus. *In situ* hybridization with antisense (*A*) and sense (*B*) *N*-syndecan RNA probes in hippocampal sections. Light microscopic (*C*) and electron microscopic (*D*) visualization of *N*-syndecan immunoreactivity in CA3 stratum radiatum. In (*D*), the *arrows* point to anti-*N*-syndecan immunoperoxidase labeling at the surface of neuronal processes (7500× magnification). Parallel control stainings with nonimmune rabbit IgG showed no reactivity (data not shown).

interaction of *N*-syndecan with the intracellular components might take place. To test this premise, *N*-syndecan and the components binding to it were isolated from the area CA1 of hippocampal slices 10 or 20 min after induction of LTP by HB-GAM-Sepharose precipitation (Fig. 8, legend). Interestingly, we found that association of cortactin to *N*-syndecan was repeat-edly increased within 10 min, and even more within 20 min after an LTP-inducing HFS in the area CA1 (Fig. 8*A*,*B*). An increase was seen also in the association between fyn and *N*-syndecan, however, this was significant only 20 min after LTP induction (Fig. 8). The observed changes in the association of *N*-syndecan to cortactin/fyn are in agreement with the hypothesis that endogenous *N*-syndecan is involved in the molecular mechanisms of LTP stabilization.

DISCUSSION

Recent imaging studies have shown an amazingly high mobility of dendritic spines in neuronal cell culture (Fischer et al., 1998), suggesting that morphological rearrangements occurring at a temporal scale of a few minutes could contribute to the mechanisms underlying the expression of LTP. Consistently, manipulation of cell–matrix contacts, which can have a profound influence on cellular morphology, affects already the early steps of LTP. The most rapid effect is caused by antibodies against adhesion molecules of the Ig superfamily (NCAM and L1), which reduce already the post-tetanic potentiation caused by the LTP-inducing high-frequency stimulation (Lüthi et al., 1994). Inhibition of cadherin family-mediated cell adhesion considerably reduces LTP within 10 min, but does not affect the size of post-tetanic potentiation (Tang et al., 1998). Integrin-mediated processes seem to be involved in the later steps of LTP stabilization, because, in contrast to NCAM and cadherins, block of integrin-mediated adhesion reduces LTP also when applied after the LTP-inducing stimulus (Bahr et al., 1997; Stäubli et al., 1998).

The data presented here indicate that heparin-type glycans, a group of molecules involved in the modulation of cell–matrix association, have a critical role in activity-dependent synaptic plasticity. Modulating interactions of endogenous components with heparan sulfates by enzymatic removal of HS or by addition
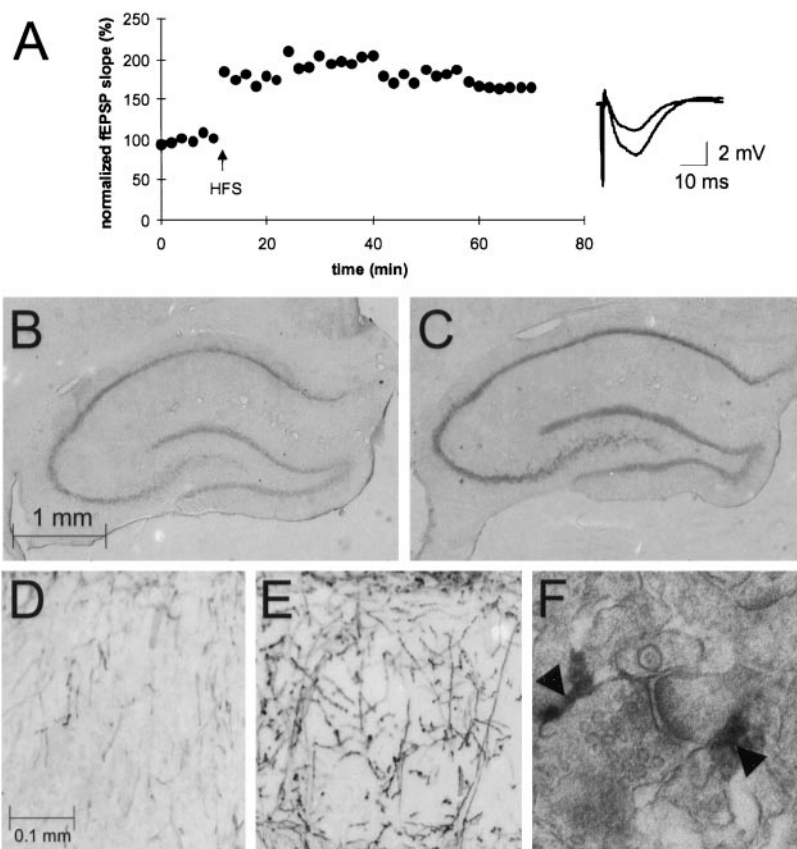
Figure 6. Changes in the expression of _N_-syndecan after LTP induction. _In vivo_ recording showing the effect of 100 Hz/sec Schaffer collateral stimulation on the fEPSP slope. Sample fEPSPs before and 1 hr after the HFS are shown on the _right_ (_A_). _In situ_ hybridization with antisense _N_-syndecan probes in hippocampus of a control animal (_B_) and after induction of LTP (_C_). Note that these sections are not comparable to Figure 5 because of different duration of color development. Light microscopic pictures from the area CA1 showing a low level of _N_-syndecan immunoreactivity in control animals (_D_) and the enhanced staining of neuronal fibers after HFS (_E_). Electron microscopic micrograph showing _N_-syndecan immunostaining at the periphery of presynaptic and postsynaptic structures (_arrows_) in the CA1 dendritic area of a stimulated hippocampus (_F_) (15,000× magnification).
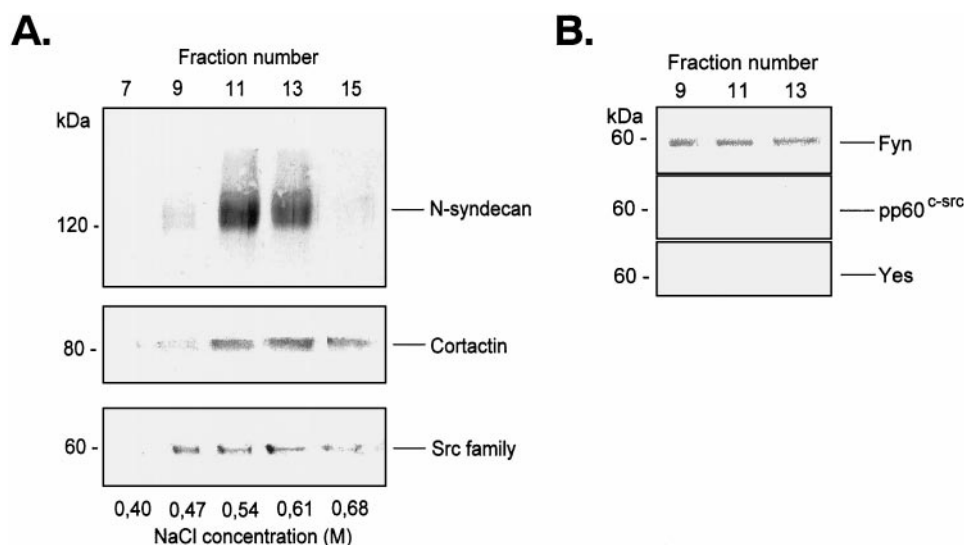

Figure 7. Hippocampal _N_-syndecan copurifies with cortactin and the tyrosine kinase fyn. _A_, Western blot of the HB-GAM affinity-purified hippocampal extracts showing copurification of _N_-syndecan, cortactin, and an src family kinase to fractions 9–13. The polyclonal antibody against src family (SRC-2) recognizes fyn, c-src, and yes-kinases. _B_, Immunoblotting of the fractions with monoclonal antibodies detects fyn but no other src family kinases in the _N_-syndecan containing hippocampal fractions.

of soluble heparin-type glycans inhibited LTP. These manipulations had no effect on the baseline synaptic responses or on the immediate post-tetanic potentiation after HFS, indicating that heparin-dependent interactions are specifically needed for the stabilization of long-term potentiation.

HSs are thought to act by regulating the assembly of active signaling complexes at the cell surface by interacting with several molecules (e.g., NCAM, FGFs, and HB-GAM) (Fig. 9). In cell culture, soluble heparin inhibits the biological activity of HB-GAM (already at a concentration of 0.1 μg/ml) (Kinnunen et al., 1996) as well as adhesion mediated by NCAM (100 μg/ml) (Cole

et al., 1986), while it enhances activation of FGF receptors in a manner that is concentration-dependent (Rapraeger, 1993; Lindahl et al., 1994). The cellular effects of soluble heparin are profoundly affected by its sulfation pattern (Maccarana et al., 1993; Kinnunen et al., 1996). In the present experiments, the requirements in the sulfation of heparin for inhibition of LTP were similar to those reported previously for its binding to HB-GAM (Kinnunen et al., 1996). Although the structural requirements in heparin are somewhat different for its binding to HB-GAM and basic fibroblast growth factor (bFGF), these proteins can act as competitive ligands for HS (shown for _N_-syndecan, see
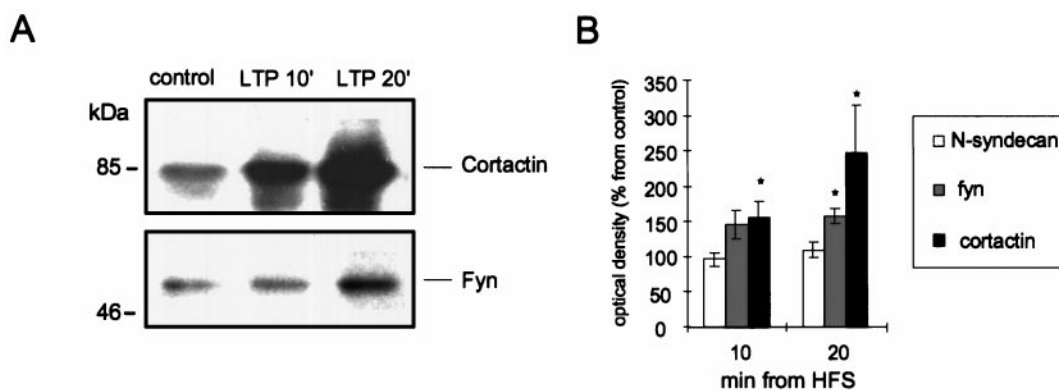
Figure 8. Association between *N*-syndecan and cortactin/fyn is increased by an LTP-inducing HFS. *A*, Western blot from HB-GAM–Sepharose-precipitated extracts from the area CA1 of hippocampal slices, which have been maintained under control conditions or in which LTP has been induced by HFS. *N*-syndecan and components associated to it were displaced from HB-GAM–Sepharose by heparin (10 μg/ml). The amount of cortactin was markedly increased after 20 min but already increased 10 min after HFS. An increase in the amount of *N*-syndecan-associated fyn was also detected. *B*, Quantification of *N*-syndecan, cortactin, and fyn from immunoblots of four independent experiments. *N*-syndecan was detected similarly in the samples from control and stimulated slices (*$p < 0.05$; Student's *t* test).
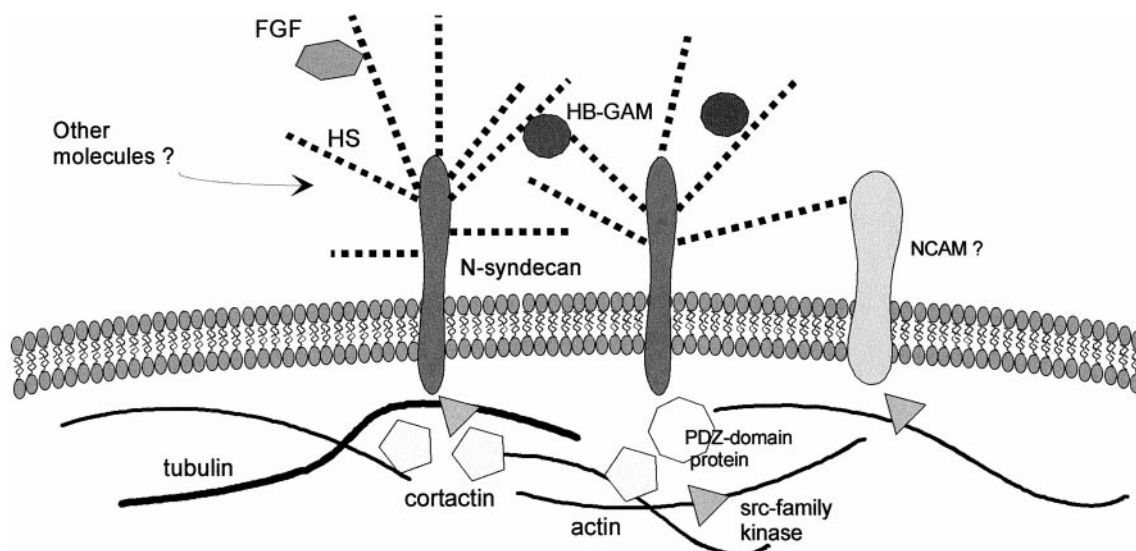

Figure 9. A schematic picture depicting interactions of *N*-syndecan with intracellular and extracellular molecules in the regulation of neuronal plasticity.

Raulo et al., 1994). In hippocampus, application of HB-GAM and antibodies against NCAM inhibit LTP (Lüthi et al., 1994; Lauri et al., 1998), and FGF enhances LTP (Seifert et al., 1990; Ishiyama et al., 1991). Interestingly, NCAM antibodies affect post-tetanic potentiation, but FGF, HB-GAM, heparin, and *N*-syndecan do not. It appears that HSs can regulate LTP through multiple ligand interactions and that the cellular consequences of an HFS might be critically dependent on the balance between different heparin-binding molecules available.

Heparan sulfate-containing extracellular proteins reported to be expressed in hippocampus include at least agrin (O'Connor et al., 1995) and syndecan-2 (Hsueh et al., 1998). Agrin is a key element inducing postsynaptic as well as presynaptic differentiation in the developing neuromuscular junction (Gautam et al., 1996; Kleiman and Reichardt, 1996). In the CNS, agrin expression is developmentally regulated and enhanced by neuronal activity (O'Connor et al., 1995; Cohen et al., 1997), suggesting a role for agrin in the differentiation of central synapses. In a recent publication by Hsueh et al. (1998), syndecan 2 was reported to be expressed in adult hippocampal neurons and to localize to syn-

aptic structures. In the present study, however, *N*-syndecan (syndecan-3) was identified as a prominent heparan sulfate proteoglycan in hippocampus. On anti-heparan sulfate immunoblots, a smear-like band comigrating with *N*-syndecan was the major HS-containing component in adult hippocampal tissue (data not shown). Other bands of the sizes of ~400 and 80 kDa were also recognized, these might represent agrin-type proteins and syndecan-2. Additional HS-containing proteins might be expressed in amounts too low to be detected by this method. Nevertheless, *N*-syndecan seems to represent the most prominent cell–surface HS carrier protein in adult hippocampus.

N-syndecan is a transmembrane HSPG, which binds bFGF (Chernousov and Carey, 1993) and has been proposed to regulate neurite growth and axonal guidance in the developing nervous system by acting as a receptor or co-receptor for HB-GAM (Raulo et al., 1994; Nolo et al., 1995; Kinnunen et al., 1996). In addition, *N*-syndecan can rapidly mediate localization of mRNA to cell processes in response to extracellular matrix contact in a manner that is dependent on tyrosine kinase activity (Fages et al., 1998). Although the total amount of *N*-syndecan in adult brain is

low (Carey et al., 1992; Nolo et al., 1995), we found that a high regional expression of both *N*-syndecan mRNA and its protein product is maintained in the adult hippocampus, and this expression is enhanced by an LTP inducing HFS. Furthermore, rat brain *N*-syndecan inhibited LTP when applied in a soluble form to the CA1 dendritic area, consistently with the idea that *N*-syndecan is part of the machinery supporting synaptic plasticity in hippocampus.

Approximately half of the apparent molecular mass of *N*-syndecan is comprised of heparan sulfates, which are exceptionally heparin-like in their structure, especially because of their very high proportion of both 2–0- and 6–0-sulfated monosaccharide residues (Kinnunen et al., 1996). The heparin-type glycan structure of *N*-syndecan appears biologically important because both selective 2–0-desulfation and 6–0-desulfation destroyed the ability of glycans to inhibit LTP. Brain *N*-syndecan is more heterogeneous in its carbohydrate structure than heparin, for which reason a higher concentration of soluble *N*-syndecan than heparin is required for specific inhibition of HS-dependent cellular processes. Interestingly, the concentrations of *N*-syndecan and heparin that were found to inhibit LTP are quite similar to those inhibiting HB-GAM-induced neurite outgrowth in forebrain neurons (Kinnunen et al., 1996). Furthermore, both the HB-GAM-induced neurite outgrowth (Rauvala et al., 1994) and LTP are inhibited by the heparinase that hydrolyzes brain *N*-syndecan. These findings are compatible with the view that *N*-syndecan interacts with HB-GAM/FGF-type ligands to regulate LTP.

In cultured brain neurons, *N*-syndecan-mediated neurite extension is dependent on the interaction of the cytosolic tail of *N*-syndecan with src kinase/cortactin (Kinnunen et al., 1998). In adult hippocampus, *N*-syndecan was associated to cortactin and the tyrosine kinase fyn. In addition, an ~100 kDa protein, detected in Western blotting by a monoclonal antibody against the PDZ domain, coprecipitated with *N*-syndecan as well as cortactin from hippocampal extracts (S. Lauri, T. Kinnunen, and H. Rauvala, unpublished results). The molecular size of this protein matches that of a recently identified syndecan-binding protein CASK/LIN-2 (Hsueh et al., 1998) that belongs to the PDZ domain-containing family of guanylate kinases that are thought to act as a scaffold at the membrane and coordinate localization of multiple proteins in the synaptic structures (Sheng, 1996; Craven and Bredt, 1998). The association of cortactin/fyn to *N*-syndecan increased already 10 min after an LTP-inducing stimulation, and, because shorter time periods were not screened, it is possible that the association was enhanced even faster. Determination of the causal relation between expression of LTP and the post-tetanic increase in the size of the intracellular complex associating to *N*-syndecan warrants further studies. However, the change in the association as such suggests that *N*-syndecan mediates transmembrane signals between extracellular heparin-binding molecules and src-type kinases after induction of LTP. The involvement of src family kinases in synaptic plasticity is supported by the recent findings showing that src activity is increased by HFS, and activation of endogenous src causes synaptic potentiation (Lu et al., 1998). Furthermore, Grant et al. (1992) have reported that fyn −/−, but not c-src-deficient mice have a specific defect in hippocampal structure and loss of LTP.

Tyrosine phosphorylation by src family kinases can directly influence the function of NMDA- and AMPA-type glutamate receptors (Yu et al., 1997; Lu et al., 1998). In addition, src-type kinases are proposed to regulate cellular morphology by phos-

phorylating cytoskeleton-associated components (Thomas et al., 1995; Lowell and Soriano, 1996). One of these src substrates is cortactin, which modulates cross-linking of filamentous actin in cell processes in a manner that is dependent on phosphorylation state of cortactin (Huang et al., 1997). Cortactin is also known to accumulate at developing neuromuscular synapses (Peng et al., 1997). The activity-dependent coupling between *N*-syndecan and cortactin suggests that *N*-syndecan participates in the mechanisms of cytoskeletal reorganization on induction of LTP and provides evidence for a novel mechanism connecting neuronal activity to the modulation of neuronal connectivity (Fig. 9).

A pathological overexpression of HSs has been observed in the brains of Alzheimer's patients. Biochemical studies have shown that HS protects Aβ peptide from degradation by proteases (Gupta-Bansal et al., 1995). On the other hand, Aβ (1–40) inhibits the function of heparanases (Bame et al., 1997), providing an explanation for the accumulation of both HS and amyloid in the nervous system of the Alzheimer's patients. Taken together, the present findings suggest important implications for HSs in the neuronal dysfunction observed in Alzheimer's disease.

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