Versican G3 Domain Regulates Neurite Growth and Synaptic Transmission of Hippocampal Neurons by Activation of Epidermal Growth Factor Receptor

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Versican is one of the major extracellular matrix (ECM) proteins in the brain. ECM molecules and their cleavage products critically regulate the growth and arborization of neurites, hence adjusting the formation of neural networks. Recent findings have revealed that peptide fragments containing the versican C terminus (G3 domain) are present in human brain astrocytes. The present study demonstrated that a versican G3 domain enhanced cell attachment, neurite growth, and glutamate receptor-mediated currents in cultured embryonic hippocampal neurons. In addition, the G3 domain intensified dendritic spines, increased the clustering of both synaptophysin and the glutamate receptor subunit GluR2, and augmented excitatory synaptic activity. In contrast, a mutated G3 domain lacking the epidermal growth factor (EGF)-like repeats (G3ΔEGF) had little effect on neurite growth and glutamatergic function. Treating the neurons with the G3-conditioned medium rapidly increased the levels of phosphorylated EGF receptor (pEGFR) and phosphorylated extracellular signal-regulated kinase (pERK), indicating an activation of EGFR-mediated signaling pathways. Blockade of EGFR prevented the G3-induced ERK activation and suppressed the G3-provoked enhancement of neurite growth and glutamatergic function but failed to block the G3-mediated enhancement of cell attachment. These combined results indicate that the versican G3 domain regulates neuronal attachment, neurite outgrowth, and synaptic function of hippocampal neurons via EGFR-dependent and -independent signaling pathway(s). Our findings suggest a role for ECM proteolytic products in neural development and regeneration.

During brain development or neural regeneration, the extracellular matrix (ECM) is dynamically cleaved by matrix metalloproteinase (1). The ECM molecules and their proteolytic products, via communications with their interacting partners (2), fundamentally regulate growth and arborization of neurite processes of neuronal cells, thus adjusting the formation of functional neuronal networks. Chondroitin sulfate proteoglycans (CSPGs) are the major ECM components in the brain and are organized into perineuronal nets. Expression of CSPGs inhibits experience-dependent neural plasticity, whereas their degradation reactivates neural plasticity (3), suggesting a critical role for CSPG metabolism in neural development and plasticity (4). However, the underlying mechanism for the functions of CSPG metabolites remains largely unclear.

Versican, one type of CSPG molecule, was originally isolated from human fibroblasts and developing limb buds in the chicken (5, 6). Later this molecule was found to be highly expressed in the developing and matured mammalian brain (7, 8). Four isoforms of versican (V0, V1, V2, and V3) have been identified in various tissues (7, 9). V0, the full-length versican, contains both N- (G1) and C-terminal globular domains (G3), together with a large central CS region that is encoded by two exons producing CSα and CSβ domains (Fig. 1A). The exons of CSα and CSβ can be alternatively spliced, thus generating three splice variants of versican; V1 lacks the CSα domain, V2 is devoid of the CSβ domain, and V3 contains neither CS domain. Studies have demonstrated that the tandem repeats in the G1 domain mediate binding of versican to hyaluronan (10, 11), whereas the central CS exons contain sites for glycosaminoglycan modification (12). More specifically, the G3 domain consists of two epidermal growth factor-like sequences, a carbohydrate recognition domain, and a complement binding protein-like motif that is structurally similar to the selectin family (Fig. 1A). The expression levels of versican V1 are high in the embryonic brain (8), whereas V2 is the dominant isoform within the mature central nervous system (8, 9). This suggests distinct roles for different versican isoforms during brain development. With respect to this notion, various studies have dem-

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Expression and Purification of Versican G3 Domains—The construction of recombinant versican G3 and a G3 fragment lacking the EGF-like motifs (G3ΔEGF) (Fig. 1A) has been described previously (16, 19). Briefly, cDNAs corresponding to the G3 and G3ΔEGF domains of chicken versican (6) were subcloned in a mammalian expression vector (pcDNA3). The leading peptide of link protein (nucleotides 1–180) was joined with the EGF domains to allow secretion of the EGF peptides. The purified versican peptides from the culture medium were determined by immunoblot with the antibody 4B6. Ctrl, control. C, the constructs of G3 and the vector (Vec.) were expressed in primary cortical astrocytes. The expression of G3 in the cell lysate and conditioned medium was also confirmed by immunoblot with the antibody 4B6.

The purity of G3 and G3ΔEGF peptides were analyzed on SDS-PAGE and Western blots (Fig. 1B) probed with the monoclonal antibody 4B6 that recognizes an epitope in the leading protein (21, 22). To study the effects of the versican G3 peptides on neuronal morphology and function, the peptides were added into the media at different time points or coated on the culture dishes/coverslips (see below).

Versican G3-conditioned Medium—Primary cultures of cortical glial cells were made following a modified protocol described previously (23). Briefly, cells in the embryonic rat cortex were dissociated by mechanical trituration. The cortical cells were plated in dishes (Falcon) at a density of 5 × 10⁴ cells/cm² and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. On the 14th day in vitro (DIV), the cells were stripped and replated for further culture. Under these conditions, only glial cells continued to proliferate. At 90% confluence, the glial cells were transiently transfected with G3 or vector, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. One day after the transfection, the medium was replaced with fresh B27-supplemented neurobasal medium (Invitrogen). The conditioned media were collected 3 days after cell transfection. The expression of G3 in the cortical glial cells and the secreted G3 in the conditioned medium were confirmed by immunoblot using 4B6 (Fig. 1C). According to our previous results (16), the concentration of the G3 products in the media was ∼1.0 μg/ml.

Coating Culture Dish/Coverslip with the Versican Peptides—The 12-well plastic dishes (Nunc, Roskilde, Denmark) that contain an 18-mm-diameter glass coverslip or 35-mm dishes were filled with the purified G3 solution or the protein purification buffer serving as a control. The peptide and control solutions were left in the dishes overnight (for about 15 h) at 4 °C and removed on the next morning. Following a rinse with PBS, the glass coverslips and dishes were then recoated with poly-d-lysine (PDL). For another set of experiments, dishes/coverslips were coated with PDL alone.

ELISA—The amount of G3 peptide bound to coverslips was determined by ELISA (colorimetric) assay as previously described (24) with modifications. In brief, after removing the G3 peptide, the coverslips were gently rinsed with PBS and then blocked with 2% bovine serum albumin at 4 °C for 1 h. The coverslips were incubated with the monoclonal antibody 4B6 overnight. Afterward, the coverslips were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Amersham Biosciences), horseradish peroxidase substrate o-phenylenediamine dihydro-chloride (Sigma-Aldrich) was added to produce a color reaction. The optical density was measured using a spectrophotometer (Ultrospec 1000; Amersham Biosciences).

Preparation and Treatment of Cultured Hippocampal Neurons—Dissociated embryonic hippocampal neurons were cultured on coverslips/dishes that were coated in different materials as described above. The procedures for cell dissociation and culture were described previously (25). Briefly, hippocampal cells from Wistar rat embryos at day 18 were isolated by mechanical trituration and then plated in control and G3-coated dishes at a density of 6 × 10⁴ cells/cm² in standard plating medium, which contains neurobasal medium (Invitrogen), B27 (1:50, Invitro-
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To examine the effect of versican peptides on neuronal cell attachment, morphology, and function, versican peptides were included in the plating medium at a final concentration of 50 ng/ml. The neurons were incubated at 37 °C in an atmosphere containing 5% CO2. Eighteen hours after plating, the plating medium was replaced with culture medium containing neurobasal medium supplemented with B27 (50:1) and 1-glutamine (0.5 mM). This culture medium supports neuronal growth while restricting the expansion of other cell types (26). To separate the effect of G3 peptides on cell attachment from the effect on neuronal morphology and function, the peptides (50 ng/ml) were added in the culture medium on the 3rd DIV. In some experiments, the specific EGFR inhibitor AG1478 (Calbiochem, San Diego, CA) was included in the plating or culture medium. Neurons were used after transfection, the neurons were washed with DPBS and fixed with 3.7% paraformaldehyde mixed with 4% sucrose in DPBS. Images of randomly selected individual GFP-expressing neurons on control and G3-coated coverslips were taken (in each group, n ≥ 30 cells) using a confocal microscope (Carl Zeiss) at 63× and/or 100× magnifications.

The digital images of neurites and dendritic spines from randomly selected image fields were analyzed using the Image J program (National Institutes of Health). For analyzing the neurite length of neurons 18 h after plating, the longest neurite of an individual neuron was measured. For 7th DIV neurons, the neurite density was semi-quantitatively analyzed by measuring the neurite-covered area (pixels). In some experiments, the fluorescent area of individual GFP-expressing neurons was analyzed. To determine the density of dendritic spines, the length of randomly selected secondary dendrites of GFP-expressing neurons was measured, the number of spines on the dendrites was counted, and the averaged number of spines/20-μm length of neurite was determined.

**Analysis of Neurite Growth and Dendritic Spines**—To examine the short (18th h) and long (7th DIV) term effects of versican peptides on the growth of neurites and dendritic spines, digital images of neurons in different conditions were taken using an inverted light microscope (Carl Zeiss, Göttingen, Germany) equipped with a digital camera (Nicon Coolpix 4500). For studying the growth of dendritic spines, neurons (7th DIV) were transfected with a green fluorescent protein (GFP)-expressing plasmid using Lipofectamine 2000. Twenty-four hours after transfection, the neurons were washed with DPBS and fixed with 3.7% paraformaldehyde mixed with 4% sucrose in DPBS. Images of randomly selected individual GFP-expressing neurons on control and G3-coated coverslips were taken (in each group, n ≥ 30 cells) using a confocal microscope (Carl Zeiss) at 63× and/or 100× magnifications.

**Immunoblot**—To test the effect of G3 on the expression of synaptic and intracellular signaling proteins, we treated neurons with the G3-conditioned medium at different time points. In some dishes, 0.5, 5.0, and 20 μM AG1478 were included in the medium 30 min before and during the G3 stimulation. The vector-conditioned medium was used as a control. For the assays of synaptic proteins, we treated the neurons with the conditioned media 24 h after plating, and the neuronal cells were used on the 7th DIV. The general procedures of Western blotting of neuronal proteins were the same as previously described (25). The following commercially available antibodies were used: anti-NR1 (BD Bioscience, Franklin Lakes, NJ), anti-PSD-95 (Affinity BioReagents, Golden, CO), anti-synaptophysin (Synaptic Systems, Göttingen, Germany), and anti-β-actin (Sigma-Aldrich). Semi-quantitative analysis of Western blot was performed by means of a GS800 densitometer (Bio-Rad). The blot films were scanned, and the band densities were calculated using the Quantity One program (Bio-Rad). In some experiments, the values of blot densities were normalized to the levels of respective β-actin blots.

**Immunocytochemistry**—The procedure for immunohistochemistry was as previously described (25) with modifications. For immunostaining the surface GluR2 subunit, anti-GluR2 (Chemicon, Temecula, CA; 1:2000) was added to the culture medium and incubated with neurons in the incubator for 2 h. Neurons on coverslips were then fixed with 3.7% paraformaldehyde and 4% sucrose in DPBS for 30 min. To stain intracellular GluR2, synaptophysin and/or MAP2 (microtubule-associated protein 2), neurons were permeabilized in 0.1% Triton X-100 for 15–30 min, blocked in 5% horse serum or 5% bovine serum albumin for 1 h, and then incubated with anti-GluR2, anti-synaptophysin (Clone 7.2; Synaptic Systems; 1:1000 dilu-
tion), or anti-MAP2 (Chemicon; 1:400) at room temperature for 2 h or at 4 °C overnight. CY3- or fluorescein isothiocyanate-conjugated secondary antibodies were added for incubation at 4 °C for 1 h. After rinsing with DPBS, the coverslips were mounted for confocal microscopic examination. The visual fields under a confocal microscope (Carl Zeiss) were randomly selected by blindly moving the cell culture coverslip. Digital images were taken with a 63× or 100× objective lens. To determine the densities of synaptic clusters in control and treated neurons, the length of randomly selected dendrites was measured via Image J, and the number of the immunoreactive protein clusters was counted. Thus, the number of protein clusters/20-μm length of dendrite was obtained. Twenty to forty individual neurons in each group were analyzed.

Statistical Analysis—Unless specified otherwise, statistical analysis was performed with Sigmaplot software (SPSS, Chicago, IL). The data were expressed as the means ± S.E. and examined using Students’ unpaired or paired t tests whenever appropriate. A p value <0.05 was considered significant.

RESULTS

The Versican G3 Domain Promotes Neural Cell Attachment and Neurite Growth—As previously reported (27), we found that more than 98% of embryonic hippocampal cells grown in the described culture conditions were neurons based on their morphology and their immunoreactivity to MAP2, a neuronal dendritic marker (not shown). With respect to the morphology of developing neurons, the neurites were seen in most neuronal cells by the 18th h after plating (left panel of Fig. 2A). Given that the versican G3 domain increases the adhesion in several cell types (28, 29), we initially investigated the effects of the versican peptides on the attachment and outgrowth of neurites. Notably, inclusion of the G3 or G3ΔEGF peptide (50 ng/ml) in the plating medium (Ctrl), in medium with purified G3 peptide (G3), and in medium with the purified G3ΔEGF solution (G3ΔEGF), B, plotted is the averaged number of attached cells/image field under different plating conditions (control, 19 ± 2; n = 8 image fields; G3, 56 ± 3; n = 10; **, compared with control, p < 0.01; G3ΔEGF, 35 ± 4; n = 10; *, compared with control, p < 0.05; #, compared with G3, p < 0.05). C, plot summarizes the length of the longest neurite in neurons grown in different media (control, 29 ± 5; n = 19 neurons; G3, 66 ± 10; n = 45; *, p < 0.05; G3ΔEGF, 34 ± 6; n = 36). D, plot is the result of ELISA of G3 immunoreactivity (arbitrary unit) on coverslips (control, 0.33 ± 0.33; G3, 4.4 ± 0.86, p < 0.01), demonstrating that G3 could be firmly coated on the coverslip/dish. E, shown are the 18-h neurons on dishes coated with PDL alone (control) or together with the G3 peptide. F, shown is the averaged number/image field of attached cells on control and G3-coated coverslips (control, 21 ± 2; n = 7 image fields; G3, 27 ± 3; n = 11); *, p < 0.05). G, the bar graph summarizes the averaged length of the longest neurites of individual neurons on control and G3-coated dishes (control, 59.2 ± 6.6 μm, n = 26 neurons from four dishes of two batches of culture; G3, 134.4 ± 8.6 μm, n = 26; **, p < 0.01). H, example images of individual GFP-expressing neurons on the control and G3-coated coverslips. Note the difference in neurite densities between the two conditions. I, plotted is the averaged fluorescent area of individual GFP neurons grown on the control and G3-coated coverslips (control, 1751 ± 123 pixels/field, n = 21 neurons; G3, 3268 ± 309 pixels/field, n = 21; **, p < 0.01). J, confocal images illustrate the fine neurite structures of GFP-expressing neurons on the control and G3-coated coverslips on the 8th DIV. The neurite structures within the rectangle of the representative neurons is enlarged and shown below. K, shown are the numbers of spines/20 μm of neurite on the control and G3-coated dishes (control, 0.9 ± 0.2, n = 30 neurons; G3, 2.5 ± 0.3, n = 30; ***, p < 0.0001).
The G3 peptide selectively enhances glutamate-evoked currents. A, panel 1, typical traces of glutamate (100 μM) currents evoked in neurons grown in control (Ctrl) medium, medium containing G3 or medium containing G3ΔEGF 18 h after plating. Panel 2, plot summarizing the peak amplitude of glutamate currents recorded 18 h after plating from neurons cultured under different conditions (control, 7.0 ± 1.5 pA, n = 6 neurons; G3, 52 ± 10 pA, n = 5; **, p < 0.01; G3ΔEGF, 10 ± 3 pA). B, panel 1, representative traces of glutamate-evoked currents recorded from neurons (8th DIV) in dishes coated with PDL alone (control) or together with G3. Panel 2, plot is the normalized glutamate currents (standardized to the averaged peak amplitude of currents recorded in control neurons; G3, 154 ± 21% of control, n = 6; *, p < 0.05). C, the plot shows the values of normalized peak currents that were evoked by NMDA (I_{NMDA}, G3, 161 ± 20% of control, n = 14; *, p < 0.05). D, panel 1, shown are the representative traces of GABA (100 μM)-evoked currents in neurons (8th DIV) grown in control and G3-coated dishes. Panel 2, plotted are the values of normalized amplitude of GABA currents (I_{GABA}). E, shown are example traces of glutamate current (panel 1), as well as the values of membrane capacitance (panel 2, control, 15.8 ± 1.4 pF, n = 6 neurons; G3, 24.1 ± 1.3 pF, n = 6; *, p < 0.05), current amplitude (panel 3, control, 113 ± 37 pA, n = 6; G3, 492 ± 43 pA, n = 6; **, p < 0.001), and current capacitance ratio (pA/pF, panel 4, control, 6.5 ± 1.5 pA/pF, n = 6; G3, 20.1 ± 1.3 pA/pF, n = 6; **, p < 0.001) obtained from neurons (2nd DIV) grown in control and G3-coated dishes.

The action of the G3 peptide on neuronal morphogenesis led us to examine whether this peptide played a role in regulating neuronal function, specifically the activity of transmitter receptors. Glutamate is the major excitatory neurotransmitter in the brain. Thus, we began by measuring glutamate-evoked currents at the 18th h after plating in neurons grown in control medium or medium containing G3 or G3ΔEGF peptide (Fig. 3A,panel I). The peak amplitude of glutamate-evoked currents was approximately four times larger in G3-treated neurons than in...
in neurons grown on the G3-coated dishes was significantly larger than controls (Fig. 3B, panel 2). Similarly, the N-methyl-d-aspartate (NMDA; 100 μM)-evoked current was also enhanced in neurons cultured on G3-coated dishes (Fig. 3C). Moreover, in the presence of 2 μM MK801 (an NMDA subtype glutamate receptor antagonist), the glutamate current in the G3-treated neurons was still larger than the controls (148 ± 18.5% of control, n = 4). These combined data indicated that the function of both the NMDA and non-NMDA subtypes of glutamate receptors were up-regulated by G3. In contrast, GABA-evoked currents were unaffected by G3 (Fig. 3D, panels 1 and 2).

The G3-induced enhancement of glutamate currents could result from increased expression of functional glutamate receptors or might simply reflect the enlarged dimension of cells as a result of neurite growth. To investigate the underlying mechanism, we measured the membrane capacitance, an index of cell size, while examining the glutamate current in the same neuron on the 2nd DIV (about 18–24 h after plating; Fig. 3D, panel 1). The neurons were used at the 2nd DIV because their neurites were relatively short (Fig. 2, A and E), allowing a fairly accurate measurement of membrane capacitance. The experiments revealed that the capacitance of neurons on the G3-coated dish increased about 50% in comparison with the controls (Fig. 3E, panel 2), whereas the glutamate currents in the G3-treated neurons was four times larger than in controls (Fig. 3E, panel 3). Thus, the “density” (pA/pF) of glutamate currents in the G3-treated neurons increased dramatically (Fig. 3E, panel 4), suggesting more functional receptors on the cell surface.

To investigate whether the G3-enhanced neurite growth and receptor function are secondary to the increase in cell density, we added G3 directly to existing cultures on the 3rd DIV, thus avoiding effects on cell attachment. Remarkably, the G3 treatment still increased neurite growth (Fig. 4, A and B) and glutamate currents (Fig. 4, C and D) by the 7th DIV. This indicates...
that the effect of G3 on neuronal growth and function is not simply due to the increase in cell density in the culture dishes.

The Versican G3 Up-regulates Neurite Growth and Neuronal Function by Activation of EGFR—The EGFR-like domains of some ECM proteins transduce signals for cell growth and differentiation (31). We proposed that the G3 domain of versican regulated neural growth and function possibly via mechanisms associated with its EGF motifs, because G3-EGF lacked these actions (Figs. 2A and 3A). To test this, we included the selective EGFR antagonist AG1478 (0.5 and 5.0 μM) in the medium when plating cells on the G3-coated dishes and examined the cell morphology and function at the 18th h after plating. We found that AG1478 did not interfere with the G3-induced increase in cell attachment (Fig. 4E; cell number/field in control, 19 ± 2.7; G3, 56 ± 3.8; G3+AG1478, 50 ± 4.5; p > 0.05) but drastically suppressed the G3-induced enhancement of neurite outgrowth (Fig. 4, E and F). Moreover, AG1478 significantly suppressed the G3-induced enhancement of glutamate currents (Fig. 4, G and H). These findings suggest that versican G3 modulates neuronal growth and function, at least partially, by activating EGFR.

To test this concept further, we examined whether activating EGFR enhances neural growth and function. Notably, the addition of 50–100 ng/ml EGF in the culture medium for 18 h significantly increased neurite growth (Fig. 5, A and B), and glutamate-evoked currents (control, 74 ± 19 pA, n = 6 neurons; EGF, 188 ± 47 pA, n = 6; p < 0.05). In addition, immunoblots revealed that the levels of phosphorylated EGFR (pEGFR), but not the total EGFR, rose drastically 30 min after adding EGF (20 ng/ml). The effect of EGF was completely blocked by 20 μM AG1478 (Fig. 5C). These results confirmed that EGFR was expressed in hippocampal neurons under our culture conditions and that activation of EGFR up-regulated neural growth and functions.

We next examined whether versican G3 could activate EGFR. Treating the neurons with G3-conditioned medium for 5–10 min increased the levels of pEGFR, whereas the total EGFR remained stable (Fig. 5D). Moreover, the levels of phosphorylated ERK (pERK), but not the total ERK, rose drastically 30 min after the G3 treatment (Fig. 5E). The activation of EGFR by G3 was transient, returning to control levels within 30 min (Fig. 5D). In contrast, the G3-activated ERK activity lasted much longer, because the levels of pERK remained high 3 h after treatment with G3 (Fig. 5E). Importantly, the G3-induced augmentation of pERK was greatly suppressed by 5.0 μM AG1478 (Fig. 5F). These results demonstrated that the G3 domain of versican was able to activate the EGFR-ERK pathway in hippocampal neurons.

The Versican G3 Domain Promotes Glutamatergic Synaptic Formation and Transmission—The G3-induced increase in the density of glutamate receptor-mediated current (Fig. 3E) strongly suggests an increase in the surface expression of these
receptors. We therefore studied the expression of the glutamate receptor subunits GluR2 and NR1 in neurons grown in control and G3-coated dishes on the 11th DIV (Fig. 6). We demonstrate that coating the dishes with the G3 peptide increases the number of GluR2 subunit clusters on the cell surface (Fig. 6, A and B). Using immunoblot analysis, we found that the total protein levels of NR1 subunits also increased in neurons treated with the G3-conditioned medium (Fig. 6C).

The increase in dendritic spines and glutamate receptor clusters in G3-treated neurons suggests an increase in synapse formation. We therefore employed immunocytochemistry to analyze the expression profile of the presynaptic protein synaptophysin. Remarkably, the number of synaptophysin clusters juxtaposed with dendrites was greatly increased in neurons on G3-coated dishes in comparison with controls (Fig. 7, A and B). In addition, immunoblots confirmed that the levels of synaptophysin (Fig. 7C) as well as the postsynaptic protein PSD-95 (Fig. 7D) were increased in neurons treated with G3. These results demonstrate that G3 increases synapse formation.

We also performed double staining of G3-treated neurons (by adding 50 ng/ml in medium at the 3rd DIV) with synaptophysin and GluR2 antibodies on the 11th DIV. As seen in neurons grown on G3-coated dishes, G3 enhanced the number of clusters of synaptophysin and GluR2 but did not change the ratio of co-localization of the two synaptic proteins (supplemental Fig. S1). This result suggests that G3 increases glutamatergic synaptic formation without altering the localization of synaptic and extrasynaptic receptors.

To further assess the effect of the versican G3 domain on glutamatergic synaptic transmission, we studied mEPSCs in neurons grown in the G3-coated and control dishes on the 11th DIV. Both the amplitude (Fig. 8, A; B, panel 1; and C, panel 1) and frequency (Fig. 8, A; B, panel 2; and C, panel 2) of mEPSCs in neurons grown on G3-coated dishes were increased in comparison with controls. Taken together, these data demonstrated that the G3 domain of versican promotes excitatory synapse formation and transmission.

**DISCUSSION**

An emerging notion is that some specific domains of ECM molecules activate certain growth factor receptors with intrinsic signaling activity (32). For example, the EGF repeats in versican G3 domain have been demonstrated to be involved in cell proliferation and differentiation (16). Expanding on this notion, we now report two major findings derived from our study. First, the versican G3 domain promotes neurite growth and facilitates glutamatergic synaptic transmission in hippocampal neurons. Second, the G3 peptide modulates the neuronal attachment, morphology, and function via EGFR-dependent and -independent signaling pathways.

**Versican Regulates Neurite Growth and Synaptic Function**

A novel finding in this study was that the versican G3 domain
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FIGURE 8. The versican G3 product enhances excitatory synaptic activity. A, shown are representative traces of mEPSCs recorded from hippocampal neurons (11th DIV) grown on dishes coated with PDL alone (control) or together with the G3 peptide. Note the increase in both the amplitude and frequency of mEPSCs in the G3-treated neurons. B, cumulative probability plots for the amplitude (panel 1) and interval (panel 2) of mEPSCs recorded from a control and a G3-treated neuron. C, the amplitude (panel 1) and frequency (panel 2) of mEPSCs recorded from neurons under the two culture conditions were analyzed and plotted (panel 1, control, 26 ± 4 pA, n = 7; G3, 46 ± 5 pA, n = 7; *, p < 0.05; panel 2, control, 1.3 ± 0.2 Hz, n = 7; G3, 1.9 ± 0.3 Hz, n = 7; *, p < 0.05). Ctrl, control.

enhanced the neurite extension of neuronal cells. With regard to the role of versican in neurite growth, V2, the major isoform of versican in the adult brain (8, 9, 33), is demonstrated to be inhibitory (13, 34–36). V2-mediated inhibition likely occurs through its GAG-attached CS region, because removing the GAG chains attenuates the inhibitory activity of CSPG on axonal regeneration (37). On the other hand, the versican V1 isoform is highly expressed in embryonic brains (8) in which vigorous neurite growth occurs. In addition, the neurite outgrowth of cultured hippocampal neurons is facilitated by the V1-conditioned medium (14). These findings indicate that different isoforms of versican may play distinct roles in regulating neurite growth. In addition, previous studies have shown that neurite growth occurs in concert with ECM cleavage, and the cleavage products possess remarkable biological activities that are absent from the parent molecules (38). Indeed, our study and previous reports demonstrated that the G3 domain exhibits novel biological activity (15–18).

Neurite growth is accompanied by neurotransmitter receptor targeting to the specific membrane domain. Interestingly, while enhancing neurite growth, the versican G3 up-regulated glutamate receptor-mediated currents. The enhancement of glutamate receptor function likely reflected an integrated action of the G3 peptide on the neurons. First, G3 intensified neurite growth, providing a larger dendritic compartment for glutamate receptor manifestation. Second, G3 increased the density of glutamate currents, indicating that G3 enhanced glutamate receptor function rather than simply enlarging the size of the cell. Because the total expression levels of receptor proteins and the compactness of receptor clusters increased significantly in treated neurons, we propose that the G3 peptide up-regulated glutamate receptor function, at least partially, by enhancing the expression and targeting of receptors to the cell surface. Nevertheless, the possibility that G3 modulates glutamate receptor channel activity via protein phosphorylation should not be ruled out.

Versican G3 increased the number of dendritic spines and clusters of the presynaptic protein synaptophysin as well as PSD-95, a postsynaptic signaling protein present at glutamatergic synapses. These results indicate a role for the versican domain in synapse formation and neural circuit establishment. This idea was supported by the observation that spontaneous synaptic activity increased in the G3-coated dishes. The results also demonstrate a sustained action of the versican G3 fragment on glutamatergic synaptic structure and function. On the other hand, rapid ECM proteolysis (39) could underlie the prompt growth of dendritic spines (40) and insertion of glutamate receptors (24) demonstrated to rapidly occur following the induction of synaptic plasticity (i.e. long-term potentiation). Versican G3 may play a role in such prompt regulation of synaptic function, because our unpublished result showed that treating the neurons with the G3 products for 20 min significantly increased glutamate currents. Moreover, the present study demonstrated a swift elevation of pEGFR following G3 treatment (Fig. 5). Further studies will be required to determine whether the versican G3 fragment may play a role in fast synaptic plasticity.

It was intriguing to observe that the versican G3 peptide selectively enhanced the function of glutamate but not GABA receptors. Most glutamate receptors are located on the distal dendrites, whereas A-type GABA receptors are densely positioned on the perikarya or primary dendrites of neurons. These two types of receptors specifically interact with distinct intracellular anchoring and signaling proteins. It is plausible that the versican G3 domain selectively regulates the expression and targeting of glutamate receptors by activating specific signaling pathway(s).

Versican G3 Exerts Biological Actions via EGFR-dependent and -independent Pathways—The initial results of this study showed that the effects of G3 on neurite growth and glutamatergic activities are associated with its EGF motifs, because G3ΔEGF lacked these functions. ECM components can act as active sites for signal transduction (41). For example, the tenascin-derived EGF repeats are able to bind to EGF receptor (42)
with concomitant signals. In this regard, the versican G3 domain can enhance endothelial cell adhesion (15) via its EGF-like motif (16). Importantly, versican V1 enhances the neurite growth, EGFR expression, and ERK phosphorylation in PC12 cells (14). Given that EGFR is widely expressed in the mammalian brain (43, 44) and EGFR activity regulates neural cell differentiation (45), neurite growth (46–48), and guidance (48), we tested a possibility that versican G3 regulates neural morphogenesis and function by activating EGFR. We found that 1) G3 swiftly increased the phosphorylation of EGFR, indicating an activation of the receptor (49); 2) the G3 peptide rapidly increased the phosphorylation of ERK, a downstream signaling mediator of EGFR activation (50, 51); and 3) the G3-induced ERK phosphorylation could be effectively suppressed by selective EGFR inhibition. These results showed that the versican G3 peptide was capable of rapid activation of the EGFR signaling pathway.

Neurite growth and synaptic protein targeting are associated with protein phosphorylation. In this regard, blockade of EGFR significantly decreased neurite growth and glutamate receptor-mediated currents in neurons on the G3-treated dishes. These results indicate that the G3 peptide regulates neurite growth and synaptic function, at least partially, via activation of the EGFR-ERK signaling pathway.

On the other hand, the G3-enhanced neuronal attachment persisted in the presence of EGFR inhibitor, indicating that versican G3 enhanced neuronal cell attachment through EGFR-independent pathway(s). The versican peptides enhanced cell attachment possibly by means of interacting with other adhesion molecules, because the highly conserved C-terminal (G3) domain of CSPGs can interact with various extracellular matrices. For example, the aggrecan G3 proteins can interact with tenasin and fibulin (52). More interestingly, the presence of EGF motif in the aggrecan G3 proteins enhances the affinity of these interactions (52). Consistent with this notion, we found that the versican G3 peptide was more efficient than G3∆EGF in enhancing neuronal cell attachment.

The Significance of ECM Proteolytic Products in Neural Development and Plasticity—Versican is highly expressed by oligodendrocytes (53) in the brain (8, 54). Versican V2 participates in the formation of perineuronal gel layer in the adult brain (55), stabilizing synapses and inhibiting neural plasticity. Conversely, degradation of CSPGs reactivates neural plasticity (3). In this regard, ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) cleave the central CS region of versican (56–58), releasing G1- and G3-containing fragments. Versican products regulate various cellular functions by interacting with cell surface receptors such as β1-integrin and P-selectin glycoprotein ligand-1 (18, 28, 59), or with other ECM molecules including hyaluronan, tenasin, fibulin-1, fibrillin, fibronectin, and selectins (10, 15, 60–63). The present study demonstrates that a synthesized versican G3 domain up-regulates neural morphogenesis and synaptic transmission through EGFR-dependent and -independent pathways. These findings support a physiological role for the proteolytic products of versican in neural development and regeneration.

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REFERENCES

1. Kazmarcheck, L., Lapinska-Dzwonek, J., and Szymczak, S. (2002) EMBO J. 21, 6643–6648
2. Juliano, R. L., and Haskill, S. (1993) J. Cell Biol. 120, 577–585
3. Pizzorusso, T., Medini, P., Berardi, N., Chierzi, S., Fawcett, J. W., and Maffei, L. (2002) Science 298, 1248–1251
4. Williams, C., Villegas, M., Atkinson, R., and Miller, C. A. (1998) J. Comp. Neurool. 390, 268–277
5. Zimmermann, D. R., and Ruoslahti, E. (1989) EMBO J. 8, 2975–2981
6. Shimomura, T., Nishida, Y., Ito, K., and Kimata, K. (1993) J. Biol. Chem. 268, 14461–14469
7. Dours-Zimmermann, M. T., and Zimmermann, D. R. (1994) J. Biol. Chem. 269, 32992–32998
8. Milev, P., Maurer, P., Chiba, A., Mevissen, M., Popp, S., Yamaguchi, Y., Margolis, R. K., and Margolis, R. U. (1998) Biochem. Biophys. Res. Commun. 247, 207–212
9. Schnalfeldt, M., Dours-Zimmermann, M. T., Winterhalter, K. H., and Zimmermann, D. R. (1998) J. Biol. Chem. 273, 15758–15764
10. LeBaron, R. G., Zimmermann, D. R., and Ruoslahti, E. (1992) J. Biol. Chem. 267, 10003–10010
11. Matsumoto, K., Shionyu, M., Go, M., Shimizu, K., Shinomura, T., Kimata, K., and Watanabe, H. (2003) J. Biol. Chem. 278, 41205–41212
12. Ito, K., Shinomura, T., Zako, M., Ujita, M., and Kimata, K. (1995) J. Biol. Chem. 270, 958–965
13. Schnalfeldt, M., Bandtlow, C. E., Dours-Zimmermann, M. T., Winterhalter, K. H., and Zimmermann, D. R. (2000) J. Cell Sci. 113, 807–816
14. Wu, Y., Sheng, W., Chen, L., Dong, H., Lee, V., Lu, F., Wong, C. S., Lu, W. Y., and Yang, B. B. (2004) Mol. Biol. Cell 15, 2093–2104
15. Zheng, P. S., Wen, J., Ang, L. C., Sheng, W., Viloria-Petit, A., Wang, Y., Wu, Y., Kerbel, R. S., and Yang, B. B. (2004) FASEB J. 18, 754–756
16. Zheng, Y., Cao, L., Yang, B. L., and Yang, B. B. (1998) J. Biol. Chem. 273, 21342–21351
17. Wu, Y., Zhang, Y., Cao, L., Chen, L., Lee, V., Zheng, P. S., Kiani, C., Adams, M. E., Ang, L. C., Paiwand, F., and Yang, B. B. (2001) J. Biol. Chem. 276, 14178–14186
18. Zheng, P. S., Vais, D., Lapierre, D., Liang, Y. Y., Lee, V., Yang, B. L., and Yang, B. B. (2004) J. Cell Sci. 117, 5887–5895
19. Yang, B. L., Cao, L., Kiani, C., Lee, V., Zhang, Y., Adams, M. E., and Yang, B. B. (2000) J. Biol. Chem. 275, 21255–21261
20. Binette, F., Cravens, J., Kahoussi, B., Haudenschild, D. R., and Goetinck, P. F. (1994) J. Biol. Chem. 269, 19116–19122
21. Kiani, C., Lee, V., Cao, L., Chen, L., Wu, Y., Zhang, Y., Adams, M. E., and Yang, B. B. (2001) Biochem. J. 354, 199–207
22. Lee, V., Chen, L., Paiwand, F., Cao, L., Wu, Y., Inman, R., Adams, M. E., and Yang, B. B. (2002) J. Biol. Chem. 277, 22279–22288
23. Ruzicka, B. B., Fox, C. A., Thompson, R. C., Meng, F., Watson, S. J., and Akil, H. (1995) Brain Res. Mol. Brain Res. 34, 209–220
24. Lu, W., Man, H., Ju, W., Trimble, W. S., MacDonald, J. F., and Wang, Y. T. (2001) Neuron 29, 243–254
25. Dong, H., Xiang, Y. Y., Farchi, N., Ju, W., Wu, Y., Chen, L., Wang, Y., Hochner, B., Yang, B., Soreq, H., and Lu, W. Y. (2004) J. Neurosci. 24, 8950–8960
26. Brewer, G. I., Torricelli, J. R., Eavee, E. K., and Price, P. J. (1993) J. Neurosci. Res. 35, 567–576
27. Brewer, G. I. (1995) J. Neurosci. Res. 42, 674–683
28. Wu, Y., Chen, L., Zheng, P. S., and Yang, B. B. (2002) J. Biol. Chem. 277, 12294–12301
29. Yang, B. L., Yang, B. B., Erwin, M., Ang, L. C., Finkelstein, J., and Yee, A. J. (2003) Life Sci. 73, 3399–3413
30. Zhang, W., and Benson, D. L. (2000) Hippocampus 10, 512–526
31. Engel, J. (1989) FEBS Lett. 251, 1–7
32. Tran, K. T., Griffith, L., and Wells, A. (2004) Wound. Repair Regen. 12, 262–268

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33. Bignami, A., Perides, G., and Rahemtulla, F. (1993) J. Neurosci. Res. 34, 97–106
34. Asher, R. A., Morgenstern, D. A., Shearer, M. C., Adcock, K. H., Pesheva, P., and Fawcett, J. W. (2002) J. Neurosci. 22, 2225–2236
35. Niederost, B. P., Zimmermann, D. R., Schwab, M. E., and Bandtlow, C. E. (1999) J. Neurosci. 19, 8979–8989
36. Schweigreiter, R., Walmsley, A. R., Niederost, B., Zimmermann, D. R., Oertle, T., Casademunt, E., Frentzel, S., Dechant, G., Mir, A., and Bandtlow, C. E. (2004) Mol. Cell Neurosci. 27, 163–174
37. Bradbury, E. J., Moon, L. D., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., and McMahon, S. B. (2002) Nature 416, 636–640
38. Labat-Robert, J. (2004) Aging Res. Rev. 3, 233–247
39. Reeves, T. M., Prins, M. L., Zhu, J., Povlishock, J. T., and Phillips, L. L. (2003) J. Neurosci. 23, 10182–10189
40. Engert, F., and Bonhoeffer, T. (1999) Nature 399, 66–70
41. Gruenbaum, L. M., and Carew, T. J. (1999) Learn. Mem. 6, 292–306
42. Swindle, C. S., Tran, K. T., Johnson, T. D., Banerjee, P., Mayes, A. M., Griffith, L., and Wells, A. (2001) J. Cell Biol. 154, 459–468
43. Werner, M. H., Nanney, L. B., Stoeckel, C. M., and King, L. E. (1988) J. Histochem. Cytochem. 36, 81–86
44. Gomez-Pinilla, F., Knauer, D. J., and Nieto-Sampedro, M. (1988) Brain Res. 438, 385–390
45. Lillien, L. (1995) Nature 377, 158–162
46. Boonstra, J., Moolenaar, W. H., Harrison, P. H., Moed, P., van der Saag, P. T., and De Laat, S. W. (1983) J. Cell Biol. 97, 92–98
47. Morrison, R. S., Kornblum, H. I., Leslie, F. M., and Bradshaw, R. A. (1987) Science 238, 72–75
48. Garcia-Alonso, L., Romani, S., and Jimenez, F. (2000) Neuron 28, 741–752
49. Llorens, F., Garcia, L., Itarte, E., and Gomez, N. (2002) FEBS Lett. 510, 149–153
50. Han, S. W., Hwang, P. G., Chung, D. H., Kim, D. W., Im, S. A., Kim, Y. T., Kim, T. Y., Heo, D. S., Bang, Y. J., and Kim, N. K. (2005) Int. J. Cancer 113, 109–115
51. Navolanic, P. M., Steelman, L. S., and McCubrey, J. A. (2003) Int. J. Oncol. 22, 237–252
52. Day, J. M., Olin, A. I., Murdoch, A. D., Canfield, A., Sasaki, T., Timpl, R., Hardingham, T. E., and Aspberg, A. (2004) J. Biol. Chem. 279, 12511–12518
53. Levine, J. M., Reynolds, R., and Fawcett, J. W. (2001) Trends Neurosci. 24, 39–47
54. Popp, S., Andersen, J. S., Maurer, P., and Margolis, R. U. (2003) Dev. Dyn. 227, 143–149
55. Murakami, T., and Ohtsuka, A. (2003) Arch. Histol. Cytol. 66, 195–207
56. Westling, J., Gottschall, P. E., Thompson, V. P., Cockburn, A., Perides, G., Zimmermann, D. R., and Sandy, J. D. (2004) Biochem. J. 377, 787–795
57. Sandy, J. D., Westling, J., Kenagy, R. D., Iruea-Arispe, M. L., Verscharen, C., Rodriguez-Mazaneque, J. C., Zimmermann, D. R., Lemire, J. M., Fischer, J. W., Wight, T. N., and Clowes, A. W. (2001) J. Biol. Chem. 276, 13372–13378
58. Russell, D. L., Doyle, K. M., Ochsner, S. A., Sandy, J. D., and Richards, J. S. (2003) J. Biol. Chem. 278, 42330–42339
59. Kawashima, H., Atarashi, K., Hirose, M., Hirose, J., Yamada, S., Sugahara, K., and Miyasaka, M. (2002) J. Biol. Chem. 277, 12921–12930
60. Aspberg, A., Binkert, C., and Ruoslahti, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10590–10594
61. Kawashima, H., Hirose, M., Hirose, J., Nagakubo, D., Plaa, A. H., and Miyasaka, M. (2000) J. Biol. Chem. 275, 35448–35456
62. Olin, A. I., Morgelin, M., Sasaki, T., Timpl, R., Heinegard, D., and Aspberg, A. (2001) J. Biol. Chem. 276, 1253–1261
63. Isogai, Z., Aspberg, A., Keene, D. R., Ono, R. N., Reinhardt, D. P., and Sakai, L. Y. (2002) J. Biol. Chem. 277, 4565–4572