A Synthetic Peptide Ligand of Neural Cell Adhesion Molecule (NCAM), C3d, Promotes Neuritogenesis and Synaptogenesis and Modulates Presynaptic Function in Primary Cultures of Rat Hippocampal Neurons*

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The neural cell adhesion molecule (NCAM) plays a key role in morphogenesis of the nervous system and in remodeling of neuronal connections accompanying regenerative and cognitive processes. Recently, a new synthetic ligand of NCAM, the C3-peptide, which binds to the NCAM IgI module, has been identified by means of combinatorial chemistry (Ronn, L. C. B, Olsen, M., Ostergaard, S., Kiseliov, V., Berezin, V., Mortensen, M. T., Lerche, M. H., Jensen, P. H., Soroka, V., Saffell, J. L., Doherty, P., Poulsen, F. M., Bock, E., Holm, A., and Saffells, J. L. (1999) Nat. Biotechnol. 17, 1000–1005). In vitro, the dendrimeric form of C3, termed C3d, disrupts NCAM-mediated cell adhesion, induces neurite outgrowth, and triggers intracellular signaling cascades similar to those activated by homophilic NCAM binding. The peptide may therefore be expected to regulate regeneration and synaptic plasticity. Here we demonstrate that in primary cultures of hippocampal neurons: 1) C3d induces a sustained neuritogenic response, the neuritogenic activity of the compound being dependent on the dose, starting time, and duration of peptide application; 2) the peptide triggers the neuritogenic response by forming an adhesive substratum necessary for NCAM-mediated neurite formation and elongation; 3) C3d promotes synapse formation; and 4) C3d modulates the presynaptic function, causing a transient increase of the function at low (2 and 5 μM) doses and a reduction when applied at a higher concentration (10 μM). The effect of the peptide is dependent on the activation of the fibroblast growth factor receptor. We suggest that C3d may constitute a useful lead for the development of compounds for treatment of various neurodegenerative disorders.

The neural cell adhesion molecule (NCAM) plays a key role in morphogenesis of the nervous system (2) and in remodeling of neuronal connections associated with regenerative and cognitive processes (for review, see Ref. 3). The extracellular part of NCAM binds to a variety of ligands, the most important being the NCAM molecule itself. Homophilic NCAM binding is thought to involve the first five immunoglobulin (IgI–IgV) modules of NCAM with a double reciprocal interaction between the IgI and IgII modules of two interacting NCAM molecules (4, 5). Upon homophilic NCAM binding, intracellular signaling cascades, including the Ras-mitogen-activated protein kinase (Ras-MAPK) and phospholipase Cγ-associated pathways, are activated (6), resulting in neurite outgrowth. Antibody interventional studies have shown NCAM necessary for the induction and maintenance of long term potentiation and for stable memory retention in vivo (7, 8), suggesting that NCAM is involved in synaptic plasticity.

The modulation of processes of neuronal differentiation and plasticity through NCAM has been impeded by the absence of small synthetic agonists mimicking homophilic or heterophilic interactions of NCAM. However, recently, a new synthetic ligand of NCAM, the C3-peptide, has been identified by means of combinatorial chemistry. The dendrimeric tetramer of this peptide (C3d) binds to the IgI module of NCAM with a dissociation constant similar to that of the natural homophilic ligand, the IgII module (1, 4). In vitro, C3d disrupts NCAM-mediated cell adhesion, induces neurite outgrowth, and triggers intracellular signaling cascades similar to those activated by physiological homophilic NCAM binding (1, 6). In vivo, C3d has been demonstrated to induce amnesia in a passive avoidance learning paradigm in the adult rat and to prevent NCAM internalization in a 3–4-h period following task acquisition (9), indicating that the peptide is capable of modulating synaptic function.

In this study, we investigate the kinetics of the neuritogenic effect of C3d and show that by providing an adhesive substratum for neurite promotion, the peptide induces both a persistent neuritogenic response from primary hippocampal neurons and the formation of functional synapses, with synaptogenesis accelerated in cultures grown on C3d versus control (poly-L-lysine) substratum. We also show that C3d affects the presynaptic function in primary hippocampal neurons in a concentration- and time-dependent manner, and this effect is dependent on the activation of FGF receptor.

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† The abbreviations used are: NCAM, neural CAM; CAM, cell adhesion molecule; FGF, fibroblast growth factor; FGFR, FGF receptor; Ras-MAPK, Ras-mitogen-activated protein kinase; CREB, cAMP-response element-binding protein; PLL, poly-L-lysine; BSA, bovine serum albumin; DIV, days in vitro.
EXPERIMENTAL PROCEDURES

Preparation of Peptides—The C3 undecapeptide (ASKKPKRNKIA) and the control peptide CSala, in which Lys-6 and Arg-7 were substituted by Ala, were synthesized as dendrimers composed of four monomers coupled to a lysine backbone by Prof. Arne Holm (Royal Agricultural and Veterinary University, Copenhagen, Denmark).

Primary Cultures of Rat Hippocampal Neurons—Hippocampal neurons were prepared from embryonic day 19 rat embryos as described in (10). Neurons were seeded at a density of 10,000 cells/cm² in eight-well LabTek culture chambers with a growth surface of Permanox (Nunc, Roskilde, Denmark) or fibronectin (Sigma). For coating, slides were incubated with fibronectin diluted in H₂O and dried over night at room temperature in a flow bench to reach a final surface concentration of 0.01–20 ng/cm². For C3d immobilization, culture chambers were preincubated with various concentrations of the peptide for 2 h at 37 °C. Before cell seeding, culture chambers were washed in phosphate-buffered saline and blocked with 1% bovine serum albumin (BSA). After plating, cultures were maintained at 37 °C, 5% CO₂ in Neurobasal medium containing 20 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.4% w/v BSA (Sigma), supplemented with B27 (all from Invitrogen).

Analysis of Neurite Outgrowth—For analysis of neurite outgrowth, cultures were fixed with 4% v/v formaldehyde, stained for 20 min with 0.04% w/v Coomassie Blue R 250 in 45% v/v ethanol and 45% v/v acetic acid (BSA). After plating, cultures were maintained at 37 °C, 5% CO₂ in Neurobasal medium containing 20 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.4% w/v BSA (Sigma), supplemented with B27 (all from Invitrogen).

Analysis of Neurite Outgrowth—For analysis of neurite outgrowth, cultures were fixed with 4% v/v formaldehyde, stained for 20 min with 0.04% w/v Coomassie Blue R 250 in 45% v/v ethanol and 45% v/v acetic acid, and subsequently analyzed employing computer-assisted microscopy as described previously (11). In each experiment, the length of neurites from 150–200 neurons was determined.

Analysis of NCAM Expression—The expression of NCAM was analyzed during culturing. To estimate the amount of NCAM in hippocampal cells 0–30 min after trypanosinization, 10⁶ cells in suspension were collected for lysis after cell dissociation in DNAse I. The cells were pelleted at 1000 × g for 2 min and resuspended in lysis buffer (10 mM Tris-HCl, 2% v/v Triton X-100, pH 8, containing a mixture of protease inhibitors: 0.8 µM apronin, 50 µM bestatin, 15 µM E-64, 20 µM leupeptin, and 10 µM pepstatin A; set III, Calbiochem). For analysis of NCAM expression at 1-24 h after dissociation, hippocampal neurons were plated in 60-mm tissue culture dishes (Nunc) at a density of 40,000 cells/cm² and maintained in culture for 1, 2, 5, 6, or 24 h. Cells were scraped off in 0.5 ml of Neurobasal growth medium, pelleted at 1000 × g for 2 min, and solubilized in lysis buffer. Cell extracts were briefly sonicated (10–15 s, 40 watts), clarified by centrifugation at 20,000 × g for 2 min at 4 °C, and treated with Endo-N (1 µg/ml, recombinant MBP-fusion protein, Protein Laboratory, Institute of Molecular Pathology, Panum Institute, Copenhagen, Denmark) to remove the NCAM polyanisolation. SDS-PAGE and immunoblotting were performed as described in Ref. 12. Protein bands were visualized using enhanced chemiluminescence substrate Western Dura (Pierce) and processed with the GenTools software package (Syngene, Cambridge, UK).

Immunofluorescence—Visualization of filamentous actin (F-actin) was performed as described in Ref. 13. For double immunostaining for synaptophysin and GAP43, cultured cells were fixed in 4% v/v paraformaldehyde in sodium phosphate buffer (0.1 M NaH₂PO₄, 50 mM sucrose, 0.4 mM CaCl₂, pH 7.1) for 30 min at room temperature, blocked with 10% v/v normal goat serum, 1% w/v BSA in phosphate-buffered saline for 1 h, and washed with 1% BSA containing 0.2% w/v saponin. For double immunostaining for the postsynaptic density protein PSD-95 and NCAM, cells were fixed in methanol/acetic acid (1:1 v/v) for 5 min at −20 °C and washed with 1% BSA. Neurons were subsequently incubated with the following primary antibodies diluted in buffer: antiglycophosphatidylinositol monospecific antibody (IG; diluted 1:500, Sigma) and rabbit anti-rat-GAP43 polyclonal antibodies (1:1000, prepared as described previously (14)), or anti-PSD-95 monoclonal antibody (IG; 1:500, clone 6G6, Affinity Bioreagents, AH Diagnostics) and rabbit anti-rat-NCAM polyclonal antibody (1:1000, Protein Laboratory). Incubation with primary antibodies was performed at 4 °C overnight. Bound antibodies were detected with Texas Red-conjugated goat anti-rabbit antibody (1:200, Molecular Probes). Slides were mounted with Prolong Antifade mounting medium (Molecular Probes) and scanned with a MultiProbe 2001 Laser scanning Confocal Microscope (Amersham Biosciences) equipped with an oil immersion ×60 1.4 NA or ×100 1.4 NA objective (Nikon, Tokyo, Japan).

Mass Spectrometry and Determination of Amount of C3d in Culture Medium—To determine the amount of C3d in culture medium at different times after addition of the peptide, 200-µl samples containing 1 or 10 µM of the peptide dissolved in Neurobasal medium, were incubated in eight-well LabTek tissue culture chamber slides at 37 °C, 5% CO₂ in the absence or presence of plated hippocampal neurons. At various times (0, 1, 6, or 24 h), aliquots were collected. Mass spectrometric determination of the amount of C3d in the samples was performed using an Esquire liquid chromatography/mass spectrometer. The mass spectrometer was an Esquire ion trap MS analyzer (Bruker Daltonik GmbH, Bremen, Germany) connected to an HP1100 system (Agilent Technologies, Palo Alto, CA) with a Vydac low trifluoroacetic acid reverse phase column (21 × 150 mm). The solvent system comprised two solvents: (a) water with 0.02% v/v trifluoroacetic acid and (b) acetonitrile/water (90:10) with 0.02% trifluoroacetic acid. A gradient of B was steadily increasing from 0 to 50% over 30 min of acquisition with a flow rate of 0.250 ml/min. Angiotensin II (Bachem, Bubendorf, Switzerland, 10 µM) was used as internal standard to control the volume of the injection and to each sample to determine the total mass of 1 µM. The total injection volume was 50 µl. Quantification of the C3d amount was performed by isolation of the MS signal at 1073,8 Da [M + 5H]⁺ and angiotensin II at 1046.0 Da [M + H]⁺. The total counts for each sample were found by integration of the isolated MS signal.

To quantify the rates of C3d clearance from the culture medium based on the obtained experimental data (concentrations of C3d after various times of incubation), a mathematical model was developed (see “Appendix”). The model adequately described the experimentally obtained time course of C3d clearance from the culture medium and was used for estimation of rate constants of C3d degradation and sedimentation (see “Results”).

Labeling and Quantitative Analysis of Functionally Active Synapses—FM1-43 labeling of functional synapses was performed as described previously by Ryan et al. (15). Briefly, 10 days in vitro (DIV) hippocampal neurons were incubated in the presence of 2 µM of the fluorescent styryl membrane probe FM1-43 (Molecular Probes) and 90 mM KCl for 60 s followed by washing 3–4 times in normal saline for 5 min each to remove surface-bound FM1-43. Normal saline contained 137 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.3 mM CaCl₂, 20 mM H₂PO₄, 4 mM KH₂PO₄, 4 mM NaHCO₃, 0.3 mM NaH₂PO₄, 5.6 mM d-glucose, 20 mM HEPES, pH adjusted to 7.2 with NaOH. After loading, a field containing labeled punctate-like areas (0.3–3 µm²) was chosen. Pixel intensities from each object were averaged to obtain a measure of the local fluorescent intensity in the image. To evaluate the rate of turnover of synaptic vesicles, the synapses loaded with FM1-43 were destained by 150-s stimulation with 90 mM KCl in FM1-43-free solution. Fluorescence images of FM1-43-loaded synapses were obtained with a MultiProbe 2001 laser scanning confocal microscope, >60 1.4 NA objective, at an excitation wavelength of 488 nm. Images were quantified using the ImageSpace software package (Amersham Biosciences). To evaluate the rate of FM1-43 unloading, the experimentally obtained time courses of synaptic destaining were being represented by a single exponential function, exp(−kt) where k is the rate of destaining and t is the time elapsed after the start of KCl application.

Statistics and Graphical Presentations—Statistics and graphical presentations were carried out using the Origin version 5.0 software package (OriginLab, Northampton, MA). Statistical evaluations were performed using a two-sided Student’s t test. The results are given as mean ± S.E. Unless otherwise stated asterisks indicate the statistical significance: *, p < 0.05; **, p < 0.01; ***, p < 0.001 as compared with control.

RESULTS

Time Course of NCAM Expression after Trypanosinization—The induction of neurite outgrowth from hippocampal neurons by C3d takes place upon its binding to NCAM-IgG (1). Thus, the amount of NCAM expressed on the cell surface at the time when C3d is added to the culture medium might be of importance for its neuritogenic activity. We therefore first analyzed the expression of the two NCAM isoforms, 180 kDa NCAM and 140 kDa NCAM, at different times after trypsinization of hippocampal cells (see “Experimental Procedures” for details). In Fig. 1, it can be seen that the amount of 180 kDa NCAM and 140 kDa NCAM was significantly less at 0–3 h as compared with 24 h in vitro. During this period, the level of expression of 180 kDa NCAM and 140 kDa NCAM amounted to ~20% and ~50%, respectively, as compared with the level of expression at 24 h in vitro. Thus, the receptor for C3d binding, although reduced, was present even shortly after trypsinization.

C3d Induces Persistent Neurite Outgrowth from Hippocampal Neurons—Treatment of hippocampal neurons with C3d for
24 h has been shown to induce a neuritogenic response with a bell-shaped dose-response relationship, the maximal activity being observed at a peptide concentration of 0.54 μM (1). Accordingly, in our experiments, 1 μM C3d induced profound neurite outgrowth (Fig. 2). The time responses of neurite outgrowth induced by various doses of the peptide (0.3, 1, 3 μM, Fig. 3A) were very similar, with the strongest induction of neurite outgrowth being observed at 1 μM C3d.

The neuritogenic effect of C3d also depended on the time of application after cell seeding and the total time of exposure to the peptide. In a first set of experiments, C3d was added at different times after cell seeding (0–24 h, Fig. 3B) and, for each time point, the average length of neurites was estimated after 24 h of exposure to the peptide. For all peptide concentrations, the amplitude of the neuritogenic response was highest if the peptide was applied 0–2 h after plating. When treatment with C3d started 24 h after seeding, no neuritogenic effect was observed (Fig. 3B), indicating that the later treatment with C3d starts after plating, the smaller was the magnitude of the neuritogenic response induced by the NCAM ligand.

In a second set of experiments, hippocampal neurons were treated with C3d in various concentrations for 1, 2, 4, 8, or 24 h starting from the time of seeding (time 0), and subsequently the average neurite length was estimated at 24 h in vitro (Fig. 3C). It can be seen that the maximal neuritogenic activity of C3d varied significantly depending both on exposure time and on the peptide concentration. To determine the maxima of the obtained experimental curves, sets of experimental data were interpolated with the Bezier polynomials, and the time of exposure to C3d resulting in a maximal neuritogenic response at any given concentration was plotted against the employed dose (Fig. 3D). From the graph, it can be seen that the higher the concentration of C3d, the shorter was the time of exposure resulting in a maximal neuritogenic stimulation.

Taken together, these results highlight two aspects of the neuritogenic effect of C3d: 1) C3d triggering of intracellular signaling pathways may only be possible shortly after cell seeding, with a “time window” in the range of 2 h, and 2) C3d may promote neurite extension by forming an adhesive peptide substratum. In the latter case, the same “optimal” surface concentration of C3d could be achieved either by a short exposure to high concentrations of the peptide or by longer exposures to lower doses. This would explain the observation that the optimal time of exposure to C3d decreases with the rise of peptide concentration (Fig. 3D).

C3d Exerts Its Effect by Forming an Adhesive Substratum for Neurite Promotion—To test the above hypothesis, hippocampal neurons were grown in culture chambers coated with C3d. The immobilized peptide strongly stimulated neurite outgrowth in a dose-dependent manner (Fig. 4A). In contrast to soluble C3d, the dose-response curve for immobilized peptide did not demonstrate a decrease at high peptide concentrations, reaching instead a plateau at [C3d] = 3 μM, probably due to a saturation of the binding sites on the plastic surface at this concentration (Fig. 4A). Moreover, addition of soluble C3d to cultures of hippocampal cells inhibited neurite outgrowth induced by immobilized C3d (not shown). These results indicated that at high C3d concentrations, the soluble peptide presumably interfered with cell-substrate adhesion, thus preventing neurite promotion.

If this was the case, one might expect that readdition of the peptide to the culture medium would competitively inhibit C3d-induced neurite outgrowth. Indeed, treatment of hippocampal neurons twice with C3d at times 0 and 24 h led to a marked decrease of the neuritogenic response when estimated at 48 h. The effect was even more pronounced when C3d was applied at times 0, 24, and 48 h, and the response was measured at 72 h as compared with control cultures treated with C3d only once, at the time of seeding (Fig. 4B). To test whether the observed effect was C3d-specific, we...
Fig. 2. Confocal images of rat hippocampal neurons grown in low density culture for 1, 2, or 3 days in the absence (A, 3 DIV) or presence (B–D, 1, 2, or 3 DIV, respectively) of 1 μM C3d. Immunostaining for filamentous (F) actin is shown.

plated hippocampal neurons onto a fibronectin substratum (0.02–20 μg/cm²) and treated them with 1 μM C3d at time 0 or at times 0 and 24 h. The average length of neurites was measured after 48 h. Cultures grown on fibronectin, but not treated with C3d, were used as controls. From Fig. 4C, it can be seen that fibronectin stimulates neurite outgrowth in a dose-dependent manner with a threshold of 0.05 μg/cm². C3d added at time 0 induced an additional neuritogenic effect over that exerted by fibronectin. A second application of the C3d (24 h) abolished the neuritogenesis induced by the peptide without affecting the rate of fibronectin-induced neurite outgrowth (Fig. 4C). Thus, repetitive treatment with C3d specifically modulated C3d-, but not fibronectin-induced, neurite outgrowth.

These results indicate that C3d induces the neuritogenic response not only by triggering intracellular signaling pathways but also by forming an adhesive substratum and subsequent interaction with NCAM expressed on the plasma membrane. Soluble C3d might competitively disrupt this interaction, resulting in a significant decrease of the neuritogenic response.

Pharmacodynamic Properties of C3d in Hippocampal Cell Culture—To directly confirm the above hypothesis, we investigated the time course of the C3d concentration in culture medium with and without hippocampal cells using mass spectroscopy. The time courses of the peptide concentration in the medium, [C3d], normalized to the corresponding initial values (1 and 10 μM), represented biphasic curves with a fast initial [C3d], which fall at 0–6 h of the incubation followed by a slower decrease at 6–24 h (Fig. 5). The rate of C3d clearance from the culture medium in the absence of cells was higher for 1 μM than for 10 μM C3d. This indicated that at least two mechanisms with different time scales contributed to the [C3d], decrease in the culture medium over time. We presumed that the process of fast initial (0–2 h) C3d clearance from the culture medium reflected the adsorption of the peptide to the plastic surface since the slower disappearance of 10 μM rather than 1 μM C3d at short times suggested the presence of a saturating clearance process, which would not be the case if the peptide simply was degraded in the medium. At longer (6–24 h) times, a slower clearance was observed, which probably reflected C3d degradation. To estimate the rates of these processes, we constructed a mathematical model comprising both mechanisms (see “Appendix”). The model adequately described the experimental data (Fig. 5, solid curves), allowing us to estimate time constants of C3d degradation (τ₁) and sedimentation (τ₂), which were found to be 10 h for both peptide doses and 5.2 h for 1 μM C3d or 0.4 h for 10 μM C3d (see “Appendix” for details).

In the presence of hippocampal neurons, 1 μM C3d was cleared from the culture medium significantly faster (Fig. 5). However, the time course of the C3d clearance could not be approximated by the biexponential curve since the peptide was degraded faster during the first hours in culture than at 6–24 h. This indicated that C3d was also cleaved by the cells, the rate of cell-induced C3d degradation decreasing by time in culture. This might be due to the binding of C3d to the cell receptors (NCAM) followed by cleavage of the peptide by cell proteases or shedding and/or endocytosis of NCAM together with the bound peptide at short times after cell dissociation.

C3d Promotes Synapse Formation in Primary Hippocampal Neurons—Since C3d peptide induced persistent neurite outgrowth terminating in the formation of a neuronal network approximately at day 5 in vitro, it seemed important to elucidate how rapidly the induced neuritogenic response resulted in formation of functional synapses. This process has been shown to correlate strongly with the acquisition of focal accumulations of synaptic vesicle proteins such as synaptophysin (17). Thus, synaptophysin-positive spots provide an accurate quantifiable estimate of presynaptic terminals (18). We investigated the time course of expression of synaptophysin in primary hippocampal neurons plated onto substrata consisting of C3d, the Ig2 module of NCAM, or poly-L-lysine (PLL). Spontaneous neuritogenesis on PLL was used as a control. Cells were immuno-
stained for synaptophysin after 3, 4, 5, 6, and 8 days in culture. Fig. 6 shows double immunostaining for the neuronal membrane protein GAP43 and the presynaptic marker, synaptophysin, of neurons grown either on C3d (A, C) or PLL (B, D) substratum at 5 (A, B) and 8 (C, D) days in vitro. At 5 DIV, cells grown on the two substrata had similar morphology (Fig. 6, A and B). However, punctate patches of synaptophysin immunoreactivity on cell bodies and dendrites were more numerous in cultures grown on C3d than on control substratum (compare Fig. 6, A and B). At 8 DIV, no difference was seen (compare Fig. 6, C and D). To obtain a quantitative estimate of the rate of synapse formation, we evaluated the number of synaptophysin-positive spots/unit of neurite length in the cultures, grown on the two substrates. From Fig. 7A, it can be seen that hippocampal neurons cultivated on either C3d or the NCAM-Ig2 module developed synapses significantly faster than cells cultivated on PLL. The difference in the number of formed synapses was considerable at 3–6 DIV but disappeared by 8 DIV (Fig. 7A). The time course of expression of the postsynaptic density marker PSD-95 in cultures grown on C3d was similar to that of synaptophysin with a punctate PSD-95 immunoreactivity appearing at approximately 5 DIV (not shown). The observed effect could not be attributed to the enhanced encounter rate between neurites in C3d- versus PLL-stimulated cells since at any time in vitro, the average neurite length in cultures grown on PLL somewhat exceeded that in cultures grown on C3d or Ig2 (Fig. 7B). Thus, both the natural NCAM ligand, NCAM-Ig2, and the peptide mimetic C3d promoted synapse formation in primary hippocampal neurons.

C3d Affects the Presynaptic Function in Primary Hippocampal Neurons—NCAM is supposed to participate in synaptic connectivity by mediating adhesion between pre- and postsynaptic sites and modulating a series of intracellular signaling cascades (see Ref. 3 for review). Accordingly, recent studies suggest that NCAM is involved in synaptic plasticity (19), which in part depends on the presynaptic events mediating the release of neurotransmitter, termed the presynaptic function (20). The presynaptic function reflects the probability of transmitter release in response to presynaptic depolarization, and it can be directly imaged using the fluorescent styryl dye FM1-43 (21). The dye is taken up into synaptic vesicles in presynaptic terminals in an activity-dependent manner (loading) as a result of endocytosis following presynaptic stimulation and transmitter release. Subsequent rounds of exocytosis caused by presynaptic stimulation lead to the release of dye from the presynaptic boutons (unload-FIG. 3. The neuritogenic activity of C3d, time- and dose-response studies. A, effect of 0.3 μM (●), 1 μM (□), and 3 μM (▲) C3d on the formation of neurites from primary hippocampal neurons depending on time in vitro. ■ control. In each experiment, the length of neurites from 150–200 neurons was estimated at each time point. B, dependence of neurite outgrowth stimulated by 0.3 μM (●), 1 μM (□), and 3 μM (▲) C3d on the time of C3d addition after seeding of cells. For each point, the average neurite length was measured 24 h after the beginning of C3d treatment. C, effect of the C3d-peptide on neurite outgrowth: dependence on time of exposure. Cultures were treated with 0.3 μM (●), 1 μM (□), 3 μM (▲), 10 μM (○), and 30 μM (□) C3d for 1, 2, 4, 8, or 24 h from the time of seeding. The average neurite length was measured 24 h after seeding. Interpolating curves were derived based on five independent experiments. D, dependence of time of exposure to C3d resulting in maximal neuritogenic response on the applied concentration of C3d.
Thus, the rate of FM1-43 unloading provides a direct index of presynaptic function (15, 20). To study the effect of C3d on the presynaptic function in primary hippocampal neurons, cells were grown on a PLL substratum for 10 days and then treated with different concentrations of C3d (2, 5, and 10 μM, dissolved in culture medium) for 3 or 48 h. Control cultures were treated with culture medium only. Both in control and treated cultures, FM1-43 fluorescence appeared as puncta-like synaptic spots along dendrites (Fig. 8). C3d significantly changed the kinetics of destaining of synapses with KCl. Fig. 8 shows confocal images of FM1-43-stained hippocampal cultures, treated with different doses of C3d before (A–C) and 100 s after (D–F) the start of stimulation with high KCl. It can be seen that both in control cultures (A and D) and in cultures treated with 2 μM C3d (B and D), the fluorescence of synaptic puncta was significantly lowered after 100 s of stimulation with KCl. Conversely, for 10 μM C3d, the decrease in fluorescence intensity after 100 s of KCl treatment was very small (compare C with F). From the obtained time courses, we calculated the rate of FM1-43 unloading (Fig. 9A) and the size of the recycling vesicle pool (Fig. 9B) at various C3d concentrations applied for 3 or 48 h. The second parameter represented a difference in fluorescence, ΔF, between fully stained and destained (300 s after the start of KCl stimulation) synaptic puncta (15, 22). From Fig. 9, it can be seen that after 3 h of incubation, both parameters exhibited a slight increase with 2 and 5 μM C3d, whereas a higher peptide concentration (10 μM) caused a more than 2-fold decrease in the rate of FM1-43 unloading and the size of the recycling vesicle pool. At 48 h of incubation with C3d, a slight increase was observed for the rate of synapse destaining at 2 μM C3d and a pronounced inhibition at 10 μM C3d. As regards ΔF, only 10 μM C3d had an inhibitory effect after 48 h of incubation. Neither the rate of synapse destaining nor ΔF was affected by the treatment of cultures with 10 μM C3ala, the control peptide.
with two alanine substitutions and no neuritogenic activity (1) (Fig. 9, A and B, insets). These results indicate that the presynaptic function was transiently increased by low to moderate concentrations of C3d and suppressed by high peptide doses.

Since C3d, like NCAM itself, has been shown to activate intracellular signaling acting via FGFR, we checked whether blocking of this receptor would prevent the decrease in synaptic function caused by treatment with high peptide concentrations. Indeed, the inhibitory effect induced by 3 h of application of 10 μM C3d on both ΔF and the rate of synapse destaining was rescued by about 80% in the presence of the inhibitor of FGFR 1, SU5402, the application of SU5402 alone being without effect (Fig. 9, A and B, insets). Thus, C3d-induced modulation of presynaptic function was dependent on FGFR.

**DISCUSSION**

The dynamic regulation of adhesion is critical for proper axon growth and has been shown to take place through either internalization/recycling (23) or shedding (24) of CAMs expressed on the cell surface, whereas the ligand is constituted by substrate molecules. We suggest that C3d exerts its neuritogenic effect by forming an adhesive substratum promoting the neurite extension. First, the neuritogenic effect of C3d depended on the time of application with little or no neuritogenic response if the peptide was added more than 4 h after plating. The possible explanation of the observed effect of C3d is probably that neurite induction by NCAM ligands can only be achieved when these are presented on a growth surface or associated to the extracellular cell matrix. If C3d is added 2–4 h after seeding, when the cells are attached to the plastic substratum and, thus, no peptide substratum can be formed underneath, poor neuritogenesis is observed.

Second, the time course of C3d clearance from the culture medium indicates the presence of surface adsorption with different time scales for 1 and 10 μM C3d (Fig. 5). Low doses of the peptide may fail to form a C3d substratum sufficient for neurite promotion, whereas high concentrations of C3d will result in a
saturation of the peptide substratum and thus in a residual amount of the peptide remaining in solution. This residual soluble peptide may competitively disrupt the interaction between cellular NCAM and substratum-bound C3d, thereby preventing effective neurite extension. The hypothesis is consistent with the bell-shaped dose-response relationship for C3d added at time 0 and is supported by the observation that the magnitude of the neuritogenic response to C3d depends on the time of exposure to the ligand. Indeed, at a low concentration (0.3 μM), C3d forms a slowly saturating substratum without notable interference with the cell-substratum adhesion. This makes an optimal exposure time as long as 24 h. When the concentration of the peptide is higher (3–30 μM) and decreases more slowly, a competitive inhibition of C3d-induced neurite outgrowth is seen due to deadhesion caused by residual soluble peptide in the medium. Therefore, the strongest neuritogenic response for high (3–30 μM) C3d doses is achieved when using short exposure times (1–4 h, Fig. 3), enough for the formation of a substratum for neurite outgrowth while avoiding interference of the soluble C3d with cellular adhesion.

According to the proposed hypothesis, repetitive application of C3d would competitively abolish C3d-induced neurite outgrowth but not outgrowth triggered by ligands of other adhesion molecules. Indeed, in our experiments, C3d-triggered neuritogenesis was inhibited by repetitive treatment with the peptide, whereas the neuritogenic response to fibronectin, a

Fig. 8. Confocal images of FM1-43-stained hippocampal cultures, treated with 2 or 10 μM C3d for 3 h, before (A–C) and 100 s after (D–F) start of stimulation with high KCl. A and D, control cultures; B and E, 2 μM C3d; C and F, 10 μM C3d. Bars, 5 μm.

Fig. 9. C3d changes the total number of synaptic vesicles released. A, dependence of the rate of puncta destaining on the applied C3d concentration after 3 (■) or 48 h (○) of incubation with the peptide. Inset, the effect of the blocking of FGFR on the rate of puncta destaining. C3d in a concentration of 10 μM was applied for 2 h either in the absence (C3) or in the presence (C3 + SU) of FGF receptor inhibitor SU5402, 50 μM. The application of the control peptide with two alanine substitutions (C3ala, 10 μM) or SU5402 alone (SU, 50 μM) had no effect. B, dependence of the difference in fluorescence intensity (ΔF) between fully stained and fully destained puncta on the applied C3d concentration after 3 (■) or 48 h (○) of incubation with the peptide. Inset, the effect of the blocking of FGFR on the ΔF. C3d in a concentration of 10 μM was applied for 3 h either in the absence (C3) or in the presence (C3 + SU) of FGF receptor inhibitor SU5402, 50 μM. The application of the control peptide with two alanine substitutions (C3ala, 10 μM) or SU5402 alone (SU, 50 μM) had no effect.
ligand of integrins, was unaffected (Fig. 4C). Thus, C3d, like NCAM itself, exerted its neuritogenic effect not only by triggering intracellular signal transduction pathways as shown before (6) but also by providing a substratum for cellular adhesion, the latter being crucial for the induction of neurite outgrowth.

Neurite outgrowth, initiated by C3d, resulted in the formation of neuronal network and functional synapses (Fig. 6, A and C). Moreover, hippocampal neurons cultivated on C3d or Ig2 substrata showed accelerated synapse formation as compared with that in cultures grown onto PLL (Fig. 7). The formed synapses were functional by 6–8 DIV, as judged from the fact that they accumulated the fluorescent lipophilic dye FM1-43 during KCl-induced depolarization and released it upon subsequent KCl-induced synaptic firing. Thus, C3d affects the rates of both neurite outgrowth and synapse formation.

C3d has been shown to inhibit the processes of memory acquisition (9), probably by affecting synaptic plasticity. The latter is known to be partially dependent on the rate of presynaptic endocytosis (25). We found that when applied in low to moderate concentrations (2–5 μM), C3d had a modest transient stimulatory effect on presynaptic function after 3 h of incubation, whereas a high concentration of the peptide steadily suppressed it. To our knowledge, this is the first demonstration of an NCAM-mediated modulation of the presynaptic function.

Synaptic plasticity in primary hippocampal neurons is associated with cAMP/protein kinase A-dependent activation of the transcription factor CREB, which in turn regulates gene expression during long term potentiation (26), in particular enhancing the number of active presynaptic terminals (27). Recent data suggest that the cAMP/protein kinase A pathway is activated upon homophilic NCAM stimulation (28) and upon depolarization-induced Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (29), resulting in CREB phosphorylation. Treatment with C3d itself induced phosphorylation of CREB in PC12 cells and primary hippocampal neurons (29) and increased the intracellular Ca\(^{2+}\) concentration by ~20 nM at a dose of 5.4 μM (30). We suggest that Ca\(^{2+}\) influx and activation of protein kinase A induced by C3d may activate CREB, enhancing the expression of synaptic proteins, which results in synaptic strengthening.

At high concentrations, C3d has been shown to steadily increase the intracellular Ca\(^{2+}\) concentration in primary hippocampal neurons up to 170–180 nM (30), probably significantly changing the submembrane Ca\(^{2+}\) concentration in presynaptic terminals. Such a rise of [Ca\(^{2+}\)]\(_{e}\) may affect interactions between CAMs and the cytoskeleton and/or activate the intracellular protease calpain, causing rapid uncoupling of synaptic contacts (16). The inhibitory effect of C3d on presynaptic function was due to its signaling properties, not to a deatheseeffect per se, since this inhibition was rescued by blocking FGF receptor activation (Fig. 9), an essential component of NCAM-mediated signal transduction.

In conclusion, the recently identified synthetic peptide-ligand of NCAM, C3d, induces persistent neurite outgrowth by forming an adhesive substratum for neurite extension and promotes synapse formation in primary hippocampal neurons. Furthermore, NCAM stimulation by C3d modulates the presynaptic function in long term neuronal cultures, predominantly through an interaction of NCAM with the FGF receptor. The detailed mechanism of NCAM-mediated modulation of the presynaptic function will need further clarification.

**APPENDIX**

In this section, we describe the determination of the rates of C3d sedimentation and degradation in culture medium. Assuming that the decrease in C3d concentration in the culture medium is caused by two basic mechanisms (see “Results”), 1) C3d sedimentation due to surface adsorption and 2) C3d degradation in the volume, we propose a model sufficient for studies of local dynamics of [C3d] in the culture medium. The local dynamics of C3d concentration in the medium (C\(_{f}\)) and the concentration of the bound C3d in the thin layer near the substratum (C\(_{b}\)) are described by the following simultaneous ordinary differential equations,

\[
d\frac{dC_f}{dt} = -k_1 C_f - k_2 C_f (C_b - C_f) - k_3 C_f (C_b - C_f)\]  
\[
d\frac{dC_b}{dt} = -k_1 C_f (C_b - C_f)\]  

(Eq. 1)

where C\(_{b}\) is the equilibrium concentration of the bound C3d in the thin layer near the substratum, μM; k\(_{1}\) is the rate of C3d degradation, 1/h; k\(_{2}\) is the rate of C3d sedimentation, 1/μM h; the initial conditions are C\(_{f}(0) = C_{f0}^0, C_{b}(0) = 0\). Based on the biphasic shape of the obtained experimental curves, we assumed that at short times (0–2 h), the sedimentation process dominates over degradation. Therefore, considering k\(_{1}\) ≈ 0, we can rewrite Eq. 1 as shown in Eq. 2.

\[
d\frac{dC_f}{dt} = -k_2 C_f (C_b - C_f)\] 
\[
d\frac{dC_b}{dt} = -k_1 C_f (C_b - C_f)\]  

(Eq. 2)

The solutions of this set are shown in Eqs. 3 and 4:

\[
C_f(t) = \text{Lim}_{C_f(t)} C_f(t) = C_{f0}^0 \frac{1}{1 + k_2 C_{b0}^0}.
\]

C\(_{b}(t) = \text{Lim}_{C_b(t)} C_b(t) = \frac{k_2 C_{b0}^0}{k_2 C_{b0}^0 + 1} \frac{C_{f0}^0}{1 + k_2 C_{b0}^0} - 1.
\]

(Eq. 3)

\[
C_f(t) = \frac{C_{f0}^0}{1 + k_2 C_{b0}^0}.
\]

(Eq. 4)

From the experimental data, at t = t\(_{2}\) = 1 h, for 1 μM C3d, C\(_{f}(t = 0) = C_{f0}^0 = 1 μM, C_{b}(t = t_{2}) = C_{f0}^0 = 0.84 μM; and for 10 μM C3d, C\(_{f}(t = 0) = C_{f0}^{10} = 1 μM, C_{b}(t = t_{2}) = C_{f0}^{10} = 92.9 μM. Therefore, from Eqs. 3 and 4, we can write the equations shown in Eqs. 5 and 6.

\[
C_f(t) = C_{f0}^0 \frac{1}{1 + k_2 C_{b0}^0} \frac{C_{f0}^0}{1 + k_2 C_{b0}^0}.
\]

(Eq. 5)

\[
C_f(t) = \frac{C_{f0}^0}{1 + k_2 C_{b0}^0} \frac{C_{f0}^0}{1 + k_2 C_{b0}^0} \frac{k_2 C_{b0}^0}{k_2 C_{b0}^0 + 1} \frac{C_{f0}^0}{1 + k_2 C_{b0}^0} - 1.
\]

(Eq. 6)

With given values of the parameters C\(_{f0}^0, C_{f0}^{10}, C_{f0}^{10}, k_{2}, t_{1}\), a solution of Eq. 5 for k\(_{2}\) does not exist. From Eq. 6 comes Eq. 7.

\[
C_f(t) = \frac{(C_f(t) + C_{b}(t) - C_{f0}^{10})}{C_f(t) + C_{b}(t) - C_{f0}^{10}} = \frac{C_f(t) + C_{b}(t) - C_{f0}^{10}}{C_f(t) + C_{b}(t) - C_{f0}^{10}} = C_f(t).
\]

(Eq. 7)

Solving Eq. 7 numerically, we obtain the following approximations: C\(_{b}\) = 0.654 μM, k\(_{2}\) = 0.26 1/μM h. Defining the time constant of sedimentation t\(_{2}\) so that C\(_{f}(t = t_{2}) = C_{b0}^0 (e - 1)/e, from Eq. 7, we derive Eq. 8.

\[
t_{2} = \frac{1}{k_2 C_{f0}^0} \ln \left( \frac{C_{f0}^0 + C_{b}(t) - C_{f0}^{10}}{C_{f0}^0} \right)
\]

(Eq. 8)

From Eq. 8, t\(_{2}\) = 5.2 h for 1 μM C3d, and t\(_{2}\) = 0.4 h for 10 μM.
C3d. Thus, at longer times (6–24 h), it is mainly the degradation process that contributes to the time course of 10 μM C3d clearance. Based on this, we can determine the unknown rate of degradation \( k_1 \). Considering that \( C_\text{C3d} \sim C_\text{C3d} \) at \( t > 6 \) h, from Eq. 1, we obtain \( C_\text{C3d}(0) = C_\text{C3d}^0 \), \( C_\text{C3d}(0) = C_\text{C3d}^0 \), which gives the solution \( C_\text{C3d} = C_\text{C3d}^0 \exp(-k_1 t) \). From this equation we can derive the rate of C3d degradation in the culture medium \( k_1 \) using the experimental data. Defining \( t_6 = 6 \) h, \( t_{24} = 24 \) h, \( C_\text{C3d}(t_6) = C_\text{C3d}^0 = 8.0 \) μM, \( C_\text{C3d}(t_{24}) = C_\text{C3d}^0 = 5.6 \) μM, obtain \( k_1 = \ln(C_\text{C3d}^0/C_\text{C3d}(t_{24})/(t_{24} - t_6)) = 0.02 \) h\(^{-1}\). Thus, the time constant of C3d degradation in the culture medium in the absence of cells is \( \tau_1 = 1/k_1 = 50 \) h for both peptide concentrations, and the time constants of sedimentation \( \tau_2 = 5.2 \) h for 1 μM C3d and \( \tau_2 = 0.4 \) h for 10 μM C3d.

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