Vasoactive Intestinal Peptide and PACAP38 Control N-Methyl-D-aspartic Acid-induced Dendrite Motility by Modifying the Activities of Rho GTPases and Phosphatidylinositol 3-Kinases*

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Dendrite morphogenesis is highly dynamic and characterized by the addition and elongation of processes and also by their selective maintenance, retraction, and elimination. Glutamate can influence these events via N-methyl-D-aspartic acid (NMDA) receptors. The neuropeptides vasoactive intestinal peptide and pituitary adenyl cyclase-activating polypeptide-38 (PACAP38) affect neurogenesis and differentiation in the developing nervous system. We report here that the peptides and NMDA acted synergistically on dendrite and branch formation. In stage III hippocampal neurons, NMDA increased not only the addition but also the elimination of new dendrites and branches by activating Rac and Cdc42 and phosphatidylinositol 3-kinases, respectively. When applied alone, the neuropeptides did not influence dendrite or branch formation. However, they reduced the elimination of newly formed dendrites and branches caused by NMDA by preventing the NMDA-induced activation of phosphatidylinositol 3-kinases. This led to the formation of persistent dendrites and branches. Additional time-lapse studies on the dynamics of dendrite elongation showed alternating periods of elongation and retraction. Phosphatidylinositol 3-kinases increased the velocities of dendrite elongation and retraction, whereas the neuropeptides prolonged the periods of elongation. By modifying NMDA-induced activation of Rho GTPases and phosphatidylinositol 3-kinases, vasoactive intestinal peptide and PACAP38 could play an important role in the control of dendrite growth and branching during development and in response to neuronal activity.

To integrate synaptic input, neurons develop a specific dendritic branching pattern that determines their function (1). Neuronal activity modifies the formation and stabilization of dendritic processes (2, 3). Dendrites are motile structures that contain high concentrations of filamentous actin. By controlling the stability and assembly of the actin cytoskeleton, members of the Rho family of small GTPases regulate neuronal morphogenesis (4). Rac and Cdc42 facilitate the outgrowth of dendrites, dendritic branches, filopodia, and spines, whereas RhoA and Rho kinase (ROCK) attenuate it (5–9). In Xenopus optic tectal neurons, the neurotransmitter glutamate changes the activity of Rho GTPases by acting on ionotropic NMDA (NMDAR) and L-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors. It facilitates dendrite formation by inhibiting RhoA and activating Rac (10). Because calcium signaling plays an important role in dendrite formation (11, 12), mainly the effects of NMDAR stimulation seem to be important. In hippocampal neurons, the guanine exchange factor Tiam1 couples NMDARs to the activity-dependent development by activating Rac1 and inhibiting RhoA (13, 14).

In vitro, class I phosphatidylinositol 3-kinases (PI3Ks) support neurite formation by producing membrane-bound phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (15–17). PI3Ks stimulate dendrite and branch outgrowth by inhibiting the RhoA/ROCK pathway and facilitate dendrite and branch elimination (18). The small GTPase Ras forms a transient complex with PI3Kβ and activates it (19). Ca2+ influx through NMDARs can regulate the neuron-specific Ras GTPase-activating protein SynGAP via the Ca2+/calmodulin-dependent protein kinase-II (20).

Vasoactive intestinal polypeptide (VIP) and pituitary adenyl cyclase-activating peptide-38 (PACAP38) belong to the secretin-glucagon-VIP family of peptides (21). VPAC1 and VPAC2 receptors are shared by VIP and PACAP38, whereas only PACAP38 binds with high affinity to the PAC1 receptor (PAC1, R) (22). In the developing rat brain, mainly PAC1, Rs are expressed (23). VPAC1, Rs are expressed in the neuroepithelium beginning with embryonic day 11 (24); compared with PAC1, Rs, their density is three times lower. VPAC2, Rs are even less expressed (23). PACAP38 and VIP act as growth factors in the developing brain by acting mainly via PAC1, Rs (21, 25–29). In the adult brain, PACAP38 enhances NMDAR function via PAC1, R (30).

We have studied the NMDA- and VIP/PACAP38-induced dendrite and branch morphogenesis in cultured hippocampal neurons.
neurons of the rat. Our biochemical analysis provided evidence that only the synergism of NMDA and VIP/PACAP38 induced changes in Rho GTPase, and PI3K activities led to a pronounced dendritic development. In contrast, activation of NMDAR alone enhanced dendritic motility by activating Rac, Cdc42, and PI3Ks but did not promote dendritogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—NMDA and LY294002 were from Tocris (Köln, Germany). Y-27632, H89, KN93, KN92, nimodipine, and dizzocilpine (MK801) were from Calbiochem, and wortmannin was from Sigma. The expression plasmids encoding the GST-PAK-CRIB domain and the pGEXC21 plasmid were gifts of Dr. J. G. Collard (Amsterdam, Netherlands). Poly-l-lysine, Dulbecco’s modified Eagle’s medium, fetal bovine serum, and StartV medium were from Biochrom (Berlin, Germany); B27 supplement was from Invitrogen; PACAP38, VIP, and the VPAC1R agonist, Arg^{16}-secretin, were from Phoenix Pharmaceuticals (Karlruhe, Germany).

**Neuronal Cell Culture**—Primary cultures of hippocampal neurons were prepared from brains of embryonic rats at day 17. Dissociated cells were seeded at a density of $3 \times 10^4$ cells/cm² on 10-mm glass coverslips or for biochemical experiments on 10-cm cell culture dishes (Greiner Bio-One, Solingen, Germany). Both were coated with poly-l-lysine. The incubation medium consisted of Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and, StartV medium were from Biochrom (Berlin, Germany); B27 supplement was from Invitrogen; PACAP38, VIP, and the VPAC1R agonist, Arg^{16}-secretin, were from Phoenix Pharmaceuticals (Karlruhe, Germany).

After 1 day in culture, the incubation medium was replaced by serum-free differentiation medium, which consisted of StartV medium, supplemented with B27, HEPES, pH 7.3. Cultures were incubated at 37 °C in a humidified atmosphere with low oxygen conditions to mimic physiological conditions (6.5% CO₂ and 9% O₂) (31). Within hours after plating, hippocampal neurons developed 4–5 neurites and progressed through stages I and II, according to Bradke and Dotti (36). Under these culture conditions, most neurons remained dissociated; only a few formed clusters. The cells showed a density of $\sim 4 \times 10^4$ cells/cm² at stage III when experiments were performed.

**Cytotoxic Staining**—Cultured hippocampal neurons were fixed for 12 min with 4% paraformaldehyde in a cytoskeletal stabilizing buffer (CSB) containing 80 mM Pipes, 5 mM EGTA, 1 mM MgCl₂, and 4% polyethylene glycol (M, 35,000) (32). After washing with CSB, neurons were permeabilized for 2 min with 0.1% (v/v) Triton X-100 in CSB. Normal goat serum was used to block non-specific binding. For β-tubulin III staining, cells were incubated with a monoclonal mouse anti-β-tubulin III antibody (1:4000; Sigma); for p-Akt staining, neurons were incubated with a rabbit monoclonal anti-phospho-Ser^{473} Akt antibody (1:100; Cell Signaling, Beverly, MA). The resulting immune complexes were visualized with an Alexa 488-conjugated F(ab)² fragment of goat anti-mouse or anti-rabbit IgG (Molecular Probes, Heidelberg, Germany). For F-actin staining, cells were incubated with Alexa 594-conjugated phalloidin (Molecular Probes, Heidelberg, Germany) and washed with PBS.

**Preparation of Transfection Vectors and Cell Transfection**—The coding regions of the RhoAN19 and Rac1N17 genes were excised from the plasmid pGEX with BamHI/EcoRI and inserted in-frame into the BglII/EcoRI sites of pEGFP-C1 (Clontech). The coding region of the Cdc42N17 gene was excised from the plasmid pcdNA3 with BamHI/EcoRI and inserted in-frame into the BglII/EcoRI sites of pEGFP-C1. The plasmids coding for the dominant-negative YFP-p110γ K833R and the constitutive active p110γ CAAX (where A4 is aliphatic amino acid) were provided by Bernd Nürnberg (Düsseldorf, Germany). The YFP-p110γ K833R and the p110γ CAAX constructs were functionally tested in a culture of rat astrocytes where they inhibited and elevated the PI3K-dependent phosphorylation of Ser^{473}Akt. All plasmids were confirmed by restriction digest analysis and sequencing. Cells were transfected for 1.5 h at 2.5% CO₂ using the calcium phosphate/DNA co-precipitation procedure. After transfection, neurons were grown for 16 h prior to use. To increase transfection efficiency, we used DIV4 hippocampal neurons. Under these conditions, the calcium phosphate/DNA co-precipitation procedure, which does not change neuronal morphology (33), produced transfection rates of 3–8%.

**Expression of GST-PAK-Cdc42/Rac Interactive Binding (CRIB) Domain and Expression of GST-C21 Protein**—GST-C21 contains the N-terminal 90 amino acids of the RhoA-binding region of the RhoA effector Rhotekin. The GST fusion proteins were expressed in *Escherichia coli* BL21 cells grown at 37 °C. Expression was induced by adding 0.1 mM isopropyl β-D-thiogalactoside at $A_{600}$ 1.0. Two hours after induction, the cells were collected and lysed by sonication in lysis buffer (50 mM Tris-Cl, pH 8.0, 2 mM MgCl₂, 2 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 14,000 × g, and the supernatant was used for purification of the GST-PAK-CRIB-/GST-C21 domain by affinity purification using glutathione-Sepharose beads (Amerham Biosciences). Beads loaded with the GST fusion proteins were washed twice with GST-fishing-buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1% (v/v) Nonidet P-40, and 25 μg/ml aprotinin) at 4 °C.

**GST-PAK-CRIB Domain/GST-C21 Domain Pulldown Experiments**—Approximately $1 \times 10^6$ neurons were used for Rac or for Cdc42 experiments; $\sim 4 \times 10^6$ neurons were used for the RhoA experiments. Cells were treated with drugs as indicated under “Results.” After addition of 250 μl of GST-fishing-buffer, cells were harvested. The detergent-soluble supernatant was recovered after centrifugation for 15 min at 14,000 × g and at 4 °C. GTP-Rac, GTP-Cdc42, or GTP-RhoA proteins were precipitated for 1 h with 20 μl of GST-PAK/GST-C21 fusion protein at 4 °C. The complexes were washed three times with ice-cold PBS, resuspended, and boiled with Laemmlı buffer. Bound Rac, Cdc42, and RhoA proteins were detected by Western blotting using anti-Rac1 (1:1000; BD Biosciences), anti-Cdc42 (1:1000; Upstate, Milton Keynes, UK), or anti-RhoA antibodies (1:400; Santa Cruz Biotechnology, Santa Cruz, CA).

**Phosphorylation Assay for Akt**—After drug treatment, cells were washed twice with PBS. Ice-cold lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1% (v/v) Nonidet P-40, and 25 μg/ml aprotinin) was added, and the neurons were harvested. The detergent-soluble supernatant was recovered after centrifugation for 15 min at 14,000 × g and at 4 °C. Cell lysates were separated by SDS-PAGE and subjected to Western blot analysis. To determine the phosphorylation of
Akt, anti-phospho-Ser473 Akt and anti-Akt antibodies were used (1:1000; Cell Signaling, Beverly, MA). Densitometric analysis of films was performed with the ImageQuant TL software (American BioSciences, Sunnyvale, CA).

**Time-lapse Light Microscopy**—To determine the dynamics of branch formation as well as dendrite elongation, hippocampal neurons were plated on poly-l-lysine-coated glass-bottom microwell dishes (MatTek, Ashland, MA) and used for time-lapse light microscopy. Neurons were incubated in a custom-made chamber at 37 °C in a humidified atmosphere (6.5% CO₂, 9% O₂), staged on a Zeiss Axiovert 200 microscope system equipped with a digital camera (Coolsnap HQ; Roper Scientific, Tucson, AZ). Differential interference contrast (DIC) images were acquired every 60 s with a Zeiss ×40; A:1.4 oil immersion objective for 8 h. To minimize phototoxicity, an automatic shutter was used (100 ms/frame). The Metamorph 6.5.2 software (Universal Imaging, Downingtown, PA) was used to acquire and process the resulting stacks of images. For analysis of branch formation, we distinguished dendritic branches from filopodia using the following parameters: branches have a lifetime longer than 20 min, whereas filopodia have a mean lifetime of ~10 min in immature dendrites (34). Typically filopodia are less than 5 μm in length (35). The number of branches detected by DIC microscopy corresponded to that of tubulin-containing branches found in immunohistochemical experiments. After acquisition of the time-lapse movies, neurons were fixed and stained for β-tubulin III and F-actin to ensure that the appropriate structures were analyzed and that the respective neuron was in a healthy condition. For analysis of dendrite elongation, we tracked primary dendritic length in intervals of 20 min. We analyzed the movies of at least 10 neurons for each treatment group.

**Morphometry of Fixed Cells**—The dendrites were distinguished from the axon of a stage III hippocampal neuron by use of morphological criteria (36). Developing axons contain microtubules up to their tips, whereas dendrites contain microtubules only in their proximal part (37). Axons have few or no axonal branches. In addition, we performed TAU-2 staining to confirm the presence and identity of axons. To quantify the axonal branches. In addition, we performed TAU-2 staining to confirm the presence and identity of axons. To quantify the axonal branches, we tracked primary dendritic length in intervals of 20 min. We analyzed the movies of at least 10 neurons for each treatment group.

**Confocal Microscopy**—A Zeiss LSM 510 visible confocal system was used to acquire z-stacks of representative neurons, afterward vertical projections were performed. When the growth cone at the tip of a neurite was not visible in the vertical projection, a horizontal projection was performed to confirm its existence.

**Statistical Analysis**—The Mann-Whitney U test and the Kruskal-Wallis test (for multiple comparisons) were used. If samples showed normal distribution, analysis of variance and Scheffe’s test or Student’s t test were used.

**RESULTS**

**NMDA Induces Dendritogenesis Only in Combination with VIP/PACAP38 or ROCK Inhibition**—In *Xenopus* tectal neurons, the development of the dendritic arbor requires glutamate receptor activity (39). In our experiments we used cultured hippocampal neurons that were at stage III of differentiation. After 2 days in vitro (DIV2), they had formed an axon and several dendrites (36, 40). Added to the hippocampal neurons for 8 h, NMDA (5 μM) increased the number of filopodia from 0.96 ± 0.19/30 μm in controls to 2.78 ± 0.28 filopodia/30 μm (p < 0.001), indicating that it changed the dynamics of the actin cytoskeleton. However, NMDA had minimal or no effects on the average number of dendrites and branches (Fig. 1A and Table 1). In *Xenopus* tectal neurons, the glutamate-induced decrease in RhoA activity is important for the dendrite and branch formation (10). In contrast, in our hippocampal neurons NMDA did not reduce the level of GTP-bound RhoA (Fig. 2A), which represents RhoA activity (41).

VIP and PACAP38 decrease RhoA activity in rat astrocytes (42, 43). Therefore, we examined whether the peptides facilitated dendrite and branch formation in our hippocampal neurons. In concentrations from 0.1 to 100 nM, both peptides did not change the average numbers of dendrites and branches, when applied alone over a period of 8 h (only the values for 1 nM VIP and 10 nM PACAP38 are shown in Table 1). At these concentrations, the peptides also did not change dendritic arborization when applied for up to 3 days (data not shown). In addition, VIP (1 nM) or PACAP38 (10 nM) did not reduce the level of GTP-bound RhoA (Fig. 2A). In contrast, the combination of NMDA (5 μM) with 1 nM VIP or 10 nM PACAP38 decreased the amount of GTP-bound RhoA to 70 and 45% of controls, respectively (Fig. 2A). The combinations of NMDA with VIP or PACAP38 also significantly increased the average number of dendrites and branches (Fig. 1A and Table 1).
The low concentration of VIP necessary for this effect indicated that a VPAC receptor was involved. Combined with NMDA, the VPAC1R agonist \( [\text{Arg}^{16}] \text{secretin} \) (10 nM) indeed increased the average number of dendrites and branches (Table 1). In contrast, the VPAC1R antagonist \( [\text{Ac-His}^{1},\text{D-Phe}^{2},\text{Lys}^{15},\text{Arg}^{16},\text{Leu}^{27}] \text{VIP}^{(1–7)} \) was ineffective (data not shown). Because most effects of VPAC receptors are mediated by cAMP and the subsequent activation of protein kinase A, we tested the effect of the cAMP-dependent protein kinase inhibitor \( (R_{p})-\text{cAMP} \). However, it did not prevent the effects of VIP plus NMDA on dendritogenesis (Table 1). Taken together, these results suggested that VIP and PACAP38 affected dendritogenesis in hippocampal neurons via atypical VPAC receptors.

Next, we studied whether NMDA and the peptides induced changes in dendrite morphology, when applied for less than 8 h. A pulse of NMDA (30 \( \mu \text{M} \)) lasting for 3 to 15 min was indeed sufficient to induce changes in dendrite morphology, when the peptides were subsequently added, and the neurons were fixed 8 h later (Fig. 1B). A 3-min pulse of NMDA followed by VIP slightly increased the average number of dendrites but had no effect on branches. A 10-min pulse of NMDA significantly increased the numbers of dendrites and branches. A 15-min pulse of NMDA was nearly as effective as the treatment with NMDA for 8 h (compare Fig. 1B and Table 1). To examine, whether the continuous treatment with VIP was necessary, we stimulated the cultures for 15 min with NMDA (30 \( \mu \text{M} \)) in the presence of VIP (1 nM). Then, we washed the cells and immediately added VIP again for 30 min up to 8 h, when the cells were analyzed (Fig. 1C). When applied for 1 h, VIP did not cause significant changes. A 2-h treatment with VIP only slightly increased the average number of dendrites. After 4 h, however, the numbers of dendrites and branches did not differ from those found after stimulation with VIP and NMDA for 8 h (Fig. 1C and Table 1). Similar results were obtained with PACAP38 (data not shown). Taken together, these data showed that a short pulse of NMDA was sufficient to induce a delayed increase in the number of dendrites and branches, if VIP or PACAP38 were subsequently
present. In contrast to NMDA, the peptides had to be applied for more than 1 h to support the dendritic changes.

Because the combination of NMDA and peptides decreased RhoA activity, we studied whether the RhoA/ROCK pathway was indeed involved in the NMDA-induced dendritogenesis. The ROCK inhibitors Y-27632 (10 μM) and H-1152 (100 nM) were used in these experiments at concentrations known to cause complete inhibition (44, 45). When applied alone, only Y-27632 slightly raised the average number of dendrites, whereas both inhibitors did not change the number of branches (Table 1). Together with NMDA, Y-27632 as well as H-1152 enhanced the number of dendrites and branches (Table 1). Compared with NMDA plus VIP or NMDA plus Y-27632, the triple combination of NMDA, VIP, and Y-27632 did not have a stronger effect on dendrite and branch formation (data not shown), suggesting that VIP and Y-27632 did not have additive effects.

By using the combination of NMDA and Y-27632, we tested whether NMDA acted indeed via ionotropic NMDA receptors. The NMDA receptor channel blocker dizocilpine (100 nM) prevented the effects of NMDA plus Y-27632 (Table 1).

Next, we studied whether the time course of the effect of Y-27632 corresponded to those of the peptides. Already a 3- or 5-min pulse of NMDA significantly increased the numbers of dendrites and branches when Y-27632 was present during the subsequent 8 h (Fig. 1D). When NMDA was applied for 15 min, Y-27632 had to be present for 1 h to produce branches and for 2 h to generate dendrites (Fig. 1E). Taken together, the ROCK inhibitors and the peptides had very similar effects on dendrite and branch formation.

To further confirm the role of RhoA in NMDA-induced dendrite formation, we transiently transfected the hippocampal neurons with an EGFP fusion protein of dominant-
negative (dn) RhoA (Table 2). To increase the transfection efficiency, we used the neurons after 4 days in culture. They had formed already numerous dendrites and branches, some of which had attached to other neurites. Because primary dendrites longer than 10 μm could be easily identified, we counted these extensions. Neurons expressing only EGFP showed an average number of 5.2 dendrites (Table 2). Only the combination of NMDA and Y-27632 further increased the number of dendrites, whereas Y-27632 (10 μM) or NMDA (5 μM) applied alone had no effect. These findings indicated that the expression of EGFP did not compromise the morphological responses. 24 h after the transfection procedure, hippocampal neurons transfected with dnRhoA had developed significantly more dendrites, i.e. the average number of 7.2, than the EGFP controls. This finding was in agreement with an earlier observation (46) but was in contrast with our data with the ROCK inhibitors, which had no effect of their own (Table 1). We can only speculate that RhoA effectors in addition to ROCK restrained the protrusion of neurites. In the neurons transfected with dnRhoA, NMDA alone further increased the number of primary dendrites (Table 2), confirming that RhoA inhibited the effects of NMDA on dendrite formation.

**TABLE 1**

| No. of dendrites | No. of branches |
|------------------|-----------------|
| Control          | 3.1 ± 0.25      |
| NMDA             | 3.6 ± 0.11*     |
| VIP              | 3.4 ± 0.13      |
| PACAP38          | 3.3 ± 0.10      |
| [Arg^{10}]Secretin | 3.5 ± 0.11     |
| NMDA + VIP       | 4.7 ± 0.14abc   |
| NMDA + PACAP38   | 5.1 ± 0.22abc   |
| NMDA + [Arg^{10}]Secretin | 5.2 ± 0.09abc |
| NMDA + (R/^)cAMP | 3.3 ± 0.16      |
| NMDA + Y-27632 + NMDA + KN-93 | 3.6 ± 0.26 |
| NMDA + Y-27632 + NMDA + Nm2odipine | 3.1 ± 0.23 |
| NMDA + Nm2odipine | 5.0 ± 0.19abc |
| NMDA + Y-27632 + Nm2odipine | 4.9 ± 0.18abc |
| Y-27632          | 3.7 ± 0.13*     |
| H-1152           | 3.3 ± 0.17      |
| NMDA + Y-27632   | 4.7 ± 0.12abc   |
| NMDA + H-1152    | 4.6 ± 0.20abc   |
| Dizocilpine      | 3.3 ± 0.12      |
| NMDA + Y-27632 + Dizocilpine | 3.5 ± 0.20 |

* Value shows significant difference (p < 0.05) compared with controls.
* Value shows significant difference (p < 0.05) compared with NMDA.
* Value shows significant difference (p < 0.05) compared with VIP, PACAP38, or [Arg^{10}]Secretin, Y-27632, and H-1152, respectively. The effects of the combination of NMDA + VIP + Rp-cAMP did not differ from those of NMDA + VIP (p < 0.05).

**TABLE 2**

| No. of dendrites |
|------------------|
| EGFP             | 5.2 ± 0.37 |
| EGFP + Y-27632   | 5.6 ± 0.41 |
| EGFP + NMDA      | 6.2 ± 0.44 |
| EGFP + NMDA + Y-27632 | 9.3 ± 0.74* |
| dnRhoA           | 7.2 ± 0.37 |
| dnRhoA + NMDA    | 10.3 ± 0.85* |
| Y-27632          | 4.4 ± 0.33 |
| Y-27632 + NMDA   | 4.1 ± 0.31 |
| Y-27632 + NMDA   | 4.2 ± 0.33 |
| Y-27632 + Y-27632 | 4.5 ± 0.24 |
| Y-27632 + Y-27632 | 4.7 ± 0.23 |
| Y-27632 + Y-27632 | 4.8 ± 0.29 |
| Y-27632 + Y-27632 | 4.9 ± 0.29 |
| Y-27632 + Y-27632 | 5.2 ± 0.45 |

* Value shows significant difference (p < 0.05) compared with respective controls.

Next, we investigated whether Y-27632 and the peptides affected the activities of the GTPases. As was to be expected from previous studies (10, 18, 47), the ROCK inhibitor increased the level of GTP-bound Rac but not that of GTP-bound Cdc42 (Fig. 2B). VIP and PACAP38 enhanced the level of GTP-bound Rac to 162 and 145% of controls, respectively, whereas the combination of NMDA and VIP or PACAP38 increased the activity of Rac to 294 and 260%, respectively (Fig. 2C). This increase was also higher than that induced by NMDA, i.e. 181% of controls, indicating synergistic effects. VIP and PACAP38 also increased the level of GTP-bound Cdc42. Here, the combination of NMDA with VIP or PACAP38 was not more effective than the agents alone (Fig. 2C).

NMDA can activate Rac and Cdc42 via the Ca^{2+}/calmodulin-independent protein kinase II (CaMKII) (11). In our hippocampal neurons, the CaMKII inhibitor KN-93 indeed blocked the NMDA-induced GTP binding of Rac and Cdc42 but not the effects of VIP. The related inactive substance KN-92 was without effect (Fig. 2D). KN93 also prevented the dendritogenesis induced by NMDA in its combination with VIP or Y-27632 (Table 1), whereas KN92 had no effect (data not shown). In contrast, the L-type voltage-gated calcium channel blocker nimodipine (1 μM) did not change the effects of NMDA plus VIP or Y-27632 on dendritogenesis (Table 1), suggesting that NMDA exerted its effects via CAMKII but independent of L-type channels.

Finally, we studied the NMDA-induced dendrite formation in hippocampal neurons transiently transfected with EGFP fusion proteins of dnRac1 or dnCdc42. These neurons did not show dendrite formation in response to NMDA, Y-27632, or the combination of both substances (Table 2).

Taken together, these data provided evidence that NMDA, VIP, and PACAP38 activated Rac and Cdc42 via different mechanisms.
mechanisms. Their activation seemed to be linked to the observed changes in dendritogenesis.

**VIP Modulates NMDA-induced Dendritic Branch Motility**—Neurites are formed in a dynamic process consisting of addition and elimination. During the process of addition, an extension starts to protrude from the perikaryon or a dendritic shaft and grows to a certain length. The extension may persist but often is retracted or even eliminated. To study how NMDA, VIP, and Y-27632 influenced these dynamics, we performed time-lapse microscopy over a period of 8 h. Because we had observed the most pronounced changes in the number of branches, we analyzed these structures. In the course of the study, we observed that an area of the dendritic shaft would extend only 1 branch during the observation period. If this branch was eliminated, no second branch would emerge from this site. Thus, the number of added branches reflected the number of sites along the dendrites that were able to produce branches. To distinguish between filopodia and branches, extensions were only counted as branches if they were longer than 5 μm and persisted for more than 20 min. Whenever tested, β-tubulin III immunostaining confirmed the presence of microtubules in such structures. It has to be mentioned that branches growing out rapidly were more likely to reach the threshold of 5 μm than those with slow elongation.

Because VIP and PACAP38 had produced nearly identical results in all previous experiments, we used only VIP. In controls, an average number of 3.7 branches was added, but 3.0 were again eliminated resulting in a ratio of 1.32 (Fig. 3B). NMDA (5 μM) strongly increased the motility. It enhanced the average number of added branches to 11.7 and that of eliminated branches to 11.0 (Fig. 3A). The resulting ratio of 1.07 demonstrated why the strong increase in motility did not result in an increased number of branches (Fig. 3B, compare Fig. 1A and Table 1).

VIP or Y-27632 alone did not change the number of added and eliminated branches, but they strongly affected the motility when combined with NMDA (Fig. 3B). In the presence of VIP plus NMDA, the average number of added branches was still increased to 11.5, but now branch elimination was strongly reduced (Fig. 3B). The resulting ratio of 1.71 was in agreement with the net addition of branches observed (Fig. 1A and Table 1). The combination of Y-27632 and NMDA produced similar changes in motility (Fig. 3B).

**PI3Ks Influence Dendrite Motility**—In hippocampal neurons, inhibition of PI3K activity reduces the rate of addition but also that of elimination of dendrites and branches (18). To examine whether PI3Ks were involved in the observed changes in branch motility, we initially used wortmannin and LY294002, two chemically unrelated inhibitors of PI3Ks, at the lowest possible concentrations. Because both agents had similar effects, we continued the experiments only with LY294002. Compared with controls, LY294002 (20 μM) reduced the average number of added branches. In addition, these branches were much shorter and extended at a slower velocity. LY294002 strongly reduced the average number of eliminated branches. Consequently, the ratio of both values increased to 1.90, so that additional branches were formed (Fig. 3B). When LY294002 was combined with VIP or Y-27632, the number of added branches was similar to that in controls. It remained open whether VIP and Y-27632 induced more outgrowth sites or accelerated the outgrowth slowed down by LY294002 so that more branches became longer than 5 μm. The ratios of added to eliminated branches rose to 2.65 and 3.65, respectively, and branch formation was pronounced. LY294002 strongly reduced branch elimination induced by NMDA and slightly reduced branch addition, resulting in an increase of the ratio to 1.76. Now, numerous branches were formed. Addition of VIP or Y-27632 to this combination enhanced branch addition but not elimination. The resulting ratios of 3.42 and 2.85, respectively, explained the large increase in net formation of branches (Fig. 3B). An additional experiment confirmed that LY294002 not only facilitated the formation of branches, as shown in Fig. 3, but also that of dendrites (Fig. 4).

**Recombinant Isoforms of PI3Kγ Affect the Numbers of Persistent Branches**—Because wortmannin and LY294002 can also act on non-PI3Ks (48), we overexpressed in DIV4 hippocampal neurons a fusion protein containing YFP and a dominant-negative mutant of the catalytic subunit of PI3Kγ, which is p110γ K833R. Compared with EGFP controls, neurons expressing p110γ K833R showed no morphological differences compared with controls (Fig. 5A). In these neurons, NMDA, VIP, or Y-27632 increased the numbers of dendrites to an extent comparable with that induced by LY294002 (Figs. 4B and 5B). NMDA applied together with VIP or Y-27632 produced an even higher increase in the number of dendrites. The wild type form of subunit p110γ can be stably expressed in the absence of its adaptor subunit. We used a recombinant protein of p110γ, which contained a CAAX box at its C terminus, to facilitate membrane attachment, so that it was constitutively active. This p110γ CAAX mutant can be inhibited with wortmannin or LY294002. Y-27632, NMDA, and wortmannin applied alone as well as Y-27632 plus NMDA did not change the number of primary dendrites in cells expressing the p110γ CAAX mutant. NMDA plus Y-27632 were able to increase the number of primary dendrites (Fig. 5, C and D) only when wortmannin was applied. Taken together, these data showed that PI3Ks were involved in dendrite and branch formation induced by NMDA, mainly by causing the elimination of newly added extensions.

**VIP Inhibit the NMDA-induced PI3K Activity**—Because VIP and Y-27632 reduced the elimination of new branches generated by NMDA, we examined whether they affected PI3K activity. We measured the phosphorylation of Akt at Ser473 (p-Akt), which depends on PI3K activity. NMDA raised the level of p-Akt to 243% of controls (Fig. 6A), confirming previous reports (14, 49). Although Y-27632 and VIP alone did not change the level of p-Akt, they indeed reduced the increase caused by NMDA to 133 and 137% of controls, respectively (Fig. 6A).

p-Akt has been found in axonal growth cones together with mPar3/mPar6 (46, 50). In view of our data that PI3Ks regulated dendrite and branch formation, we examined the localization of p-Akt after treatment of the neurons with NMDA and VIP. In control neurons, p-Akt fluorescence was strong in the perikaryon and 1 growth cone, which probably was the axonal growth cone (Fig. 6B). After NMDA application, strong p-Akt...
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A

![Images of NMDA-induced dendrite motility at different times (0h to 8h).]

B

| Treatment                  | Number of added or eliminated branches/neuron | Added / eliminated branches |
|----------------------------|-----------------------------------------------|----------------------------|
| control                   | ![Graph showing number of branches](image)     | 1.32 ± 0.17                |
| NMDA                      | ![Graph showing number of branches](image)     | 1.07 ± 0.02 a              |
| VIP                       | ![Graph showing number of branches](image)     | 1.25 ± 0.05                |
| NMDA + VIP                | ![Graph showing number of branches](image)     | 1.71 ± 0.08 a,b            |
| Y-27632                   | ![Graph showing number of branches](image)     | 1.26 ± 0.10                |
| NMDA + Y-27632            | ![Graph showing number of branches](image)     | 1.77 ± 0.08 a,b            |
| LY294002                  | ![Graph showing number of branches](image)     | 1.90 ± 0.03 a              |
| NMDA + LY294002           | ![Graph showing number of branches](image)     | 1.76 ± 0.05 a,b            |
| LY294002 + VIP            | ![Graph showing number of branches](image)     | 2.65 ± 0.34 a              |
| LY294002 + Y-27632        | ![Graph showing number of branches](image)     | 3.65 ± 0.36 a              |
| NMDA + LY294002 + VIP     | ![Graph showing number of branches](image)     | 2.85 ± 0.16 a,b,c          |
| NMDA + LY294002 + Y-27632 | ![Graph showing number of branches](image)     | 3.42 ± 0.35 a,b,c          |
NMBA-induced branch addition by reducing the activity of Cdc42.

VIP and Y-27632 Modulate Dendrite Elongation—VIP and Y-27632 applied alone did not change dendrite and branch formation but elongated the existing dendrites (Fig. 1A). Therefore, we analyzed the dynamics of dendrite elongation. In time-lapse experiments, we tracked the tips of the growth cones and measured their distance to the starting point every 20 min over a period of 8 h (Fig. 3A). NMDA and VIP exerted typical effects on the course of the growth cone tips over 8 h (Fig. 7A). In controls, long periods of elongation were interrupted by short periods of retraction. VIP had similar effects but caused net elongation over the 8-h period. In contrast, NMDA often reversed the direction of outgrowth, so that net elongation was not observed (Fig. 7A). When NMDA was applied together with VIP, the reversals of growth direction occurred less often, so that net elongation was possible. LY294002 alone slowed down the movement of the tips. Applied together with NMDA plus VIP, it nearly prevented retraction so that a pronounced net elongation was observed (Fig. 7A). Taken together, these data suggested that elongation was not only determined by the ratio of outgrowth and retraction periods but also by the velocity of both processes.

To quantify these changes, we summed up the total distances covered during all outgrowth or retraction periods and calculated the net elongation. We also counted the reversals of growth direction during the 8-h observation period as well as the percentage of time spent for outgrowth and retraction. From these data we calculated the average velocity of outgrowth and retraction.

VIP and Y-27632 affected the dynamics of dendrite elongation in a very similar manner. The results obtained with both agents are separately shown in Fig. 7, B and C, and Fig. 8, A and B, respectively, but will be described together. In control neurons, primary dendrites reversed the direction of growth ~7 times within 8 h and grew out during 52% of the total time. The

Due to increased PI3K activity along the dendrites. Mean ± S.E. n = 50. a shows significant difference (p < 0.05) compared with controls; b shows significant difference (p < 0.05) compared with NMDA; and c shows significant difference (p < 0.05) compared with NMDA + LY294002.

FIGURE 4. The PI3K inhibitor LY294002 supports dendrite and branch formation. Hippocampal neurons were treated with NMDA (5 μM), LY294002 (20 μM), VIP (1 nM), and Y-27632 (10 μM) for 8 h. A, neurons were stained for F-actin (red) and β-tubulin III (green) (bar is ~20 μm). B, quantification of the experiment. Dendrites and branches containing β-tubulin III were counted. White columns, number of primary dendrites; gray columns, number of dendritic branches. Mean ± S.E. n = 50. a shows significant difference (p < 0.05) compared with controls; b shows significant difference (p < 0.05) compared with NMDA; and c shows significant difference (p < 0.05) compared with NMDA + LY294002.

Fluorescence was found in all neurites and growth cones. VIP applied together with NMDA reduced the intensity of p-Akt fluorescence in the neurites. The PI3K inhibitor wortmannin abolished the p-Akt fluorescence in the neurites and reduced it in the cell body (Fig. 6B). These data suggested that NMDA increased PI3K activity along the dendrites.

Because LY294002 or wortmannin reduced the NMDA-induced branch addition, we also examined whether they affected the NMDA-induced Rac and Cdc42 activation. Wortmannin did not affect the increase in GTP-bound Rac1 caused by NMDA but pronouncedly diminished the respective increase in GTP-bound Cdc42 (Fig. 6C). LY294002 had a similar effect (data not shown). These findings suggested that the PI3K inhibitors attenuated the

FIGURE 3. Time-lapse analysis of the effect of NMDA (5 μM), VIP (1 nM), Y-27632 (10 μM), and LY294002 (20 μM) on the addition and elimination of dendritic branches. DIC images were taken every minute over 8 h. A, the series of DIC micrographs shows the effects of NMDA on the same dendrite during the 8-h observation period (bar is ~10 μm). Arrows point at a dendritic branch that grows out and retracts; asterisks show the tip of the dendrite (for detailed analysis of dendrite elongation see also Figs. 8 and 9). B, quantification of newly added and eliminated branches per neuron over 8 h. Branches with a life span of less than 20 min were excluded to avoid the counting of dendritic filopodia. White columns, newly added branches counted over 8 h; gray columns, eliminated branches. The ratios of newly added and eliminated branches are shown at the right. Mean ± S.E.; n = 10; a shows significant difference (p < 0.05) to controls; b shows significant difference (p < 0.05) to NMDA; and c shows significant difference (p < 0.05) to NMDA + LY294002.
Because VIP and Y-27632 prevented the increase in PI3K activity caused by NMDA, we studied the effect of a PI3K inhibitor on elongation. Compared with controls, LY294002 nearly halved the reversals of growth direction and increased the total time of outgrowth. In addition, LY294002 nearly abolished retraction and strongly reduced the velocities of outgrowth and retraction. In combination with VIP or Y-27632, LY294002 mainly reduced the velocity of outgrowth, but even more that of retraction so that net elongation was still higher than in controls. Compared with NMDA alone, the combination of NMDA and LY294002 showed velocities of outgrowth and retraction that were close to control values. However, it increased the time of outgrowth and thus reduced the total retraction distance so that net elongation occurred. When VIP or Y-27632 was added to this combination, the reversals of growth direction were further decreased so that total outgrowth time increased to 80.4%. Under these conditions, the velocity of outgrowth remained unchanged compared with the combination of LY294002 and NMDA, but the velocity of retraction was reduced. Together, these effects nearly abolished the retraction so that net elongation was similar to outgrowth.

**DISCUSSION**

New dendrites and branches are initiated and then extended. Thereafter, they may be stabilized or retracted and even eliminated. In the present study with hippocampal neurons, which were at stage III of differentiation, NMDA induced the initiation of new dendrites and branches. After initiation, these structures were stabilized or retracted. In the absence of NMDA, the average distance of total outgrowth was 14.6 μm, of which 12.8 μm were again retracted. Compared with controls, VIP and Y-27632 decreased the reversals of growth direction by ~36% and increased the total time of outgrowth to 76%. They did not change the velocities of outgrowth and retraction compared with controls. Together, these changes resulted in an increased net elongation of 17.5 μm. Compared with controls, NMDA increased the velocities of outgrowth and retraction from 4.0 to 7.9 μm/h and from 5.1 to 6.2 μm/h, respectively. NMDA enhanced the average numbers of growth reversals to 11.5 without changing the total time of outgrowth. The distances of outgrowth and retraction were increased to 29.4 and 24.1 μm, respectively, so that a significant increase in elongation was not observed. When applied in combination with NMDA, VIP and Y-27632 prevented the increase in the velocities of outgrowth and retraction caused by NMDA as well as in the reversals of growth direction. The increase in the total time of outgrowth induced by the combination of NMDA and VIP or Y-27632 increased net elongation but only to the level caused by VIP or Y-27632 alone.
tion and extension of dendrites and branches by enhancing the activities of Rac and Cdc42. The simultaneous increase in PI3K activity induced by NMDA caused the elimination of the newly extended neurites. When applied together with NMDA, the peptides VIP and PACAP38 prevented the NMDA-induced activation of PI3Ks and thus decreased the elimination of newly added dendrites and branches. In our neurons, NMDA and the neuropeptides affected the formation of primary dendrites as well as branches, suggesting that similar mechanisms mediated both types of extensions. In addition, the peptides mediated the elongation of existing dendrites.

During the subsequent 8 h, a 15-min pulse of NMDA caused the addition of numerous dendrites and branches but also their retraction and elimination. As shown by the inhibitory effect of dizocilpine, NMDA acted via NMDA receptors. NMDA exerted its effects in embryonic hippocampal neurons after 2 days in culture, in which NMDARs are already present but not yet organized in functional synapses (51–53). In contrast to the voltage-gated calcium channel blocker nimodipine, the CaMKII inhibitor KN-93 blocked the dendrite and branch formation caused by NMDA in the presence of VIP or Y-27632. These findings suggested that Ca2+ influx through NMDA receptor-gated channels activated CaMKII, which is known to initiate and stabilize dendritic arborization (54, 55). In the signal pathway initiated by CaMKII, the GTPases Rac and Cdc42 seemed to play a prominent role.

In Xenopus tectal neurons and in retinal neurons, active Rac and Cdc42 are essential for branch addition (10, 56, 57). In our hippocampal neurons, dendritogenesis was indeed no longer inducible when they expressed dnRac1 or dnCdc42, suggesting that both GTPases were necessary. Whether both GTPases had to be increased in their activity was not further investigated. It is noteworthy, however, that a selective increase in Cdc42 activity, as induced by forskolin, enhances branch addition in hippocampal neurons (18).

The influx of calcium through NMDAR channels can affect regulators of Rho GTPases, such as Tiam1, p250 GAP, or Kalirin (14, 58, 59). CaMKII is involved in the regulation of Tiam1 and p250 GAP. KN93 indeed prevented the NMDA-induced activation of Rac and Cdc42, suggesting that NMDA initiated branch addition by acting on
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A

![Graphs showing the effect of different treatments on dendrite motility](image)

B

![Bar chart comparing different treatments on dendrite motility](image)

C

| Points of growth-reversion | control | VIP | NMDA | NMDA+VIP | LY294002 | VIP+LY294002 | NMDA+LY294002 | NMDA+VIP+LY294002 |
|---------------------------|---------|-----|------|----------|-----------|--------------|-----------------|-------------------|
| % of time outgrowth       | 51.8±2.8| 75.5±4.1| 54.2±3.1| 80.6±3.3| 69.6±3.9| 66.0±2.6| 66.0±3.5| 80.4±3.3         |
| Velocity of outgrowth     | 4.0±0.51| 4.1±0.48| 7.9±1.10| 3.3±0.28| 0.5±0.13| 2.8±0.30| 4.8±0.56| 4.2±0.71          |
| Velocity of retraction    | -5.1±0.69| -4.6±0.69| -6.2±0.71| -3.3±1.20| -0.6±0.27| -1.3±0.34| -4.5±0.35| -1.3±0.42          |
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CaMKII, which then caused the activation of Rac and Cdc42. Our experiments with VIP and PACAP38 showed, however, that activation of Rac and Cdc42 per se was not sufficient to cause branch addition. Only activation of the GTPases via NMDARs and CAMKII seemed to occur in the spatial context of additional factors necessary to produce dendrite and branch formation (see also Refs. 11 and 14).

PI3Ks are crucial for branch addition under basal conditions (18). In the present study, the reduction in spontaneous branch addition caused by the PI3K inhibitors wortmannin and LY294002 was overcome by VIP as well as by Y-27632. Whether these agents affected the initiation or the subsequent outgrowth remained unclear. PI3Ks also seemed to contribute to the NMDA-induced branch addition. Thus, NMDA activated PI3Ks, and PI3K inhibitors reduced branch addition. Moreover, an immunocytochemical analysis performed after NMDA application showed p-Akt along all dendrites. Thus, PI3K activity was enhanced at sites where branches were formed.

Although NMDA caused dendrite and branch outgrowth, VIP/PACAP38 and Y-27632 supported arborization by preventing the elimination of the newly formed extensions. The agents had no effect of their own but only blocked the NMDA-induced elimination of new branches by inhibiting the NMDA-induced PI3K activation. In a previous study, we have shown that PI3Ks can increase branch elimination (18). Now, we obtained evidence that the PI3Ks can be regulated in their activity by the neurotransmitter NMDA and the neuromodulators VIP and PACAP. Underlining the important role of PI3Ks in elimination, we found more dendrites and branches compared with the respective controls whenever we applied PI3K inhibitors. Moreover, the dominant-negative form of the PI3K γ-facilitated the NMDA-induced dendrite formation, although we cannot exclude that other isoforms may be also involved, such as α, β, or δ. In time-lapse experiments, LY294002 strongly reduced the elimination of branches, confirming that PI3Ks enhanced the elimination of newly added extensions.

In addition, our results showed that PI3K activity was co-regulated by RhoA and its effector ROCK. When used alone, the ROCK inhibitor Y-27632 affected neither branch addition and elimination nor PI3K activity. However, Y-27632 prevented the NMDA-induced increase in PI3K activity and reduced the elimination of new branches caused by NMDA, suggesting that ROCK contributed to the NMDA-induced activation of PI3Ks, which led to increased elimination (see also Fig. 9). In line with this hypothesis is the previous observation that overexpression of a dominant-negative form of ROCK inhibits hyaluron receptor-induced PI3K activation in MBA-MD-231 cells (60). When our hippocampal neurons expressed dnRhoA, NMDA indeed increased dendrite formation, confirming the inhibitory role of RhoA reported previously (see Introduction).

In all experiments performed on dendrite and branch formation, the peptides VIP and PACAP38 acted like Y-27632. Together with NMDA, VIP and PACAP38 reduced RhoA activity. Therefore, we propose the hypothesis that inhibition of ROCK with Y-27632 or the reduction in RhoA activity caused by VIP/PACAP38 attenuated the NMDA-induced activation of PI3Ks and thus branch elimination (Fig. 9).

The present data suggest that NMDA initiates dendrite and branch formation by activating Cdc42, Rac, and PI3Ks. The subsequent stimulation of VPAC receptors or ROCK inhibition prevents the elimination of the newly formed extensions. It is tempting to speculate that protein complexes are involved, which are similar to those that mediate axon generation (46, 50). Whether such complexes contain NMDA and VIP/PACAP receptors has to be examined in future investigations.
VIP/PACAP38 and Y-27632 also elongated existing dendrites. Time-lapse analysis of these effects provided important information on the dynamics of this process by showing the prominent role of PI3Ks. Compared with controls, LY294002 strongly reduced both outgrowth and retraction velocity and also increased the total time of outgrowth. NMDA, which activated PI3Ks, enhanced both velocities and left the ratio of outgrowth and retraction times unaffected so that no elongation was observed. VIP and Y-27632 used alone did not change PI3K activity and also did not affect the velocity of outgrowth and retraction. However, they caused elongation by enhancing the total outgrowth time. The mechanism of this effect remained open to speculation. Both VIP and Y-27632 enhanced Rac activity. In view of the strong evidence that links Rac to increased outgrowth (11), we consider the GTPase as a candidate to regulate the outgrowth periods.

PAC1, VPAC1, and VPAC2 receptors are expressed in the developing brain (23, 25). In addition, PACAP38 and VIP have been shown to promote neurite outgrowth and elongation (61, 62). In the present study, VIP and PACAP38 facilitated dendrite and branch formation. The effect of VIP on PI3K depends on ROCK. The combination of VIP and NMDA decreases the activity of ROCK and thus of PI3K. Under these conditions, NMDA no longer enhances PI3K activity. Y-27632 inhibits ROCK and thus affects PI3K activity when combined with NMDA.

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