Spatial Changes in Calcium Signaling during the Establishment of Neuronal Polarity and Synaptogenesis

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Abstract. Calcium imaging techniques were used to obtain a clear although indirect evidence about the distribution of functional glutamate receptors of NMDA and non-NMDA type in cultured hippocampal neurons during establishment of polarity and synaptogenesis. Glutamate receptors were expressed and were already functional as early as one day after plating. At this stage NMDA and non-NMDA receptors were distributed in all plasmalemmal areas. During the establishment of neuronal polarity, responses to either types of glutamate receptors became restricted to the soma and dendrites. Compartmentalization of glutamate receptors occurred at stages of development when synaptic vesicles were already fully segregated to the axon. Formation of synapses was accompanied by a further redistribution of receptors, which segregated to synapse-enriched portions of dendrites. Receptor compartmentalization and dendritic redistribution as well as accumulation of synaptic vesicles at synaptic sites occurred also in neurons cultured in the presence of either the sodium channel blocker tetrodotoxin or glutamate receptor antagonists. These results indicate that signals generated by neuronal electrical activity or receptor activation are not involved in the establishment of neuronal polarity and synaptogenesis.

THE cellular machinery responsible for cell–cell communication reaches its maximum efficiency and spatial complexity in neurons. In these cells, mechanisms for both the release and the reception of neurotransmitter coexist. In most cases, neurons adopt the strategy of spatially divide of two functions by establishing sorting mechanisms which lead to the formation of an axon, which conducts action potentials and releases neurotransmitters, and several dendrites, specialized to receive and integrate the chemical signals.

Polarity of neurons is established at early stages of neurite outgrowth. The differentiation of axonal and dendritic processes results in a variety of peculiar shapes that have great importance for both the neuronal signaling properties and pattern of connectivity. When synaptic contacts are formed, a further redistribution of organelles and cytoskeletal proteins in specific neuronal districts takes place (Fletcher et al., 1991; Sanes, 1989; Burgoyne, 1991). Interactions between cells result therefore in the formation of highly specialized surface and cytoplasmic domains.

The study of the mechanisms by which neurons control the localization of synaptic components is an issue which has been rarely addressed in the central nervous system, mostly because of the lack of suitable experimental models. From this stand point, cultured hippocampal neurons present unique advantages in the study of neuronal development and synaptogenesis. When maintained in primary culture, they acquire characteristic axons and dendrites by a stereotyped sequence of developmental events (Dotti et al., 1988). A few hours after plating, neurons develop a few short minor processes (developmental stage 2 of Dotti et al., 1988); after 1–2 d in culture, one of these processes begins to elongate rapidly, becoming the cell axon (stage 3) and only subsequently (3–5 d in culture) the remaining minor processes become dendrites (stage 4). Synaptic vesicle proteins, which are already expressed by hippocampal neurons at stage 2, become markedly concentrated in the axon as soon as it starts elongating (Fletcher et al., 1991; Matteoli et al., 1991, 1992). Eventually the neurons form synaptic glutammatergic contacts where synaptic vesicles are clustered and which are physiologically active from day 6–7 in culture (Bartlett and Banker, 1984*a*,*b*).

At the postsynaptic level, several subtypes of ionotropic glutamate receptors are present. These receptors can be functionally divided in NMDA- and non-NMDA subtypes; the non-NMDA receptors can be further pharmacologically distinguished based on their sensitivity to AMPA and kainate (Gasic and Hollman, 1992; Nakanishi, 1992). NMDA- and non-NMDA glutamate receptors colocalize and cluster at synaptic sites in fully differentiated hippocampal neurons in culture (Bekkers and Stevens, 1989). Recently, it has been shown by immunocytochemical methods that AMPA-

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selective subunits of the non-NMDA receptors are already expressed by non-polarized neurons and that they undergo a progressive dendritic redistribution during development in culture (Craig et al., 1993). It is not known whether functional NMDA receptors are also expressed at early stages of development and whether their accumulation at synapses takes place synchronously with the other types of glutamate receptors.

In this study a calcium imaging approach was used to monitor possible changes in glutamate receptor distribution during development and synaptogenesis of hippocampal neurons in culture. This approach is possible because activation of the NMDA receptor is followed by an increase of $[Ca^{2+}]_i$ which is due to influx of the ion through the receptor channels once the Mg^{2+} block is removed; activation of non-NMDA receptors causes depolarization, and, provided that voltage-operated calcium channels (VOCCs)[1] are present, an increase of $[Ca^{2+}]_i$. Therefore, calcium imaging in combination with pharmacological tools, is a potentially powerful method to obtain a map of functional ionotropic glutamate receptor distribution in living hippocampal neurons in vitro.

This study shows that both NMDA and non-NMDA glutamate receptors undergo a parallel redistribution during the establishment of neuronal polarity which occurs independently on the formation of functional contacts between neurons. Synapse formation, on the other hand, promotes a further, intradendritic compartmentalization of receptors. These events also occurred in cultures treated with tetrodotoxin (TTX) or receptor blockers, indicating that the formation of the synapse and the control of the receptor location in the specific domains of the neuronal membrane are independent on both neuronal electrical activity and activation of the receptors themselves.

Materials and Methods

Hippocampal Cell Culture

Primary neuronal cultures were prepared from the hippocampi of 18-d-old fetal rats as described by Banker and Cowan (1977) and Bartlett and Banker (1984*a*). Briefly, hippocampi were dissociated by treatment with trypsin (0.25% for 15 min at 37°C), followed by trituration with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-L-lysine-treated glass coverslips in MEM with 10% horse serum at densities ranging from 10,000 cells/cm^2 to 20,000 cells/cm^2. After a few hours, coverslips were transferred to dishes containing a monolayer of cortical glial cells (Booher and Sensenbrenner, 1972), so that they were suspended over the glial cells but not in contact with them (Bartlett and Banker, 1984*a*). Cells were maintained in MEM (GIBCO BRL, Gaithersburg, MD) without sera, supplemented with 1% HL1 (Ventrex, Portland, ME), 2 mM glutamine and 1 mg/ml BSA (neuronal medium), both in the presence or in the absence of either TTX (2 μM) or CNQX (10 μM) and APV (100 μM). TTX, CNQX, and APV were added to the cultures at day 0 and changed every 2–3 d.

Fura-2 Videomicroscopy

Neurons were loaded for 30 min at 37°C with 2–4 μM Fura-2 pentacetoxymethylester in Krebs-Ringer solution buffered with Hepes (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes/NaOH, pH 7.4), washed in the same solution to allow deesterification of the dye and transferred to the heated stage of the microscope, where temperature was maintained at 35°C throughout the experiment. Incubations were carried in 2 ml of either the same medium or in its Mg^{2+} free version, which contained no $MgCl_2$ and was supplemented with 1 μM glycine. Stimulation with NMDA was performed always in the Mg^{2+} free/glycine medium. For high K^+ solutions, KCl was substituted for NaCl. In the Na^+ free solutions both NaCl and KCl were isosmotically substituted for choline chloride. Rapid addition of the various reagents was obtained by loading appropriate volumes of 100× concentrated solutions into a syringe connected to the incubation chamber via a small tube. Aspiration to the syringe of 1 ml incubation medium followed by reintroduction of this mixture into the chamber yielded accurate and rapid (<1 s) delivery and mixing of the agents. Fluorescent digital imaging was used to determine the spatial distribution of $[Ca^{2+}]i$ as detailed elsewhere (Grohovaz et al., 1991). Briefly, cells were observed with an inverted Zeiss IM35 microscope equipped with a calcium imaging unit. Fluorescence images were obtained by alternatively illuminating cells at 345 nm and 380 nm wavelength and emission filtered with a 418-nm long pass filter; images (345 nm or 380 nm) consisted of 0.125 MBytes each and were acquired rhythmically at 2 Hz frequency. Fluorescence and bright field images were collected with an Intensified CCD camera (Hamamatsu Photonics) and the camera output was analyzed by a digital image processor (Argus 100; Hamamatsu Photonics, Hamamatsu, Japan) where video frames were digitized and integrated in real time on four 0.5 MByte memory boards. The digital data were then transferred at high rate via a connecting board placed on the VME bus of Argus 100 into a Motorola 68020 based host computer and stored in two 300 MByte hard disks. The time resolution of the measurements was 1 s. Background and calibration images were similarly acquired at the two wavelengths, the calculation of Ca^{2+} concentration was carried out pixel by pixel on pairs of corresponding 345 and 380 images according to Grynkiewicz et al. (1985). To obtain accurate definition of cell boundaries, bright field images were used to prepare masks matching the cell shape. The masks were superimposed to the final calcium images before visualization on the monitor as 16 level pseudocolor images.

Immunocytochemistry

After calcium videomicroscopy, cells were fixed in 4% formaldehyde in 0.1 M phosphate buffer containing 0.12 M sucrose for 25 min at 37°C. Fixed neurons were detergent permeabilized with 0.2% Triton X-100 and incubated with rabbit antibodies against the synaptic vesicle protein synaptophysin followed by rhodamine-conjugated anti-rabbit antibodies, or with mouse antibodies against the cytoskeletal protein MAP2 followed by fluorescein-conjugated anti-mouse antibodies (Matteoli et al., 1991, 1992). Coverslips were mounted in 70% glycerol in phosphate buffer containing 1 mg/ml phenylendiamine. Cells were photographed with Kodak TMAX 400 film on a Zeiss Axiophot microscope equipped with epifluorescence microscopy.

Materials

Most of the chemicals used were purchased from Tocris Neuramin (Bristol, UK). Fura-2 was from Calbiochem Behring Corp. (La Jolla, CA). Polyclonal antibodies directed against rat synaptophysin were a kind gift of Dr. R. Jahn (Yale University, New Haven, CT). Anti MAP2 antibodies and anti-rabbit rhodamine-conjugated antibodies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Anti-mouse fluorescein-conjugated antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Results

Neuronal Ca^{2+} Responses to Glutamate Agonists

10-d old hippocampal neurons in culture were loaded with the Ca^{2+} sensitive dye Fura-2 and analyzed by calcium imaging techniques. Application of 50 μM NMDA, 50 μM kainate, 10–20 μM quisqualate and 50 mM KCl always induced increases in $[Ca^{2+}]_i$. Responses to NMDA were blocked by 100 μM APV, a specific NMDA antagonist and not by 100–200 μM cadmium (Cd^{2+}), a blocker of VOCCs (number of cells examined, n = 27) (Fig. 1 *A*). Responses elicited by kainate and quisqualate/AMPA (not shown) were specifically inhibited by 10 μM CNQX, a non-NMDA receptor blocker, and not by APV (Fig. 1 *B*). Apparently, changes

1. *Abbreviations used in this paper*: TTX, tetrodotoxin; VOCC, voltage-operated calcium channel.

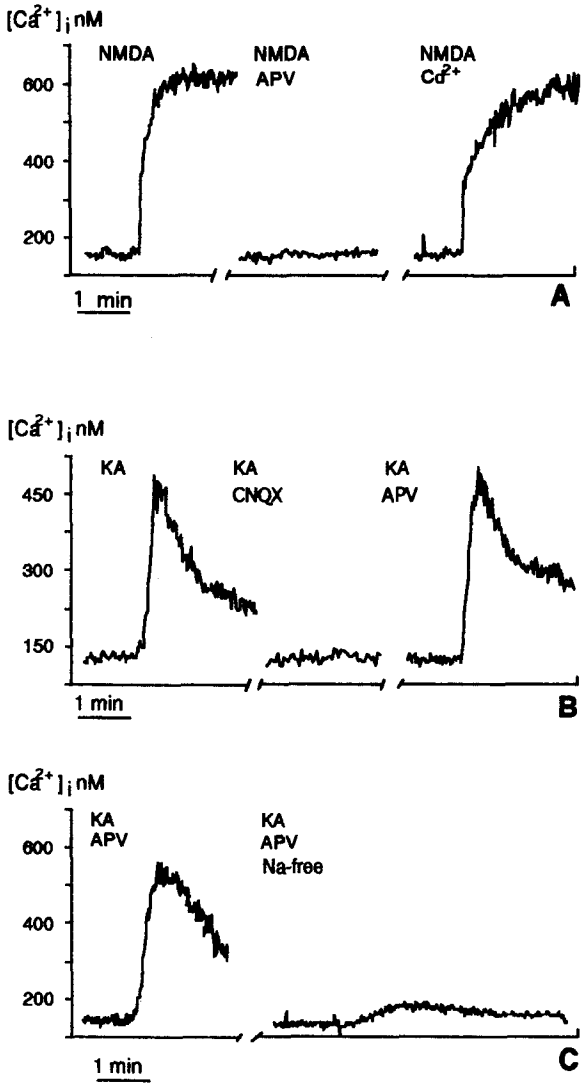

Figure 1. Pharmacological specificities of $[Ca^{2+}]_i$ responses induced by activation of NMDA and non-NMDA glutamate receptors in hippocampal neurons in vitro. Temporal plots of $[Ca^{2+}]_i$ responses induced in the soma of 10-d old hippocampal neurons by glutamate agonists under different experimental conditions. (*A*) $[Ca^{2+}]_i$ increases induced by stimulation with 50 μM NMDA in the absence and in the presence of 100 μM APV or 200 μM Cd^{2+}. (*B*) $[Ca^{2+}]_i$ responses to subsequent treatments with 30 μM kainate, 30 μM kainate in the presence of 10 μM CNQX, and 30 μM kainate in the presence of 100 μM APV. (*C*) $[Ca^{2+}]_i$ increases produced by subsequent treatments with 30 μM kainate and 100 μM APV in a control and in a Na^+-free medium.

in $[Ca^{2+}]_i$ were produced mostly by activation of VOCCs, since kainate responses were almost completely inhibited by prevention of cell depolarization by removal of extracellular sodium ions (n = 23) (Fig. 1 *C*). However, under these conditions, a small but significant increase in $[Ca^{2+}]_i$ was still detectable, suggesting that part of the calcium influx was through the kainate receptor channel. Finally, responses to 50 mM KCl were entirely due to VOCCs activation since they were not inhibited by either 100 μM APV or 10 μM CNQX or a combination of both, but blocked by 100–200 μM Cd^{2+} (not shown).

When hippocampal pyramidal cells were investigated in

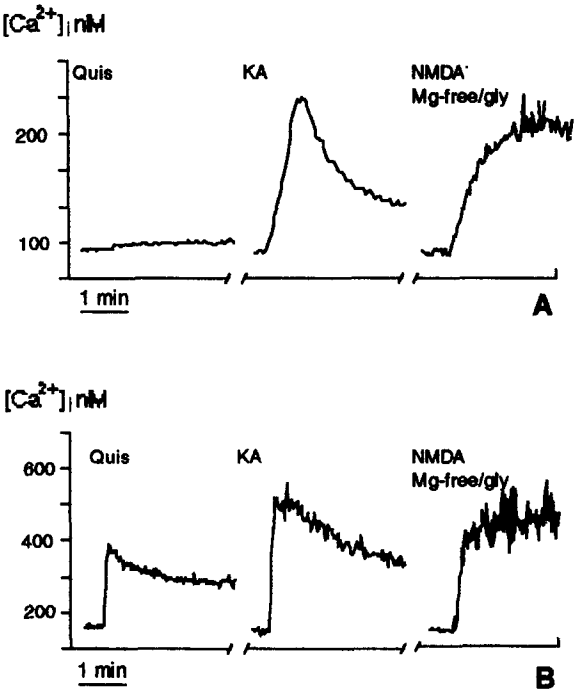

Figure 2. NMDA and kainate elicit increases in $[Ca^{2+}]_i$ early during hippocampal neuron development in vitro. Temporal plots of $[Ca^{2+}]_i$ changes induced in the cell body by 20 μM quisqualate (*Quis*), 50 μM kainate (*KA*), and 50 μM NMDA in single 2-d old (*A*) and 10-d old neurons (*B*).

the absence of extracellular Ca^{2+}, no $[Ca^{2+}]_i$ responses were even observed, even after treatment with quisqualate or 1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD), which are known as activators of the metabotropic glutamate receptors. These results indicate that the metabotropic glutamate receptors coupled to inositol phosphate/Ca^{2+} signal transduction are not expressed or functional to a detectable level in our cultured neurons.

KCl- and NMDA-induced Changes in $[Ca^{2+}]_i$ at Different Developmental Stages

Unpolarized neurons. A few hours after plating, neurons have several short undifferentiated processes (stage 2 of Dotti et al., 1988). Later on, one of these processes starts elongating at a faster rate and differentiates as an axon (stage 3). During the first three days in culture, a clear axonal and dendritic polarity is not established yet and the axons do not form synaptic contacts with adjacent cells. At this stage, challenge with NMDA, kainate, and KCl elicited changes in $[Ca^{2+}]_i$ in all cells studied (n = 39). On the other hand, only two of the same cells responded to 10–20 μM quisqualate or 50 μM AMPA (Fig. 2 *A*). The spatial distribution of $[Ca^{2+}]_i$ changes was analyzed in 27 out of 39 neurons exposed in sequence to NMDA, kainate and KCl, with 15 min of resting interval between each stimulus. Although $[Ca^{2+}]_i$ responses to the glutamate receptor agonists or to depolarization varied in intensity in individual cells, they always occurred diffusely, simultaneously (temporal resolution of the system: 1 s), and to a same extent in the soma and in the processes, including the putative axons (Fig. 3, *A–C*).

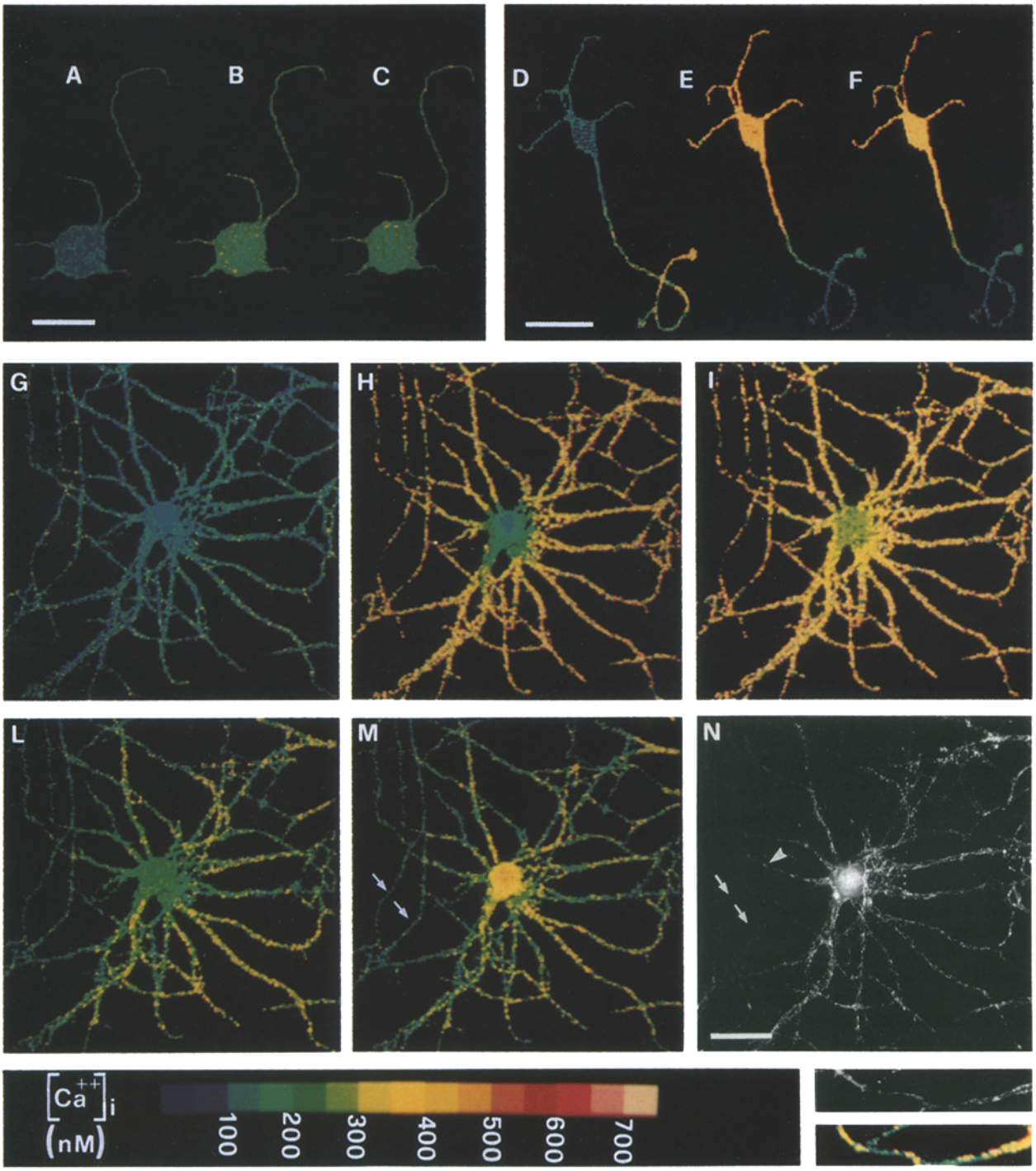

Figure 3. Developmental compartmentalization of glutamate receptor–dependent $[Ca^{2+}]_i$ responses. All the color figures are pseudocolor representations of $[Ca^{2+}]_i$ in neurons at different developmental stages. (*A–C*) Stimulation with 50 μM kainate (*B*) and 50 mM KCl (*C*) in a 2-d old neuron. (*A*) Resting $[Ca^{2+}]_i$. Notice that $[Ca^{2+}]_i$ changes produced by exposure to both stimuli occur diffusely throughout all neuronal compartments. (*D–F*) $[Ca^{2+}]_i$ responses to 50 mM KCl (*D*), 20 μM quisqualate (*E*), and 30 μM NMDA (*F*) in a polarized 9-d old neuron. $[Ca^{2+}]_i$ changes produced by depolarization with KCl appear to be localized to the axon, whereas responses to quisqualate and NMDA are segregated in the somato-dendritic compartment. (*G–M*) $[Ca^{2+}]_i$ responses produced by 50 mM KCl (*H* and *I*) and by 30 μM NMDA (*L* and *M*) in a 16-d old neuron. Images were taken after 2 (*H*), 3 (*I*), 3 (*L*), and 5 (*M*) s after agonist addition. (*G*) Resting $[Ca^{2+}]_i$. Both stimuli produce increases in $[Ca^{2+}]_i$ which originate from the periphery. Notice that $[Ca^{2+}]_i$ increases produced by stimulation with NMDA are restricted to a subset of neuronal processes. (*N*) Immunostaining of the same neuron with antibodies to synaptophysin. Bright dots represent sites of synaptic contacts. $[Ca^{2+}]_i$ changes produced by NMDA are selectively distributed in those processes where synapses are localized. (*Inset*) Detail of a process stimulated with NMDA and stained for synaptophysin. Bars: (*A–C*) 16 μm; (*D–F*) 30 μm; (*G–N*) 38 μm; (*inset*) 14 μm.

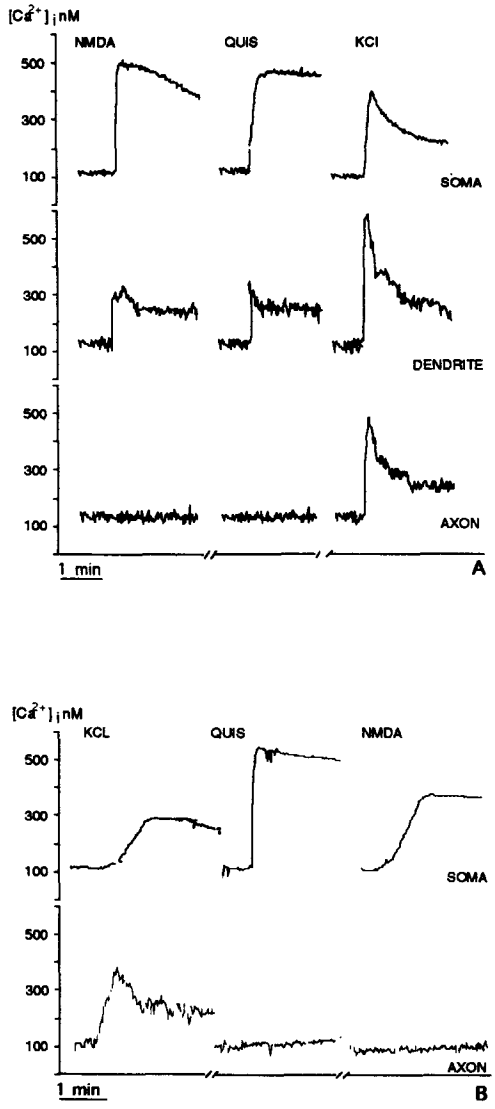

Figure 4. Temporal plots of $[Ca^{2+}]_i$ responses induced in the soma, dendrites, and axonal processes of the 9-d old (*B*) and 16-d old (*A*) neurons illustrated in Fig. 3.

Polarized neurons. After 8–10 d in culture, neurons exhibit a clear axonal and dendritic polarity (stage 4). At this stage, the axon is recognizable as the longest process. Fully polarized, isolated neurons responded to stimulation with all glutamate agonists, including AMPA/quisqualate, with a sudden increase in $[Ca^{2+}]_i$ (n = 52) (Fig. 2 *B*). The spatial distribution of $[Ca^{2+}]_i$ changes produced by glutamate agonists was analyzed in 18 neurons. $[Ca^{2+}]_i$ increases were found to be constantly segregated in the somato-dendritic region. In contrast, the $[Ca^{2+}]_i$ increases induced by KCl occurred in all neuronal processes. Extreme examples of these different distribution patterns are illustrated in Figs. 3, *D–F* and 4 *B*, which show pseudocolor representations and temporal plots, respectively, of $[Ca^{2+}]_i$ changes induced in an isolated, polarized neuron by the sequential application of 50 mM KCl (Fig. 3 *D*), 20 μM quisqualate (Fig. 3 *E*) and 30 μM NMDA (Fig. 3 *F*). Stimulation with glutamate agonists produced a persistent (up to 2 min) increase in $[Ca^{2+}]_i$ in the somato-dendritic regions of the neuron, without any $[Ca^{2+}]_i$ changes in the most distal portion of the axon. In contrast, depolarization of the same cell induced $[Ca^{2+}]_i$ changes in the distal axon (Fig. 3 *D*); later on, $[Ca^{2+}]_i$ increased also in dendrites and cell body (Fig. 4 *B*). In all the cells stimulated with KCl at this and later stages of differentiation, the rate of increase and the peak values of $[Ca^{2+}]_i$ were constantly smaller in the soma than in the processes.

Both the distribution and the intensity of the responses to KCl and glutamate agonists were maintained when the same cell was repeatedly stimulated, irrespectively of the sequence of application of the agents.

Neurons with synaptic contacts. After 14–16 d in culture, neurons form an extensive network of well differentiated and polarized processes. Axons are thin and form functional synaptic contacts with neuronal cell bodies and dendrites. At this stage, spontaneous $[Ca^{2+}]_i$ oscillations are detectable in a percentage of neurons (~5–10%, depending on neuronal preparation). Antibodies against synaptic vesicle proteins stain the cell body and the presynaptic compartment of the axon, whereas the intersynaptic portion of the axon and the dendrites remain unstained. Dendrites can be labeled with antibodies against the cytoskeletal protein MAP2 and their profile is usually outlined by synaptic contacts immunostained with antibodies to synaptic vesicle proteins (Fletcher et al., 1991; Matteoli et al., 1991, 1992).

A typical spatial distribution of $[Ca^{2+}]_i$ changes produced by activation of glutamate receptors and by depolarization in neurons at this developmental stage is shown in Fig. 3, *G–M*. Stimulation with 50 mM KCl produced a sudden increase in $[Ca^{2+}]_i$ in all the processes present in the field while in the cell body the kinetic was slower (Fig. 3, *H–I*). When the same cell was challenged with 30 μM NMDA, only a subset of the processes was responsive (Fig. 3, *L* and *M*). During the following tens of seconds, $[Ca^{2+}]_i$ was maintained at a high level in the same processes and increased in the cell body (Fig. 3, *L* and *M*). In the unresponsive processes $[Ca^{2+}]_i$ remained at the basal levels for the entire experiment (3 min). A similar distribution of $[Ca^{2+}]_i$ responses was observed when the same neuron was stimulated with quisqualate (Fig. 4 *A*) or kainate (not shown). Temporal plots of $[Ca^{2+}]_i$ changes in different neuronal districts after KCl and glutamate agonist stimulation is illustrated in Fig. 4 *A*. These patterns of responses to depolarization and to glutamate agonists were the same in all the neurons studied at this stage (n = 18).

The unresponsive processes were invariably thin long and of uniform diameter, suggesting that they were axons. This was further confirmed in 5 of the 18 cells by labeling with antibodies against the synaptic vesicle protein, synaptophysin, which stain presynaptic boutons formed by axons on neuronal cell body and dendrites (Fig. 3 *N*). The unresponsive processes (an example is indicated by *arrows* in Fig. 3, *M* and *N*) were invariably negative except when crossing dendrites (*arrowhead*), and were not stained by antibodies directed against the microtubule-associated protein MAP2 (not shown), a well established marker for the somatodendritic compartment.

$[Ca^{2+}]_i$ did not rise with a same rate in all the dendrites, and, in some cases, even along the length of an individual dendrite (see for example *inset* in Fig. 3). To determine whether a correlation was present between local $[Ca^{2+}]_i$ increases taking place immediately after the stimulation and

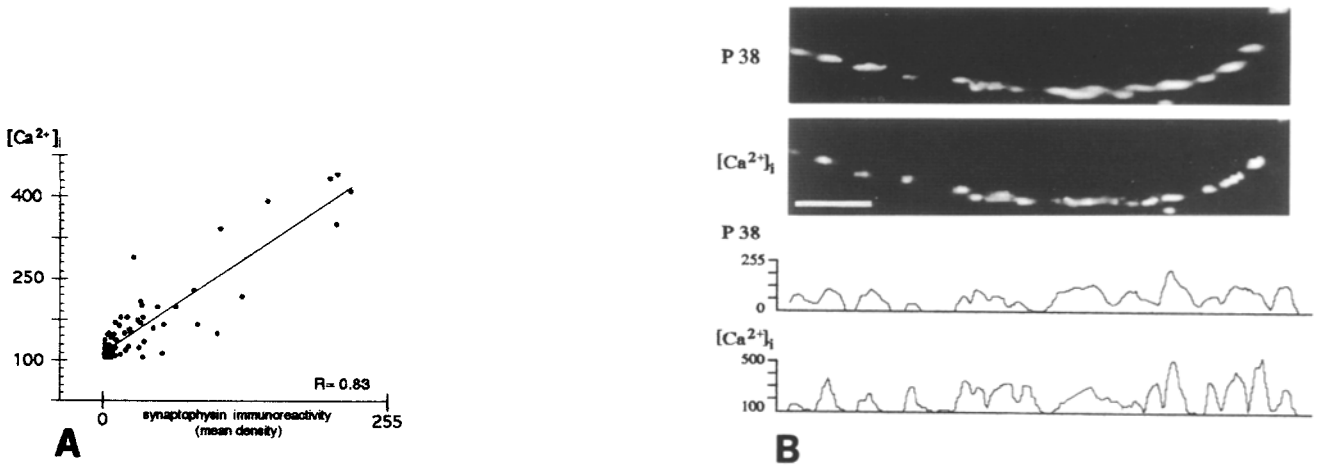

Figure 5. Intradendritic distribution of NMDA receptors is related to the presence of synaptic contacts. (*A*) Plot showing the relationship between intensity of immunostaining for synaptophysin and mean $[Ca^{2+}]_i$ induced by NMDA in the same portions of 14–16-d old neurons. Two cells were examined; correlation coefficient was 0.83, $p < 0.001$. (*B*) On some of the dendrites, the sites of origin of NMDA-induced $[Ca^{2+}]_i$ changes and the clusters of synaptic vesicles were almost perfectly matching (Bar, 6 μm). The graphs beneath the two figures represent their density profiles. Both in *A* and *B*, $[Ca^{2+}]_i$ data were from images obtained 5 s after addition of 30 μM NMDA to the medium.

synaptic contacts, the intensity of the synaptophysin immunofluorescence signal along a process was compared with the local $[Ca^{2+}]_i$ levels present briefly after exposure to NMDA. For this analysis, we used the $[Ca^{2+}]_i$ images obtained 4 s after addition of NMDA, when $[Ca^{2+}]_i$ changes were still discrete. The data from two cells are shown in Fig. 5 *A*. For each point in the graph, the X-value represents the mean intensity of the synaptophysin fluorescence signal in a fixed area whereas the Y-value represents the mean $[Ca^{2+}]_i$ levels in the same area of the neuron. These data indicate that $[Ca^{2+}]_i$ levels produced by NMDA were lower in those portions of processes where vesicle density was also low (correlation coefficient = 0.83, $p < 0.001$). In some of the processes there was a striking spatial correlation between synaptic boutons, identified by the antibodies, and spots of high $[Ca^{2+}]_i$ (Fig. 5 *B*). Similar results were obtained when kainate instead of NMDA was used (not shown).

$[Ca^{2+}]_i$ Changes in Neurons Cultured in the Presence of TTX or Glutamate Antagonists

Spontaneous electrical activity and activation of postsynaptic receptors by released glutamate occurs in hippocampal neurons in culture (Abele et al., 1990; Malgaroli and Tsien, 1992; Verderio, C., S. Coco, G. Fumagalli, and M. Matteoli, manuscript in preparation). To investigate whether neuronal activity played a role in the stage-dependent compartmentalization of glutamate responses, neurons were cultured in the presence of the sodium channel blocker, TTX. TTX (2 μM) was added to the culture medium of neurons starting from day 1 and replaced every 2–3 d; after 14–16 d, hippocampal neurons exhibited abundant, synaptic contacts outlining the neuronal cell body and dendrites (Fig. 6, *B* and *D*). Moreover, in some cases, dendritic spines were detectable (not shown). For Ca^{2+}-imaging studies, TTX was removed 1 h before cell observation. When these neurons were stimulated with glutamate agonists (40 μM kainate, in the case of the neuron of Fig. 6, *A–D*), $[Ca^{2+}]_i$ changes, as in the case of controls, were selectively localized in the somatodendritic compartment (Fig. 6 *A*) as revealed by MAP2 staining (Fig. 6 *B*). Double labeling of the same neurons with antibodies to the synaptic vesicle protein synaptophysin (Fig. 6 *D*) showed that $[Ca^{2+}]_i$ increases were particularly prominent in the synapse-enriched portions of dendrites ($n = 5$). On the other hand, stimulation with 50 mM KCl elicited changes in $[Ca^{2+}]_i$ distributed in all cellular compartments (Fig. 6 *C*). A site of synaptic contact formed by an axon with a MAP2 positive dendrite and showing a higher $[Ca^{2+}]_i$ response to kainate is indicated by arrows.

To further investigate the possible role of receptor activation in the compartmentalization of glutamate-induced $[Ca^{2+}]_i$ changes, neurons were continuously cultured in a medium containing 10 μM CNQX. In some experiments 100 μM APV was also added to the extracellular medium. Under these conditions, no relevant changes in the number and location as well as in the temporal developmental of synaptic contacts were observed during the 14–16 d of culture. Abundant synaptic contacts were formed on neuronal soma and dendrites, and dendritic spines were often detectable (not shown). A detailed quantification of these synaptic parameters is currently in progress. In some cases, when the receptor antagonists were removed from the medium, $[Ca^{2+}]_i$ oscillatory activity was resumed (Fig. 6 *H*), indicating that the functionality of glutamate receptors had been indeed blocked by the pharmacological treatment. In APV-CNQX-treated neurons, as for the cases of control and TTX-treated neurons, KCl-induced $[Ca^{2+}]_i$ increases localized in all processes, whereas activation of glutamate receptors was followed by significant $[Ca^{2+}]_i$ increase in the cell body and dendrites but not in the axons ($n = 4$). Fig. 6, *E–G* shows a cell cultured for 16 d in the presence of the glutamate antagonist CNQX. One hour before Ca^{2+}-imaging study, the glutamate receptor antagonist was removed. In the long thin bona-fide axon of this cell, $[Ca^{2+}]_i$ did not significantly change during a challenge with 40 μM kainate (Fig. 6 *F*) and raised to >600 nM when stimulated with 50 mM KCl (Fig. 6 *G*).

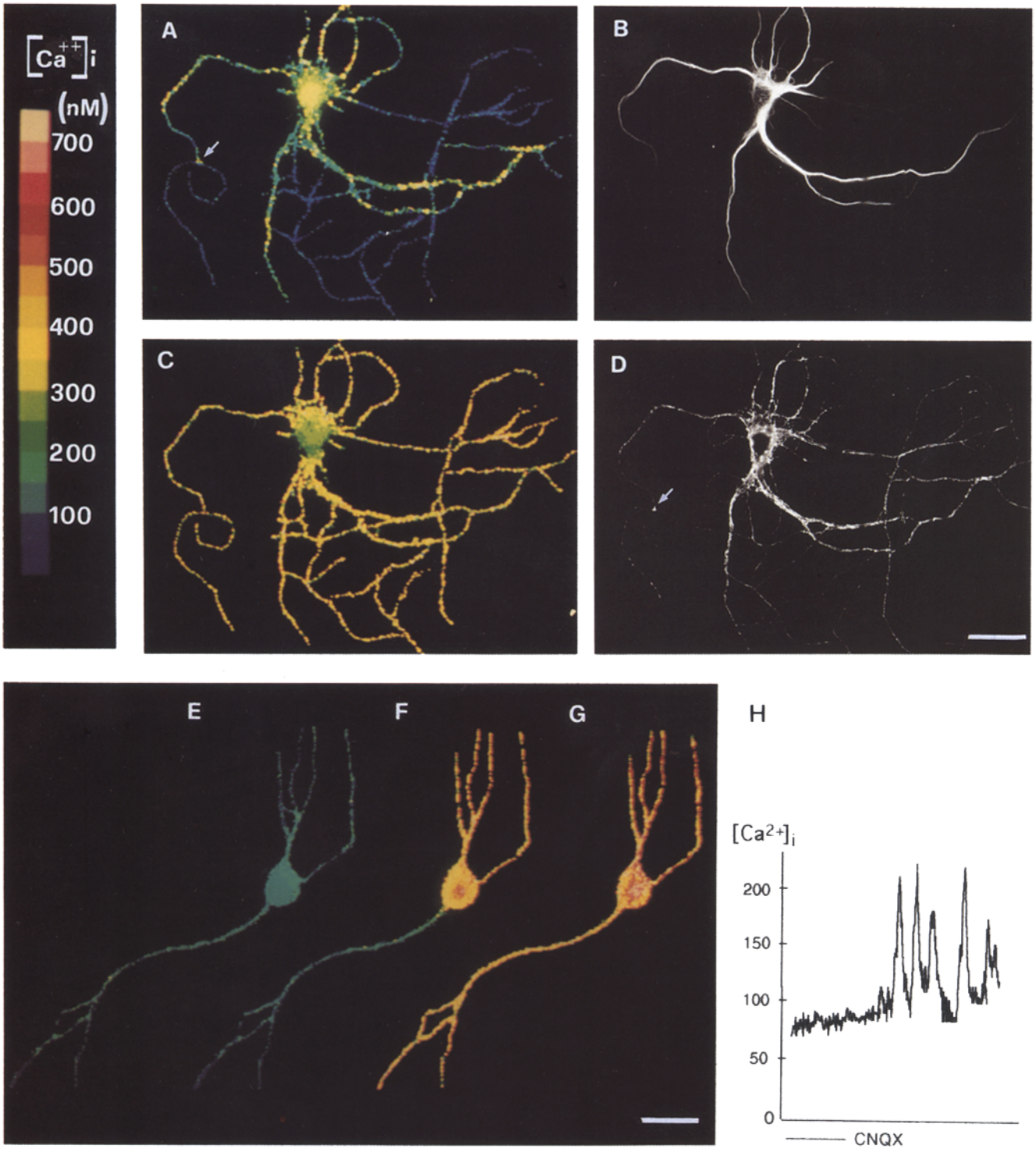

Figure 6. Receptor distribution during development is independent of neuronal activity and receptor activation. Localization of glutamate-induced $[Ca^{2+}]_i$ changes in neurons grown in the presence of TTX or CNQX. (*A* and *C*) $[Ca^{2+}]_i$ changes induced by 40 μM kainate (*A*) or 50 mM KCl (*C*) in the same neurons grown for 13 d in the presence of 2 mM TTX in the extracellular medium. $[Ca^{2+}]_i$ changes induced by kainate are restricted to the somatodendritic region, as revealed by staining of the same neuron with antibodies against MAP2 (*B*). Double labeling with antibodies against the synaptic vesicle protein synaptophysin (*D*) indicates that $[Ca^{2+}]_i$ changes arise from and are more prominent in those regions where synaptic contacts are more abundant. (*E–G*) $[Ca^{2+}]_i$ increases induced by stimulation with 40 μM kainate (*F*) or 50 mM KCl (*G*) in a neuron grown for 16 d in the presence of 100 μM APV and 10 μM CNQX in the extracellular medium. $[Ca^{2+}]_i$ increases produced by kainate are selectively localized in the somatodendritic region whereas changes induced by KCl are distributed to all neuronal compartments. (*H*) Temporal plot showing that neurons are able to resume their $[Ca^{2+}]_i$ oscillatory activity when glutamate antagonists are removed from the medium. Bars: (*A–D*) 25 μm; (*E–G*) 23 μm.

Discussion

Spatially resolved Ca^{2+} measurements demonstrated that cytosolic Ca^{2+} signaling can be controlled differently in local regions of fully differentiated mammalian neurons (Connor et al, 1988; Lipscombe et al., 1988; Muller and Connor, 1991; Regehr and Tank, 1992; Miyakawa et al., 1992). We have therefore used Ca^{2+}-imaging to obtain information about distribution and function of VOCCs and ionotropic glutamate receptors during establishment of neuronal polarity and synaptogenesis in hippocampal neurons in vitro. This approach has unique advantages over immunocytochemical methods, which do not provide information about receptor functionality, and over electrophysiology, which may allow monitoring of the responses induced by receptor activation only in microdomains of neuronal membrane.

Ca^{2+}-imaging can provide a clear although indirect evidence about the distribution of functional VOCCs and NMDA-receptors which are highly permeable to calcium ions. The distribution of the non-NMDA receptors, which are little permeable to divalent cations, could be inferred by imaging of $[Ca^{2+}]_i$ transients induced by depolarization-dependent activation of VOCCs, strategically located in close proximity of the receptors themselves. Accordingly, $[Ca^{2+}]_i$ changes induced by AMPA/kainate were almost completely abolished by removal of Na^+ ions from the medium.

This study shows that, during development in culture, both NMDA and non-NMDA glutamate receptors are progressively excluded from the axon, by mechanisms which appear to be independent of both spontaneous neuronal electrical activity and glutamate receptor activation. Eventually, in parallel with synaptogenesis, glutamate receptors become enriched in the synaptic portions of the dendrites.

Surface Compartmentalization of Glutamate Receptors during the Establishment of Neuronal Polarity

A first accomplishment of this study is the finding that hippocampal neurons express functional glutamate receptors of both NMDA and non-NMDA types as early as 1 d after plating. At this stage, $[Ca^{2+}]_i$ changes induced by activation of both types of glutamate receptors, as well as of VOCCs, occurred diffusely in all neuronal surface compartments. The homogeneous distribution of $[Ca^{2+}]_i$ responses was maintained in the neurons for at least 3–4 d of culture when almost all neurons were endowed with a long thin axon (stage 3 neurons). When a complete polarization was well established (stage 4 neurons, 9–14-d old neurons in this study), the $[Ca^{2+}]_i$ changes produced by activation of both NMDA and non-NMDA glutamate receptors were already segregated in the somato-dendritic compartment. In these cells a surprisingly steep calcium gradient was constantly seen at the axon hillock. Though mechanisms for local control of calcium have already been described in neurons (Muller and Connor, 1991; Guthrie et al., 1991; Silver et al., 1990), we want to point out that the steep gradients seen in our experiments persisted for the entire length of receptor stimulation (up to three minutes), indicating that the axon is indeed provided with a very efficient calcium-buffering system.

In all cases, the subcellular distributions of the $[Ca^{2+}]_i$ changes induced by NMDA and non-NMDA agonists were superimposable in single cells, regardless of whether the ion influx was through the receptor channel (NMDA receptor) or through VOCCs activated by the AMPA/kainate receptor-induced depolarization.

We cannot rule out the possibility that part of the responses induced by non-NMDA agonists were due to activation of presynaptic receptors. On the other hand, kainate-induced $[Ca^{2+}]_i$ increases were never seen to occur or propagate along the length of the axons of well differentiated neurons. This argues against a relevant participation of the presynaptic compartment in generating the calcium signal induced by the non-NMDA agonists. Moreover, a dendritic, postsynaptic compartmentalization of AMPA-selective subunits has been recently demonstrated by immunocytochemistry (Craig et al., 1993). Our results complement those data by showing, first, that glutamate receptors are in fact functional even before their clustering at synapses, and, second, that a similar redistribution occurs for both NMDA and non-NMDA receptors. On the other hand, the lack of receptor-induced $[Ca^{2+}]_i$ changes at the tip of the growing axon argues against a functional relevance of the AMPA subunits detected on these surface domains by immunocytochemistry (Craig et al., 1993). Alternatively, possible differences could be accounted for by a certain degree of variability in neuronal maturation rate.

At difference with receptor activation, responses to depolarization occurred in all neuronal processes. This clearly indicates that functional VOCCs are distributed throughout the entire neuronal plasmamembrane. Our pharmacological approach does not allow to discriminate among the various VOCCs subtypes which are expressed in these cells (Jones et al., 1989; Ozawa et al., 1989; Takahashi et al., 1989; Westenbroek et al., 1990; Fisher et al., 1990; Mogul et al., 1993). A different distribution of some of these channels has been shown to occur in hippocampus and in cortical neurons (Jones et al., 1989; Westenbroek et al., 1990; Benke et al., 1993; Wheeler et al., 1994). It cannot be excluded therefore that different domains of the membranes of hippocampal neurons are endowed with specific VOCC subtypes.

Compartmentalization of Presynaptic Synaptic Vesicles and Postsynaptic Glutamate Receptor Occurs at a Different Time

Our results indicate that compartmentalization of the ionotropic glutamate receptors occurred at a developmental stage when axonal sorting of presynaptic vesicles was already fully established. Also in the case of virus-infected hippocampal neurons, sorting of HA and VSV glycoproteins to the axonal and dendritic compartments, respectively, occurred at late stages of development (Dotti and Simons, 1990). It appears therefore that the sorting of integral membrane proteins to specific domains of the neuronal plasmamembrane and of presynaptic vesicles to the axon rely on different cellular machineries. It is noteworthy that synaptic vesicles and vesicles containing neurotransmitter channels have been shown to interact with specific and different members of the microtubular motor kinesin family (Hall and Hedgecock, 1991; Gho et al., 1992). Differences in the temporal expression of specific cellular motor may then account for the asynchronous compartmentalization of the pre- and the postsynaptic elements. In addition, a differential axonal vs dendritic distribution can also be achieved and maintained by inhibition of diffusion in the axon of receptors already in-

serted in the perikarial plasmamembrane. Indeed a functional barrier to diffusion of lipids has been envised at the level of the axon hillock in polarized neurons (Kobayashi et al., 1992).

Relocation of Glutamate Receptors at Sites of Synaptic Contacts

At later stages of development, when synaptic contacts were formed, glutamate agonist-induced $[Ca^{2+}]_i$ elevations originated mostly from discrete portions of the dendrites facing the presynaptic boutons. Electrophysiological and immunocytochemical evidences indicate that clustering of glutamate receptors occurs indeed at synaptic contacts (Bekkers and Stevens, 1989; Jones and Baughman, 1991; Craig et al., 1993).

The cellular and molecular mechanisms responsible for receptor aggregation underneath presynaptic membrane have been described in some detail for the acetylcholine receptor in muscle: here clustering of the receptor in the postsynaptic membrane is the result of interaction with a specific intracellular protein, the 43 K protein, and cytoskeletal elements (Froehner, 1993) as well as with endplate-associated extracellular protein, such as agrin (McMahan, 1990; Hoch et al., 1993). Agrin is widely expressed in the CNS and a functional analogue of 43 K, gephyrin, has been described to promote and sustain clustering of the glycine receptor in cultured spinal neurons (Kirsch et al., 1993*b*). Several forms of gephyrin are widely expressed in the CNS (Kirsch et al., 1993*a*), suggesting that members of this family may sustain clustering of glutamate receptors as well. Primary cultures of hippocampal neurons, by providing a source of neurons synchronized in the formation of synaptic contacts, characterized by distinguishable and accessible pre- and postsynaptic compartments, represent therefore an interesting model for the characterization of molecular mechanisms involved in the process of synapse formation and stabilization in the CNS.

The Distribution of Glutamate-induced Ca^{2+} Transients Is Independent of Neuronal Activity

At the neuromuscular junction, neuromuscular transmission and muscle activity play relevant roles in the maturation and maintainance of several physiological, biochemical, morphological and metabolic properties of the muscle, the postsynaptic apparatus, and the acetylcholine receptor (Hall and Sanes, 1993). Activity also has a major role in the acquisition of the mature anatomy and physiology of the CNS: during the early postnatal period it affects not only the overall structure of axon and dendrites but also the synaptic structure of neurons (Frank, 1987; Contantine-Paton et al., 1990; Fields and Nelson, 1992; Hockfield and Kalb, 1993).

Hippocampal neurons in vitro are spontaneously active: variations of transmembrane potential in the form of both miniature endplate potentials and propagating action potentials have been described and are due to activation of glutamate receptors by presynaptically released neurotransmitter (Abele et al., 1990; Malgaroli and Tsien, 1992). Fluctuations of $[Ca^{2+}]_i$ can be detected by Ca^{2+}-imaging when Mg^{2+} is removed from the medium (Abele et al., 1990) or even in normal medium in a limited number of cells (this study). The reason why only a few cells show spontaneous $[Ca^{2+}]_i$ changes is obscure and is now under investigation. Nonetheless, the spontaneous $[Ca^{2+}]_i$ oscillations are blocked by the sodium-channel blocker TTX and by glutamate receptor antagonists suggesting that they are dependent, at least in part, by receptor activation.

Spontaneous activity at the early stages of differentiation, before synapses are formed, have never been observed in the present study. To our knowledge, the issue had never been addressed. On the other hand, hippocampal neurons recycle synaptic vesicles (Matteoli et al., 1992; Mundigl et al., 1993) and release glutamate (Verderio, C., S. Coco, G. Fumagalli, and M. Matteoli, manuscript in preparation) even before the establishment of synaptic contacts. In principle, one cannot rule out that activation of glutamate receptors also occurs before synaptogenesis, though at a subthreshold level.

To properly test the hypothesis that spontaneous electrical activity or receptor activation have any role in the spatial control of glutamate receptor distribution, we cultured hippocampal neuron in the continuous presence of TTX or receptor blockers (CNQX alone or in combination with APV). Under these conditions, neurons developed an apparently normal synaptic network, with abundant synaptic boutons along dendritic spines and also the soma. In some cases, when the receptor antagonists were removed from the medium, $[Ca^{2+}]_i$ oscillatory activity was resumed indicating that the antagonists had indeed blocked the postsynaptic receptors. In all these cells the responses to NMDA, non-NMDA glutamate agonists and depolarization were spatially distributed as in control cells cultured without inhibitors. Analysis of the sites of origin of $[Ca^{2+}]_i$ changes showed that most of the responses originated from limited portions of the dendrites which were coincident with the presence of presynaptic boutons.

These results indicate that the axonal exclusion of ionotropic glutamate receptors and their clustering at synaptic sites are independent of neurotransmitter receptor activation or cell depolarization. Whether this is due to TTX-insensitive presynaptic release of some trophic factor or is an intrinsic property of neurons, it cannot be determined by this study. However, the similarities which exist between hippocampal neurons and muscle cells in the modifications of neurotransmitter receptor distribution during development can be emphasized. In muscle, AChRs are initially randomly distributed on the plasmamembrane. Then, even before synaptic contacts are formed, part of the AChR molecules is bridled and clustered. When synaptic contacts are formed with motoneurons, receptors become highly concentrated at the tips of the junctional folds and almost completely disappear from the extrajunctional portions of the muscle membrane (for a review see Hall and Sanes, 1993). The hypothesis that clusters of glutamate receptors can form spontaneously in absence of presynaptic contacts is now under investigation.

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