Hippocampal Synaptogenesis in Cell Culture: Developmental Time Course of Synapse Formation, Calcium Influx, and Synaptic Protein Distribution

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The formation of chemical synapses between hippocampal neurons in primary cell culture was studied using electrophysiology, calcium imaging, and immunocytochemical approaches. Inhibitory and excitatory synapses formed within 12 d in cell culture (DIC) that were sensitive to the N-type calcium channel blocker ω-conotoxin GVIA (ω-CgTx). At 4 DIC, immature connections were present in which spontaneous, but rarely evoked, synaptic currents were detected. At both 4 and 12 DIC, the synaptic proteins rab3a, synapsin I, and synaptotagmin were present in hippocampal neurons, but the subcellular distribution changed from one in which immunoreactivity was initially distributed within soma and neurites to a punctate varicose appearance. Correlated with the transformation from immature to mature synaptic states was the onset of ω-CgTx–sensitive calcium influx. Taken together, these data suggest that the expression of functional ω-CgTx–sensitive calcium influx is temporally coincident with synapse formation, and that during the maturation of the synapse there is a redistribution of synaptic proteins.

[Key words: synaptic proteins, synaptotagmin, synapsin, rab3a, synaptogenesis, calcium channel, conotoxin, ω-CgTx]

Much of our knowledge about synaptogenesis has been gained from studies of neuromuscular development where agrin and aria, synthesized in motoneurons, regulate the expression and aggregation of postsynaptic ACh receptors (Falls et al., 1990; McMahan, 1990). For the development of fast synaptic transmission, it is also necessary that presynaptic macromolecules are synthesized, transported to the presynaptic terminal, and arranged with the appropriate mutual spatial relation. For example, it is necessary for the calcium channel to become localized in close proximity to the secretory vesicle to supply calcium in a sufficiently high concentration to stimulate secretion (Simon and Llinas, 1985; Zucker and Fogelson, 1986). It is also unclear whether different calcium channels are subject to the same regulation during synaptogenesis. For example, are L-type channels that do not stimulate transmitter release subject to similar reg-

ulation as N-type channels that do supply calcium to stimulate synaptic transmission (Takahashi and Momiyama, 1993)? Little information is available concerning the regulation of these presynaptic developmental events.

Studies of nerve–muscle synapse formation have shown that the synaptic target supplies retrograde signals that control the appropriate development of presynaptic machinery. Muscle cells manipulated into contact with growth cones of neurons derived from the *Xenopus* neural tube cause a local increase in resting calcium level (Dai and Peng, 1993), a rapid induction of secretion (Xie and Poo, 1986), and a local reorganization of the growth cone such that the quantal content of evoked synaptic transmission is augmented (Sun and Poo, 1987). In *Helisoma*, muscle fibers activate presynaptic protein kinase A, which causes an elevation of the resting calcium level of neuronal growth cones, and an enhancement of the presynaptic calcium influx during action potentials (Funte and Haydon, 1993; Zoran et al., 1993). After many hours of contact, the calcium sensitivity of the secretory apparatus is then increased (Zoran et al., 1991). These regulatory events lead to the effective coupling of presynaptic action potentials to secretion.

In contrast to studies of neuromuscular synapse formation, there is a paucity of studies of the regulation of synaptogenesis between central neurons. Central neurons can contain many types of calcium channels, yet only a restricted subset stimulates transmitter release (Takahashi and Momiyama, 1993). *A priori*, one would expect a differential regulation of these different subsets of calcium channels during synaptogenesis. Furthermore, it is unclear when specific calcium channels develop in relation to the onset of synapse formation, when and where synaptic associated proteins are expressed and when excitation becomes coupled to secretion. Using dissociated hippocampal cell cultures, the goal of this study is to elucidate the temporal relation between the appearance of functional synaptic transmission, L- and N-type calcium channels, and synaptic protein distribution. This study demonstrates that while many components of the presynaptic neuron are expressed early in cell culture the development of ω-CgTx–sensitive N-type, but not nifedipine-sensitive L-type, calcium influx is temporally correlated with the delayed detection of evoked synaptic transmission.

Some of these data have appeared in preliminary form (Basarsky et al., 1992).

Materials and Methods

Cell culture. Hippocampi were dissected from 1–4-d-old Sprague–Dawley rats. Tissue was incubated for 1 hr at 37°C in Ca^{2+}/Mg^{2+}-free Earle's balanced salt solution (EBSS; pH 7.35; GIBCO) containing papain (20

U/ml; Sigma), HEPES (10 mM), L-cysteine (0.2 mg/ml), glucose (20 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Tissue was washed once with fresh EBSS and then placed in EBSS (pH 7.35) containing HEPES (10 mM) and trypsin inhibitor (10 mg/ml, type II-O; Sigma) for 5 min. After being rinsed, hippocampi were mechanically dispersed by triturating through a fire-polished glass pipette. Cells were plated into poly-L-lysine (1 mg/ml, MW 100,000; Sigma)–coated glass-bottomed dishes. Cultures were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere. Culture medium consisted of Eagle's minimum essential medium (Earle's salts, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and containing 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, 14 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin (pH 7.35). The dissociation procedure was modified from previously described procedures (Leifer et al., 1984; Huettner and Baughman, 1986; Mattson and Kater, 1989). To suppress proliferation of non-neuronal cells, arabinosylcytosine (ARA-C; 5 μM) was added after 2–3 d in culture. Cultures were fed once a week by exchanging 30% of the medium with fresh medium.

Electrophysiology. Conventional dual whole-cell recording techniques (Hamill et al., 1981) were employed to stimulate and record synaptic currents from cultured hippocampal neurons that had been grown in cell culture for 1–21 d. All experiments were performed at room temperature (22–24°C). Normal external saline contained (in mM) NaCl, 140; HEPES, 10; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; pH 7.35 with NaOH. Pipette solutions contained (in mM) K-gluconate, 140; EGTA, 10; Mg-ATP, 4; GTP, 0.1; HEPES, 10; pH 7.35 with KOH. The osmolarity of the external saline was adjusted with sucrose to be 10 mOsm higher than the internal pipette solution. Pipettes fabricated from 1.5 mm o.d. borosilicate glass had D.C. resistances of 30–7 MΩ. Postsynaptic cells were voltage clamped at a range of −80 to −30 mV to test for the presence of excitatory and inhibitory synaptic transmission. Presynaptic cells were held in current clamp at −60 mV. The presence of spontaneous synaptic events was assayed at holding potentials of −70 and −40 mV in the first 7 min after whole-cell access. Evoked connections between neuronal pairs were measured by current injection to induce action potentials in the putative presynaptic cell and monitoring postsynaptic responses in voltage-clamped postsynaptic neurons. Evoked connections were considered monosynaptic if the synaptic delay was less than 5 msec from the peak of the presynaptic action potential to the onset of the postsynaptic response and the synaptic latency remained unchanged with repetitive stimulation. This was confirmed in some experiments by elevating external divalent cations by 2 mM to reduce neuronal excitability. All drugs were administered via bath application.

Pharmacology test protocols consisted of 20 action potentials stimulated at 2 sec intervals. To reduce variability in synaptic responses, the first response was discarded, and the remaining 19 episodes were averaged.

Data was acquired from two Axopatch 1-C amplifiers using pCLAMP software (v. 5.51, Axon Instruments) and Labmaster hardware (TL-1, Scientific Solutions) after filtering at 1 kHz. Records were also digitized and stored on VCR tape (Vetter PCM/VCR).

CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) was purchased from Tocris Neuramin; ω-CgTx GVIA, picrotoxin, and TTX (tetrodotoxin) were from Sigma.

Immunocytochemistry. Presence of synapse-specific proteins was determined by indirect immunocytochemistry procedures. Primary monoclonal antibodies directed against synaptotagmin (CL 41.1) and rab3a (CL 42.2) were generously provided by R. Jahn, and a polyclonal antibody (CG-454/455) directed against synapsin I was generously supplied by A. Czernik. The antibodies were used at the following dilutions: synaptotagmin (1:250), rab3a (1:50 and 1:500), and synapsin I (1:250). Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After permeabilization with Triton X-100 and incubation with BSA (5%) and goat serum (5%) to prevent nonspecific binding, primary antibodies were added and the cells were incubated overnight at 4°C. After washout of the primary antibodies, goat anti-mouse and goat anti-rabbit rhodamine conjugated antibodies (1:200; Fisher) were added and the preparation was incubated for 1 hr at room temperature. In some preparations, after immunochemical staining, the cells were washed with PBS containing 0.1% $NaBH_4$ for 30 min at room temperature to reduce autofluorescence (Beisker et al., 1987).

Visualization was accomplished using two separate imaging systems. For most experiments, conventional epifluorescence microscopy was utilized using a silicon intensified target (SIT) camera driven by IMAGE-1/AT software (v. 4.0, Universal Imaging Corp.).

In some cases, to gain enhanced spatial resolution, a Noran Odyssey real-time laser scanning confocal microscope equipped with IMAGE-1/AT software (v. 4.0) was used. Optical sections were collected through a 60× plan-apochromatic objective that gave a single section optical thickness of 0.7 μm (full width at half-maximum, FWHM). The initial and final optical planes were specified to ensure that all immunoreactive areas were captured.

Calcium imaging. Calcium levels were estimated using fura-2/AM and ratiometric imaging techniques (Grynkiewicz et al., 1985). All measurements were made from somatic regions. Cells were loaded with fura-2/AM (2 μM) for 40 min at 37°C. One microliter of 25% (w/w) of Pluronic F-127 was mixed per 1 ml of fura-2/AM loading solution (2 μM) to aid solubilization of the ester into aqueous medium. After washing, fura-2/AM was deesterified for 40 min at 37°C. All experiments took place at 22–24°C. Normal external saline was (in mM) NaCl, 140; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 2; and HEPES, 10 (pH 7.35). In zero-calcium saline, Ca^{2+} was replaced with Mg^{2+} and 2 mM EGTA was added.

To test for the presence of voltage-sensitive calcium influx, neurons were incubated in TTX (1 μM) to prevent spontaneous action potentials, and were depolarized using elevated potassium saline that was applied by a 30 sec pressure ejection pulse from a puffer pipette (~2–3 μm opening; 5–8 psi). In this saline, KCl (50 mM) replaced NaCl.

Image processing and analysis was performed using either QFM ratiometric software and a QX-7 processor (Quantex Corp.) or IMAGE-1/FLUOR (v. 1.63g, Universal Imaging Corp.). Background subtracted ratio images (340/380 nm or 350/380 nm) were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz et al. (1985).

Calibration of fura-2 was performed *in situ* (Thomas and Delaville, 1991). Briefly, cells were permeabilized with the calcium ionophore 4-bromo-A23187 (10 μM; Molecular Probes) in the presence of 4 mM calcium to obtain R_{max}, or 0 Ca^{2+}/10 mM EGTA to obtain R_{min}. Fura-2 fluorescence was then quenched with Mn^{2+} (20 mM) to acquire background fluorescence levels for R_{min} and R_{max}. In some cases, at the end of an experiment, digitonin (40 μM) was added to measure the extent of compartmentalization of Fura-2. In all cases where this was done, compartmentalization was not significant. R_{min} was 0.17–0.21, R_{max} was 2.86–3.90, and F_0/F_s was 3.44–10.57. Similar calibration values were obtained when parallel calibrations were performed using fura-2 pentapotassium salt *in vitro*. There were no differences in the calibration values between cells at different times in culture. A K_d of 224 nM as previously reported (Grynkiewicz et al., 1985) was used. For experiments where osmolarity was increased by the addition of 300 mM sucrose, a linear relationship between ionic strength and K_d was assumed. The K_d was adjusted to 990 nM, based on previously reported values (Grynkiewicz et al., 1985).

Cells were kept for further analysis if the calcium accumulation due to the first application of high-$[K^+]$ saline exceeded 50% of the resting calcium level. Using this criteria 97% (583 of 600 tested) of neurons were responsive.
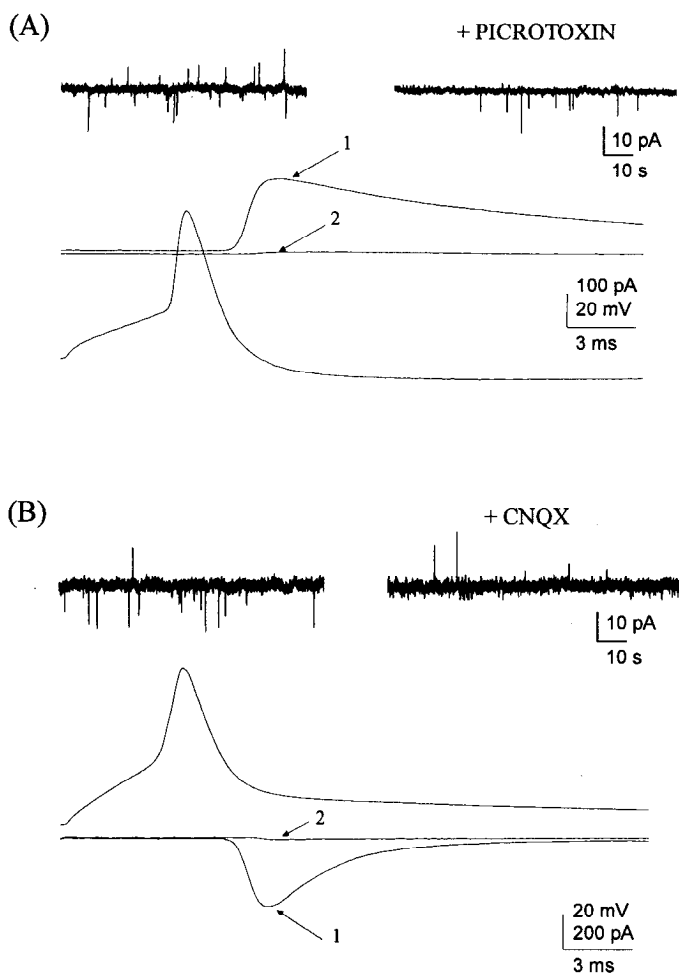
For experiments involving application of calcium channel antagonists, DMSO, or zero-calcium, high-$[K^+]$ applications were 12 min apart. DMSO (0.1%), nifedipine (5 μM), or zero-calcium saline was uniformly bath applied 5 min prior to the second application of high $[K^+]$ while ω-CgTx (100 nM) and cadmium (100 μM) were bath applied at 10 and 1–2 min prior to the second high-$[K^+]$ application.

Only cultures that remained viable for at least 2 weeks, and in which evoked connections were detected in at least 50% of all pairs assayed electrophysiologically on day 12, were included in electrophysiology, calcium imaging, and immunocytochemistry analysis.

Results

Pharmacology of synaptic currents

As an initial step in characterizing synapse formation between cultured hippocampal neurons, the pharmacological properties of action potential–evoked and spontaneous synaptic currents were examined in neurons cultured from 1 to 14 d. In normal saline spontaneous inhibitory and excitatory synaptic currents were present. While some of these spontaneous synaptic events likely arise from the generation of action potentials in synaptically connected neurons, the majority of the events are due to spontaneous release of neurotransmitter since they were still detected in the presence of TTX (1 μM). Bath application of 100

(A)

+ PICROTOXIN

| 10 pA
10 s

| 100 pA
| 20 mV
3 ms

(B)

+ CNQX

| 10 pA
10 s

| 20 mV
| 200 pA
3 ms

Figure 1. Evoked and spontaneous synaptic connections in hippocampal neurons 12–13 d after plating into culture. *A:Upper trace, left,* Recording of spontaneous inhibitory and excitatory synaptic events. *Right,* Inhibitory spontaneous synaptic events are blocked by bath application of 100 μM picrotoxin. *Lower trace,* Evoked inhibitory synaptic connections in normal saline (*1*) or with bath application of 100 μM picrotoxin (*2*). *B:Upper trace, left,* Spontaneous inhibitory and excitatory spontaneous synaptic events in a different cell than *A. Right,* Excitatory spontaneous synaptic events are blocked by bath application of 10 μM CNQX. *Lower trace,* Evoked excitatory connections in normal saline (*1*) are abolished in the presence of CNQX (*2*). Evoked responses shown are the average of 19 responses evoked at 2 sec intervals. Current and voltage traces are graphically offset for clarity.

μM picrotoxin completely and selectively abolished spontaneous and action potential–evoked synaptic inhibitory currents (Fig. 1*A*), while bath application of 10 μM CNQX completely inhibited both action potential–evoked and spontaneous excitatory synaptic currents (Fig. 1*B*). This pharmacological profile is consistent with GABA$_A$-mediated inhibitory synaptic responses and CNQX-sensitive non-NMDA-mediated excitatory synaptic events. The absence of an NMDA component may be attributed to the lack of glycine and the presence of 2 mM MgCl$_2$ in the bath saline.

Pharmacology of presynaptic calcium channels

Since action potentials must admit calcium to trigger transmitter release (Katz, 1969; Mulkey and Zucker, 1991), we examined which calcium channel subtypes mediate calcium influx necessary for neurotransmitter release in 12–13-d-old hippocampal

neurons. The specific N-type calcium channel antagonist ω-CgTx was used to determine the contribution of calcium influx through N-type channels in mediating transmitter release. Bath application of 100 nM ω-CgTx differentially reduced excitatory and inhibitory synaptic currents. In the presence of ω-CgTx, excitatory synaptic currents were inhibited 58 ± 7% (mean ± SEM, *n* = 6), while inhibitory synaptic currents were reduced by 87 ± 3% (*n* = 15) of their initial amplitude (Fig. 2*B,C*). Thus, N-type calcium channels are required for inhibitory synaptic transmission and contribute calcium for excitatory synaptic transmission. In contrast, the L-type calcium channel antagonist nifedipine (5 μM) did not inhibit either excitatory (−36 ± 21%; *n* = 6) or inhibitory (−9 ± 6%; *n* = 14) synaptic connections (Fig. 2*A*). The incomplete inhibition of excitatory synaptic transmission supports other observations that multiple subtypes of calcium channels supply calcium to stimulate transmitter release (Takahashi and Momiyama, 1993).

## Time course of synapse development

Having characterized the properties of inhibitory and excitatory synaptic transmission in culture, we examined the temporal pattern of synapse development. Initially, the development of functional hippocampal synapses was examined using electrophysiological approaches. Cultures were assayed for synaptic events at 2 d intervals ranging from day 2 through day 14 after plating into culture (Fig. 3). Spontaneous synaptic events were detected as early as day 2 in culture. By day 4, spontaneous synaptic events were detected in 32 ± 13% of all cells assayed, while evoked connections were only detected in 11 ± 4% of all synaptic pairs assayed. On day 12, spontaneous events were detected in 86 ± 17% of all cells assayed, and evoked connections were detected in 75 ± 22% of all cell pairs assayed. The temporal pattern of development shows that the appearance of evoked synaptic transmission was delayed in comparison to spontaneous synaptic events (Fig. 3).

Based on this developmental profile we used day 4 and day 12 cultures to represent immature and mature synaptic states. We chose day 4 as it was a time when spontaneous synaptic events were substantially present but evoked connections were still sparse. In contrast, day 12 cultures represented a more synaptically mature state, as determined by the increased number of cells with spontaneous and evoked connections.

Availability of releasable neurotransmitter

Considering the paucity of spontaneous synaptic events at day 4 in culture, we determined if synaptic events could be induced in cells that were initially synaptically silent ("silent cells") by the application of high-osmolarity saline. Application of high-osmolarity saline has been shown to cause an increase in spontaneous transmitter release (Fatt and Katz, 1952; Hubbard et al., 1968; Malgaroli and Tsien, 1992; Manabe et al., 1992). Imaging of intracellular calcium levels during the application of high-osmolarity saline did not reveal a significant difference in the emission ratio of Fura-2 due to excitation at 350 and 380 nm (*n* = 23; *p* > 0.7, Student's *t* test). However, if the K_d of fura-2 is adjusted for the change in ionic strength that occurs due to the shrinkage of the presynaptic terminal in the presence of high-osmolarity saline (Delaney et al., 1991) a calcium accumulation of 292 ± 27 nM was observed.

In 12-d-old cultures, application of high-osmolarity saline in the presence of TTX reliably produced a large increase in spontaneous synaptic events (Fig. 4*A*). Both inhibitory and excitatory

(A) 5 μM NIFEDIPINE
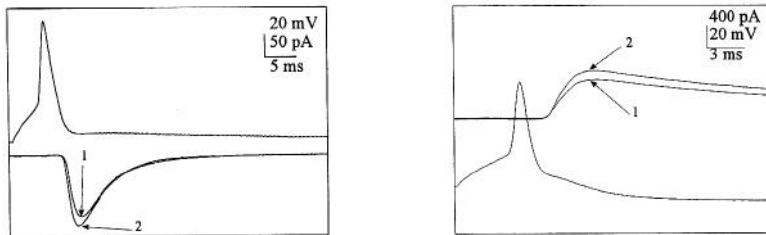
(B) 100 nM CONOTOXIN
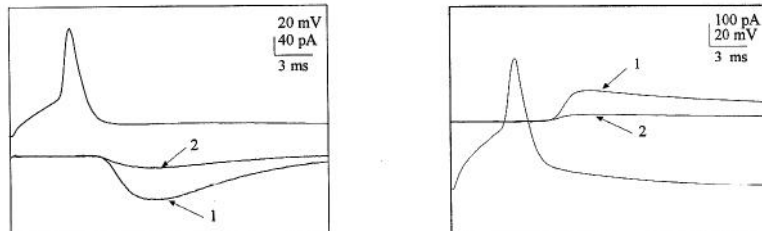
(C)
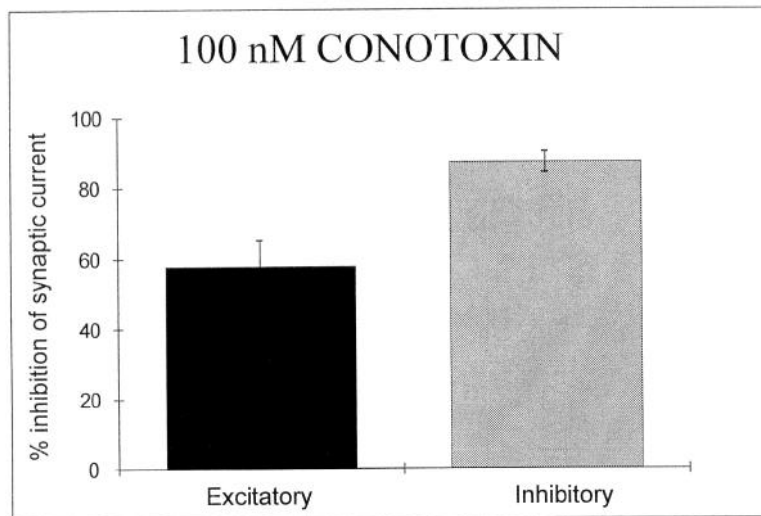
Figure 2. Evoked synaptic responses in 12 DIC hippocampal neurons are not reduced by the L-type calcium channel antagonist nifedipine, but are differentially sensitive to the N-type calcium channel antagonist ω-CgTx. The averaged response at 10 min post antagonist addition was compared against the initial averaged response. *A* and *B*, Postsynaptic excitatory (*left*) and inhibitory (*right*) responses in the absence (*1*) or presence (*2*) of 5 μM nifedipine (*A*) or 100 nM ω-CgTx (*B*). Current and voltage traces are graphically offset for clarity. Cumulative effect of ω-CgTx data from 6 excitatory and 15 inhibitory synaptic pairs is shown in *C*.
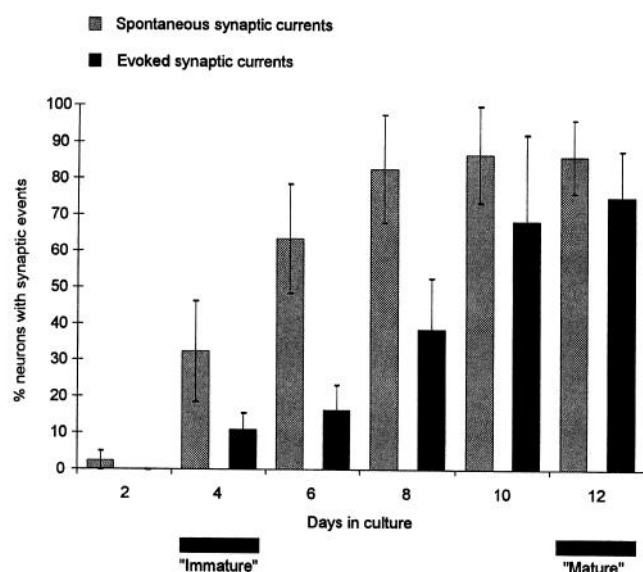
spontaneous events were induced at 12 DIC. In 4-d-old cultures, spontaneous synaptic events were absent in 27 out of 38 cells examined. Application of high-osmolarity saline to these 27 cells without spontaneous synaptic events resulted in the detection of spontaneous transmitter release in seven of these cells (Fig. 4*B*) with inhibitory (*n* = 3) and excitatory (*n* = 4) events induced. Thus, even in 26% of preparations in which no synaptic events were initially detected, postsynaptic receptors and presynaptic release apparatus were present.

Development of synapse-specific proteins

In the functional synapse, the release of neurotransmitter is dependent on the coupling of calcium channels with a calcium responsive secretory apparatus. As a first step in understanding the rate limiting steps in the development of functional synapses we performed immunocytochemistry to ask whether synaptic

proteins are expressed in a temporal pattern similar to that of functional synapse formation. A multitude of synapse-specific proteins implicated in neurotransmitter release have been characterized (for review, see Sudhof and Jahn, 1991; Jessell and Kandel, 1993). We determined if the pattern of expression of the synaptic vesicle protein synaptotagmin (p65), and the vesicle associated proteins synapsin I, and rab3a were developmentally regulated. In immature cultures, synaptotagmin immunoreactivity was localized primarily to the soma with some staining throughout neuritic processes (Fig. 5*A*). In contrast, in mature cultures the staining profile was strikingly different, with the appearance of intense punctate staining along neuritic processes (Fig. 5*C*). This punctate staining is characteristic of synaptic release sites in hippocampal neurons (Bekkers and Stevens, 1989; Fletcher et al., 1991). The appearance of distinct puncta with maturation was also evident for synapsin I, and rab3a (Fig. 6).

To further investigate the cellular localization of these syn-

aptic proteins, confocal microscopy was utilized. Optical sectioning revealed the presence of synaptotagmin immunoreactivity in the soma in a pattern consistent with its presence in the Golgi apparatus and throughout the neurites in immature cultures (73%, $n = 74$) (Fig. 5B). In contrast, in mature cultures synaptotagmin immunoreactivity was observed only on the surface of the somatic region, consistent with neuritic–somatic contact sites (100%, $n = 79$) (Fig. 5D). Synaptotagmin immunoreactivity in Golgi was not detected in these mature cultures.

←

Figure 3. Developmental profile of spontaneous and evoked synaptic connections. Hippocampal cultures were assayed for the presence of spontaneous and evoked synaptic currents at various times after plating. The percentage of cells where spontaneous or evoked connections could be detected are shown as a function of days in culture. The *horizontal bars* arbitrarily represent the developmentally immature state at 4 DIC and the more synaptically mature state at 12 DIC. Each bar represents the mean ± SEM from a minimum of 33 cells or 30 synaptic pairs at each time point for spontaneous or evoked synaptic events, respectively.
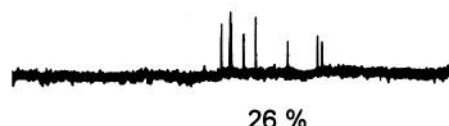
(A) 12 day

## (B) 4 day


Figure 4. Application of high-osmolarity saline to synaptically mature and immature neurons causes neurotransmitter release. *A,* Application of saline enriched with 300 mM sucrose and 1 μM TTX reliably produced a large increase in spontaneous synaptic events in day 12 neurons. A typical increase in excitatory events is shown. *B, Left* shows the absence of spontaneous synaptic events in 71% of 4-d-old cells examined. *Right,* Application of high-osmolarity saline to these silent cells produced synaptic events in 26% of these cells. An increase in spontaneous inhibitory events is shown, but both excitatory and inhibitory events were induced (see text). High-osmolarity saline was delivered by picospritzer-driven pressure ejection from a patch pipette for 30 sec directly onto the neuronal somata. Constant bath perfusion of normal saline with 1 μM TTX was maintained throughout.
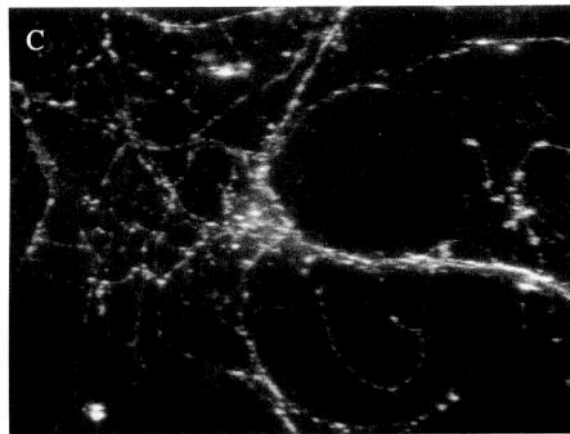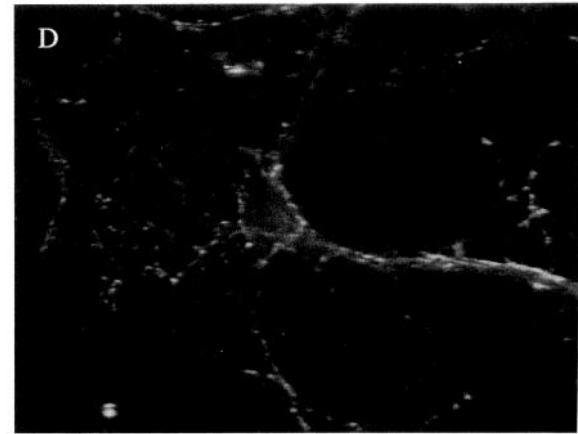
4 Day
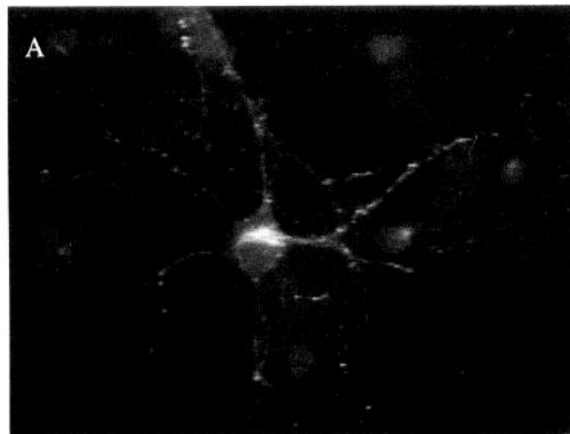
Top View Reconstruction
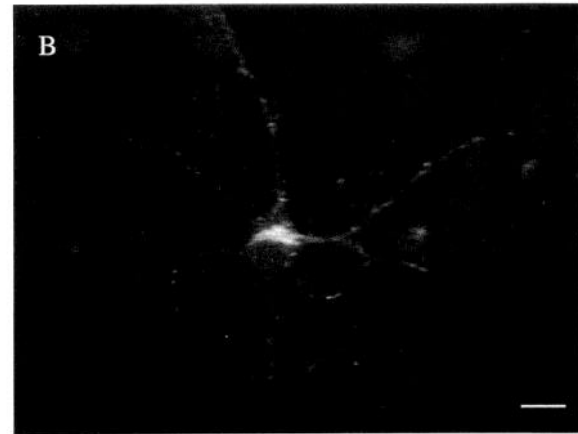
Single Optical Section

12 Day

Top View Reconstruction

Single Optical Section

Figure 5. Confocal microscopy of synaptotagmin immunoreactivity. *A,* Top view of a composite image reconstructed from 40 optical sections taken at 0.25 μm vertical increments from a 4-d-old culture. Intense Golgi and diffuse neuritic staining is apparent. *B,* A single optical section from the middle of the stack of sections used to construct the top view shown in *A* demonstrating that somatic staining is intracellular. *C,* Top view of a composite image reconstructed from 40 optical sections taken at 0.35 μm vertical increments from a 12-d-old culture. Distinct punctate staining is present on neurites and the soma. *D,* Single optical section from the middle of the stack of sections used to construct the top view in *C* reveals that the somatic staining is not intracellular and is likely due to neurite contact on the soma surface. Scale bar, 10 μm.

Neuritic staining was exclusively localized to neuritic contact sites. A similar pattern of staining was seen for rab3a and synapsin I. However, in immature cultures these proteins did not show Golgi accumulation, but instead a diffuse pattern throughout the soma and neurites was seen (Fig. 6).

These patterns of immunoreactivity are consistent with previous studies (Fletcher et al., 1991; Matteoli et al., 1991), and in addition we have shown that the immature immunoreactivity profile is temporally correlated with a functionally immature synaptic state of the neurons.

Development of voltage-dependent calcium influx

Since the ω-CgTx, but not the nifedipine-sensitive calcium channel is involved in evoked synaptic transmission, we have determined the temporal pattern of expression of these two channel types in relation to the formation of functional synaptic connections. As an initial step, the development of high-[K^+]-evoked calcium influx was examined using conventional calcium imaging techniques. Fifty millimolar K^+ saline was applied

to neurons from a puffer pipette while monitoring the calcium levels of neuronal somata. At all times in culture, high [K^+] caused an enhancement of neuronal calcium levels that required the presence of calcium in the bathing saline. After greater than 6 d in culture high [K^+] caused a greater change in the calcium level of neurons. For example, at 4 d in culture voltage-dependent calcium accumulation due to application of high [K^+] was 224 ± 10 nM while by 12 d in culture it was 466 ± 28 nM (Fig. 7).

To further examine the relation between the development of calcium influx and synaptogenesis we studied the contribution of N-type and L-type calcium channels to calcium influx. Using synaptically immature (4 DIC) and mature cultures (12 DIC) we determined the contribution of each channel type to voltage-dependent calcium accumulation (Fig. 8). Neurons were depolarized with two applications of elevated [K^+] separated in time by 10 min. Repeated applications of [K^+] reliably elevated calcium levels that are due to calcium influx since omission of calcium from the bathing medium reduced calcium accumu-
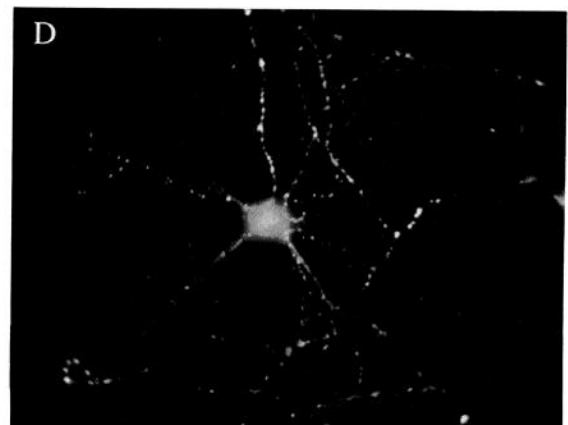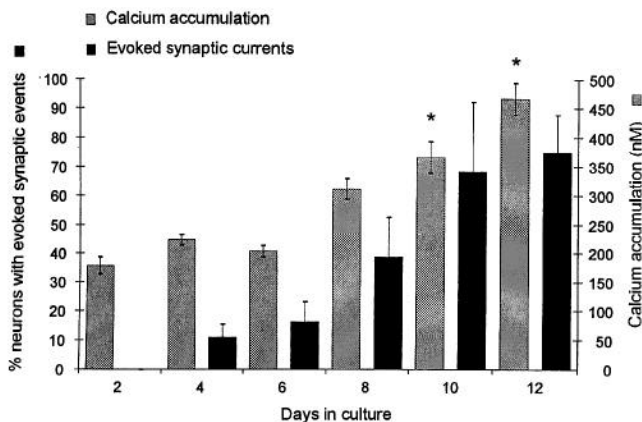
4 Day

rab3a
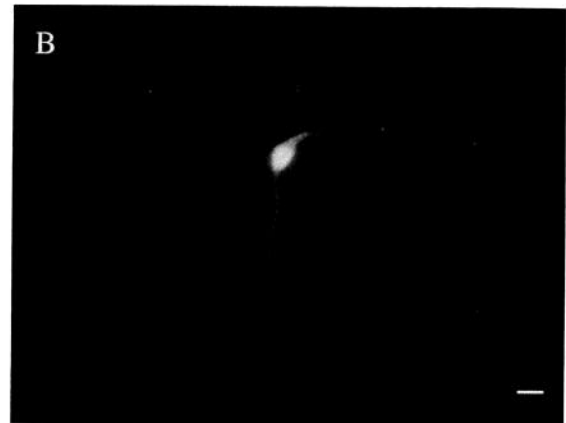
Synapsin I

12 Day

rab3a

Synapsin I

Figure 6. Conventional epifluorescent immunoreactivity profiles of rab3a and synapsin I. *A* and *B*, Distribution of rab3a (*A*) and synapsin I (*B*) from a 4-d-old culture. Somatic and neuritic staining is evident. Punctate varicose staining of rab3a (*C*) and synapsin I (*D*) is apparent in 12-d-old cultures. Scale bar, 10 μm.


Figure 7. Potassium-evoked calcium accumulation increases in a pattern parallel to that of synapse formation. A statistically significant increase in calcium accumulation was first observed at 10 DIC as compared to 4 DIC. Points represent mean ± SEM. Calcium accumulation was determined from a minimum of 39 cells at each time period. Statistical significance (*) was established at $p < 0.01$ using one-way ANOVA with post hoc Scheffe's test.

lation (98 ± 1%, $n = 30$) or cadmium (100 μM), a general calcium channel antagonist, reduced calcium accumulation by 72 ± 3% ($n = 27, p < 0.01$).

In synaptically immature neurons, 76 ± 2% ($n = 36$) of the calcium influx is mediated by L-type nifedipine-sensitive channels (Fig. 8). The nifedipine carrier DMSO (0.1%) did not attenuate calcium influx ($n = 33$). In contrast to nifedipine, addition of ω-CgTx at this same stage in culture did not affect the voltage-dependent calcium accumulation (4 ± 3% inhibition, $n = 29$) indicating that L- but not N-type calcium channels are expressed in neuronal somata in synaptically immature neurons. In synaptically mature neurons when the calcium accumulation has increased from 224 to 466 nM, nifedipine still attenuated the calcium accumulation causing a 67 ± 2% ($n = 34$) reduction in high-[K$^+$]-evoked calcium accumulation. Additionally, at this time, an ω-CgTx–sensitive component to calcium influx has developed. Addition of 100 nM ω-CgTx attenuated the [K$^+$]-evoked calcium accumulation by 43 ± 4% ($n = 39$). This indicates that both L- and N-type calcium channels are being functionally expressed in the somata of synaptically mature neurons. Since evoked synaptic connections are sensitive to the
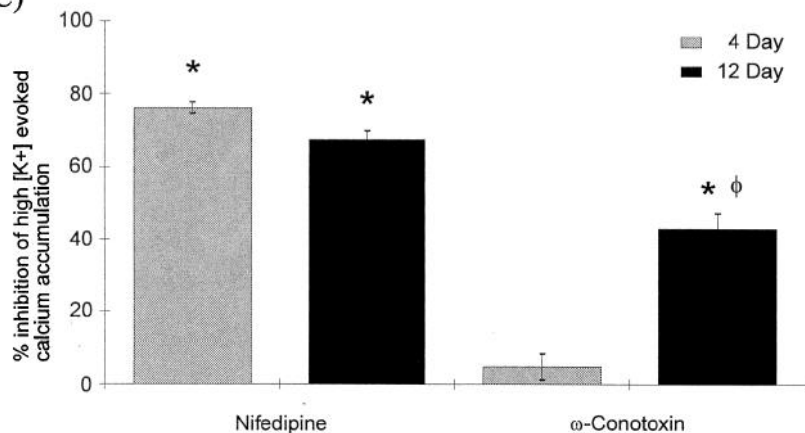
(A) 4 Day
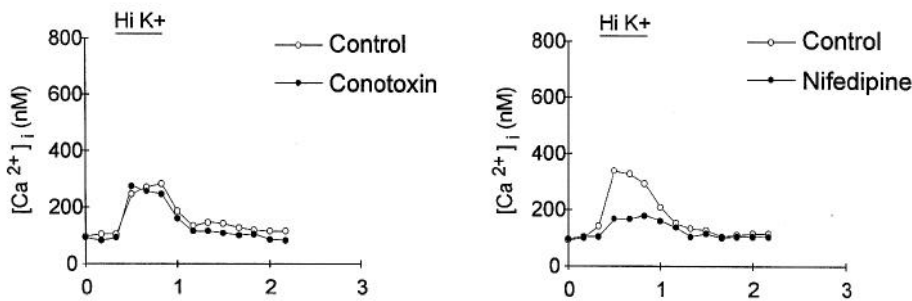
(B) 12 Day
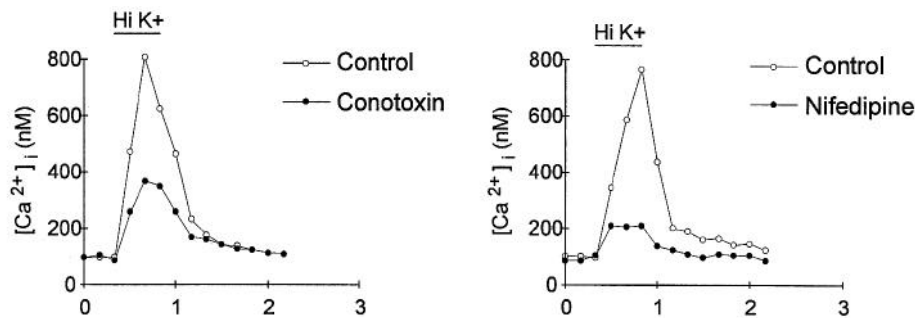
(C)

Figure 8. Calcium channel subtypes mediating calcium influx; 100 nM ω-CgTx or 5 μM nifedipine was bath applied to synaptically immature 4 d cultures or synaptically mature 12 d cultures. *A* and *B*, Calcium levels in response to high-[K$^+$] application measured from single 4- or 12-d-old cells in the presence or absence of antagonist. *C*, Summary of inhibition of high-[K$^+$]-evoked calcium accumulation. Inhibition was calculated as the percentage inhibition of calcium accumulation during the second application (ΔCa_2) of high [K$^+$] compared to the first (ΔCa_1): inhibition = $100 \times (1 - [\Delta Ca_2/\Delta Ca_1])$, where ΔCa = peak [Ca^{2+}]$_i$ subtracted from resting [Ca^{2+}]$_i$. Each culture dish was exposed to only one treatment. The significance of the effects on high-[K$^+$]-evoked calcium accumulation was established at $p < 0.01$ (*) using one-way ANOVA with post hoc Scheffe's test. Comparison of nifedipine and ω-CgTx-sensitive components at 4 and 12 DIC revealed a significant (ϕ, $p < 0.01$) increase of the ω-CgTx–sensitive component from 4 DIC to 12 DIC. Bars represent means ± SEM.

N-type calcium channel antagonist ω-CgTx, the timing of the development of functional synaptic transmission may reflect the appearance of functional N-type calcium channels.

Discussion

The formation of chemical synapses in the nervous system is the result of the complex interplay between a series of developmental events. Neuronal growth cones are responsible for guiding the extension of the axon to the target field where local interactions with potential synaptic targets regulate the development of presynaptic apparatus and the formation of the functional synapse. The interactions between future synaptic partners that subsequently lead to synapse formation are ill defined.

Much of our understanding of presynaptic development has arisen from a few model systems. For example, chick ciliary ganglion neurons and dissociated neurons from *Xenopus* spinal cord can release transmitter before contacting synaptic targets

(Young and Poo, 1983). This suggests that presynaptic neurons contain much of the machinery necessary for synaptic transmission in the absence of signals supplied by target cells. Studies on identified neurons of *Helisoma* have similarly shown that one presynaptic neuron, B5, is promiscuous and forms novel synapses with all synaptic targets tested (Haydon and Kater, 1988; Haydon and Zoran, 1989). When contact is made between such cells, functional synaptic interactions are detected within seconds of target contact, indicating that presynaptic machinery has been presynthesized in preparation for synapse formation. In contrast, another identified neuron of *Helisoma*, B19, is selective in synapse formation (Haydon and Kater, 1988; Haydon and Zoran, 1989; Zoran et al., 1989, 1990, 1991). Many hours of contact, and protein synthesis are necessary for the presynaptic neuron B19 to gain the ability to couple presynaptic action potentials with neurotransmitter release. Thus, different neurons may use different strategies during synapse formation. While

some presynthesize presynaptic apparatus, others require instructive cues from target cells to complete the developmental expression of presynaptic ion channels and synaptic proteins.

In hippocampal neurons there are several days of neuron–neuron contact before functional synaptic transmission is detected. At 4 d in culture only 11% of neuron pairs have evoked synaptic transmission while by day 12 it has increased to 75%. Correlated with this change in detectability of functional synapses are several changes in the cellular properties of the presynaptic cell. Synaptically immature preparations do express synaptic proteins rab3a, synapsin I, and synaptotagmin. However, immunocytochemistry indicates that the distribution of these synaptic markers changes from one in which there is significant somatic immunostaining to punctate staining restricted to varicose boutons in mature synaptic cultures. It is likely that this change in distribution of synaptic proteins, and associated vesicles, accounts in part for the slow acquisition of functional synaptic transmission.

Addition of high-osmolarity medium to neurons in 4 DIC cultures stimulates the release of transmitter. Because transmission can be detected in response to high-osmolarity medium at early times in culture, the development of action potential–evoked transmitter release is likely to be limited by the acquisition of presynaptic properties rather than by the presence of postsynaptic receptors. In support of this possibility, Craig et al. (1993) have shown that AMPA-sensitive channels are expressed in hippocampal neurons in the absence of contact by presynaptic axons at early times in culture. The ability to release transmitter, as detected by spontaneous synaptic currents and high-osmolarity medium, indicates that much of the presynaptic apparatus is functional. Even though much of the release apparatus is present, action potentials rarely evoke transmitter release. A number of possibilities may account for these observations. Perhaps a limited pool of vesicles contributes to this low probability of detecting synapses. Additionally, the calcium-secretion coupling mechanism or calcium influx through the appropriate calcium channels may not be fully developed at this stage in culture. An increase in neurite outgrowth could also increase the number of putative contact sites, resulting in an increase in the detectability of evoked secretion. Finally, axonal versus dendritic differentiation may be changing in parallel with synapse formation.

Inhibitory and excitatory synapses are sensitive to ω-CgTx, but not nifedipine. While other calcium channel subtypes may also be required for synaptic transmission, we chose to compare the development of one calcium channel involved directly in transmitter release (N-type) with one channel that does not stimulate release (L-type). An examination of the development of nifedipine-sensitive and ω-CgTx–sensitive calcium influx demonstrated that L- and N-type calcium channels are functionally expressed at different times in relation to synaptogenesis. L-type channels, which do not stimulate transmitter release, develop prior to the onset of functional transmission, whereas N-type channels that contribute calcium in transmitter release, appear at a later time in development when synaptic transmission has been established.

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