

Contact-Dependent Regulation of N-Type Calcium Channel Subunits during Synaptogenesis

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ABSTRACT: The developmental regulation of the N-type calcium channel during synaptogenesis was studied using cultured rat hippocampal neurons to elucidate the roles of extrinsic versus intrinsic cues in the expression and distribution of this channel. Prior to synapse formation, α_{1B} and β_3 subunits of the N-type calcium channel were distributed diffusely throughout neurites, growth cones, and somata. As synaptogenesis proceeded, the subunit distributions became punctate and colocalized with the synaptic vesicle protein synaptotagmin. Isolated neurons were also examined to test for the requirement of extrinsic cues that control N-type calcium channel expression and distribution. These neurons expressed N-type cal-

cium channel subunits, but their distributions remained diffuse. Functional ω -conotoxin GVIA-sensitive channels were expressed in isolated neurons, although the distribution of α_{1B} subunits was diffuse. The distribution of the α_{1B} subunit and synaptotagmin only became punctate when neuron–neuron contact was allowed. Thus, the expression of functional N-type calcium channels is the result of an intrinsic program while extrinsic regulatory cues mediated by neuron–neuron contact are required to control their distribution during synaptogenesis. © 1998 John Wiley & Sons, Inc.

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INTRODUCTION

Influx of calcium through voltage-dependent calcium channels plays an integral role in vesicle fusion during synaptic transmission. In neurons, many calcium channel subtypes have been identified (Tsien et al., 1988; Bean, 1989; Llinas et al., 1992; Miller, 1992). Thus far, N-, P-, and Q-type calcium

channels have been implicated in evoking fast neurotransmitter release (Wheeler et al., 1994; Luebke et al., 1993; Takahashi and Momiyama, 1993; Turner et al., 1993). Pharmacologically, the N-type channel can be distinguished from other channel types by its sensitivity to ω -conotoxin GVIA (ω -CTX GVIA), a peptide toxin isolated from the snail *Conus geographus* (Olivera et al., 1984). ω -CTX GVIA binds irreversibly to the N-type calcium channel, blocking calcium influx and transmitter release in several vertebrate neurons (Boland et al., 1994).

Witcher et al. (1993b) used ω -CTX GVIA to purify the N-type calcium channel. They found that it contains four distinct subunits of molecular weight 230, 140, 95, and 57 kD. Antibodies which recognize specific regions of these subunits demon-

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strated that the α_{1B} and β_3 subunits of the N-type calcium channel are immunologically distinct from those of dihydropyridine-sensitive calcium channels. Consistent with a role in transmitter release, the N-type calcium channel has been localized to the release face of nerve terminals using fluorescently labeled ω -CTX GVIA (Robitaille et al., 1990; Cohen et al., 1991; Torri Tarelli et al., 1991). In addition, the spatial distribution of calcium channels at the single channel level has been studied. We have observed, using atomic force microscopy, that ω -CTX GVIA binding sites are organized in clusters and short linear or parallel arrays on the release face of presynaptic nerve terminals isolated from chick ciliary ganglia (Haydon et al., 1994).

The mechanism of regulation of calcium channel distribution during synapse formation is undefined. However, it is clear that the N-type channel is critical for exocytosis immediately after synaptogenesis, although with further synaptic maturation other channel types become more important in controlling exocytosis (Scholz and Miller, 1995; Verderio et al., 1995). In the present study, we asked whether neuron–neuron contact regulates the distribution of N-type calcium channels in the presynaptic terminal of cultured embryonic rat hippocampal neurons during synaptogenesis. These experiments demonstrate many parallels between the regulation of acetylcholine receptors at the neuromuscular junction and calcium channels in the presynaptic nerve terminal. For example, we find that prior to target interaction, the subunits of the N-type calcium channels are synthesized and functional ω -CTX GVIA-sensitive calcium channels are assembled. Furthermore, the distribution of these channels remains diffuse until cues provided by synaptic contact cause them to reorganize into discrete punctate clusters.

MATERIALS AND METHODS

Hippocampal Cultures

Hippocampi dissected from E17 or E18 rat fetuses were dissociated by trypsinization [0.025% trypsin in $\text{Ca}^{2+}/\text{Mg}^{2+}$ Earle's balanced salt solution (EBSS)] for 15 min followed by trituration in modified minimal essential medium (MEM) (Eagle's minimum essential medium, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin) supplemented with 10% fetal bovine serum. The cells were then incubated for 30 min at 4°C before plating onto 12-mm-diameter circular glass coverslips coated with covalently bound poly-L-lysine (Scholz and Miller, 1995). Coverslips were then placed into 35-mm Falcon culture dishes containing

modified MEM supplemented with N2 (3 coverslips/dish). After 24 h, the medium was replaced with MEM supplemented with N2 and the cultures were maintained in a humidified 5% $\text{CO}_2/95\%$ room air incubator at 37°C. This culture method provided highly enriched pyramidal neuron cultures with <3% astrocytes, as verified by immunocytochemistry for glial fibrillary-associated protein (results not shown). Cells were plated at a density of 60–80 cells/ mm^2 such that by approximately 48 h, the majority of neurons had made contact with other neurons. For experiments where isolated neurons were necessary, low-density cultures were prepared (5–10 neurons/ mm^2). With this method, it was possible to clearly identify isolated neurons. To enhance long-term survival and to control for possible diffusible factors that may affect synaptic development, each coverslip with low-density neurons was cultured with two normal density coverslips in the same 35-mm culture dish.

Reverse-Transcriptase–Polymerase Chain Reaction (RT-PCR)

Expression of the N-type calcium channel alpha subunit gene was determined by both single-cell RT-PCR and amplification of cDNA obtained by reverse transcription of mRNA from rat hippocampal neurons in primary culture or acutely isolated hippocampal slices. The mRNA was purified using oligo-dT beads (Oligotex; Qiagen). The poly-A transcript was reverse-transcribed to first-strand cDNA using a First Strand cDNA Synthesis Kit (Pharmacia) and 10 pmol of a specific antisense primer of α_{1B} (TGC TGA GTC CCA AAG TGC).

Aliquots of cDNA template were added to the PCR reaction mixture with 10 pmol each of α_{1B} sense (TAG CCA GGT GTC CCA TCA) and antisense primers, 100 mM dNTPs, 2 mM MgCl_2 , 0.5 U *Taq* polymerase. After 30 cycles, 5 μL of PCR product was used as the template for another PCR reaction using 10 pmol each of specific primers internal to the previous primers: sense (ACC ACC ACC GCT GCC ACC), antisense: (TAG CCA TTG GGT ACA CGG).

Single-Cell PCR

Single hippocampal neurons were drawn into a glass patch-clamp pipette using negative pressure. The pipette contents were expelled into a test tube and mixed with 3.5 μL of first-strand cDNA synthesis mixture containing 45 mM Tris (pH 8.3), 68 mM KCl, 15 mM dithiothreitol (DTT), 9 mM MgCl_2 , 0.08 mg/mL bovine serum albumin (BSA), 1.8 mM of each dNTP, 10 pmol of specific α_{1B} antisense primer (see above), and 10 U of murine reverse transcriptase. The mixture was incubated at 37°C for 1 h. The reaction was terminated by incubating at 95°C for 2 min. The cDNA from the first-strand synthesis reaction was subjected to PCR as described above. Two types of controls were utilized. First, reactions were performed in the absence of reverse transcriptase. Second,

we performed the complete RT-PCR reaction on saline that was aspirated into a patch pipette from a region adjacent to a neuron. In all experiments, these controls were negative. All results were generally replicated in at least three independent experiments.

Production and Purification of Specific Polyclonal Antibodies to the α_{1B} and β_3 Subunits

Polyclonal antibodies against the N-type Ca^{2+} channel complex and specific α_{1B} and β_3 GST fusion proteins were produced as previously described (Witcher et al., 1993a). Specific sheep polyclonal antibodies (sheep 46) were produced against the purified N-type Ca^{2+} channel complex, and sheep polyclonal antibodies (sheep 49) were generated against an unique β_3 C-terminal GST fusion protein. Specific rabbit polyclonal antibodies (rabbit 95) were also produced against an α_{1B} GST fusion protein (Witcher et al., 1993a). Both the α_{1B} and β_3 GST fusion proteins were covalently coupled to CNBr-Sepharose as previously described (Witcher et al., 1995). These resins were used to affinity-purify specific polyclonal antibodies to α_{1B} and β_3 subunits. Briefly, serum containing specific polyclonal antibodies to the α_{1B} or β_3 subunits was diluted 1:10 with 20 mM MOPS, 150 mM NaCl, pH 7.4, and placed over the α_{1B} or β_3 GST fusion protein Sepharose columns. The Sepharose columns were then extensively washed with 20 mM MOPS, 150 mM NaCl, pH 7.4, and the polyclonal antibodies were eluted from the columns with 20 mM glycine, 150 mM NaCl, pH 2.4. The eluted fractions were immediately neutralized with 1 M MOPS, pH 7.6. Affinity-purified sheep 49 and rabbit 96 polyclonal antibodies were also placed on a GST Sepharose column to remove any GST antibodies. Finally, the affinity-purified polyclonal antibodies were concentrated and dialyzed in phosphate-buffered saline (PBS) containing 20% sucrose, 1 mM ethylenediamine-tetraacetic acid (EDTA), and 0.001% sodium azide.

Immunohistochemistry

Cultures were fixed in Zamboni's fixative for 60 min at room temperature followed by multiple rinses in 0.1 M PBS. After additional washing with 0.05 M Tris-buffered saline, the coverslips were incubated in 0.02% Triton-X 100 (5 min, 27°C) and blocked in 1% BSA and 1.5% normal rabbit or goat serum (60 min, 37°C). Following overnight incubation (4°C) with anti- β_3 or α_{1B} (1:500), visualization was accomplished using a biotinylated goat anti-rabbit immunoglobulin G (IgG) or rabbit anti-sheep IgG (60 min, 27°C) and avidin conjugated with fluorescein or rhodamine. The coverslips were then mounted onto microscope slides using glycerol containing *n*-propyl gallate. For double-label experiments, coverslips were incubated with either anti-MAP2 (AP-20, 1:1000), or anti-synaptotagmin (CL41.1, 1:500) in addition to incubation with the anti-calcium channel subunit antibodies.

For these experiments, MAP2 and synaptotagmin were visualized using a rhodamine-conjugated goat anti-mouse IgG. Anti-synaptotagmin (CL41.1) was generously provided by Dr. R. Jahn, MAP2 (clone AP-20) was from Boehringer, and anti-GFAP from Sigma (St. Louis, MO). Control experiments in which primary antibodies were omitted were negative.

Calcium Imaging

The presence of voltage-sensitive calcium influx in neurons >4 days in culture was examined using Fura-2 and ratiometric imaging techniques as previously described (Grynkiewicz et al., 1985; Basarsky et al., 1994). After loading with Fura-2/AM (2 μM) for 45 min at 37°C and de-esterifying for an additional 45 min, ratio images (350 nm/380 nm) were acquired at 5-s intervals using a CH-250 Photometrics liquid-cooled CCD camera and MetaFluor software (Universal Imaging). Two images were acquired for baseline Ca^{2+} levels, then two additional images were taken while neurons were depolarized with 50 mM K^+ saline. One final image was taken 1 min after normal saline flow was restored. A second depolarization was performed after a 10-min recovery period in flowing normal saline. The second depolarization involved either control (normal saline) or pharmacological solutions. The results of the two units of depolarization were compared to assess the effect of the control versus test solutions on Ca^{2+} accumulation.

Measurement of Calcium Currents

Calcium currents were measured by voltage-clamping cultured hippocampal neurons in the whole-cell mode. Patch pipettes were pulled in two stages on a Narishige PP-83 puller from 1.5-mm O.D.–1.12-mm I.D. omega dot borosilicate capillaries (FHC, Brunswick, ME). Their resistance was between 5 and 8 M Ω when filled with internal recording solution. The shank of the electrodes was coated with wax to reduce capacitance. No fire polishing was used. The internal solution consisted of (in mM): tetraethylammonium chloride (TEA-Cl) 117, MgCl_2 4.5, ethylene glycol-*bis*(*b*-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) 9, Hepes 9, phosphocreatine, adenosine 5'-triphosphate magnesium salt (Mg-ATP) 4, phosphocreatine 14, guanosine 5'-triphosphate tris salt (Tris-GTP) 0.3 [pH 7.4 adjusted with tetraethylammonium hydroxide (TEA-OH)]. The external recording medium was (in mM): BaCl_2 10 or 25, TEA-Cl 160 or 145, Hepes 10 (pH 7.4 adjusted with TEA-OH). The data were acquired and analyzed with pClamp software (Axon Instruments).

Drugs. Drugs used were TEA-Cl, TEA-OH, barium chloride, EGTA, phosphocreatine, Mg-ATP, and Tris-GTP, obtained from Sigma. ω -CTX GVIA was obtained from Peptides International (Louisville, KY).

RESULTS

mRNA for the α_{1B} Calcium Channel Subunit Is Present in Immature Neurons

Functional synaptic transmission can be detected in 17 to 18-day embryonic hippocampal pyramidal neurons after 7–8 days in culture (Doyle et al., 1996; Scholz and Miller, 1995). Around the time of synapse formation, N-type calcium channels have been shown to be the primary class of channel linked to transmitter release (Scholz and Miller, 1995). We focused on the developmental aspects of expression and function of the N-type channel and considered neurons earlier than 6 days in culture to be synaptically immature. We initially asked when mRNA for the α_{1B} subunit was present utilizing RT-PCR. We performed RT-PCR on mass-dissociated neurons from day E18 rats at times varying from zero to 14 days in culture [Fig. 1(A)]. The presence of mRNA for the α_{1B} subunit was detected at all ages examined. To confirm that the α_{1B} transcripts originated from pyramidal neurons, we performed single-neuron RT-PCR on isolated single pyramidal neurons after varying times in culture [Fig. 1(A)]. Again, α_{1B} mRNA could be detected at all ages examined. Thus, at early time points in culture, mRNA encoding the α_{1B} subunit of the N-type calcium channel is present in hippocampal pyramidal neurons.

Functional N-Type Calcium Channels Are Present in Synaptically Immature Neurons

The presence of the α_{1B} mRNA does not necessarily mean that functional N channels have been assembled and inserted into the neuronal membrane. To test for the presence of functional N channels, we measured macroscopic calcium currents using patch-clamp recordings in the whole-cell mode. To rapidly generate current–voltage relationships, we used a slow ramp from -100 to $+50$ mV as a command. Ramps were obtained before and after $5 \mu\text{M}$ ω -CTX GVIA was applied to the neuronal soma by pressure ejection [Fig. 1(B)]. Administration of ω -CTX GVIA produced a decrease in the whole-cell calcium current at all ages of neurons examined [$23 \pm 14\%$; mean \pm standard deviation (S.D.), $n = 75$]. An effect of ω -CTX GVIA could be demonstrated as early as 2–3 h after neurons were isolated and placed into culture. The actions of ω -CTX GVIA were significantly different from the effects of pressure ejection of external barium saline onto the

soma at all ages examined [$p < 0.0151$ for neurons < 1 day; $p < 0.0001$ for all other ages; two-way analysis of variance (ANOVA) with Scheffé post hoc comparison] [Fig. 1(C)]. While α_{1B} subunit mRNA and ω -CTX GVIA-sensitive calcium currents are present from the time of plating in culture, the magnitude of the ω -CTX GVIA-sensitive current density (ω -CTX GVIA current density) increases with age in culture [Fig. 1(D)]. However, the relative contribution of the ω -CTX GVIA-sensitive calcium current to the whole-cell calcium current was not affected by the age of the neuron ($p \geq 0.6156$; two-way ANOVA with Scheffé post hoc comparison).

Functional N-Type Calcium Channels Are Present in Neurites

While patch-clamp experiments demonstrated the presence of functional N-type calcium channels in the soma of hippocampal neurons, they did not allow us to determine whether functional channels were present in neurites. We used calcium imaging methods to determine the ω -CTX GVIA-sensitive contribution to depolarization-induced calcium accumulation in neurites. Bath application of ω -CTX GVIA ($1 \mu\text{M}$) caused a $51 \pm 4\%$ decrease in calcium accumulation ($n = 20$) (Fig. 2) measured in neurites of neurons maintained in culture for 4 days. In comparison to voltage-clamp experiments, ω -CTX GVIA produced a more substantial reduction in calcium accumulation than expected. Perhaps this results from a differential contribution of distinct calcium channel subtypes in neurites compared to somata. Alternatively, this quantitative difference may be due to the sustained period of depolarization (~ 10 s) employed in the calcium imaging experiments. Nonetheless, these data suggest that at this early time point in culture, prior to synaptogenesis, the mRNA detected by RT-PCR gives rise to functional ω -CTX GVIA-sensitive N-type calcium channels in the neurites of synaptically immature neurons.

Immunocytochemical Characterization of N-Type Calcium Channel Subunits in Developing Neurons

To further evaluate the distribution of N-type channels prior to synapse formation, we used immunocytochemistry to examine the expression and distribution patterns of the α_{1B} and β_3 subunits of this calcium channel. In young, synaptically immature neurons (≤ 4 days in culture), the immunoreactivity

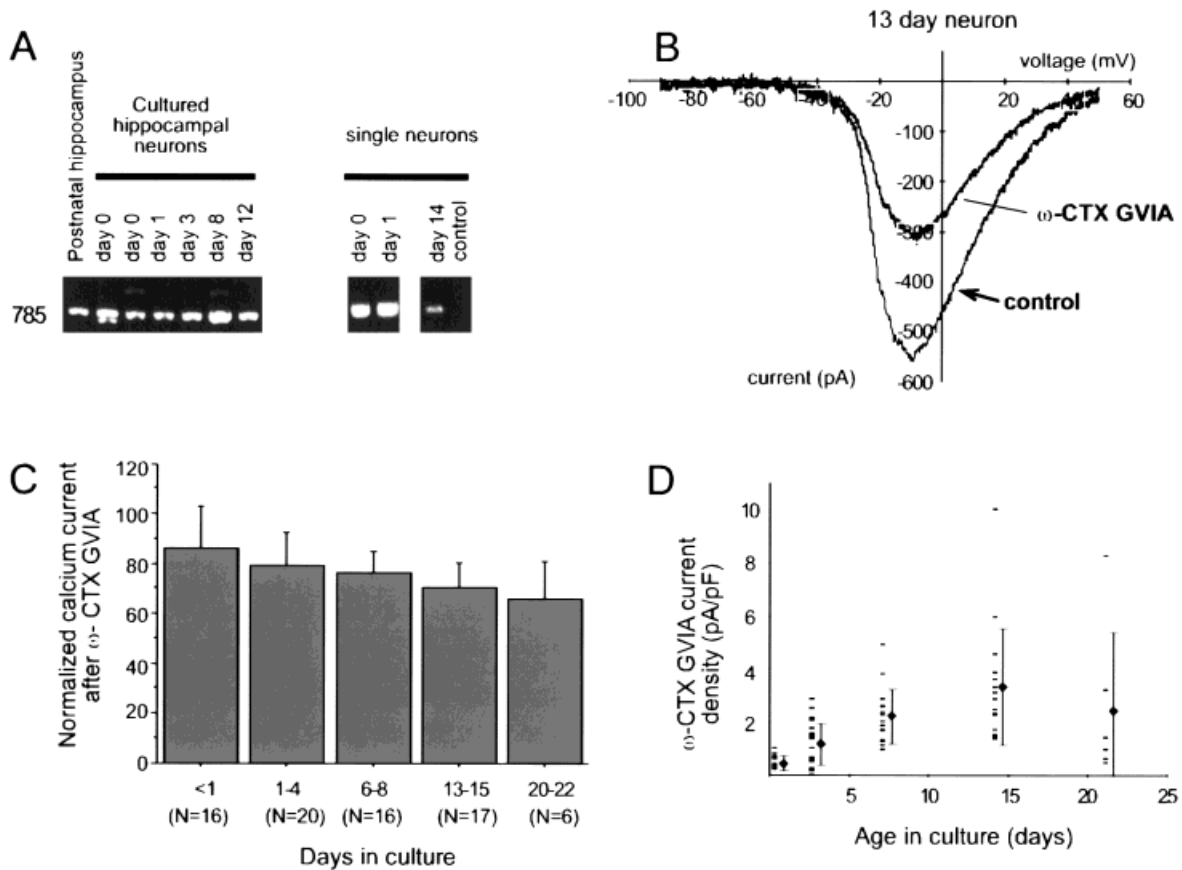


Figure 1 Functional N-type calcium channels are synthesized and inserted into the neuronal membrane prior to synapse formation. (A) m-RNA for the α_{1B} calcium channel subunit was detected using RT-PCR from postnatal hippocampus, embryonic hippocampal neuronal cultures (at least six independent experiments at each time point, with positive results in 67–100% of reactions) and from single cultured embryonic hippocampal pyramidal neurons. Control was a single 14-day neuron processed without reverse transcription. While it appears that less product is generated after 14 days in culture in single-cell PCR, quantitative comparisons are not appropriate under our conditions. (B) Whole-cell calcium currents recorded from a neuron after 13 days in culture before and after exposure to $5 \mu\text{M}$ ω -CTX GVIA. (C) Summary of the actions of ω -CTX GVIA on whole-cell calcium currents in neurons at different times in culture. Responses were normalized to the response to pressure ejection of external barium saline onto the soma of neurons of the same age. The response to ω -CTX GVIA was significantly different from that to barium saline at all ages examined ($p < 0.015$ for neurons <1 day in culture; $p < 0.001$ for all other ages; two-way ANOVA with Scheffé post hoc comparison). (D) The magnitude of the ω -CTX GVIA-sensitive calcium current density (i.e., N-type calcium current) increases with increasing age in culture. (Individual data points are indicated by dashes; offset to the right is the mean and standard deviation for each age.)

for both subunits was largely diffuse throughout the soma and neurites [Fig. 3(A,C)]. There were, however, notable regions of enhanced neurite staining. These regions were frequently observed at sites of soma or neurite contact. Synaptically mature neurons 12 days in culture also had diffuse staining throughout the soma and apical dendrite, but staining in neurites was largely punctate [Fig. 3(b,d)].

To determine whether the α_{1B} subunit is localized

in the axons or dendrites of mature neurons, we used immunocytochemistry to double label for the dendritic marker MAP2 and the α_{1B} subunit. While the α_{1B} subunit was colocalized in neurites with MAP2, there was also α_{1B} subunit immunoreactivity in many neurites that were MAP2 negative (data not shown). This nondendritic α_{1B} subunit immunoreactivity was generally punctate in appearance. Double labeling using anti- α_{1B} and anti-synaptotag-

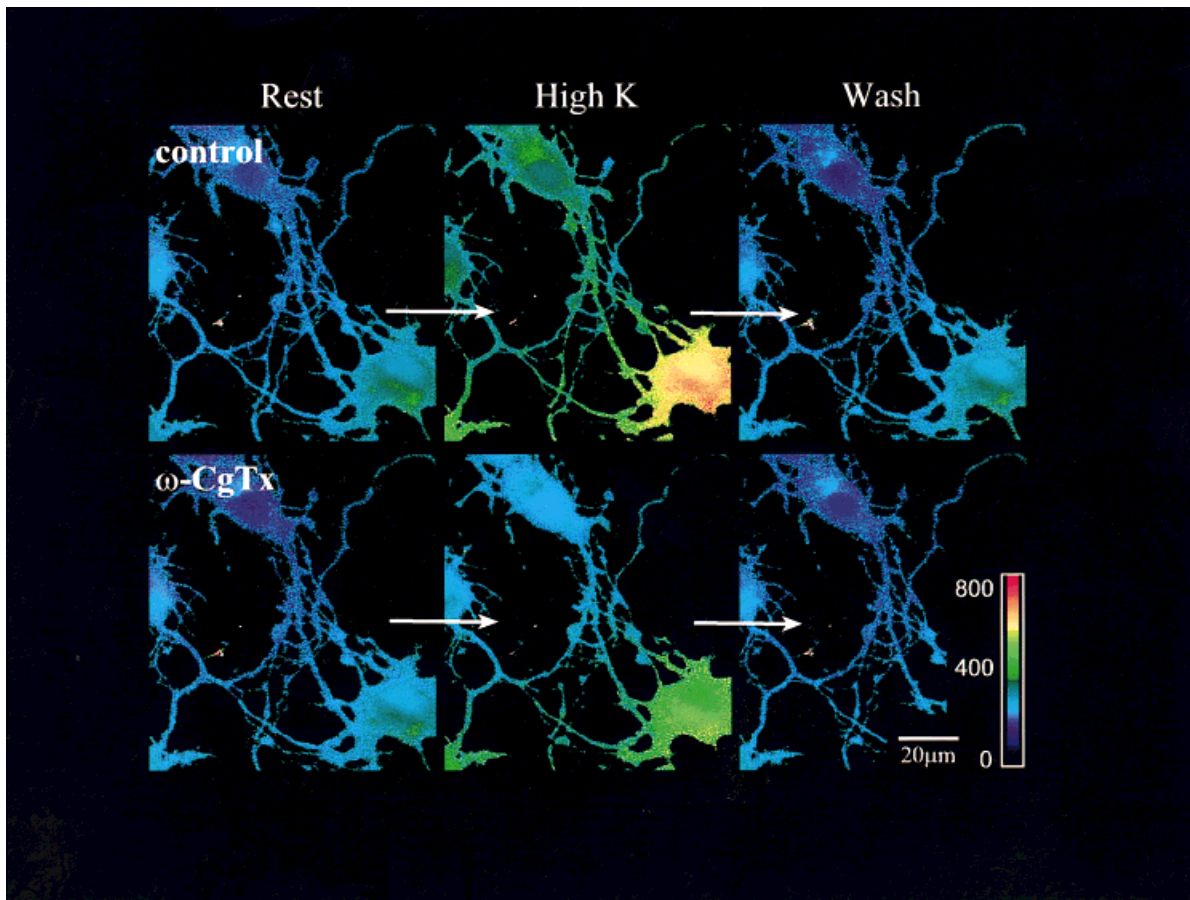


Figure 2 ω -CgTx GVIA reduces depolarization-dependent calcium accumulation in somata and neurites of hippocampal neurons. (Top) Sequential ratiometric images displaying estimated calcium levels of hippocampal neurons. Depolarization of neurons with high- K^+ saline elevates calcium throughout the neurons. (Bottom) Addition of $1 \mu M$ ω -CgTx reduces the extent of depolarization-dependent calcium accumulation both within the neurites and somata.

min revealed that synaptotagmin and the N-type calcium channel subunit α_{1B} are colocalized in mature neurons at least to the extent resolved by the diffraction-limited optics of fluorescence microscopy. Staining profiles in the neurites of neurons after 8 days in culture were punctate for both α_{1B} and synaptotagmin (Fig. 4).

A Punctate, Synaptic Distribution of the α_{1B} Subunit Requires Neuron–Neuron Interactions

Neuron–neuron contact is likely to play a key role in the regulation of N-type calcium channel expression and distribution (Jones et al., 1989). To test this hypothesis, we cultured hippocampal neurons under conditions which resulted in either isolated neurons or neurons with neuron–neuron contacts.

Immunocytochemistry was then performed to localize the α_{1B} subunit of the N-type calcium channel. After 8 days in culture, isolated neurons had α_{1B} staining that was diffuse and nonpunctate [Fig. 5(a)]. Whole-cell recording from such isolated neurons revealed the presence of functional calcium currents sensitive to ω -CTX GVIA ($32.0 \pm 7.6\%$; $n = 6$). In contrast to the diffuse immunostaining in isolated neurons, punctate α_{1B} staining was seen in the neurites of neurons 8 days in culture that were allowed to form neuron–neuron contacts [Fig. 5(d)].

Immunocytochemistry to localize synaptotagmin was also performed on isolated hippocampal neurons. Like the α_{1B} subunit, punctate synaptotagmin staining was detected in cultures where neurons were allowed neuron–neuron contact [Fig. 5(e)], but was diffuse and immature in isolated neurons

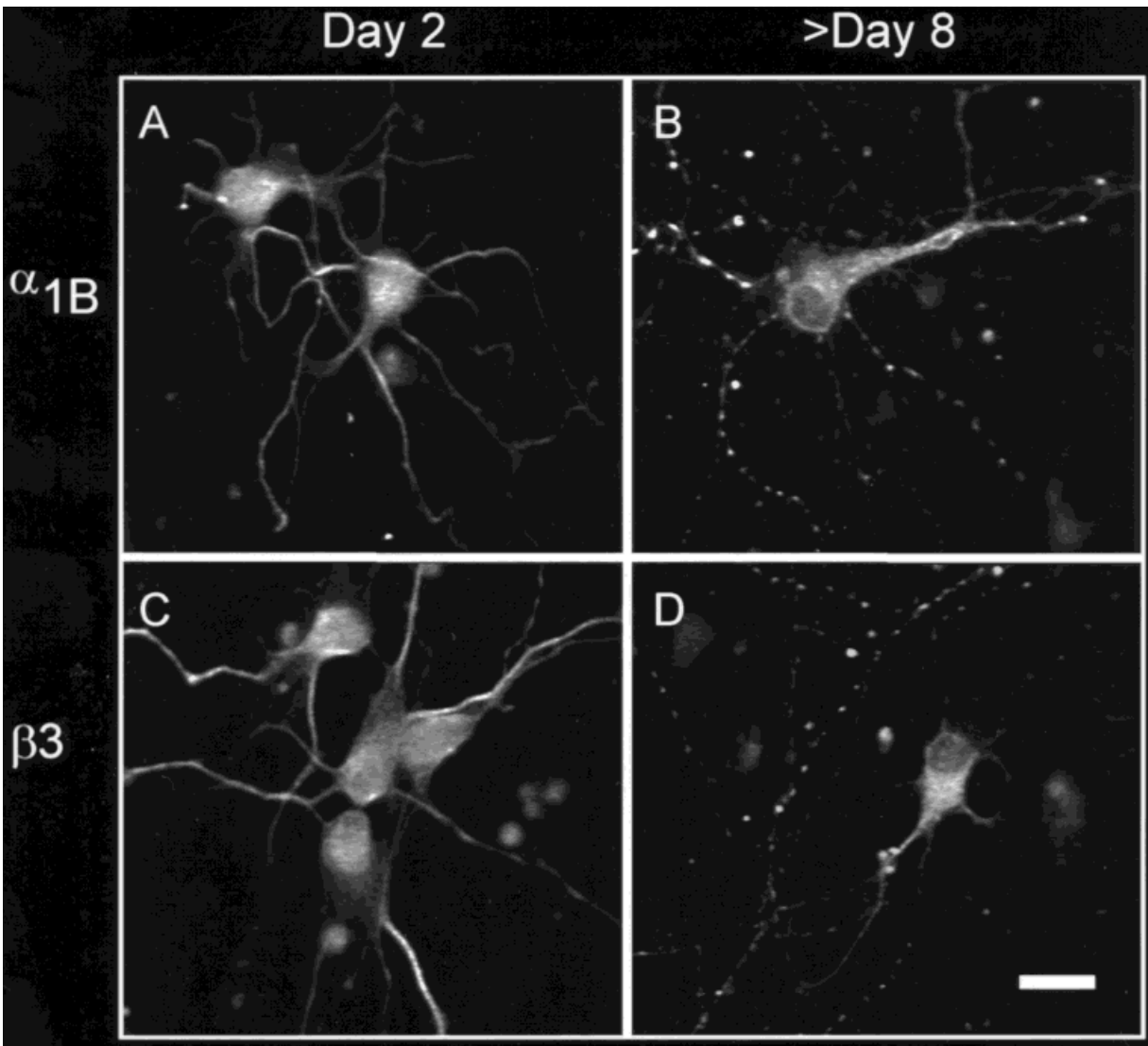


Figure 3 Expression and distribution of the α_{1B} and β_3 subunits of the N-type calcium channel during development. (A) At 2 days in culture, α_{1B} is expressed, but is distributed diffusely throughout the neuron. (B) By 8 days in culture, α_{1B} immunoreactivity becomes punctate in neurites. (C,D) Distributions of the β subunit parallel the distribution of the α_{1B} subunit. Bar = 20 μM .

[Fig. 5(b)]. These data suggest that isolated neurons are capable of expressing the α_{1B} subunit of the N-type calcium channel and synaptotagmin, but extrinsic signals are required to control their distribution.

DISCUSSION

Our understanding of the developmental mechanisms that regulate the assembly of a functional synaptic connection has, in large part, been due to

studies performed at the experimentally accessible neuromuscular junction. This system has yielded much information regarding the control of the postsynaptic acetylcholine receptor which we now know is regulated by multiple signals. For example, agrin, which can be derived from innervating neurons, induces the accumulation of the acetylcholine receptor at the site of nerve–muscle contact (Ferns and Hall, 1992; McMahan, 1990), and ARIA regulates acetylcholine receptor synthesis and tyrosine phosphorylation (Falls et al., 1993; Corfas et al., 1993). Just as the postsynaptic apparatus is regulated by antero-

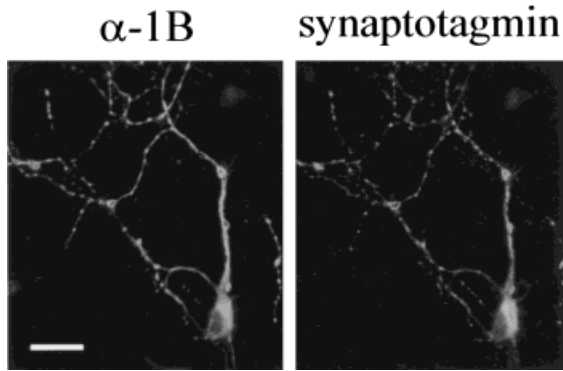


Figure 4 Double-label immunocytochemistry for α_{1B} and synaptotagmin. After 8 days in culture, α_{1B} and synaptotagmin colocalize. Bar = 20 μM .

grade signals, the presynaptic apparatus is also presumed to be regulated by retrograde signals supplied from the target (Haydon and Drapeau, 1995). Our previous studies in *Helisoma* have demonstrated that muscle contact induces a presynaptic activation of protein kinase A leading to enhancement of local presynaptic calcium influx (Funte et al., 1993). Little other information, however, is available about the regulation of calcium channels at the presynaptic terminal of mammalian neurons during synaptogenesis.

The presynaptic terminal must accumulate both calcium channels and synaptic proteins at sites that are in juxtaposition to the postsynaptic receptors, for fast synaptic transmission to occur. Using antibodies directed against the α_{1B} subunit of the N-type calcium channel, we have now demonstrated that contact between pre- and postsynaptic neurons is essential for the acquisition of mature presynaptic N-type calcium channel and synaptotagmin distributions. In the absence of local instructive cues provided by a postsynaptic neuron, individual hippocampal neurons synthesize calcium channel subunits which are transported along the neurites. These subunits form functional ω -CTX GVIA-sensitive channels that permit calcium entry during depolarization. However, contact with other hippocampal neurons is required for these subunits to organize into discrete clusters. The particular signal that mediates this regulatory control is unknown. Fletcher et al. (1994) demonstrated that a property specific to "mature" dendrites is required to induce the axonal reorganization of presynaptic vesicles in hippocampal neurons. Presumably similar signals also control the distribution of the N-type calcium channel in the axon terminals.

Westenbroek et al. (1992), using antibodies spe-

cific to α_{1B} subunits, localized N-type calcium channels in the brains of adult rats. They observed immunoreactivity on dendritic shafts and also within punctate structures located on dendrites. Reportedly, this punctate staining was particularly notable on the mossy fibers of dentate gyrus granule neurons. Using radioligand binding with conotoxin and immunoprecipitation with α_{1B} antibodies, Jones et al. (1997) demonstrated that N-type channels are expressed prenatally and are initially confined to the soma and proximal dendrites, and that punctate staining can be seen in mature neurons. Functional support for the presence of N-type calcium channels has been recently provided by whole-cell recordings performed using dendrosomes (Kavalali et al., 1997). However, the functional significance of the N-type channel in the dendrite is still uncertain. Westenbroek et al. (1992) speculated that punctate staining patterns represent N-type calcium channels in nerve terminals. Similarly, we observed that N-type calcium channel subunits are organized in punctate clusters on the neurites of synaptically mature neurons. Nevertheless, based on our data, we cannot unequivocally state that the punctate immunoreactivity of the calcium channel subunits is presynaptic. However, since the α_{1B} subunit can colocalize with the synaptic vesicle protein synaptotagmin and action potential-evoked transmitter release is reduced by ω -CTX GVIA in hippocampal cultures (Basarsky et al., 1994; Scholz and Miller, 1995), it seems likely that this immunoreactivity is due to presynaptic channels.

While the mechanisms of regulation of distribution of calcium channels and vesicles are unknown, there are many issues that now need to be resolved. For example, do calcium channels cluster at sites of neurite-neurite contact independent of synapse formation? Do calcium channels first cluster at the site of contact and act as an anchor which permits the subsequent aggregation of vesicles and synaptic apparatus? Alternatively, of course, the opposite temporal sequence may occur in which synaptic proteins first aggregate at contact sites and act as a trap for synaptic calcium channels. One might envision a role for the presynaptic plasma membrane protein neurexin in the process of aggregation of calcium channels. The neurexins, a family of proteins that have extracellular immunoglobulin motifs, are also known to bind to the vesicle protein synaptotagmin through their short cytoplasmic tail (Ushkaryov et al., 1992). Perhaps contact with the postsynaptic target induces the clustering of neurexins in the presynaptic membrane, which in turn leads to a clustering of synaptic vesicles through the syn-

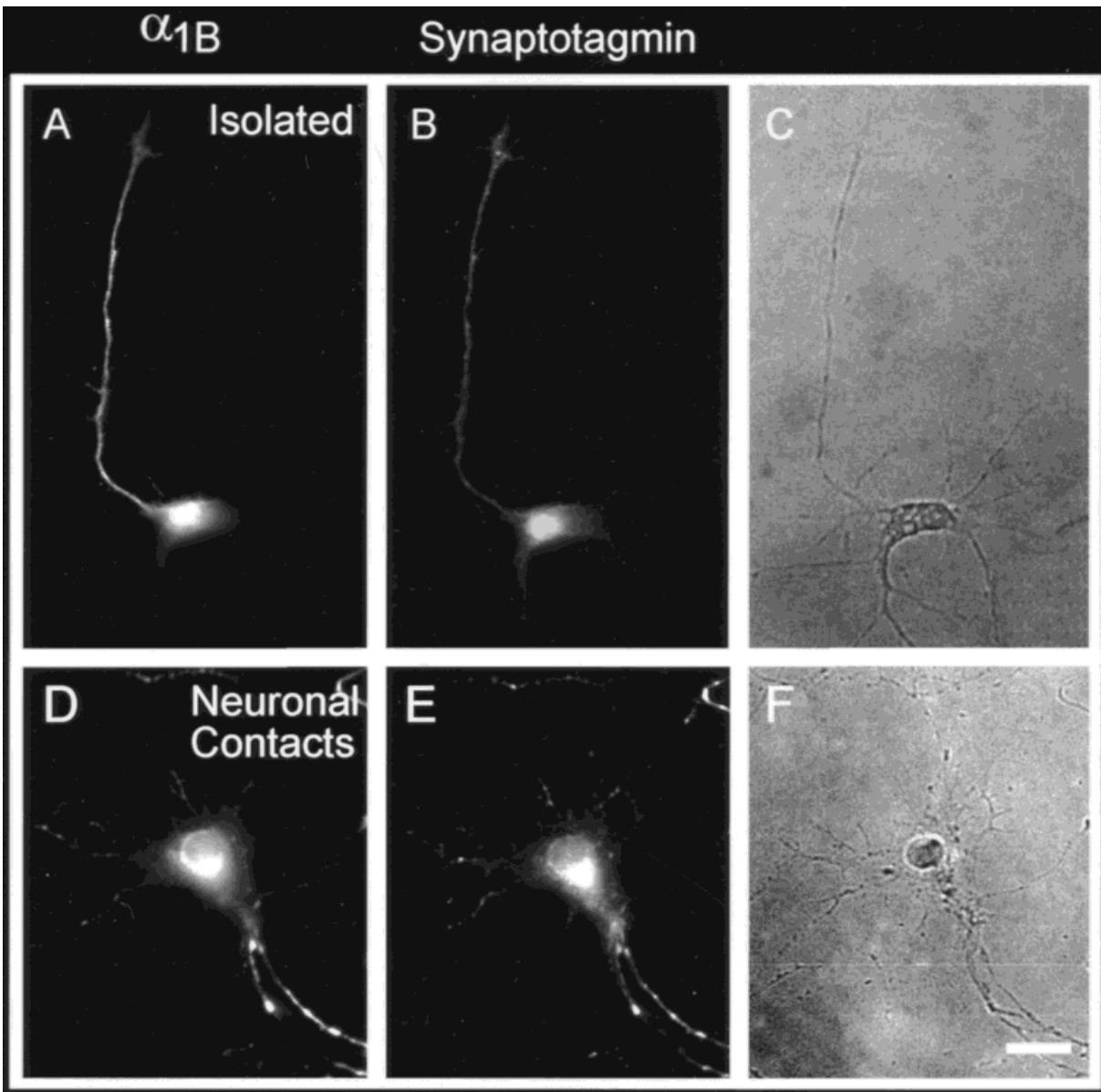


Figure 5 α_{1B} and synaptotagmin expression and distribution in isolated neurons and in neurons that were allowed neuron–neuron contacts. (A, B) α_{1B} and synaptotagmin immunoreactivities were distributed diffusely in isolated hippocampal neurons that were grown for 8 days in culture. (D,E) In contrast, α_{1B} and synaptotagmin immunoreactivities were punctate in the neurites of neurons that were grown in parallel cultures where neuron–neuron contacts were allowed. Bar = 20 μM .

aptotagmin–neurexin interaction. Such a complex, together with other synaptic proteins such as syntaxin, could then act as a calcium channel binding site to induce the local accumulation of N-type calcium channels.

A previous electrophysiological study has demonstrated that the development of action potential-evoked transmitter release is delayed in relation to

the appearance of spontaneous synaptic currents (Basarsky et al., 1994). What is the rate-limiting step in synaptogenesis between hippocampal neurons? A priori, there are four potential mechanisms that could limit the formation of synapses: the lack of (a) calcium channels, (b) secretory machinery, (c) expression of postsynaptic receptors, or (d) spatial coupling between calcium channels, secretory

machinery, and postsynaptic receptors. This study has clearly ruled out the first of these possibilities, since we have demonstrated that the calcium channel subunits are synthesized and transported and form functional channels prior to synaptogenesis. In addition, the synthesis of synaptic machinery is unlikely to be rate-limiting, since synaptic proteins have been shown to be expressed prior to the appearance of action potential-evoked transmitter release (Basarsky et al., 1994). Postsynaptically, Craig et al. (1994) showed that glutamate receptors are expressed by hippocampal neurons within about 1 day of culture. Taken together, these data suggest that the limiting step in synaptogenesis is the regulation of the tight spatial coupling of molecules within and between the pre- and postsynaptic terminal.

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