Activity-dependent Regulation of Dendritic BC1 RNA in Hippocampal Neurons in Culture

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Abstract. Several neuronal RNAs have been identified in dendrites, and it has been suggested that the dendritic location of these RNAs may be relevant to the spatiotemporal regulation of mosaic postsynaptic protein repertoires through transsynaptic activity. Such regulation would require that dendritic RNAs themselves, or at least some of them, be subject to physiological control. We have therefore examined the functional regulation of somatodendritic expression levels of dendritic BC1 RNA in hippocampal neurons in culture. BC1 RNA, an RNA polymerase III transcript that is a component of a ribonucleoprotein particle, became first detectable in somatodendritic domains of developing hippocampal neurons at times of initial synapse formation. BC1 RNA was identified only in such neurons that had established synapses on cell bodies and/or developing dendritic arbors. When synaptic contact formation was initiated later in low-density cultures, BC1 expression was coordinately delayed. Inhibition of neuronal activity in hippocampal neurons resulted in a substantial but reversible reduction of somatodendritic BC1 expression. We conclude that expression of BC1 RNA in somatic and dendritic domains of hippocampal neurons is regulated in development, and is dependent upon neuronal activity. These results establish (for the first time to our knowledge) that an RNA polymerase III transcript can be subject to control through physiological activity in nerve cells.

Increasing experimental evidence indicates that individual protein repertoires of mosaic dendritic microdomains can be regulated not only through delivery of proteins to dendritic target sites, but also through the local synthesis of selected proteins on site in dendrites (for reviews see Steward, 1995; Kindler et al., 1997; Steward, 1997). The concept that specific proteins may be manufactured locally in postsynaptic dendritic microdomains was initially prompted by the discovery of polyribosomes in such domains (Steward and Levy, 1982; Steward and Reeves, 1988). Postsynaptic accumulation of ribosomes is particularly prominent during periods of developmental or reactive synaptogenesis (Steward, 1983; Steward and Falk, 1991). More recently, dendrites of hippocampal neurons in culture have been shown to contain various factors necessary for translation, including components of the rough endoplasmic reticulum and the Golgi complex (Tiedge and Brosius, 1996; Torre and Steward, 1996). De novo synthesis of proteins has been demonstrated in isolated dendrites and growth cones of cultured hippocampal neurons, respectively (Torre and Steward, 1992; Crino and Eberwine, 1996).

The notion of dendritic translation has been substantiated by the identification in recent years of an increasing number of specific RNAs that are located in dendrites. Dendritic mRNAs encode respective cognate dendritic proteins that can be grouped into several classes, such as cytoskeletal elements, kinases, and receptors, among others (for reviews see Steward, 1994; Steward, 1995; Kindler et al., 1997; Steward, 1997). Noncoding dendritic RNAs include ribosomal RNAs (Kleiman et al., 1993), tRNAs (Tiedge and Brosius, 1996), and BC1 RNA (Tiedge et al., 1991). This latter RNA is a short untranslated RNA polymerase III transcript that is specifically expressed in neurons where it is complexed with proteins to form a ribonucleoprotein particle (RNP; Kobayashi et al., 1991; Cheng et al., 1996).[1] BC1 RNA is rapidly and selectively transported to dendrites (Muslimov et al., 1997), and it has been identified as a prominent component of postsynaptic dendritic microdomains (Chicurel et al., 1993; Rao and

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1. *Abbreviations used in this paper*: DIC, differential interference contrast; DIV, days in vitro; RNP, ribonucleoprotein particle; TTX, tetrodotoxin.

Steward, 1993). BC1 RNA (or the BC1 RNP) has been suggested to play a role in transport and/or translation of mRNAs in dendrites (Brosius and Tiedge, 1995).

The physiological significance of transporting specific RNAs to dendritic target sites for local translation may lie in the potential for enhanced and flexible spatiotemporal regulation of dendritic/postsynaptic protein pools (Brosius and Tiedge, 1995; Steward, 1995). It has in fact been shown that dendritic protein synthesis in hippocampal pyramidal cells is dependent upon neuronal activity (Feig and Lipton, 1993; Weiler and Greenough, 1993), and that such local synthesis is in turn a prerequisite for synaptic plasticity (Kang and Schuman, 1996). In this way, functional regulation of dendritic translation may be instrumental in the development and plasticity of synapses (discussed by Brosius and Tiedge, 1995; Steward, 1995; Kindler et al., 1997; Schuman, 1997; Steward, 1997).

If, as has been suggested, BC1 RNA is functionally involved in transport and/or translation of dendritic mRNAs, then it may be conjectured that the functional regulation of such mRNAs in dendrites may, at least in part, be mediated through BC1 RNA. Consequently, one would postulate that in this case BC1 RNA itself should be subject to activity-dependent regulation. The experimental test of this hypothesis was the primary objective of the present work. Using hippocampal neurons in primary culture, we show here that the developmental onset of somatodendritic BC1 expression is concomitant with initiation of developmental synaptogenesis. Furthermore, somatodendritic levels of BC1 RNA are reversibly modulated by neuronal activity. These results demonstrate that BC1 RNA is an activity-regulated RNA polymerase III transcript, with expression levels in somata and dendrites being dependent upon the developmental and physiological status of a neuron.

Materials and Methods

Cell Culture

Primary cultures of hippocampal neurons were prepared as described (Goslin et al., 1998). In brief, cells were dissociated from hippocampal tissue of 18-d-old rat embryos, and were plated onto polylysine-treated glass coverslips in MEM containing 10% horse serum. Cells were plated at nominal densities of 1,000 (low density), 4,000 (medium density), or 16,000 (high density) cells per cm^2. In some experiments (as noted in figure legends), a medium density of 6,000 cells per cm^2 was used. After attachment of cells to the substrate, coverslips were transferred to dishes containing monolayer cultures of astroglia so that neurons were facing the layer of glial cells without being in physical contact with them. Cells were then maintained in serum-free medium. 5 μM cytosine-β-D-arabinofuranoside was added on the fourth day to reduce glial proliferation. In cultures that were maintained in the presence of tetrodotoxin (TTX; Sigma Chemical Co., St. Louis, MO), TTX was added to a final concentration of 1 μM 4 h after plating in culture, and was replenished at the same concentration with every exchange of media (see also Benson and Cohen, 1996). At given time points, cells were fixed in 4% formaldehyde (made from paraformaldehyde) and 4% sucrose in PBS (140 mM NaCl, 15 mM phosphate buffer, pH 7.3) at room temperature for 20 min. Coverslips with cells were stored in 70% ethanol at −20°C until further processing.

In situ Hybridization and Immunocytochemistry

RNA probes against BC1 RNA were generated from plasmid pMK1 (Tiedge, 1991; Tiedge et al., 1991). This plasmid contains a sequence that corresponds to the nonrepetitive segment of 60 3′-most nucleotides of BC1 RNA. Probes specific for 7SL RNA were generated from plasmid pKK451-1 (Tiedge and Brosius, 1996). This plasmid contains a sequence that corresponds to part of the non-Alu central S region of 7SL RNA. ^{35}S-labeled RNA probes were transcribed from linearized templates, using T3 or T7 RNA polymerase as recommended by the manufacturer (Stratagene, La Jolla, CA). Prehybridization and hybridization steps were carried out as described (Tiedge, 1991). High stringency washes were performed at 50°C (hybridization to BC1 RNA) or 45°C (hybridization to 7SL RNA), respectively.

When immunocytochemistry was combined with in situ hybridization, the in situ part was performed first. After the final high-stringency wash, coverslips were directly transferred to PBS and rinsed for 10 min. Cells were fixed again with 4% formaldehyde (see above), rinsed in PBS with 5 mM $MgCl_2$ for 10 min at room temperature, and processed for immunocytochemistry as described (Tiedge and Brosius, 1996). All primary antibodies used in this work have been characterized before (Tiedge and Brosius, 1996). After immunocytochemistry, coverslips were processed for emulsion autoradiography.

Emulsion Autoradiography

Dried coverslips were mounted cell-side up on microscope slides with DPX (Fluka, Ronkonkoma, NY), dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with HPLC-grade water, air-dried, and exposed at 4°C for 3 wk. After photographic development (D-19 developer, 50% strength, and Rapid-Fix™; Eastman Kodak Co.), cells were coverslipped with Kaiser's glycerol jelly (Banker and Goslin, 1998). Cultures that had been used for immunocytochemistry were coverslipped with 80% glycerol containing 2.5% 1,4-diazabicyclo-[2.2.2]octane (Sigma Chemical Co.), as described (Tiedge and Brosius, 1996).

Data Evaluation

Cells were analyzed and photographed on a Microphot-FXA™ microscope (Nikon, Inc., Melville, NY), using darkfield (Ektachrome-160T film; Eastman Kodak Co.), fluorescence (Ektachrome-400 film; Eastman Kodak Co.), phase contrast, and differential interference contrast (DIC) optics. For determining expression levels by in situ hybridization, a labeling signal was considered significant if it exceeded background levels by a factor of at least three. Silver grains were classified as somatic signal if they were located within a 15-μm radius from the center of the cell body, dendritic if they were located within a 150-μm radius, but outside the 15-μm radius from the center of the cell body. If radii of two or more cells overlapped, silver grains in the overlapping areas were attributed proportionally to the respective cells. Background was determined in areas of equal size in sense strand controls, and was subtracted from signal.

To analyze dendritic extent, cells were labeled with an antibody specific for dendritic MAP2, as described previously (Tiedge and Brosius, 1996). For a comparison of TTX cultures with control cultures after 14 d in vitro, three parameters of dendritic extent were analyzed as follows. Number of primary dendrites per cell (i.e., number of MAP2-labeled processes emerging from a cell body): control cultures, 6.2 ± 1.6 (53); TTX cultures, 5.1 ± 1.5 (55). Branch points per dendrite (i.e., number of branch points per MAP2-positive process emerging from a cell body): control cultures, 4.8 ± 1.8 (58); TTX cultures, 3.9 ± 2.0 (49). Length of principal dendrites (i.e., distance in μm from the center of the cell body to the proximal-most branch point of any MAP2-positive process emerging from a cell body): control cultures, 181 ± 68 (77); TTX cultures, 145 ± 55 (60). Data are given in the format mean ± SD (numbers of cells analyzed). Similar observations have previously been reported by Benson and Cohen (1996).

Results

BC1 RNA in Somatodendritic Domains of Hippocampal Neurons in Culture

Hippocampal neurons in primary culture have been used extensively as a model system for analysis of neuronal polarity in general and differential distribution of RNAs in particular. Hippocampal neurons can be maintained in culture over extended periods of time, during which they extend well-defined axons and elaborate dendritic arbors (for reviews see Banker and Waxman, 1988; Goslin et al., 1998). In mature hippocampal neurons maintained for 21 d

in culture, we identified BC1 RNA in somatic and dendritic domains (Fig. 1). After in situ hybridization with a probe specific for BC1 RNA (Tiedge et al., 1991), autoradiographic silver grains indicating the presence of the RNA were seen labeling dendrites at considerable distances from cell bodies. No significant labeling was detected along isolated shafts of numerous axons that could be seen in mature cultures where they form a plexus composed of individual processes and small bundles. As a caveat, it should be noted that axons sometimes run along the surfaces of dendrites and somata (Goslin et al., 1988). Given the resolution limits of the techniques used, it would be impossible in such a case to detect a low-level axonal BC1 signal over a stronger dendritic one.

BC1 labeling along dendrites was strong and nonhomogeneous; clusters of silver grains separated by gaps were evident in many dendritic segments, suggesting nonuniform distribution of the RNA along dendrites (Fig. 1, *A* and *C*). Similar clusters have also been observed after microinjection into sympathetic neurons in culture of in vitro–synthesized BC1 RNA (Muslimov et al., 1997). Such clusters may be indicative of supramolecular BC1 transport structures, or of sites of preferential BC1 docking in dendritic microdomains. In line with the former scenario are observations, made with neurons as well as with other cell types (Ainger et al., 1993; Knowles et al., 1996), that RNAs transported within cellular processes may be contained within granules en route.

Dendrites and axons were identified on the basis of criteria established previously (Banker and Waxman, 1988; Dotti et al., 1988) in which dendrites were defined as rather short tapering processes that contain polyribosomes (see below), and axons were described as long, thin processes that are relatively uniform in diameter and lack polyribosomes. However, since BC1 RNA has previously been localized to dendritic layers in the rodent CNS and to neuronal processes of acutely isolated cells (Tiedge et al., 1991; Tiedge et al., 1992), it was further necessary to establish the dendritic nature of BC1-positive neuronal processes of hippocampal neurons. We therefore double-labeled such neurons with a probe for BC1 RNA and with an antibody specific for ribosomal P proteins. This antibody (which has previously been shown to label somatodendritic domains of hippocampal neurons in culture [Tiedge and Brosius, 1996]) and the anti-BC1 probe produced labeling signals that colocalized to the same somatodendritic domains (data not shown). Since ribosomes have been shown, using a variety of approaches and crite-

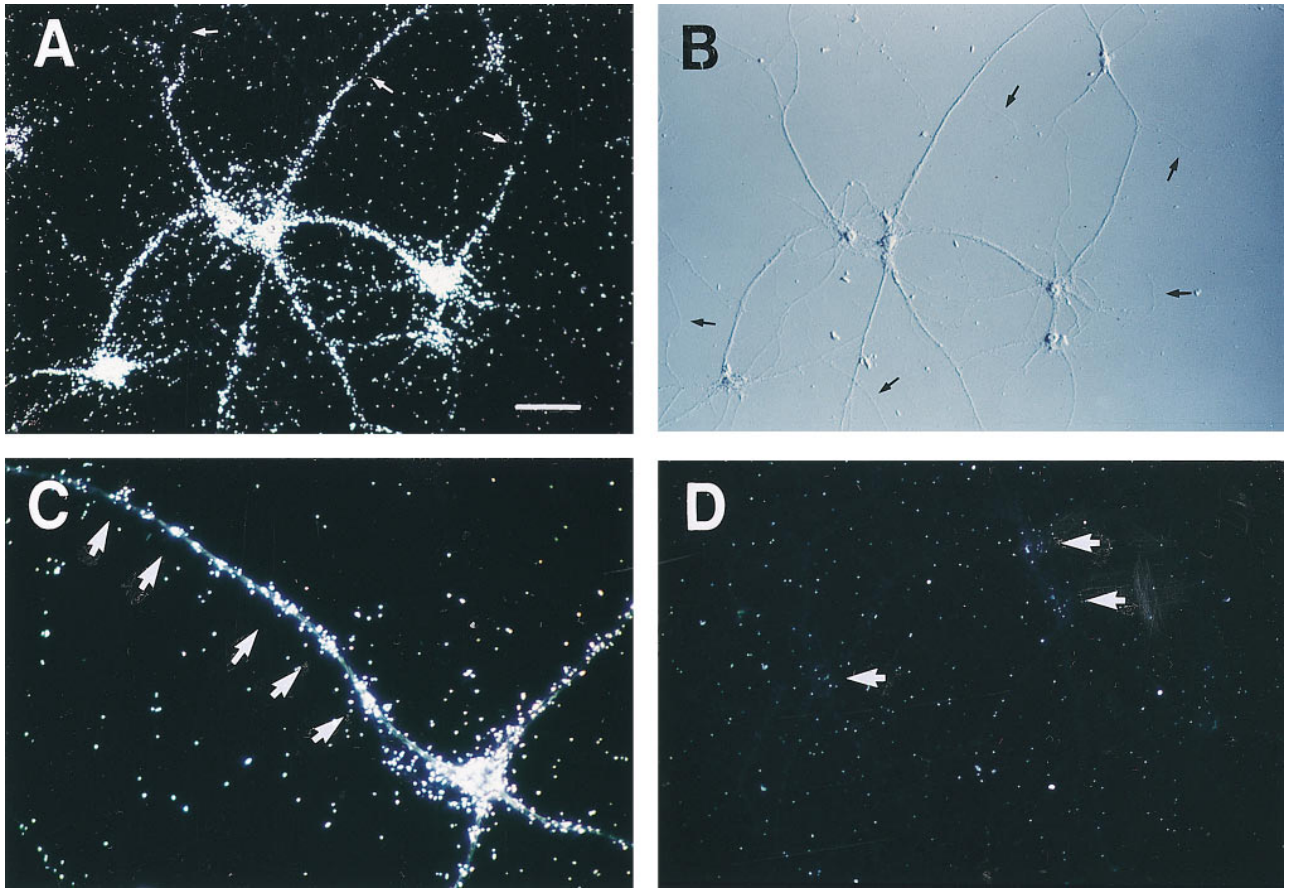

Figure 1. Localization of BC1 RNA to dendrites of mature hippocampal neurons in culture. Cells were probed for BC1 RNA by in situ hybridization; labeling signal is indicated by autoradiographic silver grains (white in dark-field photomicrographs *A*, *C*, and *D*). (*A* and *B*) BC1 labeling signal is observed over somata and dendrites, but not over axons (*black arrows* in DIC photomicrograph *B*) of hippocampal neurons in culture. Labeling was discontinuous over some dendrites: gaps are indicated by white arrows in *A*. A higher power photomicrograph (*C*) reveals clustering (*white arrowheads*) of the BC1 labeling signal over dendrites. (*D*) Sense strand control experiments did not produce significant labeling over either cell bodies (*white arrowheads*) or neurites. (*A*, *B*, and *D*) Bar, 50 μm; (*C*) bar, 25 μm.

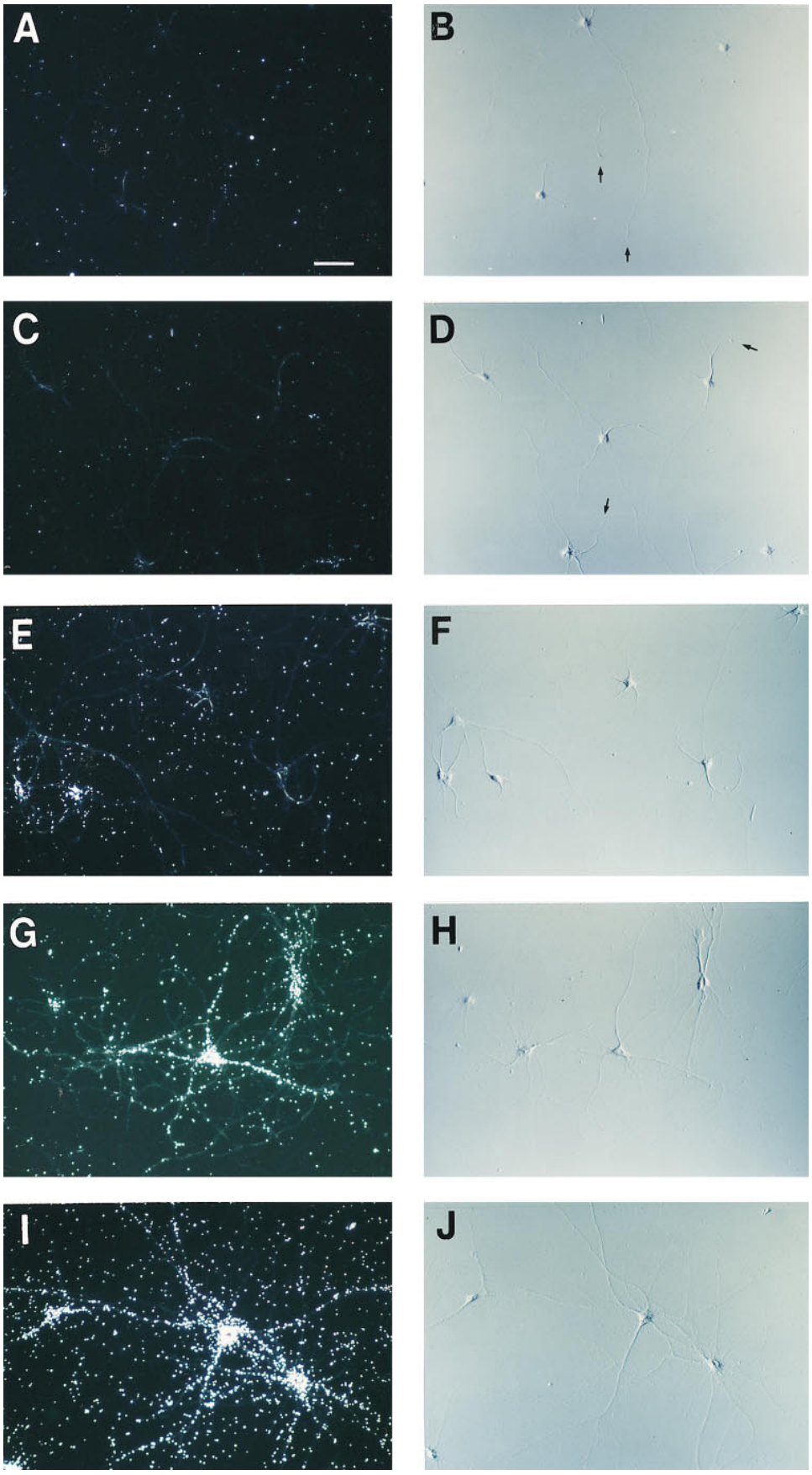

Figure 2. Development of somatodendritic BC1 expression in hippocampal neurons in culture. Cells were grown at medium density (6,000 cells per cm^2) for 2 d in vitro (DIV; *A* and *B*); 4 DIV (*C* and *D*); 7 DIV (*E* and *F*); 9 DIV (*G* and *H*); and 14 DIV (*I* and *J*). (*Left*) Dark-field photomicrographs; (*right*) DIC photomicrographs. Arrows indicate axonal (*B*) or dendritic (*D*) growth cones. Bar, 50 μm.

ria, to be localized to somata and dendrites but not to axons of mature hippocampal neurons in culture (Deitch and Banker, 1993; Tiedge and Brosius, 1996), our data further establish that BC1-positive neuronal processes were in fact dendrites. Finally, double-labeling experiments with an antibody against synaptophysin (a marker of presynaptic specializations; see Fletcher et al., 1991) indicated that BC1-positive dendritic processes were targets for synaptic contacts (not shown). In combination, these data demonstrate that in mature hippocampal neurons in culture, clusters of BC1 RNA are nonuniformly localized to dendrites that contain protein synthetic capacity and are recipients of synaptic contacts.

Development of Somatodendritic BC1 Expression

The development of axons and dendrites in hippocampal neurons in culture has been described as a series of events that are controlled by a combination of intrinsic and extrinsic determinants (Banker and Waxman, 1988; Dotti et al., 1988). The former are those that are defined as governing stereotypical developmental events such as initial process outgrowth, while the latter play a prominent role during later maturation stages, triggered for example by an increasing number of cell interactions. Dotti et al. (1988) have subdivided the development of hippocampal neurons in culture into five distinct stages. After lamellipodia formation and outgrowth of minor processes at stages 1 and 2, respectively, during the first 12 h in culture, axon outgrowth is initiated at stage 3 (after 1.5–2 d in culture), and dendrite outgrowth at stage 4 (after ~4 d in culture). The subsequent maturation of axons and dendrites (stage 5) is an open-ended process that can continue for days or weeks. To begin to understand the significance of somatodendritic BC1 expression in relation to developmental neurite outgrowth and synaptogenesis, we probed hippocampal neurons in culture at different developmental stages for expression onset and subcellular distribution of the RNA.

Fig. 2 illustrates the onset and development of BC1 expression in hippocampal neurons during the first 2 wk in culture. The initial two time points were chosen to represent neurons at the stages of initial axonal and dendritic outgrowth, respectively (corresponding to stages 3 and 4 of Dotti et al., 1988). During these developmental periods, very little specific labeling signal could be detected over somata or developing axons and dendrites. Thus, expression of BC1 RNA does not correlate with, and can therefore not be required for, the initial outgrowth of axons or dendrites of cultured hippocampal neurons. As a positive control, we probed for 7SL RNA (also called SRP RNA), a ubiquitously expressed RNA polymerase III transcript that is part of the signal recognition particle (Walter and Blobel, 1982). This RNA was detected in cell bodies of cultured hippocampal neurons at uniform and constant levels during those early stages of development at which BC1 RNA remained undetectable (not shown).

In cultures of medium density as shown in Fig. 2, BC1 RNA first became detectable towards the end of the first week in culture. After 7 d in culture, significant numbers of autoradiographic silver grains were detected over ~50% of all neuronal somata. Axons and most dendrites remained unlabeled, although a few dendrites showed beginning BC1 labeling, mostly in proximal segments. By 9 d in culture, labeling had become stronger and was by now detectable in ~75% of all cells. In most of these neurons, significant numbers of silver grains could also be seen over dendrites. This development continued over the next few days, and by day 14 in culture, labeling was robust in virtually all neurons analyzed. At the same time, dendritic labeling had become more extensive and elaborate as dendritic arborizations continued to mature, grow in complexity, and establish more synaptic contacts. We performed a quantitative analysis of the development of dendritic BC1 labeling in hippocampal neurons during the first three weeks in vitro; these data are summarized in diagrammatic form in Fig. 3.

In clear contrast to BC1 RNA, expression levels of 7SL RNA showed no significant changes during these first 2 wk in culture (not shown). In the course of the third week in culture, 7SL RNA became increasingly localized to proximal dendritic domains, while somatic labeling remained strong and overall expression levels constant (as shown by Tiedge and Brosius, 1996). Dendritic BC1 labeling continued to increase in extent (Fig. 3) and in complexity (evidenced for example by increased clustering; see Fig. 1) during the third week in culture. It is noteworthy that even in mature cultures, not all dendrites exhibited BC1 labeling. A similar observation has previously been reported with dendritic MAP2 mRNA (Kleiman et al., 1994). At no time during the development of these cells in culture was there any clear evidence for significant BC1 labeling in axons.

In summary, the above data indicate that expression of BC1 RNA in hippocampal neurons in culture is not initiated until after developing axons and dendrites have already grown to significant lengths. Thus, BC1 expression cannot be functionally related to early axonal or dendritic development. The results rather suggest that somatodendritic expression of BC1 RNA coincides with the functional maturation of dendrites, in particular with formation and/or maturation of functional synaptic connections. This hypothesis was experimentally examined as described below.

BC1 Expression and Synapse Formation

To examine whether the onset of BC1 expression in hippocampal neurons in culture was correlated with the be-

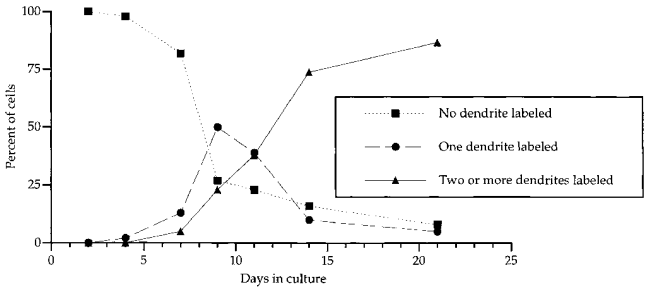

Figure 3. Quantitative analysis of dendritic BC1 delivery in hippocampal neurons developing in culture. The number of cells analyzed for each time point was as follows: 2 DIV, 214; 4 DIV, 187; 7 DIV, 171; 9 DIV, 200; 11 DIV, 298; 14 DIV, 219; 21 DIV, 238.

ginning of developmental synapse formation, we analyzed the development of BC1 expression in neurons that were maintained at different plating densities (Banker and Waxman, 1988). It has previously been shown that the rate at which presynaptic specializations form in such cultures was density-dependent (Fletcher et al., 1991). Synapse formation in low-density cultures will proceed at a slower rate than in high-density cultures because it will take an outgrowing axon more time to encounter a suitable target. Taking advantage of this fact to modulate initiation of synapse formation, we cultured hippocampal neurons at a wide range of plating densities (for a definition of low-, medium-, and high-density, see Materials and Methods), and we probed such cultures for the appearance of BC1 RNA in somata and dendrites at various stages of development. Simultaneously, using immunocytochemistry with an antibody against the synaptic vesicle protein synaptophysin, we visualized synapses as puncta of antisynaptophysin immunoreactivity (Fletcher et al., 1991).

We consistently observed substantial differences in somatic and dendritic levels of BC1 RNA as a function of cell and synapse density. In Fig. 4, this is exemplified with hippocampal neurons that had been maintained in culture for 7 d. In low-density cultures at this stage, we observed only a small number of synaptophysin puncta per neuron (Fig. 4, *A* and *B*). This number was typically below 20. Often, very few puncta or none at all were seen associated with a particular neuron; this was usually the case with cells that developed in relative spatial isolation. A BC1 signal was observed at low levels in the somata of most of those cells that exhibited synaptophysin puncta on cell bodies and/or dendrites (Fig. 4 *B*). No significant levels of BC1 RNA were detected in cells that were devoid of synaptophysin puncta. In some cases, however, BC1 RNA was also undetectable in cells that clearly exhibited at least several synaptophysin puncta (Fig. 4 *A*). These data suggest that the onset of BC1 expression is concurrent with, or immediately subsequent to, initial synapse formation.

In cultures of medium or high density at the same stage, synaptophysin puncta were far more numerous; this was paralleled by significantly higher and more widespread BC1 labeling (Fig. 4, *C* and *D*). In medium-density cul-

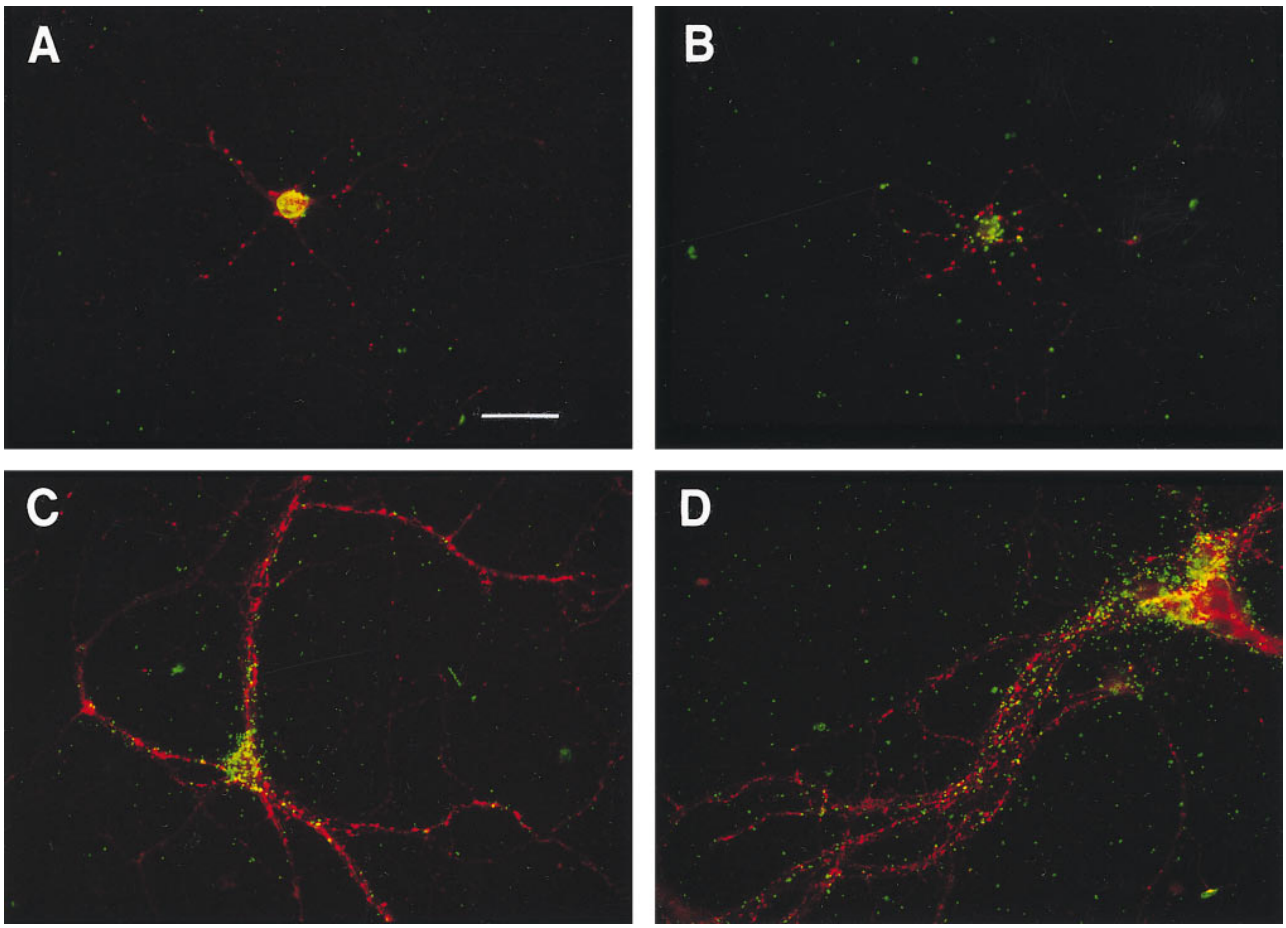

Figure 4. Expression of BC1 RNA in cultured hippocampal neurons developing at different cell densities. Hippocampal neurons were grown for 7 d in culture and were double-labeled for BC1 RNA (in situ hybridization; *green*) and synaptophysin (immunocytochemistry; *red*). A green filter was used for visualizing autoradiographic silver grains in double-exposure photomicrographs in order to differentiate them from the red immunofluorescence signal. (*A* and *B*) 1,000 cells per cm^2; (*C*) 4,000 cells per cm^2; (*D*) 16,000 cells per cm^2. At low density, beginning BC1 expression can be observed in a few cells (*B*) while the majority shows no labeling (*A*). At medium density (*C*), BC1 labeling was often observed over proximal segments of dendrites that were lined with synaptophysin puncta. At high density (*D*), BC1 labeling was frequently observed over dendrites at considerable distances from somata. Bar, 25 μm.

tures, BC1 label was mostly observed over cell bodies, but some dendrites with particularly extensive synaptophysin labeling showed first signs of a dendritic BC1 signal (Fig. 4 *C*). In total, BC1 RNA was observed in 52% of all neurons at medium density, compared with 36% of all cells in low-density cultures at the same stage. Frequently, labeled neurons were those that had their somata located in close proximity to each other. Strikingly different from low-density cultures (Fig. 4, *A* and *B*) was the somatodendritic labeling pattern of BC1 RNA in high-density cultures at 7 d in vitro (Fig. 4 *D*). Here, most neurons were labeled, many at robust levels. The difference in BC1 expression between low-density and high-density cultures was both quantitative and qualitative in nature in that in high-density cultures, the signal intensity was overall much stronger, and autoradiographic silver grains could be seen tracing dendrites for extended distances. At the same time, a significant number of synaptophysin puncta could be seen tracing dendrites in high-density cultures (Fig. 4 *D*). In contrast, expression levels of 7SL RNA, which was probed in parallel control experiments, were constant and not modulated as a function of cell density (not shown).

During the second week in vitro, both synapse density and BC1 expression increased significantly in low-density cultures, thus catching up with cells at higher density (not shown). Between days 7 and 9 in vitro, we observed a significant increase in the number of BC1-positive neurons (from 36 to 60%) in low-density cultures. After a total of 2 wk, virtually all neurons in low-density cultures were labeled for BC1 RNA at significant levels. In most of these cells, labeling clearly extended to dendrites, very similar to the situation in high-density cultures at earlier stages. Overall, our observations with low-density cultures lead us to conclude that synapse formation and BC1 expression were initiated later than in high-density cultures, and that the first appearance of BC1 RNA in dendrites was similarly delayed.

Taken together, our results indicate that synapse formation and BC1 expression are closely synchronized processes in developing hippocampal neurons in culture, and they are consistent with the notion that somatodendritic expression of BC1 RNA is correlated with the number of synaptic contacts that these neurons receive.

Activity-dependent BC1 Expression

The above data imply a correlation between developmental synaptogenesis and BC1 expression in hippocampal neurons in culture. However, they do not allow us to differentiate whether such correlation was directly causal or merely temporal. In the latter case, it would be conceivable that, for example, a diffusible factor produced at higher concentrations in high-density cultures might induce earlier onset of BC1 expression. On the other hand, in case the correlation was causal, with BC1 expression being dependent on synapse formation, it remained to be determined whether it was the physical formation of synaptic contacts or rather the functional activity of such newly formed synapses that initiated BC1 expression. To address these questions, we performed a further series of experiments in which the electrical activity of hippocampal neurons in culture was reversibly modulated.

We used TTX, a potent inhibitor of voltage-gated Na^+-channels, in cultured hippocampal neurons during the first 2 wk of development. Chronic application of TTX, which has been used extensively in hippocampal neurons in culture (see for example Craig et al., 1994; Verderio et al., 1994; Benson and Cohen, 1996), has been shown to result in complete suppression of action potentials (Craig et al., 1994), thereby preventing evoked synaptic transmission. Thus, if BC1 expression is functionally dependent upon such transmission, application of TTX can be expected to result in decreased levels of the RNA. We maintained hippocampal neurons in culture at medium density in the presence of TTX for 14 d. Such application of TTX resulted in a dramatic reduction of somatodendritic BC1 levels relative to neurons that were maintained in culture for the same amount of time in the absence of TTX (Fig. 5). After 14 d in vitro, when BC1 levels were robust in somata and dendrites of neurons in control cultures, TTX cultures showed low-level BC1 labeling that reached, on average, no more than 10% of BC1 labeling levels in control cultures. A similarly substantial TTX-induced reduction of BC1 levels could already be observed at an earlier stage of BC1 expression after 9 d in culture (not shown). The reduction in BC1 expression levels was not uniform: whereas many cells in the 14-d TTX cultures showed no labeling at all, others exhibited low-level somatic labeling, while in still others, labeling was moderate in the soma and extended into proximal dendrites. Table I provides a summary of these data on the basis of a quantitative analysis of silver grain densities in the cultures examined. From the table, it appears that the ratio between dendritic and somatic BC1 signal intensities in TTX-treated vs. untreated cultures is constant. This result may be taken to indicate that regulation of BC1 RNA through electrical activity is mediated mainly through regulation of expression in the nucleus, as this can be expected to result in similar effects on somatic and dendritic BC1 levels, respectively. On the other hand, because BC1 levels in TTX-treated cultures are only marginally above background, making the calculation of ratios difficult, it cannot be ruled out that dendritic BC1 levels may also be regulated locally, in addition to regulation through gene expression.

In clear contrast to BC1 RNA, expression of 7SL RNA was unaffected by the presence of TTX, both after 9 d (not shown) and 14 d in culture (Fig. 5). Neurons that had been treated with TTX were of healthy appearance, qualitatively indistinguishable from cells in control cultures, as has been reported earlier (Craig et al., 1994; Verderio et al., 1994; Benson and Cohen, 1996). In addition, to probe for possible effects of TTX on neuronal morphology, we monitored dendritic extent and synapse density of cells in TTX and control cultures using antibodies against dendritic MAP2 (Cáceres et al., 1986) and against synaptophysin (Fletcher et al., 1991), respectively. Compared with control cultures, we observed a moderate decrease in overall dendritic extent in TTX cultures (number of primary dendrites per cell: −18%; length of principal dendrites: −20%; branch points per dendrite: −19%; see Materials and Methods for details) and, conversely, a small increase in the density of synaptophysin-positive puncta (+ 10%; see also similar data previously reported by Benson and Cohen, 1996). However, these changes were minor in com-

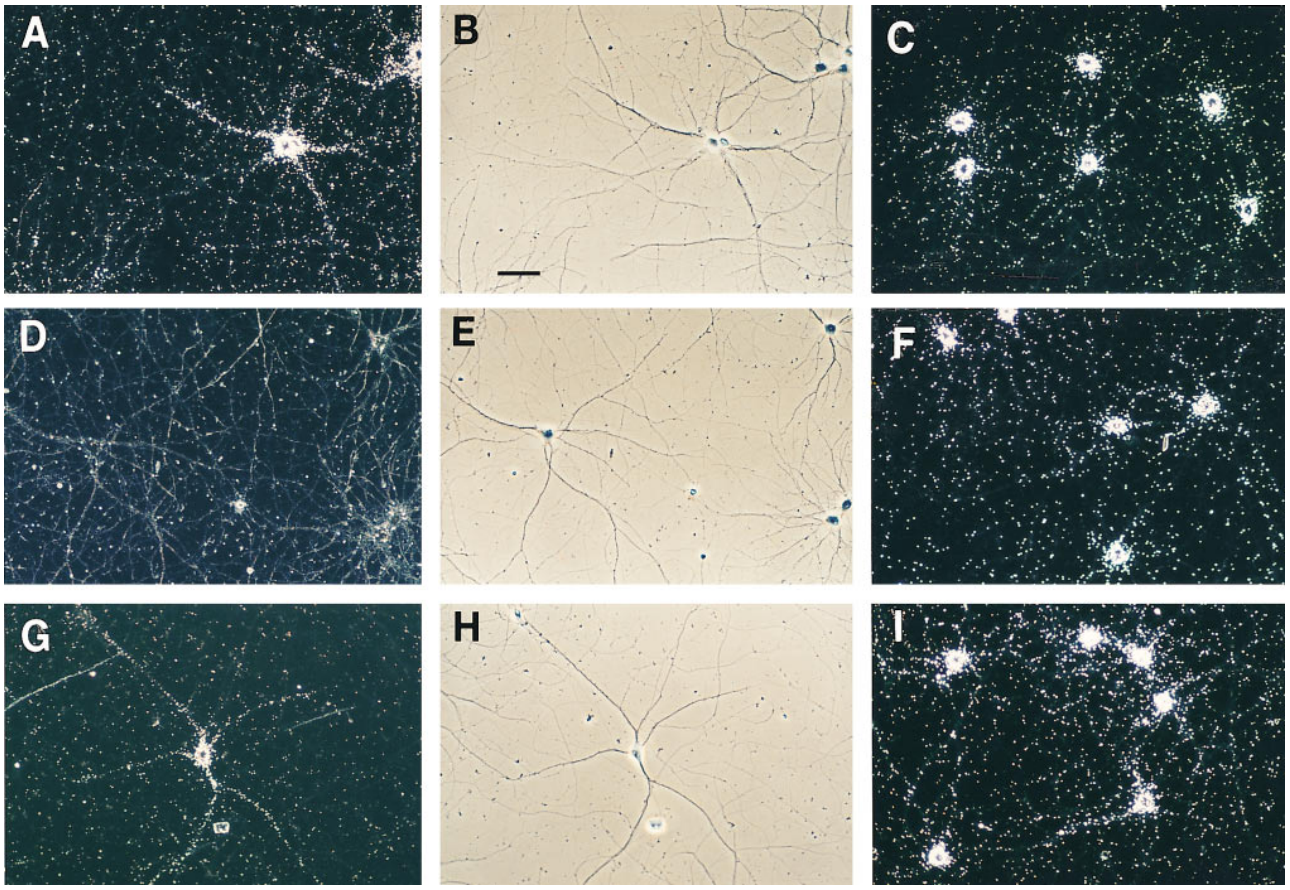

Figure 5. Regulation of BC1 expression by neuronal activity. Hippocampal neurons were grown in culture for 14 d in the absence of TTX (*A–C*), in the presence of 1 μM TTX (*D–F*), or in the presence of 1 μM TTX for the first 9 d, and in the absence of TTX for the following 5 d (*G–I*). Left (*A–G*): BC1 RNA, dark-field photomicrographs; middle (*B–H*), BC1 RNA, phase contrast photomicrographs, corresponding to dark-field photomicrographs in left column; right (*C–I*): 7SL RNA, dark-field photomicrographs. Dark field photomicrograph D (BC1 RNA in the presence of TTX) was overexposed to reveal absence of any significant labeling over cells or neurites. All cultures were grown at medium density. Bar, 50 μm. (*J*) Cultured hippocampal neurons on coverslips were exposed to autoradiographic film. Autoradiographs show BC1- and 7SL-labeling signals for cells that were grown for 14 d in the absence of TTX (1), in the presence of 1 μM TTX (2), or in the presence of 1 μM TTX for the first 9 d and in the absence of TTX for the following 5 d (3). (4) Sense strand control.

parison with (and therefore unlikely to account for) the substantial TTX-induced reduction of somatodendritic BC1 expression levels. Against the background of these observations, our data suggest that inhibition of electrical activity in developing hippocampal neurons results in a significant suppression of the developmental initiation of BC1 expression.

If regulation of BC1 expression is indeed the direct result of changes in neuronal activity, one would predict that expression of BC1 RNA would return to a normal level, and that distribution would return upon restoration of full electrical activity. This was indeed observed. When hippocampal neurons were maintained in medium-density cultures in the presence of TTX until day 9 in vitro, and then transferred to TTX-free media and maintained in culture for five additional days, we found that BC1 expression levels and somatodendritic distribution patterns had been restored, and had become indistinguishable from sister cultures that had been maintained in parallel over the entire period of 14 d in the absence of TTX (Fig. 5 *G*; Ta-

Table I. Expression of BC1 RNA in Hippocampal Neurons in Culture as a Function of Electrical Activity

	Control	TTX	TTX/Control
Somatic BC1 signal	162 ± 52 (106)	16 ± 11 (112)	165 ± 60 (236)
Dendritic BC1 signal	279 ± 93 (110)	25 ± 14 (115)	266 ± 94 (236)

Control: 14 d in vitro, no TTX; TTX: 14 d in vitro, 1 μM TTX; TTX/Control: 14 d in vitro, 1 μM TTX (first 9 d), no TTX (subsequent 5 d). Data are given in the format mean ± SD (no. of cells analyzed). Numbers for somatic/dendritic signal represent the numbers of silver grains over somatic/dendritic areas. Parameters listed were determined as described in Materials and Methods.

ble I). For further quantitative corroboration, we analyzed entire cultures on coverslips by exposure to autoradiographic film (Fig. 5 *J*) and by phosphoimaging. We obtained the following phosphoimaging data on BC1 labeling intensities (in arbitrary units): 14 d in the absence of TTX, 3.96 (± 0.02); 14 d in the presence of 1 μM TTX, 0.3 (± 0.28); 9 d in the presence of 1 μM TTX, followed by 5 d in the absence of TTX 3.95 (± 0.49). These data again confirm that overall BC1 levels were substantially reduced in TTX cultures (to <10% of control levels), but reverted to normal in cultures in which electrical activity was restored by transfer to TTX-free media. In contrast, expression of 7SL RNA in hippocampal neurons was not affected by the activity status of the cells.

The combined data thus show that somatodendritic expression of BC1 RNA in hippocampal neurons in culture is reversibly modulated by neuronal activity.

Discussion

It was our goal in this work to establish whether somatodendritic expression of neuronal BC1 RNA is subject to developmental and/or activity-dependent regulation. We identified BC1 RNA in somata and in dendrites, but not in axons, of mature hippocampal neurons in culture. In developing hippocampal neurons, BC1 RNA was not expressed during stages of initial outgrowth of axons and dendrites. Rather, the onset of BC1 expression was correlated with dendritic maturation, concurrent with or immediately subsequent to developmental synapse formation. Expression of BC1 RNA was significantly but reversibly suppressed by inhibition of electrical activity. Two major conclusions are drawn from these results: (*a*) developmental expression of BC1 RNA in somatic and dendritic domains is coordinated with synapse formation; and (*b*) somatodendritic expression levels of BC1 RNA are subject to regulation by neuronal activity.

Developmental and activity-dependent regulation of dendritic RNAs may be directly relevant for formation, growth, and/or long-lasting modulations of synaptic connections. This notion was initially prompted by the discovery that polyribosome accumulation in postsynaptic microdomains is significantly increased at times of developmental and reactive synaptogenesis in vivo (Steward, 1983; Steward and Falk, 1986). In CA1 pyramidal neurons of the developing hippocampus, the rate of synapse formation is low during the first postnatal week and increases significantly thereafter; polyribosomes are associated with a sizable fraction of such early synapses (Steward and Falk, 1991). BC1 RNA first becomes detectable in the CA1 region of the hippocampus during the second half of the first postnatal week (V. Liu, J. Brosius, and H. Tiedge, unpublished data). Thus, it appears that in vivo, developmental synapse formation, clustering of polyribosome at postsynaptic sites, and onset of BC1 expression are events that are initiated in synchrony.

Developmental synapse formation has been studied extensively in hippocampal neurons in culture (for reviews see Banker and Waxman, 1988; Goslin et al., 1998). In general, synapses begin to form in such cultures when outgrowing dendrites become receptive during the second half of the first week in vitro. This is the time at which evoked synaptic currents can first be recorded (Basarsky et al., 1994); it is also the time at which synaptic vesicle proteins such as synapsins, synaptophysin, synaptotagmin, and rab3a become clustered in presynaptic specializations, revealed as bright puncta by immunocytochemistry with specific antibodies (Fletcher et al., 1991; Basarsky et al., 1994). However, the exact time point at which these events can first be observed is variable and can be modulated by extrinsic determinants such as cell density. For this reason, we double-labeled hippocampal neurons with an anti-synaptophysin antibody to correlate the appearance of BC1 RNA with the onset of developmental synaptogenesis in cultures maintained at different densities. Results from this series of experiments led to the conclusion that BC1 expression begins concomitant with or immediately subsequent to the earliest formation of synapses. This apparent coordination of BC1 expression with developmental synapse formation prompted us to ask whether somatodendritic levels of BC1 RNA were in fact modulated as a function of neuronal activity. That this was indeed the case was demonstrated by the results of the TTX experiments: inhibition of electrical activity resulted in a substantial but fully reversible decrease of somatodendritic BC1 levels. These results thus indicate that it is not merely the physical establishment of synaptic contacts, but rather the functional activity of such contacts that is needed to initiate developmental BC1 expression. Initiation of BC1 expression during development therefore appears to be a direct reflection of activity-dependent regulation. In summary, neuronal activity is thus a necessary condition for BC1 expression in hippocampal neurons. It remains open at this time whether other factors may in addition be involved in modulating somatodendritic levels of the RNA.

What is the significance of such regulation in relation to other dendritic RNAs? BC1 RNA is the only known RNA polymerase III transcript that is located to dendrites and is regulated through synapse formation and physiological activity. Expression of other dendritic RNA polymerase III transcripts, such as 7SL RNA and tRNAs, has been shown to be independent of synaptogenesis and/or neuronal activity (this report; Tiedge and Brosius, 1996). These RNAs, as well as dendritic rRNA (Kleiman et al., 1994), are thus expressed constitutively in hippocampal neurons (notwithstanding the possibility that local concentration patterns of these RNAs in individual dendritic microdomains may still be subject to regulation). Activity-dependent regulation of BC1 RNA is therefore a feature that distinguishes this RNA as fundamentally different from other polymerase III transcripts and from polymerase I transcripts (rRNA).

Dendritic RNA polymerase II transcripts (mRNAs) form a heterogeneous class in terms of their activity-dependent regulation. The prototypical dendritic mRNA, encoding high-M_r MAP2, becomes first detectable in dendrites of hippocampal neurons after 5–7 d in culture (Kleiman et al., 1994). Before this time, MAP2 mRNA is expressed at very low levels in cell bodies. Expression of MAP2 mRNA thus seems to be correlated with dendritic maturation. Application of NMDA or of agents releasing nitric oxide has been shown to result in an increase of MAP2 mRNA levels in dendritic layers of the dentate gyrus that was independent of changes in the somatic layer (Johnston and Morris, 1994). Dendritic delivery of BDNF

and TrkB mRNAs is similarly regulated by activity in hippocampal neurons: high-potassium depolarization resulted in their increased, but RNA synthesis–independent dendritic targeting (Tongiorgi et al., 1997). These dendritic mRNAs are thus regulated at the level of dendritic targeting, but not at the level of expression. In contrast, Arc mRNA is regulated by neuronal activity both at the level of expression and dendritic delivery (Link et al., 1995; Lyford et al., 1995; Wallace et al., 1998) although it remains to be determined to which degree the latter may be consequential to the former. Not all dendritic mRNAs have been examined with respect to activity-dependent regulation, and some may not be subject to such regulation at all. Of those that are, it appears that the majority is regulated at the level of dendritic targeting.

BC1 RNA is thus unique in that it is a dendritic RNA polymerase III transcript that is regulated by neuronal activity. Such regulation may operate mainly at the level of gene expression, although additional regulation at the local dendritic level cannot be ruled out. Activity-dependent regulation of BC1 RNA may be indicative of a role of the RNA (or the BC1 RNP) at a functional interface between mRNAs and protein synthetic machinery in dendrites. In such an adapter role, BC1 RNA would interact with a subset of dendritic mRNAs and with the dendritic translational apparatus, an interaction that would be subject to activity-dependent regulation through BC1 RNA. In support of this notion is previous work (Chicurel et al., 1993; Rao and Steward, 1993) with synaptosome preparations of dendritic spines (also called synaptodendrosomes). In such preparations, BC1 RNA was identified colocalized with a subset of dendritic mRNAs, with BC1 RNA being detected as one of the most abundant RNA species. Since ribosomes have also been identified in dendritic spines (Steward and Reeves, 1988), the combined evidence indicates an interaction of BC1 RNA with synapse-associated mRNAs and ribosomes in postsynaptic microdomains. As has been suggested previously (Tiedge et al., 1991; Chicurel et al., 1993), BC1 RNA may play a role in regulating dendritic translation, including pre- or cotranslational processes such as RNA transport or docking. Activity-dependent regulation of BC1 expression would thus offer a mechanism to control availability and/or translatability of dendritic mRNAs at synaptic sites. This conjecture is presented here as a testable hypothesis that we suggest should be subject to future experimental scrutiny.

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