In neurons, activity-dependent association of dendritically transported mRNA transcripts with the transacting factor CBF-A is mediated by A2RE/RTS elements

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Running title: mRNA trafficking in neurons
Keywords: hnRNPs, RNP assembly, mRNA transport and localization, neurons, postsynaptic stimulation

ABSTRACT

In neurons certain mRNA transcripts are transported to synapses through mechanisms that are not fully understood. Here we report that the hnRNP CBF-A (CARG Box binding Factor A) facilitates dendritic transport and localization of Arc, BDNF and CaMKIIα mRNAs. In the adult mouse brain we discovered that CBF-A has a broad distribution. In the nucleus CBF-A

Supplemental Material can be found at: http://www.molbiolcell.org/content/suppl/2011/04/04/mbc.E10-11-0904.DC1.html
was found at active transcription sites, interchromosomal spaces and close to nuclear pores. In the cytoplasm CBF-A localized to dendrites as well as pre and post-synaptic sites. CBF-A was found in synaptosomal fractions, associated with Arc, BDNF and CaMKIIα mRNAs. Electrophoretic mobility shift assays demonstrated a direct interaction mediated via their A2RE/RTS elements located in the 3’UTRs. In situ hybridization and microscopy on live hippocampal neurons showed that CBF-A is in dynamic granules containing Arc, BDNF and CaMKIIα mRNAs. NMDA and AMPA postsynaptic receptor stimulation led to CBF-A accumulation in dendrites, increased Arc, BDNF and CaMKIIα mRNA levels and increased amounts of transcripts co-precipitating with CBF-A. Finally, CBF-A gene knockdown led to decreased mRNA levels. We propose that CBF-A cotranscriptionally binds RTSs in Arc, BDNF and CaMKIIα mRNAs and follows the transcripts from genes to dendrites, promoting activity-dependent nuclear sorting of transport-competent mRNAs.

INTRODUCTION

Transport and localization of mRNA transcripts to subcellular compartments occurs in many cell types and provides temporal and spatial control of gene expression. These mechanisms are dependent on the interplay between cis-acting elements located in the 3’ or 5’ untranslated regions (UTRs) of the transported mRNAs with a set of transacting factors (Rodrigues et al., 2008; Percipalle et al., 2009; Martin and Ephrussi, 2009). In fibroblasts and neurons, transport of the beta-actin mRNA to leading edge and synapses is mediated by the
cis-acting element zipcode which is recognized by ZBP1 (Eom et al., 2003; Tiruchinapalli et al., 2003; Condeelis and Singer, 2005; Huttelmayer et al., 2005). In oligodendrocytes, the myelin basic protein (MBP) mRNA is transported to the myelin compartment (Carson et al., 2001; Carson and Barbarese, 2005; Percipalle et al., 2009). In its 3'UTR, the MBP mRNA has a specific cis-acting element termed A2RE (hnRNP A2 response element) or more generally RTS (RNA trafficking sequence). This sequence is specifically recognized by the heterogeneous nuclear ribonucleoproteins hnRNP A2 and CArG Box binding factor A (CBF-A) (Ainger et al., 1993; Hoek et al., 1998; Raju et al., 2008). In mammals CBF-A is also known as hnRNP A/B. Similarly to hnRNP A2, CBF-A belongs to the A/B-type hnRNP subfamily and it is closely related to hnRNP A1 and hnRNP D, being characterized by two conserved RNA binding motifs and a C-terminal auxiliary domain involved in protein-protein interactions (Dreyfuss et al., 2002). Sequence analysis shows that CBF-A and hnRNP A2 exhibit 40 % sequence identity. Consistent with a role as transacting factor, we discovered that CBF-A is part of the same RNP complex with hnRNP A2 and in mouse oligodendrocytes, CBF-A gene silencing led to impairment of MBP mRNA trafficking. Based on these findings we proposed that A2RE/RTS recognition by CBF-A is important to sort transport-competent MBP mRNA to the final cytoplasmic destination (Percipalle et al., 2002; Raju et al., 2008; Percipalle et al., 2009).

In neurons certain mRNA transcripts are transported to dendrites for localized translation. (Bramham and Wells, 2007). In many cases dendritic localization is dependent on the UTRs
(Lisman et al., 2002; Bramham and Wells, 2007; Jambhekar and DeRisi, 2007; Andreassi and Riccio, 2009; Martin and Ephrussi, 2009). However even though these mechanisms are compatible with the establishment of excitatory synapses, it remains largely unclear how specific mRNAs are transported and localized to dendrites. RTS and RTS-like sequences were identified in a number of neuronal transcripts (Ainger et al., 1997), including Arc (activity-regulated cytoskeleton-associated protein) and CaMKIIα (Calmodulin-dependent protein kinase II) mRNAs which are believed to be dendritically transported via the A2RE/RTS pathway (An et al., 2008; Gao et al., 2008). CBF-A (or hnRNP A/B) is among the hnRNPs that are found in the RNA granules isolated from developing and adult mouse brains (Kanai et al., 2004; Elvira et al., 2006). Furthermore CBF-A is a shuttling hnRNP component of pre-mRNP/mRNP particles and in neuron lineages we discovered that CBF-A positive granules decorate dendrites (Pericipalle et al., 2002; Raju et al., 2008). However at this stage it is not known whether CBF-A associates with the putative RTS-like sequences in the UTRs of dendritically transported transcripts and whether CBF-A has a role in trafficking of neuronal mRNAs (Bramham and Wells, 2007; Chua et al., 2010).

Here we report that CBF-A directly binds the RTS sequences found in Arc, CaMKIIα and BDNF (brain-derived neurotrophic factor) mRNAs and accompanies the transcripts from gene to dendrites. Since in hippocampal neurons association is sensitive to postsynaptic receptor activation we propose that CBF-A has an important role in trafficking of RTS-containing neuronal mRNAs.
RESULTS

In vivo localization of CBF-A in the adult forebrain

CBF-A is known to be present in two different splice variants termed p37 and p42 (Dean et al., 2002). Using the polyclonal anti-CBF-A antibody ICCI directed against the unique C-terminal epitope within the p42 variant we performed immunofluorescence staining of sections of adult mouse forebrain. Confocal microscopy confirmed that CBF-A is abundantly expressed in the mouse brain (Raju et al., 2008) and it is expressed in neurons (Fig 1). In double immune labelling experiments we discovered that CBF-A has distinctive nuclear localization and its distribution correlates with the neuronal nuclear marker NeuN (Fig 1B). A fraction of CBF-A was also found outside the cell nucleus in small clusters or discrete particles, reminiscent of dendritically transported mRNA granules, with different sizes and signal intensities depending on the brain regions analyzed (Fig 1). These clusters seem to be associated with microtubulin fibres of dendrites as revealed by co-staining with the dendrite marker MAP2 (Fig 1D). Furthermore co-immunostaining with the anti-CBF-A antibody ICCI and a mouse monoclonal antibody against the presynaptic marker synapsin 1 revealed that CBF-A is located close to synapses (Fig. 1E-F). Distinctive nuclear staining as well as CBF-A positive granules along dendrites were also revealed by double immunofluorescence staining performed on rat hippocampal neurons using ICCI and the MAP2 antibody (Fig 2A), as well as antibodies to synapsin 1 and PSD95 (Suppl fig 1). These experiments were further validated with the anti-CBF-A antibody SAK22 raised against the CBF-A N-terminus (Dean et al., 2002) and known to react with both p37 and p42 splice variants (Suppl fig 2). Interestingly in double immunostainings of hippocampal neurons the signals obtained from
ICCI and SAK22 displayed a linear correlation and more than 80% overlap (Fig 2B, 2C), supporting their specificity.

To further characterize the in vivo localization of CBF-A we next carried out immuno-electron microscopy (IEM) experiments on thin sections of adult mouse brain. Sections of hippocampus incubated with the anti-CBF-A antibody SAK22 revealed that CBF-A has a widespread distribution, in agreement with the light microscopy results reported above (Fig 1). Consistent with previous data on the subcellular distribution of CBF-A in oligodendrocytes (Raju et al., 2008), the anti-CBF-A antibodies revealed high density of labelling in the nucleus of different types of brain cells, including neurons (see also Fig 1, 2 as well as Suppl fig 1, 2). The CBF-A antibodies decorated electrodense structures located in the interchromosomal space (ICS in Fig 3) and in the perichromatin area (arrowheads in Fig 2C and D) where active transcription takes place (Fakan and Puvion 1980). CBF-A was instead excluded from the patches of dense chromatin (DC in Fig 3). The location and morphology of the CBF-A-positive structures suggests that CBF-A is associated with (pre)-mRNP complexes at the sites of transcription and in the interchromosomal space. CBF-A was also found associated with electrodense structures, presumably mRNPs, at the nuclear pores and in transit to the cytoplasm (arrows in Fig 3B and 3D).

In the cytoplasm very significant immunolabeling was observed in myelinized axons (Fig 4 A-B). The SAK22 antibody also stained synapses, where immuno-gold markers were found on both pre-synaptic and post-synaptic compartments (Fig 4 E-H). The labeling density in synapses was relatively low compared to that observed in axons but was highly significant as judged by the absence of immuno-gold in sections processed in parallel with only secondary antibody (Fig 4D). We could also detect CBF-A in neuronal cytosol and over non-myelinized
processes. The labeling in axons and synapses was confirmed using the rabbit polyclonal anti-CBF-A antibody ICCI (Suppl fig 3).

In synaptosomal fractions CBF-A associates with RTS-containing mRNA transcripts

To confirm the subcellular localization of CBF-A in neurons, we fractionated brain lysates by ultracentrifugation on sucrose cushions (Fig 5A). The fractionated material was analyzed on immunoblots with antibodies against CBF-A as well as antibodies against the post-synaptic marker PSD95 (post synaptic density-95) and the nuclear marker histone H3. Consistent with the histological data and previous observations (Raju et al., 2008), CBF-A was found in nucleus and cytosol as well as in synaptosome-enriched fractions (Fig 5B).

We next applied RNA immunoprecipitation (RIP) assays to examine whether in synaptosome-enriched P2 fractions CBF-A associates with synaptic mRNAs. Synaptosomal preparations were incubated with the CBF-A antibody ICCI. Total RNA was isolated from immunoprecipitated samples of mouse brain P2 fraction (see Fig 5C) using the TRI reagent and reverse-transcribed with oligodT primers. The resulting cDNA was analyzed by semiquantitative RT-PCR using specific primers amplifying BDNF, CaMKIIα, and Arc mRNAs. As can be seen, we revealed a specific enrichment in the levels of Arc, BDNF and CaMKIIα mRNAs co-precipitated with CBF-A (Fig 5D). Densitometric quantification revealed a considerable 3-10 fold increase in the levels of BDNF, CaMKIIα and ARC mRNA co-precipitated with CBF-A in comparison to α-tubulin mRNA and control immunoprecipitations carried out with non-specific IgGs (Fig 5D, 5E). We conclude that in synaptosomal preparations CBF-A associates with Arc, BDNF and CaMKIIα mRNAs.
The 3’ UTRs of CaMKIIα and Arc mRNAs contain RTS-like sequences that are closely related to the MBP mRNA RTS (Gao et al., 2008) and we identified a similar RTS-like sequence in the 3’ UTR of BDNF mRNA (Fig 6A). To evaluate whether CBF-A bound any of these sequences, we synthesized biotinylated RNA oligonucleotides encompassing the wild-type RTS-like sequences present in Arc, BDNF, CaMKIIα mRNAs and the MBP mRNA RTS. As control we used an RNA oligonucleotide encompassing a scrambled version (scrRTS) with identical nucleotide composition as the MBP mRNA RTS but different primary sequence (see Raju et al., 2008). These RNA oligonucleotides were coupled to streptavidin-coated Sepharose beads and the beads were incubated with total mouse brain lysates. The blots in figure 6B show that wtRTS sequences precipitated endogenous CBF-A (Fig 6B cf lanes 4 to 7). Control scrRTS or mock beads which were not conjugated with any RNA oligonucleotides did not precipitate CBF-A (Fig 6B cf. lanes 2,3). As expected, similar results were obtained with endogenous hnRNP A2. On the contrary, hnRNP U which is also found in RNA granules isolated from developing and adult mouse brains and the mitochondrial protein Tom20 were not co-precipitated with any of the beads used in the RNA affinity chromatography assays (Fig 6B), altogether supporting the specificity of the assay.

We next applied electrophoretic mobility shift assays (EMSA) to determine whether CBF-A directly binds the RTS motifs. We incubated purified recombinantly expressed GST-tagged CBF-A (p37 isoform) where the affinity tag had been proteolytically removed with 33P-labeled RNA oligonucleotides encompassing Arc, CaMKIIα and BDNF wtRTSs. In parallel CBF-A was also incubated with control RNA oligonucleotides encompassing scrambled versions (scrRTS) of the wt RTS sequences. Figure 6 shows that incubation with CBF-A retarded more efficiently the electrophoretic mobility of wtRTS-containing oligonucleotides in comparison to the scrambled sequences (Fig 6C-E). In addition, gel
retardation of wtRTS-containing RNA oligonucleotides incubated with CBF-A was competed in the presence of corresponding unlabeled wtRTS oligonucleotides but not in the presence of unlabeled scrRTS sequences when added in large excess to the reaction mixture (Fig 6C-E). Finally, incubation of CBF-A with labeled scrRTS oligonucleotides was sensitive to competition with unlabeled wtRTS oligonucleotides but was not sensitive to competition with unlabeled scrRTS oligonucleotides (Fig 6C-E).

Altogether the above results indicate that CBF-A specifically associates with Arc, BDNF and CaMKIIα mRNAs through direct interactions with their RTS sequences.

In dendrites CBF-A localizes to dynamic mRNA granules

Immunofluorescence staining performed on hippocampal neurons demonstrated that CBF-A is present in dendrites (Fig 2A). If CBF-A associates with dendritic mRNAs via their RTS elements it is likely that CBF-A is found in transported granules. To evaluate the possibility hippocampal neurons were analyzed by immunofluorescence in situ hybridization (immuno-FISH) with the anti-CBF-A antibody ICCI and specific digoxigenin-labelled RNA probes hybridizing with endogenous Arc, BDNF and CaMKIIα mRNAs. Confocal microscopy revealed that CBF-A positive granules distributed along dendrites and their distributions correlated with the granular structures identified by the FISH signals using antisense probes specific for Arc, BDNF and CaMKIIα mRNA (Fig 7). Notably, no FISH signal was detected with sense RNA probes against the corresponding above transcripts, supporting the specificity of the assay (Suppl fig 4). To evaluate colocalizations of endogenous CBF-A with Arc, BDNF and CaMKIIα mRNAs, we performed unbiased statistical analysis on the fluorescence intensity levels from individual granules derived from
the corresponding confocal images, as previously described (Ma et al., 2002; Raju et al., 2008). In all cases the results revealed linear correlations between the signals from CBF-A and the RNA transcripts analyzed (Fig 7D-L). Quantification of the number of colocalization events occurring in individual particles showed that 60% to 70% of the granules simultaneously contain CBF-A and the RTS-containing Arc, BDNF and CaMKIIα mRNA. These observations suggest that CBF-A is likely to be incorporated in endogenous mRNA granules containing Arc, BDNF and CaMKIIα transcripts.

To evaluate whether in dendrites CBF-A-positive granules are dynamically transported, we transiently expressed EGFP-tagged full length CBF-A (p37 isoform) in rat hippocampal neurons. Confocal microscopy showed that the EGFP-CBF-A construct displayed both nuclear distribution and granular clusters in dendrites, overall matching the distribution observed in the case of the endogenous protein (Fig 8A). We next performed time lapse live cell microscopy on hippocampal neurons transiently expressing EGFP-CBF-A. As can be seen in figure 8B we found that a major fraction of EGFP CBF-A positive granules were immobile or stationary. A fraction of EGFP CBF-A containing granules exhibited an oscillatory movement. Finally a subset of EGFP CBF-A positive granules displayed a single direction motion, anterograde or retrograde, with a calculated speed of 0.028-0.13 μm/sec (Fig 8, B-D), similar to that measured for Staufen 1-, DDX3- and CaMKIIα mRNA-containing granules (Kohrmann et al. 1999; Elvira et al, 2006; Rook et al, 2000). Interestingly we found that in dendrites of hippocampal neurons EGFP CBF-A positive granules correlated with the FISH signal from digoxigenin-labelled RNA probes hybridizing with CaMKIIα mRNAs (Fig 8E) as well as Arc and BDNF mRNAs (not shown). Therefore taken altogether these observations suggest that in hippocampal neurons a fraction of CBF-A
is incorporated in dynamically transported granules which are likely to contain dendritic mRNAs.

**Postsynaptic receptor activation leads to increased levels of CBF-A in dendrites**

Treatment of hippocampal neurons with the selective agonists NMDA (N-methyl-D-aspartic acid) and AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) mimics the effects of the neurotransmitter glutamate, leads to synaptic activation and upregulates dendritic mRNA transcripts eventually leading to synaptic consolidation (Bramham and Wells, 2007; Tian et al., 2007). CBF-A is present in postsynaptic densities (Fig 4). Therefore since CBF-A binds to Arc, BDNF and CaMKIIα mRNAs, upon NMDA and AMPA treatment we expect to find increased CBF-A protein levels in dendrites. To test this possibility we treated hippocampal neurons with the agonists NMDA or AMPA and the corresponding antagonists APV [(2R)-amino-5-phosphonovaleric acid)] and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) as negative controls. After 4h treatment we monitored the distributions of CBF-A and dendrite marker MAP2 by immunofluorescence and confocal microscopy. We found that both NMDA (Fig 9A) and AMPA (not shown) induced increased levels of CBF-A along dendrites (Fig 8A). On the contrary APV treatment (Fig 9A) or treatment with CNQX (not shown) did not induce any changes in the CBF-A distribution. To quantify the effects we randomly selected multiple dendritic regions from ten independent hippocampal neurons untreated or treated with antagonists or agonists and in all cases we measured CBF-A signals intensities. We discovered that NMDA and AMPA induced approximately a 2 fold increase in the levels of CBF-A in dendrites in comparison to untreated cells (Fig 9B-C). Only marginal effects were
detected upon treatment with the respective agonists (Fig 9B, 9C). We conclude that in dendrites trafficking and localization of endogenous CBF-A is sensitive to post-synaptic receptor stimulation.

**Association of CBF-A with dendritic mRNA transcripts is activity-dependent**

During mRNA biogenesis hnRNP proteins such as CBF-A are cotranscriptionally associated with nascent transcripts, facilitate RNP assembly and in many cases accompany the mRNA from gene to polysomes (Visa et al., 1996; Daneholt, 2001; Dreyfuss et al., 2002; Percipalle et al., 2002).

CBF-A is an abundant nuclear protein, localizes to dendrites and its distribution is activity dependent. Since the distribution of Arc, BDNF and CaMKIIα mRNAs is sensitive to NMDA and AMPA treatment (Steward et al., 1998; Bramham and Wells, 2007), we reasoned that CBF-A association with transported mRNAs may be intrinsically sensitive to synaptic stimulation. To start proving this hypothesis we stimulated rat hippocampal neurons with NMDA. Next, total RNA was extracted and reverse transcribed with oligodT primers. The resulting cDNA was analyzed by quantitative real time PCR (qRT-PCR) with primers amplifying Arc, BDNF, CaMKIIα mRNAs. In at least three independent experiments we found a 10-20% significant increase in the steady state levels of Arc, BDNF and CaMKIIα mRNAs, in all cases normalized against the levels of GAPDH mRNA (Fig 9D). None of the transcripts were affected upon treatment with the antagonist APV (Fig 9D), altogether supporting the view that synaptic stimulation induces increased transcriptional rates of dendritically localized mRNAs (Bramham and Wells, 2007). We next tested whether NMDA stimulation correlates with increased binding of CBF-A to Arc, BDNF and CaMKIIα mRNAs.
For this purpose total lysates from NMDA- or APV-treated rat hippocampal neurons were subjected to immunoprecipitations with anti-CBF-A antibodies. Total RNA was extracted and the cDNA analyzed by qRT-PCR. We found that the amounts of Arc, BDNF and CaMKIIα mRNAs co-precipitated with CBF-A were significantly increased in comparison to APV-treated cells (Fig 9E) or untreated cells (not shown). As expected non-specific IgGs did not pull down any of the transcripts analyzed (not shown). Altogether these findings indicate that the specific binding of CBF-A to Arc, BDNF and CaMKIIα mRNAs correlates with post-synaptic receptor stimulation and support the view that CBF-A accompanies the transcripts from gene to dendrites.

To test for an involvement of CBF-A in transport of dendritic transcripts, we next silenced the CBF-A gene by RNA interference (RNAi). Hippocampal neurons were transfected with RNA duplexes against target sequences on the CBF-A gene. Steady-state expression of endogenous CBF-A was monitored by immunofluorescence on hippocampal neurons anti-CBF-A antibodies and an anti-MAP2 antibody (Fig 10A), whereas the CBF-A mRNA levels were monitored by qRT-PCR (Fig 10B). A specific shut down of the expression resulting in a drop in endogenous CBF-A steady-state level was observed approximately 3 d after transfection (Fig 10A-B). To test the effect of CBF-A silencing on the distribution of dendritic mRNA, we performed immuno-FISH on CBF-A–silenced hippocampal neurons and monitored the distribution of CaMKIIα mRNA. We found that CBF-A gene silencing led to a drop in the levels of CaMKIIα mRNA in dendrites (Fig 10C-D), whereas in control cells transfected with unrelated RNAi oligonucleotides, dendritic localization of CaMKIIα mRNA granules was not affected (Fig 10C-D). For quantification we randomly selected dendritic regions from non-transfected as well as control and CBF-A silenced hippocampal neurons and in all cases we measured CBF-A and FISH average signals intensities. Concomitantly with an
average 50% specific reduction in the steady state expression of CBF-A we observed a
twofold drop in the levels of CaMKIIα mRNA in dendrites in comparison to controls (Fig
10D). Similar results were obtained for Arc and BDNF mRNA dendritic localization upon
CBF-A gene silencing (not shown). We conclude that in neurons CBF-A gene knockdown
specifically affects mRNA expression levels of transported mRNAs.

DISCUSSION

In the present study we found that the transacting factor CBF-A is abundantly expressed
in neurons. In the cytosol, CBF-A is present in dendrites and synapses with a characteristic
granular distribution. In synaptosomal preparations from total mouse brain lysates, CBF-A
specifically associates with Arc, BDNF and CaMKIIα mRNAs. A close look at the 3’ UTRs
of the above transcripts shows the presence of RTS elements which are related to the MBP
mRNA RTS. Consistent with previous work (Raju et al., 2008), these RTS elements are
specifically and directly recognized by CBF-A. These observations and in situ evidence that
in dendrites a fraction of CBF-A co-localizes with all three transcripts suggests that CBF-A is
genuinely present in dendritic mRNA granules, presumably with hnRNP A2 (see also Raju et
al., 2008), where CBF-A binds Arc, BDNF and CaMKIIα mRNA through their RTS
elements.

The RTS-containing 3’ UTRs of BNDF and CaMKIIα mRNAs play an essential role for
localized translation. There is evidence that in mutated mice where the protein-coding region
of CaMKIIα is intact but lacks the 3’ UTR the mRNA is restricted to the soma. These animals
show reduction of CaMKIIα transcripts in postsynaptic densities, a reduction in late-phase
long-term potentiation (LTP) and impairments in spatial memory (Miller et al., 2002). For
BDNF, the brain produces two different transcripts, with either short or long 3' UTRs. In mutated mice with truncated long 3' UTR, dendritic targeting of BDNF mRNAs is impaired and these animals display selective impairment in long-term potentiation in dendrites of hippocampal neurons (An et al., 2008). Here we report that CBF-A positive mRNA granules are dynamic in nature and their localization in dendrites is activity-dependent. Similarly to Arc, BDNF and CaMKIIα mRNAs, treatment of hippocampal neurons with NMDA and AMPA which mimic the effect of the neurotransmitter glutamate resulted in CBF-A accumulation in dendrites. CBF-A is also present in the characteristic post-synaptic densities where translation of Arc, BDNF and CaMKIIα mRNAs is believed to take place (Bramham and Wells, 2007). Therefore it is conceivable that failure in correct localization and targeting of BDNF and CaMKIIα mRNAs upon truncation of their 3’ UTRs in mutated mice partly results from deletion of their RTS elements. Similarly to BDNF and CaMKIIα mRNAs we predict that Arc mRNA lacking the RTS would not be transported to dendrites. Based on these considerations we propose that the RTS sequences in the 3’ UTRs of Arc, BDNF and CaMKIIα mRNAs are bona fide cis-acting elements required for dendritic trafficking. Given that in dendrites of CBF-A-silenced hippocampal neurons we found a twofold drop in the levels of CaMKIIα mRNA, we suggest that CBF-A recognizes RTS elements and functions as transacting factor to facilitate mRNA transport and localization in dendrites. Since association of CBF-A with the RTS-containing mRNAs is sensitive to NMDA treatment it is likely that the interplay between CBF-A and RTS elements correlates with the function of excitatory synapses.

IEM experiments demonstrated that in the cell nucleus CBF-A localizes in the perichromatin region where active transcription is believed to take place (Rouquette et al., 2010), presumably coupled to nascent pre-mRNPs as well as mature mRNPs in
interchromatin space and passages through the nuclear pore complex. Since CBF-A is a shuttling hnRNP and core component of pre-mRNP/mRNPs (Percipalle et al., 2002), these observations suggest that CBF-A is assembled into mRNP complexes in the nucleus and exported from the nucleus to the cytoplasm as part of mature mRNPs. CBF-A is known to bind the MBP mRNA RTS already in the cell nucleus (Raju et al., 2008). Therefore in view of the present findings, it is likely that RTS recognition by CBF-A is important during pre-mRNP assembly. We propose that CBF-A recognizes the RTS elements present in Arc, BDNF and CaMKIIα mRNAs to facilitate co-transcriptional pre-mRNP assembly, a mechanism that promotes the establishment of transport-competent RNPs. We suggest that co-transcriptional binding of CBF-A to RTS elements provides de facto a way to sort transported Arc, BDNF and CaMKIIα mRNPs at a very early stage during RNP biogenesis.

At later stages when granules have reached their final destinations there is evidence that hnRNP A2 phosphorylation by Fyn kinase leads to unwinding of the MBP mRNA granule for localized translation (White et al., 2008), which indicates a structural role for hnRNP A2 in the maintenance of the RNP granule. Similar mechanisms may be envisaged in neurons for dendritic RTS-mediated mRNA targeting. In any case we propose that in the cell nucleus RTS recognition by CBF-A mediates RNP packaging of Arc, BDNF and CaMKIIα mRNAs through a mechanism that probably leads to nuclear remodeling of their 3’ UTR. This in turn contributes to keep dendritic mRNAs in a state which is transport-competent and presumably translationally dormant. We speculate that these mechanisms are required for establishment of excitatory synapses and may have a long-term effect on the plasticity of neuronal contacts.
MATERIALS AND METHODS

Antibodies. The mouse monoclonal antibodies against Map2, NeuN, synapsin I and PSD95 were respectively from Sigma, Chemicon, Cell Signalling and Abcam. The rabbit polyclonal anti-CBF-A antibody ICCI was raised against the peptide YQQGYGPGYGGYDY located in the C-terminus of the p42 CBF-A splice variant as described by Raju et al. (2008). The guinea pig anti-CBF-A antibody SAK22 was designed against the peptide EEQPMETTGATEN located in the N-terminus of both p37 and p42 CBF-A isoforms as previously described (Dean et al., 2002) and purchased from Peptide Specialty Laboratories (PSL) GmbH, Heidelberg (Germany). The monoclonal antibodies against histone H3, hnRNP U (3G6) and hnRNP A2 were from Abcam (Cambridge, UK) whereas the anti-TOM20 was purchased from Santacruz Biotechnology. Non specific mouse IgGs were from DAKO. For immunofluorescence experiments the primary antibodies were revealed with species-specific fluorophore-conjugated (Cy3, Cy5 from Jackson, Alexa 488 from Molecular Probes) secondary antibodies.

Immunohistochemistry on mouse forebrain. Adult C57bl6 mice (Charles River) were transcardially perfused with PBS followed by 4% formaldehyde in PBS, and brains were dissected and post-fixed overnight at 4°C. Coronal sections were made at 35µm using a vibratrom (Leica, Germany). Staining was performed on free-floating sections. Sections were incubated with blocking solution (10% donkey serum in PBS with 0.2% Triton-X100) for 1h
at RT and then incubated at 4°C for 14h with primary antibody diluted in blocking solution. Primary antibodies against CBF-A (ICCI), Map2, NeuN, Synapsin I and PSD95 were used on the sections. Primary antibodies were revealed with Cy3, Cy5 or Alexa 488-conjugated secondary antibodies. Control sections were stained with secondary antibodies alone.

**Primary neuronal cultures and cell stimulation.** Hippocampal neurons were prepared as described in Ledda et al. (2007). Briefly hippocampi were dissected from E18-20 rat embryos and hippocampal neurons were cultured in neurobasal medium (Invitrogen) supplemented with B-27 (Gibco) on Poly-D-lysine coated cover slips or dishes. Neurons were fed once weekly by replacing half of the medium. Neuronal cell stimulation was carried out as described earlier (Tian et al., 2007). Where indicated, 18 DIV hippocampal neurons were incubated with the 20 μM NMDA (Sigma) or 20 μM APV (Sigma) or, alternatively, with 20 μM AMPA or 20 μM CNQX. In all cases treatment was performed for 4h. Cells were subsequently fixed with the 3.7% formaldehyde in PBS and further processed for immunofluorescence.

**Immunofluorescence microscopy and immuno-FISH.** In situ hybridization and immunostaining was performed as described by Raju et al. (2008). Digoxigenin-labeled RNA probes were synthesized using DIG RNA labeling mix (Roche). PCR products obtained with primer pairs amplifying Arc mRNA (forward, AGC AGC AGA CCT GAC ATC CT; reverse,
GGC TTG TCT TCA CCT TCA GC), BDNF mRNA (forward, TGG CCT AAC AGT GTT TGC AG; reverse, GGA TTT GAG TGT GGT TCT CC), CaMKIIα mRNA (forward, GAC ACC AAA GTG CGC AAA CAGG; reverse, GCG AAG CAA GGA CGC AGG) were cloned into the p-GEM-T vector (Promega) and used as a template for in vitro transcription by T7 RNA polymerase. Briefly hippocampal neurons grown on poly-D-lysine coated coverslips were fixed with 3.7% formaldehyde and hybridized overnight at 42ºC. After hybridization the cells were extensively washed three times for 30 min using 2x SSC buffer supplemented with 50% formamide. The cells were briefly fixed again and immunostaining was performed with the rabbit polyclonal antibody against CBF-A (ICCI). Detection of the hybridized probe and endogenous CBF-A was performed with anti-DIG rhodamine (Roche) and donkey anti-rabbit Alexa488-conjugated secondary antibody (Invitrogen). Where indicated the same probes were used in FISH experiments performed on rat hippocampal neurons expressing EGFP CBF-A (see below) and in hippocampal neurons subjected to CBF-A gene silencing (see below).

**Trasfection and live cell microscopy on cultured hippocampal neurons.** Rat hippocampal neurons were grown on Poly D-lysine coated glass coverslips in 60 mm dishes (corning) and transfected at DIV 9-10 with an EGFP- CBFA expression plasmid (gift from Tomas Leanderson, Lund University, Sweden) using Lipofectamine 2000 as described in the manufacturer’s handbook (Invitrogen). The following day, EGFP-CBFA expressing neurons were imaged with 40x objective using Zeiss LSM510 META laser scanning confocal
microscope equipped with heated chamber and humidity control unit at 37°C and 5% CO₂. Images were acquired every 40s and the data were analyzed using the Image J and the Volocity image analysis software.

**Immuno electron microscopy (IEM) on sections of adult mouse brain.** Adult mouse brains were fixed following Godsave et al. (2008) with 2% paraformaldehyde (PFA) and 0.2% glutaraldehyde in 0.1M PHEM buffer (25 mM HEPES, 10 mM EGTA, 60 mM PIPES, 2 mM MgCl₂, pH 7.2) during 20 h after intracardiac perfusion. Small brain pieces were post-fixed during 7 days in 1% PFA and stored in 0.5% PFA in the same buffer, washed with 50 mM glycine in PHEM buffer and embedded gradually in gelatin (2, 5 and 12% in PHEM) prior to infiltration overnight in 2.3 M sucrose. Brain pieces were frozen in liquid nitrogen and sectioned at -120°C in a cryoultramicrotome Leica EM UC6/FC6 (Leica, Vienna). The sections were retrieved in Formvar-coated gold EM grids of 200 mesh employing a mixture 1:1 of 2.3 M sucrose and 2% methyl cellulose. For immunogold labeling, the grids were sequentially incubated at 37°C in 2% gelatin for 20 min, in 50 mM glycine in PHEM, in 5% BSA in PHEM and finally in 2% BSA in PHEM. Incubation with the anti-CBF-A antibody SAK22 (1/7) and protein A gold conjugated (1/100) were also done in 2% BSA in PHEM during 1 hour and 30 min, respectively. Washes were then done in PHEM. Grids were finally post-fixed with 1% glutaraldehyde in PHEM, washed in distilled water and stained with 2% methyl cellulose – saturated uranyl acetate (9:1) for 10 min. The observations and images
were made in a Tecnai Spirit 120 kv electron microscope (FEI Company, Eindhoven, Netherlands).

**Isolation of synaptosome-enriched fraction.** Brain fractions were prepared as previously described (Huttner et al., 1983; Zhou et al., 2007). Briefly, 3 adult mice were sacrificed by cervical dislocation, and the forebrains were dissected out on ice. All of the following steps were performed at 4°C. The tissues were quickly minced and homogenized in 10X volumes of 0.32 M sucrose buffer (0.32M sucrose in 10 mM HEPES pH.7.4) supplemented with protease inhibitors (Complete, Roche) with 9 up-and-down strokes of glass homogenizer. The homogenate was centrifuged at 1000 x g for 10 min and supernatant (S1) was centrifuged again at 17,500 xg for 30 min. The pellet (P2) was dissolved into lysis buffer (0.4% NP-40, protease inhibitors in 1X PBS) and used for further experiments. For the characterization of each fractions, the same amount of proteins (20 μg/lane) was resolved by SDS-PAGE, detected by Coomassie staining and analyzed on immunoblots with antibodies against CBF-A (ICCI), PSD-95 and H3.

**Protein and RNA immunoprecipitation (RIP).** Mouse brain P2 fractions were incubated overnight at 4°C with anti-CBF-A antibody (ICCI), no antibody (mock) or nonspecific mouse IgGs (DAKO). Where indicated, protein extracts were treated with the RNAse inhibitor 100μg/ml RNaseout (Fermentas) for 15 min before incubation with antibodies. In all cases the
reaction mixes were incubated for 1 hr with Protein G-Sepharose 4B conjugate (Zymed, Invitrogen). The precipitated samples were resolved by SDS-PAGE and analyzed on immunoblot with antibodies against CBF-A. For analysis of the RNA species coprecipitated with the CBF-A antibodies, the immunoprecipitated RNA was extracted using the TRI reagent (Sigma), and reverse-transcribed using Superscript II and oligo (dT) primers as described in the manufacturer’s protocols (Invitrogen). An equal volume of RNA incubated without Superscript II was used as negative control (RT-). The samples were then analyzed by semi-quantitative PCR with primers specific to BDNF mRNA with long 3’ UTR (BDNF forward, TGG CCT AAC AGT GTT TGC AG; BDNF reverse, GGA TTT GAG TGT GGT TCT CC), CaMKIIα mRNA (CaMKIIα forward, GAC ACC AAA GTG CGC AAA CAGG; CaMKIIα reverse, GCG AAG CAA GGA CGC AGG), Arc mRNA (Arc forward, AGC AGC AGA CCT GAC ATC CT; Arc reverse, GGC TTG TCT TCA CCT TCA GC) and α-tubulin mRNA (forward, TTC GTA GAC CTG GAA CCC AC; reverse, TGG AAT TGT AGG GCT CAA CC). Quantification of PCR products was performed over 3 independent experiments using the ImageJ software.

RNA-protein interaction assays. For RNA affinity chromatography, the experiments were performed essentially as described (Hoek et al., 1998; Raju et al., 2008). The following RTS-containing RNA oligonucleotides (Thermofisher scientific, Germany) were used in the binding assays: Arc wtRTS, GCU GAG GAG GAG GAG AUC AUU; CaMKIIα, wtRTS,
AAC GCC AGU GAG CCA GGA ACU and BDNF wtRTS, AUG GAC CCA AUG AGA ACU AGU as well as the previously described MBP wtRTS and scrambled MBP scrRTS sequences (Raju et al., 2008). In all cases RNA oligonucleotides were coupled to streptavidin coated sepharose (GE Healthcare) and incubated with total mouse brain lysates. Bound proteins were resolved by SDS-PAGE and analysed on immunoblots with antibodies to CBF-A, hnRNP A2 or TOM20. Electrophoretic mobility shift assays (EMSA) were performed as described by Raju et al. (2008). Briefly, Arc wtRTS (see above for the sequence), CaMKIIα wtRTS (see above for the sequence), wtRTS BDNF (see above for the sequence) RNA oligonucleotides encompassing the wild type RTS sequences and corresponding Arc scrRTS (GGCUGAGAAUGCGGAAUAGUG), CaMKIIα scrRTS (GGCAAGGUAGCCCUAAACA) and BDNF scrRTS (GUGAACCAGGGCACUAAAUAU) RNA oligonucleotides encompassing scrambled RTS sequences were 5’end-labeled using γ-33P-ATP (GE Healthcare) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Purified recombinant CBF-A was incubated with 50 fmol of 33P-labeled wtRTS or scrRTS RNA oligonucleotides in EMSA buffer (20 mM HEPES, pH 7.6, 5 mM MgCl2, 40 mM KCl, 1 mM DTT, 5% glycerol) containing heparin (5 μg/μl) and BSA (100 μg/ml) for 30 min at room temperature. Protein–RNA complexes were resolved by native gel electrophoresis and analyzed with a Fuji-BAS 2000 phosphorimager (Tokyo, Japan).
CBF-A gene silencing. 2- or 5-DIV rat hippocampal neurons were transfected with 50 nM predesigned siGENOME smart pool (Dharmacon) against the hnRNP CBFA or control siRNA against EGFP (for the sequences see Raju et. al, 2008) using the Lipofectamine RNAiMAX reagent according to the manufacturer’s protocols (Invitrogen). Transfection was allowed for 72-96 h. Silencing was monitored by immunofluorescence staining with anti-CBF-A antibodies and confocal microscopy as mentioned above. CBF-A mRNA levels were monitored by qRT PCR, forward primer 5' CCG AACACTGGTGCAGATCA AGAG 3', reverse primer 5' ACACGACCACCAGTGCTGAGTCTGTGCT 3' (see also below).

Quantitative real time PCR. For analysis of steady state mRNA levels, total RNA was extracted from hippocampal neurons treated with NMDA, AMPA, APV or CNQX and from hippocampal neurons subjected to CBF-A gene silencing (see above). In all cases total RNA was reverse-transcribed and the cDNA analyzed by qRT-PCR on an ABI 7500 (Applied Biosystems) as recently described (Sahlgren et al., 2008). Individual amplification reactions were carried out using the Fast SYBR® Green Master Mix (Applied Biosystems) and specific primers against Arc mRNA (forward primer, CCCTGCAGCCCAAGTTCAAG; reverse primer, GAAGGCTCAGCTGCTGCTC), BDNF mRNA (forward primer, AGCTGAGCGTGTGACAGT; reverse primer, ACCCATGGGATTACACTTGG), CaMKIIα mRNA (forward primer, CCATCCTCACCACATGCTGCT; reverse primer, ATCGATGAAAGTGCCAGGCCC) and GAPDH mRNA (forward primer,
GCATCCTGCACCACCAACTG; reverse primer, ACGCCACAGCTTTCCAGAGG). In all cases GAPDH is used as internal control and all the values are normalized accordingly over five independent experiments.

ACKNOWLEDGEMENTS

We thank Carlos Ibanez for comments and Malte Wittman, Maurice Perrinjaquet, Annalisa Vicario and Debashish Das for help with rat embryos and qRT-PCR. We also thank Gema Martínez and Nieves Hernández for EM cryo-immunogold. This work was supported by grants from the Swedish Research Council (Vetenskapsrådet) and the Swedish Cancer Society (Cancerfonden) to PP and NV. NF is supported by a postdoctoral grant from the Wenner-Gren Foundations, Sweden.
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FIGURE LEGENDS

Figure 1. In vivo localization of CBF-A in the adult mouse brain. (A-F) Coronal sections.

(A-B) CBF-A is localized in neuronal nuclei and in small clusters in their proximity. (C-D) CBF-A clusters are associated with MAP2 positive dendrites in a synaptic pattern. (E-F) CBF-A clusters match the distribution of the pre-synaptic marker synapsin I but are not overlapping, suggesting preferential post-synaptic localization of CBF-A. The rectangle is shown in F. Scale bars: A, B=50µm, C, D=20µm, E=10µm, F=2.5µm.
Figure 2. Dendritic localization of CBF-A in rat hippocampal neurons. (A) Consistent with the in vivo distribution, CBF-A is found in dendrites of hippocampal neurons, as revealed by
co-immunostaining with anti-CBF-A (ICCI) and MAP2 antibodies. Scale bar, 10 μM.

Magnifications of boxed areas are approximately five fold in comparison to the corresponding overviews. (B) Double immunofluorescence staining with the anti-CBF-A antibodies ICCI and SAK22 reveals considerable overlap in nucleus and dendrites of rat hippocampal neurons. Scale bar, 20 μM. (C) Quantification of individual dendritic granules shows a linear correlation between the signals obtained with ICCI and SAK22 antibodies against CBF-A. More than 80% of the individual granules are positively labeled with both CBF-A antibodies.
**Figure 3.** Immuno-electron microscopy localization of CBF-A in the cell nucleus. Thin sections of adult mouse brain were immunostained with the anti-CBF-A antibody SAK22. (A) Overview of a nucleus from a pericyte found wrapped about precapillary arterioles showing the typical appearance of dense chromatin regions (DC) and interchromatin space (ICS). The arrows point at two distinct nuclear pores. CYT, cytoplasm. (B) The same nuclear pores as in (A) at higher magnification. Note the presence of anti-CBF-A labeling in the central regions of the pores (*arrows*). (C-D) Examples of nuclear labeling in hypoccampal neurons. The anti-CBF-A labeling is located in the interchromatin space (ICS) and in the perichromatin regions (*arrowheads*) but is excluded from the dense chromatin (DC). Note the labeling associated with a nuclear pore (*arrow in D*). The magnification bars represent 200 nm in A, B and D, and 100 nm in C.
Figure 4. Immuno-electron microscopy labeling of CBF-A in axons and synapses. Thin sections of adult mouse brain were immunostained with the anti-CBF-A antibody SAK22.
The antibody binding sites were detected with protein-A conjugated to colloidal gold particles. (A) and (B) show two examples of immunolabeling in myelinated hippocampal axons. The magnification bars represent 200 nm. \textit{ax}: axon; \textit{my}: myelin sheath. A schematic representation of a synapse is provided in (C) and shows the vesicular appearance of the pre-synaptic compartment (~\textit{pre}) and the characteristic density of the post-synaptic button (~\textit{post}). Panels (E-H) show examples of anti-CBF-A labeling in synapses in the hippocampus. CBF-A was revealed in both pre-synaptic and post-synaptic compartments. (D) A synapse taken from a negative control sample processed in parallel and incubated with only secondary antibody. The magnification bar in D-H represents 100 nm.
Figure 5. In synaptosome enriched fractions, CBF-A specifically interacts with dendritically transported Arc, BDNF and CaMKII mRNAs. (A) Preparation of synaptosome enriched fractions. (B) P2 fractions were resolved by SDS PAGE, stained with Coomassie and analyzed on immunoblots with CBF-A (ICCI), PSD95 and histone H3 antibodies. (C) CBF-A can be specifically coprecipitated from synaptosomal preparations. P2 fractions were subjected to immunoprecipitation with the CBF-A (ICCI) antibody or non-specific IgGs. Bound proteins were resolved by SDS-PAGE and CBF-A revealed on immunoblots with the ICCI antibody.
Mock, protein A Sepharose incubated with P2 fraction in the absence of antibody. (D-E) In synaptosomal preparations CBF-A is associated with BDNF, CaMKIIα and Arc mRNAs. Total mRNA precipitated with anti-CBF-A (ICCI) antibody or non-specific IgGs from synaptosomal preparations was isolated. The cDNA was analyzed with primers amplifying BDNF, CaMKIIα, BDNF and α-tubulin mRNAs. Quantification of precipitated mRNA species was by densitometric analysis in at least three successful experiments.
Figure 6. CBF-A directly binds to Arc, BDNF and CaMKIIα mRNA RTSs. (A) Sequence analysis of 3’ UTRs shows considerable homologies between MBP mRNA RTS and RTS-like
sequences in BDNF, CaMKIIα and ARC mRNAs. (B) CBF-A binds the RTS sequences found in BDNF, CaMKIIα and ARC mRNAs. Biotinylated wtRTSs from BDNF, CaMKIIα, and MBP mRNA as well as a scrambled version of the MBP RTS (scrRTS) were conjugated to streptavidin Sepharose. Beads were incubated with mouse brain lysates. Bound proteins were resolved by SDS-PAGE and analyzed on immunoblots with antibodies to CBF-A (SAK22), hnRNP A2B1 and with antibodies against hnRNP U and the mitochondrial protein TOM20 used as negative control. (C-E) RTS-binding electrophoretic mobility shift assays using $^{33}$P-labeled Arc wtRTS and scrRTS (C), BDNF wtRTS and scrRTS (D) and CaMKIIα wtRTS and scrRTS probes (E). To perform EMSA, wtRTS or scrRTS probes were incubated with purified recombinant CBF-A without affinity tag. Incubations were also performed in the presence or absence of a 50-fold excess of unlabeled competitor RNA oligonucleotides as indicated.
Figure 7. In dendrites of hippocampal neurons CBF-A exhibits a granular distribution which correlates with transported Arc, BDNF and αCaMKII mRNAs. Endogenous CBF-A and Arc
(A-C), BDNF (E-G) or CaMKII mRNAs (I-K) were simultaneously monitored by immuno-FISH and confocal microscopy. Scale bar, 10 μm. Magnifications of boxed areas are approximately five fold in comparison to corresponding overviews. Unbiased statistical quantification of individual CBF-A and Arc (D), BDNF (H) or CaMKIIα (L) RTS-positive granules based on the immuno-FISH analysis in the panels above. In all cases linear correlations between the fluorescence intensity levels of CBF-A and RTS-containing transcripts was revealed.
Figure 8. In dendrites CBF-A is present in dynamic mRNA granules. (A) 10 DIV hippocampal neurons transfected with EGFP-CBFA. Rectangular areas represent the regions
followed by live cell microscopy. Scale bar, 10 μm. (B) The bars diagram summarizes the different categories of EGFP CBF-A positive granules identified in transiently transfected neurons in terms of their movement. Particles that do not move at least three steps (as evaluated over four frames) in a row in the same direction are scored as either anterograde or retrograde. Particles that move one or two steps, stop or move back and forth, stop, are scored as oscillating. (C-D) Time course analysis of two EGFP CBF-A positive granules traced by the arrows in the time-lapse microscopy experiments (see rectangular areas in panel A). (D) Top panels, EGFP-CBF-A positive granules moving anterogradely are indicated by arrows. Bottom panels provide examples of EGFP-CBF-A positive granules which moving retrogradely (see arrows). In all cases the asterisks indicates an immobile reference point. Scale bar, 5 μm. (E) In transfected hippocampal neurons the distribution of EGFP CBF-A correlates with CaMKIIα mRNAs. EGFP CBF-A and CaMKIIα mRNA were simultaneously monitored by immuno-FISH and confocal microscopy with antibodies against GFP and an RNA probe hybridizing with CaMKIIα mRNA. Scale bar, 15 μm. Magnifications of boxed areas are approximately five fold in comparison to corresponding overviews (see arrowheads point to examples of colocalizations).
Figure 9. CBF-A association with RTS-containing Arc, BDNF and CaMKIIα mRNAs is sensitive to postsynaptic receptor stimulation. CBF-A accumulates in dendrites of hippocampal neurons upon synaptic stimulation with the agonists NMDA or AMPA. (A) Merged images and fivefold magnification of rectangular areas obtained from (A-C) untreated neurons, (D-F) APV-treated neurons, (G-J) NMDA-treated hippocampal neurons immunostained with antibodies to CBF-A and MAP2. (B-C) Quantification of NMDA and AMPA effects on the distribution of CBF-A. CBF-A intensities measured from randomly
selected dendritic areas taken from neurons treated with the indicated reagents (NMDA or APV; AMPA or CNQX) were plotted in bars diagrams with standard deviations. CBF-A levels are specifically and significantly enriched in dendrites upon NMDA treatment in comparison to untreated or AMPA treated cells (p-values were calculated by student’s t-test). AU, arbitrary units. (D) NMDA stimulation of hippocampal neurons induces increased level of Arc, CaMKIIα and BDNF mRNAs. Total RNA from untreated, NMDA- or APV-treated neurons was reverse transcribed with oligo dT primers and the cDNA analyzed by qRT-PCR with primers amplifying Arc, CaMKIIα, BDNF and GAPDH mRNAs. The bars diagram shows relative amounts of the indicated RNAs towards GAPDH mRNA determined over three independent experiments. Error bars depict standard error estimated by Student’s t-test. (E) RNA co-immunoprecipitated with CBF-A from lysates of neurons treated with NMDA or APV was analyzed by qRT-PCR. Arc, BDNF and CaMKIIα mRNA levels are specifically and significantly enriched following NMDA stimulation (p-values were calculated by student’s t-test). Error bars, standard deviations.
Figure 10. CBF-A silencing impairs dendritic mRNA localization in hippocampal neurons.

(A) Rat hippocampal neurons transfected with CBF-A specific or control siRNA oligonucleotides. Silencing is monitored by immunostaining with anti-CBF-A antibodies and with a monoclonal antibody against MAP2. Arrows point to examples of cells where CBF-A is specifically knocked down. Scale bar, 10 μm. (B) qRT-PCR was performed on cDNA derived from total RNA isolated from CBF-A silenced or control hippocampal neurons.
Significant down regulation of relative CBF-A mRNA levels was observed upon CBF-A gene silencing in comparison to untreated or control siRNA treated cells (p-values were calculated by student’s t-test). Error bars represent standard deviations. (C) In CBF-A–silenced hippocampal neurons, there is a drop in the levels of CaMKIIα mRNA in dendrites as revealed by immuno-FISH with antibodies against CBF-A and an RNA probe hybridizing with CaMKIIα mRNA. Analysis is by confocal microscopy. Scale bars, 10 μm. Panels D-F, fivefold magnifications of boxed areas in A-C. Panels J-L, fivefold magnifications of boxed areas in G-I. Overall, these experiments were repeated in triplicates. (D) The bars diagrams display signal intensities for both CBF-A and CaMKIIα mRNA obtained in immuno-FISH experiments on untreated, control siRNA and CBF-A siRNA-treated hippocampal neurons. In all cases signals intensities were measured over as many as 20-30 hippocampal neurons.