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The moonlighting protein c-Fos activates lipid synthesis in neurons, an activity that is critical for cellular differentiation and cortical development

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Running title: *Participation of c-Fos in neuronal differentiation*

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ABSTRACT

Differentiation of neuronal cells is crucial for the development and function of the nervous system. This process involves high rates of membrane expansion, during which the synthesis of membrane lipids must be tightly regulated. In this work, using a variety of molecular and biochemical assays and approaches, including immunofluorescence microscopy and FRET analyses, we demonstrate that the proto-oncogene c-Fos (c-Fos) activates cytoplasmic lipid synthesis in the central nervous system and thereby supports neuronal differentiation. Specifically, in hippocampal primary cultures, blocking c-Fos expression or its activity impairs neuronal differentiation. When examining its sub-cellular localization, we found that c-Fos co-localizes with endoplasmic reticulum markers and strongly interacts with lipid-synthesizing enzymes, whose activities were markedly increased in vitro in the presence of recombinant c-Fos. Of note, the expression of c-Fos dominant-negative variants capable of blocking its lipid synthesis–activating activity impaired neuronal differentiation. Moreover, using an *in utero* electroporation model, we observed that neurons with blocked c-Fos expression or lacking its AP-1–independent activity fail to initiate cortical development. These results highlight the importance of c-Fos–mediated activation of lipid synthesis for proper nervous system development.

Introduction

Neuronal differentiation, an intricate cellular process, comprises a series of complex and coordinated events such as cell proliferation, migration and differentiation (1-5). To reach its final structure and establish functional neuronal circuits, a neuron must promote neuritogenesis, neurite elongation, polarization, arborization and synaptogenesis (6-8). The proper function of the nervous system heavily relies on the number of multiple and targeted synaptic contacts established by each neuron. Sprouting of axon branches along the axon shaft allows multiple innervation targets (9). The overall process of neuronal differentiation determines patterns of connectivity of neuronal circuits whereas the disruption of these patterns may cause severe pathologies or developmental disorders (10,11).

Outgrowth events involve cellular volume and surface area increase, thus demanding membrane material that must be supplied according to the temporal and spatial requirements of the cells in their different developmental stages (5,6,12-14). In fact, membrane expansion can reach an increment of up to 20% according to the different steps of neuronal differentiation (13).

Considering their architecture and size, it is reasonable to hypothesize that neurons will need temporal and domain-specific mechanisms to regulate the synthesis of the components required according to their local demands. In line with this hypothesis, Tsukita and co-workers provided evidence of the three-dimensional structure of the endoplasmic reticulum (ER) in axonal processes (15). Several reports later convincingly demonstrated a synthetic machinery present in axons, with capacity to regulate the levels of proteins, lipids and other macromolecules (16,17). Consequently, the mechanisms by which neurons supply components for membrane biogenesis such as lipids in growing axons have been the subject of several studies. Using compartmented culture systems, compelling evidence emerged demonstrating that axons carry out active lipid synthesis (18-20).

The expression of *Fos*, an oncogene of the *Immediate Early Genes* (IEGs) family, is rapidly and transiently induced in different cell types by diverse stimuli (i.e. growth factors, neurotransmitters) (21-23). The proteins of the Fos family (c-Fos, Fos-B, ΔFos-B, Fra-1, Fra-2) heterodimerize mainly with those of the Jun family, comprising the well-known AP-1 transcription factors (24). Our laboratory described c-Fos as a moonlighting protein capable of achieving an additional, non-AP-1 function: it associates to the ER membranes and activates lipid synthesis through an interaction with specific lipid synthesizing enzymes (25-31). Using PC12 cells, we distinguished between the two known functions of c-Fos, that is, its AP-1 activity and its capacity to activate lipid synthesis. Initially, when cells are stimulated with nerve growth factor (NGF) to differentiate into a sympathetic neuron phenotype, c-Fos is required in the nucleus to trigger the genomic program of differentiation. However, once the cells have been primed to differentiate, c-Fos is only required at the cytoplasm, associated to the ER, activating phospholipid synthesis, for cells to continue differentiating (32).

Herein, using hippocampal neurons we evidenced that c-Fos is capable of exerting its lipid synthesis activator capacity in the nervous system and that this AP-1-independent activity is crucial for normal neuronal differentiation. Furthermore, by *in utero* electroporation we demonstrate that this role is involved in cortical development. In this direction, c-Fos could be an active player in different events required for neuronal plasticity, such as those involved in learning and memory formation.

Results

c-Fos expression during neuronal differentiation

The presence of c-Fos in the brain, and more specifically in the hippocampus, has been demonstrated almost 30 years ago by Dragunow and co-workers (33,34). We initially evaluated c-Fos expression over time in primary cultures of rat hippocampal neurons. At all times examined, c-Fos is expressed at similar levels even after 96

hours in culture, as determined by immunocytochemistry and Western Blot (Figure 1). It is important to highlight that c-Fos expression is observed not only in the nucleus but also extending to the entire cytoplasm of the neurons.

We then evaluated if c-Fos participates in the differentiation of rat hippocampal neurons. The method applied to determine the differentiation stage of the cultured cells was the one described by Dotti and co-workers (7). Briefly, cells in stage 1 of differentiation, shortly after they attach to the substrate, show motile lamellipodia developed around the periphery of the cell. At stage 2, these lamellipodia begin to transform into distinct minor processes that extend to a length of up to 10-15 µm. At stage 3, one of these minor processes begins to grow 5 to 10 times faster than the rest of them, indicating that the axon of the cell has been formed and the cell has polarized. At stage 4, a significant development of dendrites initiates and finally, at differentiation stage 5, the axonal and dendritic arbors suffer a maturation process.

To evaluate the effect of blocking c-Fos activity during this complex process, primary cultures of rat hippocampal neurons were profected at 2 hours of culture with anti c-Fos antibodies or with a non-related antibody as a control using the BioPorter system. Cells were examined 48 hours later by immunocytochemistry but only using secondary antibodies in the case of the anti-c-Fos-profected cells. As can be seen in the graphs of Figure 2A, blocking c-Fos activity impairs axon formation. We quantified different morphological features of the neurons observing that there were no changes in soma size or in the mean number of neurites per cell when comparing cells lacking c-Fos activity and the control ones. It should be noticed that we were not able to observe the development of axonal-like processes even after 48 hours of culture when c-Fos activity was impaired, a phenomenon not observed in the control cells (Figure 2A).

These results were confirmed by blocking c-Fos expression. When neurons are infected two hours after seeding with lentiviral vectors designed to express a specific shRNA against c-Fos, impaired differentiation is clearly observed (Figure 2B). When neurons are classified into the differentiation stages according to their morphology, more than 85% of the c-Fos-knocked-down neurons remained at stages 1 and 2 of differentiation as compared to the almost 50% of neurons in stages 3 and 4 of the control cultures (Figure 2C).

c-Fos co-localizes with ER markers in neuronal processes

Previous studies from our laboratory have shown that upon a subcellular fractionation of PC12 cell homogenates, c-Fos and the ER membranes are predominantly collected in the same fraction (35). This led us to examine if the c-Fos/ER co-localization observed previously can be evidenced in neurons of the central nervous system as well. To test this, we performed immunocytochemistry assays on primary rat hippocampal neurons after 48 hours of culture and, as expected, a strong co-localization of c-Fos with the ER marker Calnexin was detected (Figure 3). Surprisingly, c-Fos and the ER were found not only in the perinuclear region of the soma; defined co-localization dots at punctuate structures confined to axonal branching points were also very frequently observed.

c-Fos activation of lipid synthesizing enzymes in neurons

Previous results from our group have demonstrated that c-Fos physically interacts with and activates particular enzymes of the lipid synthesis pathways. Such is the case for CDP-diacylglycerol Synthase (CDS), Phosphatidylinositol 4 Kinase II-alpha (PI4KIIα) and Lipin1 (29,31). Taking this into consideration, it was hypothesized that c-Fos could be involved in neuronal differentiation by a lipid-activating mechanism that requires its physical interaction with specific enzymes. To this end, we examined the possible interaction of c-Fos with CDS, the enzyme that catalyzes the conversion of phosphatidic acid (PtdOH) into CDP-diacylglycerol (CDP-DAG), the first step of phosphatidylinositol-phosphate (PtdInsP)

synthesis in the ER. To infer protein-protein interactions, Förster Resonance Energy Transfer (FRET) experiments were performed (36). For this, rat primary hippocampal neurons were co-transfected to express c-Fos-mTurquoise2 and CDS-SYFP2 and examined by confocal microscopy. As shown in Figure 4A and B, a positive FRET phenomenon is observed between both proteins, indicating that there is indeed a physical interaction between them.

To study if this physical interaction leads to enzyme activation, CDS activity was assayed using cell homogenates obtained from rat hippocampal neurons as the enzyme source and ^{3}H-CTP and dioleoyl-PtdOH as substrates, with or without the addition of recombinant c-Fos to the incubates (29,37,38). At the times assayed, about 40% more CDP-DAG was synthetized in the +c-Fos incubates as compared to the control (Figure 4C). This confirms that the lipid synthesis activation mediated by c-Fos observed previously also occurs in hippocampal neurons in culture.

The association of c-Fos to CTP:phosphocholine cytidylyltransferase (CCT), the rate limiting enzyme in phosphatidylcholine (PtdCho) synthesis (Supplementary Figure 1) suggests that c-Fos could be also mediating the activation of other phospholipid pathways.

Neuronal differentiation is impaired in cells lacking cytoplasmic c-Fos

The results shown so far support the need of c-Fos to normally complete the differentiation events. However, they do not allow us to discern if the effect is due to the AP-1or the lipid synthesis activator function of c-Fos. In order to activate lipid synthesis, c-Fos associates through its N-terminal domain (NA domain, amino acids 1-138 of full length c-Fos) to the enzymes it activates (29,31,32). However, this portion of the protein is not sufficient to produce lipid synthesis activation because of the absence of the basic domain (BD, amino acids 139-159), a domain required for enzyme activation to occur. As the NA domain is not involved in c-Fos AP-1 functions, it seems reasonable to propose its use as a negative dominant of its lipid synthesis activator function. That is, the NA should interfere only with the lipid synthesizing function of c-Fos and not with its AP-1, transcriptional one. Furthermore, we have previously shown that recombinant NA is not able to activate total phospholipid synthesis and inhibits CDS activity (30,38).

Taking this into consideration, we examined if the impairment in neuronal differentiation could be linked to the lack of c-Fos-dependent lipid synthesis activity. Hippocampal neuronal cells were transfected with a construct that contains the NA domain, that does not activate lipid synthesis, or with the construct NB that contains amino acids 1 to 159 (NB domain), which is capable of activating lipid synthesis at comparable levels to those of full length c-Fos, both fused with YFP. The empty vector was used as a control. Then the differentiation stages of the transfected cells at different fixation times were analyzed. It can be seen in Figure 5A and its quantification in 5B (cells fixed at 48h) that the expression of the NA domain impairs neuronal differentiation as evidenced after a morphological analysis and stage quantification of the NA transfected cells relative to the NB or non-transfected ones. Even more, it is clear that the impairment in differentiation promoted by the NA domain is comparable to that observed when c-Fos expression is blocked (Figure 2B and C).

In order to test if this effect was due to the lipid synthesis activator capacity of c-Fos, we performed biochemical assays to assess total phospholipid labeling in homogenates obtained from hippocampal neurons primary cultures. Previous reports have shown that the γ-phosphate group of labeled [^{32}P]-ATP can be incorporated into different lipids of cell lysates assayed *in vitro*, mainly phosphatidylinositol and its derivatives and phosphatidic acid (39-44) and that recombinant c-Fos promotes an increase in their synthesis (45). When assaying this in neurons, it is clear that the addition of recombinant c-Fos to the assay promotes a significant increase in total phospholipid labeling, while recombinant NA does not (Figure 5C). However, if both c-Fos and NA are added together, the activating effect of c-Fos is abolished. These results are compatible with a competition between c-Fos and NA for

binding of c-Fos to the enzymes as shown previously in other systems (38). This is also compatible with the notion that differentiation is impaired as a consequence of the lack of the lipid synthesis activator capacity of c-Fos.

c-Fos is essential for cortical development

We next studied if c-Fos participates in cortical development *in vivo* by using *in utero* electroporation. Briefly, the vertebrate cortex is organized into layers of neurons that share functions, morphology and birthdates (46). During development, radial glia progenitors in the ventricular zone (VZ) divide asymmetrically to originate cortical pyramidal projecting neurons. Then they suffer a radial migration process towards the marginal zone and through the subventricular zone (SVZ) and lower intermediate zone (IZ). In the IZ, neurons acquire a transient multipolar morphology where they extend and retract multiple dynamic projections and move in apparently random directions (47-49). As cells approach the middle of the IZ, the genesis of the axon starts and when they reach the upper region of the IZ, they change their morphology from multipolar to bipolar and carry on radial migration (46,50,51).

Using the electroporation model, it can be visualized if the manipulated neurons, that are destined to migrate to the upper layers of the brain cortex, suffer an impairment in this process or in the establishment of neuronal polarity. Cortical progenitors at embryonic day 15 (E15) were electroporated with a specific shRNA targeting c-Fos and an analysis of the location and morphology of the progeny was performed at E19 after *in vivo* differentiation. Visualization of the electroporated cells was achieved by co-electroporating the shRNAs with a plasmid encoding the DsRed fluorescent protein.

We first analyzed differentiation of cells electroporated with a non-relevant shRNA (shControl) plus DsRed at E19. About 10% of the neurons were located in the ventricular zone/sub-ventricular zone (VZ/SVZ), 30% of cells were found migrating through the IZ and the majority (60%) had reached the top of the cortical plate (Figure 6A, left; quantification in 6B). Cells with knocked-down expression of c-Fos clearly showed an altered distribution and abnormal migration: around 80% of the cells remained arrested at the VZ/SVZ compared to the 10% in this localization in control experiments and practically no electroporated cells were observed in the upper CP (Figure 6A, right; quantification in 6B).

Electroporation assays were then performed with the deletion mutants of c-Fos to evaluate if its lipid synthesis activator function is responsible for the abnormal phenotype observed when c-Fos expression is blocked. Electroporated cells of control experiments using the empty vector show a similar localization to that shown in the controls of Figure 6A (Figure 6C, left; quantification in 6D). By contrast, cells transfected with c-Fos NA domain that acts as a negative dominant of its lipid synthesis activator function, remained arrested at the VZ/SVZ and IZ (Figure 6C, right; quantification in 6D). These results support the notion that the AP-1-independent function of c-Fos is in fact involved in cortical development.

Discussion

Although for a long time it was assumed that biosynthesis of most macromolecules in neurons was confined to the cell body (52,53), an increasing body of molecular evidence has supported the presence of functional ER resident components in axons (15-17,54-62). Together with the understanding of the complex structural features of the axonal ER, different studies provided emerging evidence of active, axon-localized lipid synthesis (18,19,63-66). In fact, in the case of phosphatidylcholine synthesis, almost 50% has been found to be locally synthesized in distal axons and it is required for axonal growth (20). Although this highlights the importance of the axonal ER in lipid homeostasis and in the maintenance of the complex structure of neurons, there have been no significant advances in the last years regarding the regulation of lipid metabolism in neurons.

Taking into consideration that the plasma membrane surface area of a developing neuron

increases up to 20% per day (13) and that the compositional differences along the membrane due to the functional differences of axons and dendrites must necessarily be maintained, the molecular mechanisms regulating localized lipid synthesis must be carefully orchestrated. Since most of the local metabolic demands for axonal development, plasticity or regeneration must be rapidly supplied, it seems reasonable to propose that, at least in part, the mechanisms involved in lipid synthesis regulation could be independent of the cells' nuclear activity. In the present work we provide evidence that confirms the existence of the AP-1-independent function of c-Fos as a lipid synthesis activator in the nervous system, particularly in rat hippocampal neurons in culture and in the developing brain cortex during embryonic stages.

The fact that c-Fos is expressed during neuronal differentiation but is not in the adult brain in non-pathological conditions (67) implies that its expression is related to developmental events. c-Fos was described as a marker of neuronal activity, specially tied to learning and memory processes (68). In the experiments presented herein where c-Fos expression or its activity are blocked, neurons do not develop an axon and remain at early stages of differentiation without a distinguishable axon even after 48 hours of culture. Until now, the effects of c-Fos on neuronal plasticity have been always linked to gene expression through its nuclear function, but given the evidence presented herein, it is possible that they are associated to processes that involve changes in lipid homeostasis. This can be particularly visualized in the experiments carried out with negative dominants of the lipid synthesis activator function of c-Fos, where the same results of impairment on differentiation in culture are observed in spite that AP-1 activity should not be affected.

This hypothesis can be extended to cortical development: when performing *in utero* experiments, the cells with no c-Fos expression remain at the ventricular and sub-ventricular zones instead of migrating to the superior layers of the cortex, an observation that implies a strong failure in normal cortical development. These results deepen our previous findings in the cerebral cortex of *fos* (-/-) mice, that show a strong reduction in the cortex thickness and a marked tendency of cells towards an undifferentiated phenotype (69). Since the *in utero* model does not have compensatory effects relying on other proteins that might fulfill the function of c-Fos, the phenotype observed in our experiments confirm the importance of this protein in cortex development. Even more, the same *in utero* experiments with negative dominants of its AP-1-independent function show that this effect is directly related to its lipid synthesis activator role. However, the changes in the localization of the NA transfected cells are not as extreme as those observed in the c-Fos knocked-down cells. Two different interpretations arise from the intermediate phenotype observed in this case: both functions, AP-1 and lipid synthesis activation are actively involved in cortical development, or the expression level of the NA domain is not enough to displace all the endogenous c-Fos molecules. It remains to be determined if the observed phenotype is a consequence of failures in polarization or migration of the cells.

c-Fos has been implicated in numerous physiological processes in the nervous system. It was the first transcription factor whose induction was proven to be dependent on the activity of the neuron (70,71), an evidence that rapidly transformed c-Fos in a marker of neuronal activity. In fact, it has been consistently demonstrated that its expression rises in the central nervous system after learning or memory trainings (68), although this changes are observed only during the first sessions of the protocols, indicating that this is probably an adaptive response. Given the results presented in this work, it seems reasonable to hypothesize that c-Fos lipid activator function could be involved in processes related to neuronal plasticity and learning processes by providing new membrane lipids for the establishment of new circuits that are no longer required once they are formed.

In light of all the above mentioned results, the role of AP-1-independent c-Fos results of vital importance for neuronal development. Its lipid

synthesis activator capacity might contribute to the high membrane expansion rates necessary for the extension of the different neuronal processes that favor polarization and the correct establishment of synaptic connections for normal nervous system function.

Experimental procedures

Cell cultures, profection and transfection. Dissociated hippocampal pyramidal neurons were prepared from fetal rat brain and cultured as described (72). Briefly, pregnant *Rattus Norvegicus* Wistar rats at 18 days post-fertilization were euthanized and prenatal pups were excised from the uterus with sterile dissecting scissors. Pups were then decapitated with sterile scissors in a laminar flow hood and the removed heads were placed in plates with sterile Hank's Balance Salt Solution (HBSS, Sigma Aldrich, St. Louis, Missouri, United States) at 4°C under a dissecting microscope. After hippocampus isolation and dissociation with Trypsin 0.25% (Thermo Fisher Scientific, Waltham, Massachusetts, United States), the tissue was dissociated with Pasteur pipettes. The cells obtained were plated onto acid washed, poly-lysine-coated glass coverslips or poly-lysine-coated plates and maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with 10% (v/v) Horse Serum (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 2 hours after which the culture media was replaced with serum-free Neurobasal medium supplemented with N2 and B27 supplements (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Cultures were maintained in a humidified 37°C incubator with 5% CO_2 during the indicated times.

Profections were performed using BioPORTER® Protein Delivery Reagent (Genlantis, San Diego, California, United States) and transfections using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) according to the manufacturer´s protocol.

Production of shRNA containing lentiviral particles. Upon desired confluence, embryonic kidney epithelial HEK 293T cells (ATCC, Manassas, Virginia, United States) were co-transfected with MISSION® c-Fos custom shRNA plasmids (clones ID TRCN0000042680 and TRCN0000042678) cloned in pLKO.1-CMV-tGFP (Sigma Aldrich, St. Louis, Missouri, United States) or with the control scrambled shRNA sequence and with compatible packaging plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Lentiviral titer was determined according to the manufacturer's protocol (MISSION®, Sigma Aldrich, St. Louis, Missouri, United States).

Preparation of recombinant c-Fos and NA. His-tagged c-Fos and NA were expressed and recovered from pDS56-HisFos-transformed BL21 cells as described previously (30,38,73).

Co-immunoprecipitation assays, electrophoresis and Western Blot (WB). For co-immunoprecipitation (co-IP) assays, 500 µg of total protein from cells treated as indicated were immunoprecipitated for 4 hours at 4°C with Protein G–Sepharose (GE Healthcare Biosciences, Chicago, Illinois, United States) with the desired antibody, washed and immunodetection performed as described below. Cell lysates (50 µg) or IPs were fractionated through SDS-containing polyacrylamide gels (12%) and electrotransferred to a nitrocellulose membrane at 300 mA during 1h. Immunodetection was carried out by blocking of membranes with 10 mM PBS containing 5% (w/v) non-fat dried milk for 1 hour at RT, followed by incubation with the desired antibody overnight at 4°C in PBS-0.1% (v/v) Tween-20 (Sigma Aldrich, St. Louis, Missouri, United States). Membranes were washed three times (10 min) with PBS-0.1% (v/v) Tween-20 and incubated for 1 hour at RT with secondary antibodies. Membranes were washed and detection performed using an ODYSSEY Infrared Imaging System (LI-COR, Lincoln, Nebraska, United States).

In vitro phospholipid labelling. *In vitro* phospholipid labelling capacity of neurons was

assayed as described previously (32,74). Briefly, reactions were incubated for 60 minutes at 37°C in a final volume of 50 µL containing 50 µg of cell homogenate protein as the enzyme source, 2.8 mM NaCl, 100 mM KCl, 10 mM $MgCl_2$, 112 mM glucose, HEPES buffer pH 7.5, 1,5 µCi of [^{32}P] ATP (specific activity 3000 Ci/mmol; PerkinElmer) and the indicated amounts of c-Fos or NA. Conditions of linearity with time and protein concentration were determined for the enzyme with 1 ng of recombinant proteins per µg of protein cell homogenate, suspended in 3 µL of elution buffer or an equal volume of elution buffer for control reactions. For the competition conditions, the ratios between c-Fos and NA were calculated according to the number of molecules of each protein. Reactions were stopped by the addition of trichloroacetic acid (TCA) and phosphotungstic acid (PTA; 5–0.5% w/v, respectively). Incubates were centrifuged and the pellet was washed three more times with TCA-PTA 5:0,5 %w/v. After a final washing step with water, the pellet was suspended in 1.5 mL of chloroform:metanol 2:1. In this phase partitioning, lipids remain in the organic phase. Phospholipid labelling was quantified in the organic phase by scintillation counting (30,74,75). A more detailed version of the protocol has been published (76).

Enzyme Activity Determinations. Total CDS activity was assayed as described by Lykidis *et al*. 1997 (37). All reactions were performed in 80 µL of final volume containing 100 µg of cell homogenate protein as the enzyme source, 0.69 µM [^{3}H]-CTP (Perkin-Elmer, Waltham, Massachusetts, United States) and 2 mM phosphatidic acid (Avanti Polar Lipids, Alabaster, Alabama, United States). Conditions of linearity with time and protein concentration were determined for the enzyme with 0.5 ng of recombinant c-Fos per µg of protein cell homogenate, suspended in 3 µL of elution buffer or an equal volume of elution buffer for control reactions (29,38). The reaction was started by the addition of 10 mM $MgCl_2$ and assays were incubated at 37°C for 1 hour. Reactions were stopped by the addition of 180 µL of chloroform/methanol/HCl (1:2:0.02% v/v). After addition of 60 µL of chloroform and 60 µL of KCl 2M, phases were separated by centrifugation. The amount of [^{3}H]-CDP-diacylglycerol synthesized was determined by liquid scintillation counting in the organic phase.

Immunofluorescence (IF) and microscopy. Cells grown on round, acid-washed coverslips were rinsed twice with ice-cold 10 mM PBS and fixed in para-formaldehyde 4% (w/v) (Sigma Aldrich, St. Louis, Missouri, United States), sucrose 4% (w/v) (Sigma Aldrich, St. Louis, Missouri, United States) in 10 mM PBS at 37°C for 10 minutes. Cells were then permeabilized with Triton X-100 0.1% (v/v) (Sigma Aldrich, St. Louis, Missouri, United States) in 10 mM PBS during 10 minutes and blocked with Horse Serum 2% (v/v) and Bovine Serum Albumin 3% (w/v) in 10 mM PBS for 2 hours at RT in a humid chamber. Samples were incubated overnight at 4°C in blocking buffer containing the desired antibody, washed twice with 10 mM PBS and incubated with secondary antibodies for 2 hours at RT, washed and mounted with FluorSave (Millipore, Burlington, Massachusetts, United States). When indicated, DAPI (4,6-diamino-2-phenylindole, dihydrochloride, Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to visualize nuclear structures.

Antibodies. The following primary antibodies were used: rabbit polyclonal anti-c-Fos antibody (Santa Cruz Biotechnology, Dallas, Texas, United States) diluted 1:200 for IF; rabbit polyclonal anti-c-Fos antibody (Sigma Aldrich, St. Louis, Missouri, United States) diluted 1:2000 for WB; mouse monoclonal anti-α-Tubulin antibody (Sigma Aldrich, St. Louis, Missouri, United States) diluted 1:3000 for WB; mouse monoclonal anti-βIII-Tubulin antibody (Sigma Aldrich, St. Louis, Missouri, United States) diluted 1:1500 for IF; goat polyclonal anti-calnexin antibody (ER marker, Abcam, Cambridge, United Kingdom) diluted 1:500 for IF; rabbit polyclonal anti-CCTβ2 antibody (Sigma Aldrich, St. Louis, Missouri, United States) diluted 1:500 for IF. The following secondary antibodies were used: IRDye 800CW goat anti-mouse antibody and IRDye 800 goat anti-rabbit antibody (LI-COR, Lincoln, Nebraska, United States) diluted 1:25000 for WB;

Alexa 488, Alexa 546 and Alexa 633 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) diluted 1:1000 for IF.

FRET analysis. Cells grown on round, acid-washed coverslips in 24 multiwell plates were transfected with c-Fos-mTurquoise2-N1, CDS-pSYFP2, CCTβ2-pSYFP2, pSYFP2-mTurquoise2 or the empty vectors using Lipofectamine 2000 according to the manufacturer´s protocol. After 24 hours of transfection, cells were rinsed twice with ice-cold 10 mM PBS and fixed in para-formaldehyde 4% (w/v) (Sigma Aldrich, St. Louis, Missouri, United States), sucrose 4% (w/v) (Sigma Aldrich, St. Louis, Missouri, United States) in 10 mM PBS at 37°C for 10 minutes. Cells were then washed three times with PBS and rinsed with Milli-Q water. Coverslips were mounted with FluorSave (Millipore, Burlington, Massachusetts, United States) and cells visualized using an Olympus FV1000 laser scanning confocal microscope with Olympus Fluoview Software (Olympus, Shinjuku, Tokyo, Japan). For FRET determinations, the sensitized emission measurement approach was used (36). The mTurquoise (donor) and SYFP (acceptor) chimeric proteins were excited with an argon laser at 458 and 515 nm, respectively. The emission channel was 470–500 nm for the donor and 530–560 nm for the acceptor. Background values were determined independently for each channel from a coverslip with non-transfected cells and then subtracted using ImageJ software. Donor spectral bleedthrough and acceptor cross excitation were calculated and corrected from single transfected cells. Mean FRET efficiency values (%E) within a cell were obtained on a pixel-by-pixel basis (31). The resulting image was then pseudo-colored to better illustrate the distribution of the calculated efficiencies in the cell.

In utero electroporation. In utero electroporation was performed as previously described (77) with minor modifications. Briefly, pregnant C57BL/6J mice at E15 days were anaesthetized with ketamine/xylazine (Laboratorios Richmond, Ciudad Autónoma de Buenos Aires, Argentina). Needles for injections were pulled from P-97 Flaming/Brownglass capillaries (World Precision Instruments, Sarasota, Florida, United States). shRNA solutions were mixed with Tripan blue 1% (v/v) at a DNA concentration of 0.5–1.5 µg/µL for each construct and injected. Five pulses of 40V (50 ms ON, 950 OFF) were applied using 5 mm electrodes and a specially manufactured electroporator (LIADE National University of Córdoba, Córdoba, Córdoba, Argentina). The embryos were placed back into the abdominal cavity which was then sutured. After 3 days post intervention, the mother was sacrificed by cortical dislocation, the embryos removed and the brains of the electroporated ones extracted and fixed with 4% (w/v) para-formaldehyde (Sigma Aldrich, St. Louis, Missouri, United States) for 24 hours. Then, they were cryo-protected by immersion in Sucrose 30% (w/v) (Sigma Aldrich, St. Louis, Missouri, United States) and finally embedded in medium for frozen tissue specimens to ensure optimal cutting temperature (O.C.T) and freezed in Nitrogen. After 3 days at -80°C, coronal cryosections of 20 µm were obtained and processed for immunofluorescence.

Microscope image acquisition. Imaging was performed on an Olympus FV1000 laser scanning confocal microscope Using Olympus Fluoview Software (Olympus, Shinjuku, Tokyo, Japan) using a 60× (1.4 NA) oil objective or a 10× (0.40 NA) air objective. Images were analyzed with ImageJ Software.

Experimental design and statistical analysis. All the statistical analysis were performed using GraphPad Prism software. Statistical significance was defined by a p value < 0.05. The statistical test performed and the number of replicates for each experiment are indicated in the correspondent figure legend.

Animal care. All the procedures were performed according to the "*Guide for the care and use of laboratory animals*" (8th Edition, USA) and the approved protocols of the *Institutional Board for Animal Welfare* (CICUAL, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba).

Data availability. All data are contained within the manuscript.

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Footnotes

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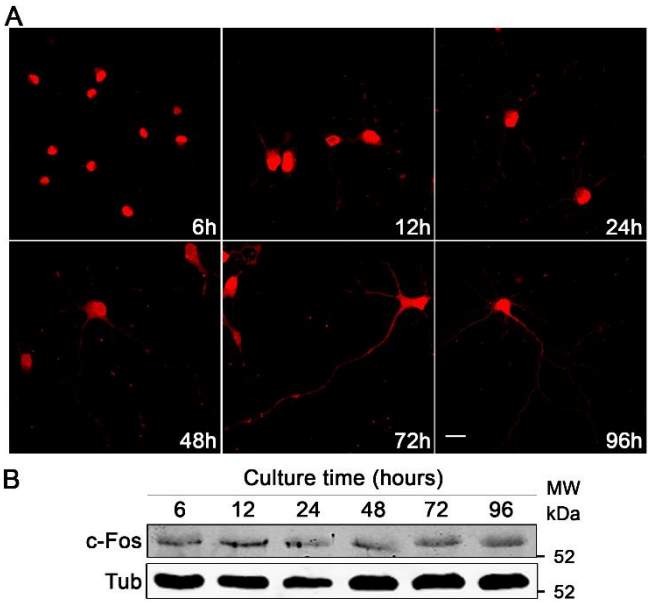
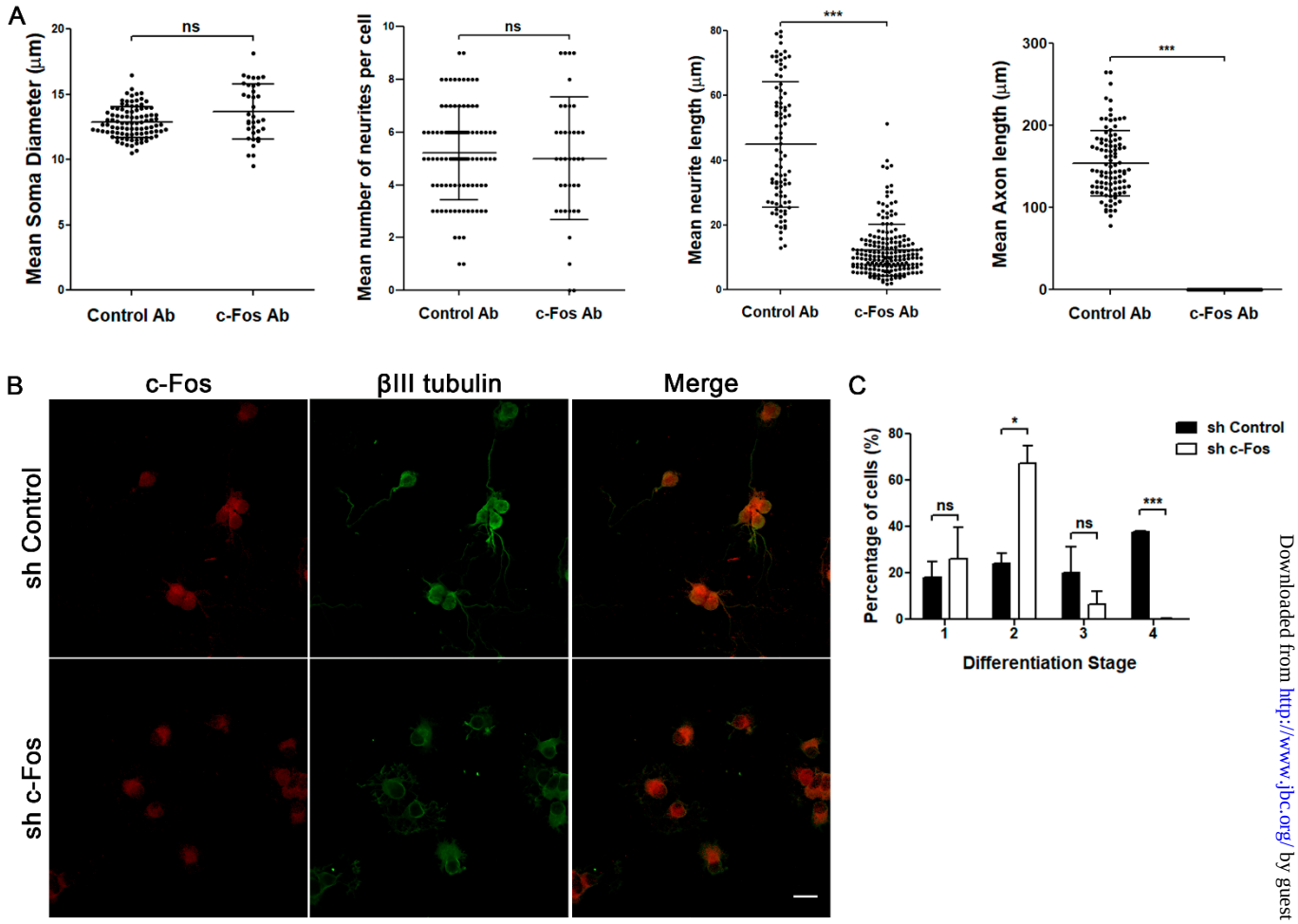
The abbreviations used are: CCT, CTP:phosphocholine cytidylyltransferase; CDP-DAG, CDP-diacylglycerol; CDS, CDP-diacylglycerol Synthase; ER, endoplasmic reticulum; FRET, Förster resonance energy transfer; IEGs, Immediate Early Genes; NGF, nerve growth factor; PI4KIIα, Phosphatidylinositol 4 Kinase II-alpha; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid.

Figure 1



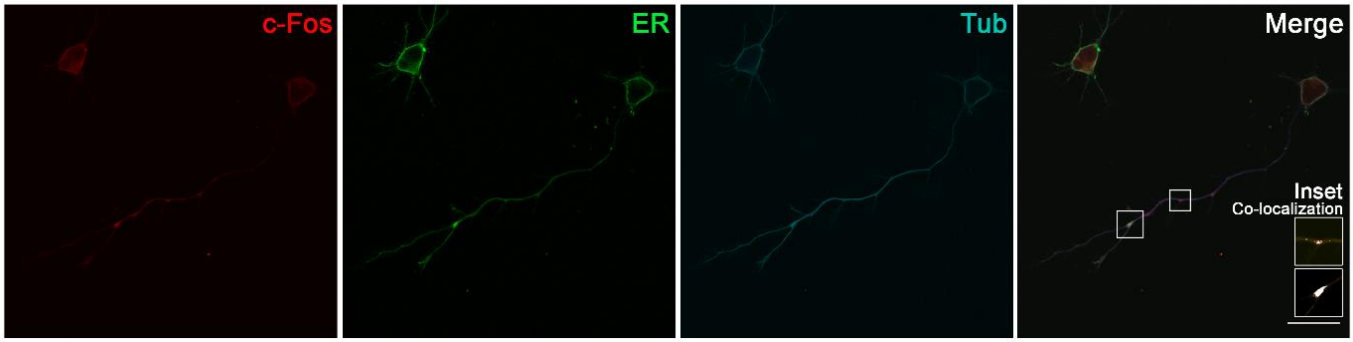
c-Fos is expressed in primary rat hippocampal neuronal cultures. **(A)** Rat primary hippocampal neurons were fixed at different times of culture and immuno-stained with an anti c-Fos antibody (red). h: hours. Scale bar: 20 µm. **(B)** Western Blot of hippocampal cell lysates harvested at different times of culture. Membranes were immuno-stained with an anti c-Fos antibody (upper panel) and an anti-Tubulin antibody (lower panel) as a loading control and secondary antibodies suitable for near infrared fluorescence. The obtained images were converted to grey scale and inverted. The results of one of three independent experiments are shown.

Figure 2



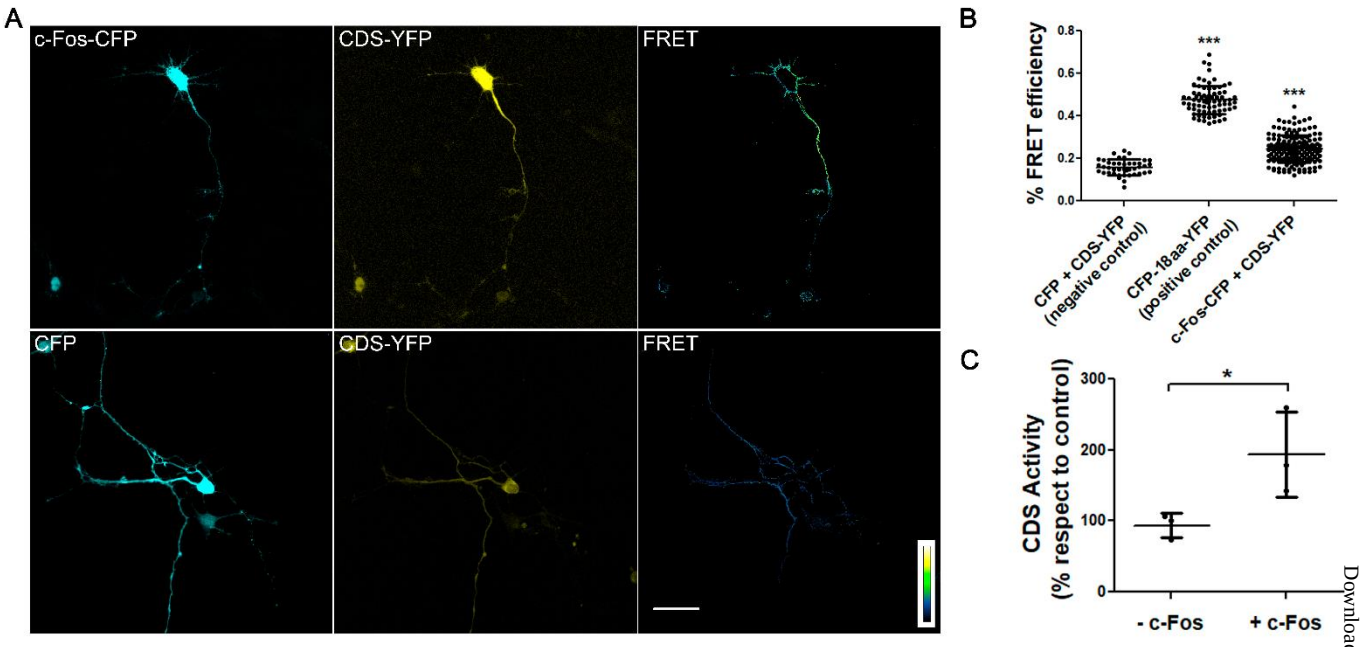
Blocking the activity or the expression of c-Fos impairs neuronal differentiation. **(A)** Cells were profected with anti c-Fos (c-Fos Ab) or with a non-related anti mouse IgG antibody as a control (Control Ab) 2 hours after seeding using BioPorter and then were fixed after 48 hours of culture. The different morphological aspects were quantified from microscopy images, using ImageJ software, and are shown as the mean ± SD in each case. Student's t test statistical analysis was performed using GraphPad Software. ***p< 0.001; n.s.: non-significant; in each experiment, n=40 cells from each condition were examined. Results of one of three independent experiments performed are shown. **(B)** Cells were infected at the initiation of the culture with lentiviral particles designed to express an anti c-Fos shRNA or a shRNA with a scrambled sequence of c-Fos as a control. After 48 hours of culture cells were fixed and immune-stained with an anti c-Fos antibody (red, first column) and an anti βIII-Tubulin antibody (green, second column). The third column shows the merge between both labels. Scale bar: 20 µm. **(C)** Morphological quantification of neuronal differentiation stages in both c-Fos and scrambled infected cells was performed using ImageJ software. The graph shows the mean number of cells ± SD in each case. Student's t test statistical analysis was performed using GraphPad Software. *p< 0.05; ***p< 0.001; n.s.: non-significant; n=198 cells for scrambled infected cells, n=254 cells for c-Fos infected cells. Results of one of three independent experiments performed are shown.

Figure 3



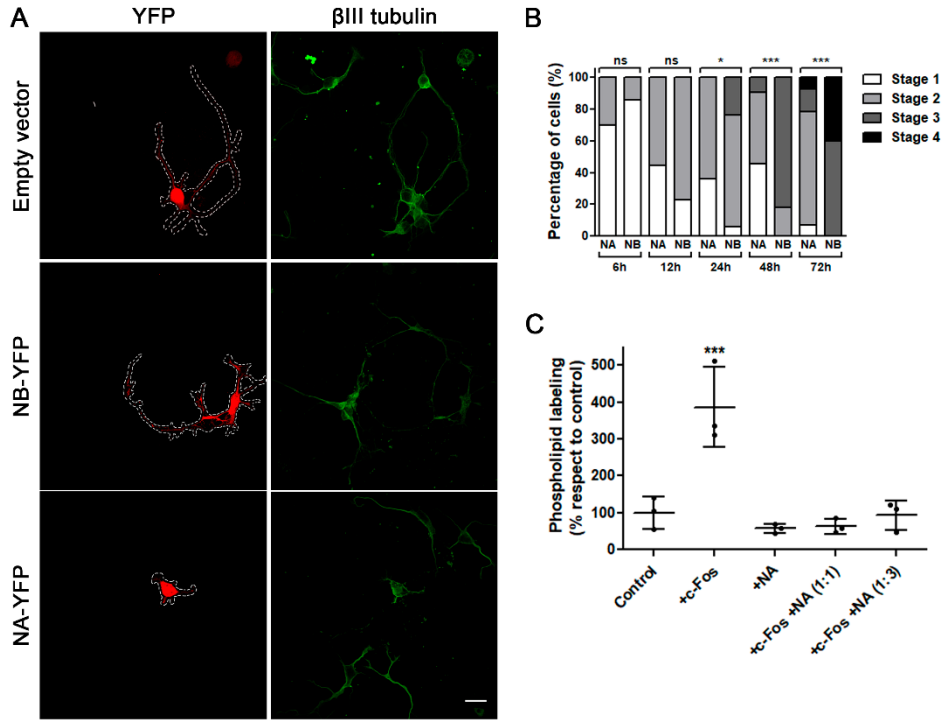
***c-Fos co-localizes with ER markers*.** Immunocytochemistry of c-Fos (red), the ER marker Calnexin (green) and βIII-Tubulin (cyan) of rat hippocampal neurons at 48 hours of culture. The fourth panel shows the merged images between the three labels. A Pearson's R value of 0,81 was calculated for co-localization of both labels (Coloc2, ImageJ). An inset is shown with a co-localization analysis between c-Fos and the ER, where the co-localizing pixels are colored in white (Colocalization Finder, ImageJ). Scale bar: 20 µm. The results of one of three independent experiments are shown.

Figure 4



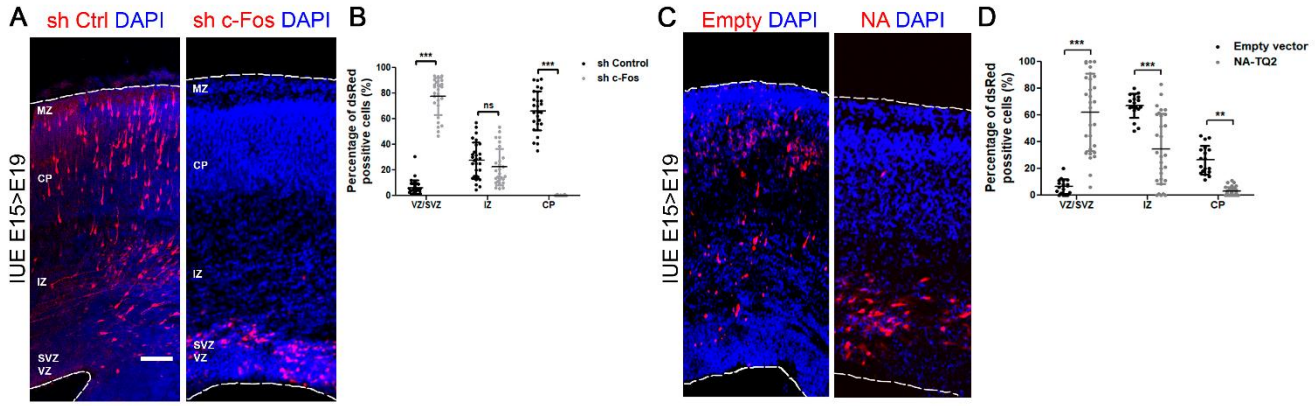
c-Fos activates and physically interacts with CDS. **(A)** Upper row, neurons were co-transfected at 24 hours of culture to express c-Fos-CFP (first panel) and CDS-YFP (second panel) and examined by confocal microscopy at 48 hours. FRET images were obtained by the sensitized emission method and pseudo-colored using ImageJ software (third panel). The lower row shows a negative control with cells co-transfected with the CFP empty vector and CDS-YFP. Scale goes from no FRET (black) to maximum FRET (yellow). Scale bar: 30 µm. **(B)** The graphic shows the quantification of the mean efficiencies ± SD for the donor/acceptor pairs shown in the images, with one-way ANOVA analysis and Tukey's post-test. ***p<0.001; n=25 cells for each condition. The results of one of three independent experiments are shown. **(C)** Evaluation of CDS activity through the measurement of the incorporation of [^{3}H]-CTP into CDP-DAG in neurons homogenates in the presence of c-Fos (+ c-Fos). Elution buffer was used as a control (- c-Fos). Results are the mean of three independent experiments performed in triplicate. The results are expressed as the mean ± SD, with t-Student analysis. *p<0.05.

Figure 5



***NA deletion mutant of c-Fos impairs differentiation in neuronal cultures and abrogates c-Fos-dependent lipid synthesis activation*. (A)** Neuronal cultures were transfected at seeding with NA-YFP (pseudo-colored red, lower row), or NB-YFP (pseudo-colored red, middle row) or the empty vector as a control (pseudo-colored red, upper row) and were fixed after 48 hours of culture. Cells were subjected to immunofluorescence against βIII-tubulin (green, second column). Scale bar: 20 µm. **(B)** Morphological quantification of neuronal differentiation stages in both NA-YFP and NB-YFP transfected cells at different fixation times. The results of one of three independent experiments are shown. Normality Kolmogorov-Smirnov test was performed, where the deviation from the distribution respect to the NB transfected cells was evaluated. *p<0.05; ***p<0.001; n.s.: non-significant; n=15 from each condition were examined. **(C)** Evaluation of ^{32}P-phospholipid labelling capacity of neuron homogenates in the presence of c-Fos (+c-Fos), NA (+NA) or both (+c-Fos +NA 1:1 and +c-Fos +NA 1:3). Elution buffer was used as a control (Control). Results are the mean of three independent experiments performed in triplicate. The results are expressed as the mean ± SD, with one-way ANOVA analysis. ***p<0.001 respect to control condition.

Figure 6



Expression of c-Fos is involved in cortical development. **(A)** Embryo brains were electroporated with a specifically designed shRNA to block c-Fos expression (sh c-Fos) (right) or a control scrambled shRNA (left) at E15 and analyzed at E19. IUE: *in utero* electroporation; VZ: ventricular zone; SVZ: subventricular zone; IZ; intermedial zone; CP: cortical plate; MZ: marginal zone. Scale bar = 100 µm. **(B)** Quantification of the distribution of dsRed-positive cells in the CP, IZ and VZ/SVZ. The mean ± SD of the quantification of at least 20 coronal cryo-sections from three independent experiments performed independently is shown, with two way Anova statistical analysis with Bonferroni post-test. *** $p < 0.001$; n.s.: non-significant. **(C)** Embryo brains were electroporated with a vector designed to express the NA domain of c-Fos fused to CFP or the empty vector as a control at E15 and analyzed at E19. **(D)** Quantification of the distribution of dsRed-positive cells in the CP, IZ and VZ/SVZ. The mean ± SD of the quantification of at least 20 coronal cryo-sections from three independent experiments performed independently is shown, with two way Anova statistical analysis with Bonferroni post-test. *** $p < 0.001$; **$p< 0.001$.

The moonlighting protein c-Fos activates lipid synthesis in neurons, an activity that is critical for cellular differentiation and cortical development

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