Eicosapentaenoic acid modulates the synergistic action of CREB1 and ID/E2A family members in the rat pup brain and mouse embryonic stem cells

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Title. Eicosapentaenoic acid modulates the synergistic action of CREB1 and ID/E2A family members in the rat pup brain and mouse embryonic stem cells

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ABSTRACT

The aim of this study was to investigate the molecular mechanism by which
eicosapentaenoic acid (EPA) may exert neuroprotective effects through an “EPA-
cyclic AMP response element-binding protein (CREB)” signaling pathway. The
current study reveals that EPA modulates the exquisite interplay of interaction of
CREB1 with the inhibitor of DNA binding (ID) and E2A family members, thereby

delivering mechanistic insights into specific neural differentiation program. In this scenario, our work provides evidence for the capability of CREB1 to sequester ID:E2A family members in brain tissues and neural differentiating mouse embryonic stem cells (mESCs) through formation of a [CREB1]₂:ID2:E47 tetrameric complex. In essence, the molecular function of CREB1 is to dynamically regulate the location-specific assembly or disassembly of basic-helix-loop-helix (bHLH):HLH protein complexes to mediate the activation of neural/glial target genes. Together, these findings support the one-to-many binding mechanism of CREB1 and indicate that EPA treatment potentiates the integration of CREB dependent signaling with HLH/bHLH transcriptional network, adding specificity to the CREB1-mediated gene regulation during neural/glial differentiation. Our current research on the EPA-CREB axis could reveal new molecular targets for treating neurogenerative disease.

KEYWORDS:

Lipid signaling; Embryonic stem cell; Eicosapentaenoic acid; Transcription factor CREB; HLH protein; Multiprotein complex

ABBREVIATIONS:

bHLH, basic-helix-loop-helix; Ca²⁺, calcium(II) ion; CREB, cyclic AMP response element-binding protein; EPA, eicosapentaenoic acid; GPR40, G-protein coupled receptor 40; ID, inhibitor of DNA binding; LIF, leukemia inhibitory factor; mESC, mouse embryonic stem cells; p-CREB1, CREB1 phosphorylation; PLP, proteolipid protein, PPAR, peroxisome proliferator-activated receptor; PUFAs, polyunsaturated fatty acids; RA, retinoic acid; RXR, retinoid X receptor; t-CREB1, total-CREB1.

57 1. INTRODUCTION

58 The benefits of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid
59 (22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) have been reported in various
60 neurological disorders [1, 2]. We recently showed that diets enriched with EPA
61 ameliorated the clinical course of experimental autoimmune encephalomyelitis
62 induced in Dark Agouti rats by both triggering myelin repair mechanism and
63 modulating the autoimmune response [2]. In addition, we found that EPA prevents
64 total brain and myelin damage in the cuprizone rat model of multiple sclerosis based
65 on the selective toxicity for oligodendrocytes [3]. However, the underlying
66 mechanisms responsible for the beneficial effects of PUFAs are not well clarified.
67 The neuroprotective effects of EPA and docosahexaenoic acid may result from a
68 variety of overlapping mechanisms including modulation of membrane fluidity and
69 permeability [4, 5], alterations in pro- and anti-inflammatory response [6, 7], and
70 regulation of transcription factor activities and gene expression [8-10]. Interestingly,
71 non-esterified EPA and docosahexaenoic acid may regulate gene expression via
72 retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR)
73 [11-13].

74 In C6 glioma cells the effects of EPA treatment on myelin gene expression
75 appeared more pronounced than those of docosahexaenoic acid and these effects are
76 likely mediated via cyclic AMP-dependent pathway [14]. In vivo studies indicate that
77 early events involving CREB1 phosphorylation (p-CREB1) are coincident with the
78 activation of myelin gene expression in EPA treated rat pup brain [15]. Concordantly,
79 an interesting “PUFA-G-protein coupled receptor 40 (GPR40)- CREB” signaling
80 pathway has been hypothesized for adult primate neurogenesis [16]. Probably distinct
81 signaling pathways control specific temporal phases of p-CREB1 into specific gene
82 expression programs [17]. However, p-CREB1 alone is not sufficient for target gene
83 activation, as shown by genome-wide analysis of CREB target gene activation [18].
84 Only a minor part of signals that promote p-CREB1 may induce gene activation [19].
85 A physiologically relevant mechanism could be CREB1 interaction with regulatory

86 partners for stable recruitment of the transcriptional apparatus to the promoter of
87 selective CREB1 target genes. The identification of these regulatory partners may
88 allow the determination of the potential contribution of CREB1 interacting factors
89 and their activation in the regulation of stage-specific and signal-dependent
90 developmental responses in immature or mature neural/glial cells from different brain
91 regions.

92 Recent studies have suggested that PUFA enhances rat neural stem cells by
93 regulating bHLH transcription factors [20]. Several lines of evidence suggest that ID
94 family members, categorized as Class V HLH factors [21], are involved in self-
95 renewal, proliferation and adhesion to the niche of neural stem cells during
96 development [22, 23]. Interestingly, a recent work suggests a more sophisticated role
97 of Class I bHLH (E proteins) such as E12 and E47 binding partners of ID proteins in
98 orchestrating neural stem cell differentiation [24]. However the precise mechanism
99 behind E proteins regulation in differentiating neural stem cells is currently unknown.

100 The CRE-DNA binding complexes and the protein partners of CREB1 were
101 investigated in the rat pup brain and mESCs before and after EPA treatment. Our
102 results suggest that EPA stimulates neural/glial differentiation by modulating the
103 levels of HLH/bHLH transcription factors and promoting a direct cross-talk between
104 CREB1 isoforms and HLH/bHLH family members. We propose that a
105 [CREB1]₂:ID2:E47 tetrameric complex plays a key role in an EPA-CREB signaling
106 pathway during neural differentiation. The identification of ID2 and E2A as
107 regulatory partners of CREB1 in brain tissues and retinoic acid (RA) differentiating
108 mESC, reveals new insights to explain how CREB1 can elicit distinct and stage-
109 specific transcriptional programs during neurogenesis and in other differentiation
110 process.

111
112
113

114 **2. Materials and Methods**

115 *2.1. Animal Treatment*

116 All experiments were performed according to the European Community Council
117 Directive of 24 November 1986 (86/609/ECC) and Italian legislation (DL 116/92)
118 for the treatment and handling of animals. The Italian Ministry of Health approved
119 this study (DM 151/2004-B). Sprague-Dawley rats were obtained from a Charles
120 River colony. Litter size was adjusted to eight pups upon parturition. In our previous
121 investigation [15] we calibrated the dose response effect of EPA in stimulating PLP
122 gene expression. We found that PLP gene expression was more pronounced in rats
123 treated with 10 mM EPA. For this reason, all the experiments were carried out under
124 this experimental condition. In detail, four pups from each litter were injected
125 intracerebroventricularly on postnatal day 2 with 3 μ l of 10 mM EPA (Sigma, St.
126 Louis, MO) in Dulbecco's modified Eagle's high-glucose medium (DMEM;
127 Invitrogen Life Technologies, Bethesda, MD) as described [15, 25]. The four
128 remaining control pups received 3 μ l of DMEM. At 1h, 6h, and 24 h after EPA
129 injection, pups were sacrificed by decapitation. The brains were carefully removed
130 and dissected into medulla, cerebellum, and cerebral cortex. The isolated regions,
131 pooled for each experiment, were quickly frozen in liquid nitrogen for nuclear protein
132 extraction. At least three different litters were analyzed at specific time points.

133 *2.2. Cell culture*

134 The feeder-independent mESC line CGR8 was cultered in Glasgow's Modified
135 Eagle's Medium (GMEM) supplemented with 10% fetal calf serum, 2 mM L-
136 glutamine, 1% nonessential amino acid solution, 1 mM sodium pyruvate, 0.1 mM 2-
137 mercaptoethanol (Sigma), 100 U/mL penicillin, 100 mg/mL streptomycin, and 1000
138 U/mL human leukemia inhibitory factor (LIF) as previously described [26]. ESC
139 differentiation in monolayer was induced by withdrawal of LIF for 24 h, followed by
140 treatment with 10^{-6} M RA for 24hr. In some experiments, 10 mM EPA was added to

141 the complete ESC medium in the LIF or RA treated ESC culture and the cells were
142 collected at the indicated time points.

143 2.3. Immunoblotting

144 The nuclear extracts from cortex, cerebellum, and medulla were obtained as
145 previously reported [15]. The different brain regions were homogenized in a buffer
146 containing 10 mM sodium HEPES (pH 7.9), 10 mM KCl, 0.5 mM EDTA, 0.1 mM
147 EGTA, 5 mM DTT, 0,5% Nonidet P-40 (Sigma-Aldrich, Saint Louis, MO) and
148 protease inhibitor cocktail (Sigma-Aldrich).

149 In addition, the nuclear extract from undifferentiated and RA differentiated ESCs
150 were done as previously described [27]. The protein concentrations of all extracts
151 were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).
152 Proteins (20-25 µg per lane) were resolved by SDS-PAGE, and electroblotted onto
153 nitrocellulose membranes for 2 hr at 4°C. After blocking with 5% nonfat dry milk in
154 TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) overnight at 4°C, the blots
155 were incubated with specific primary antibodies at different incubation time from 2hr
156 to overnight depending on the antibody used. Primary antibodies for immunoblotting
157 were: CREB1 (catalog number 05-767; Upstate), p-CREB (Ser¹³³) (catalog number
158 06-519; Upstate) CREB (catalog number 9197; Cell Signaling Technologies), p-
159 CREB (Ser¹³³) (catalog number 9198; Cell Signaling Technologies) and Id1 (C20)
160 (catalog number sc-488), Id2 (C20) (catalog number sc-489), Id3 (C20) (catalog
161 number sc-490), Id4 (L20) (catalog number sc-491), E12 (H208) (catalog number sc-
162 762), E47 (N-649) (catalog number sc-763) (all from Santa Cruz Biotechnology).
163 Secondary anti-mouse or anti-rabbit antibody coupled to horseradish peroxidase were
164 from Bio-Rad Laboratories. Positive signals were revealed by an enhanced
165 chemiluminescence (ECL) detection kit (SuperSignal Substrate, Pierce) according to
166 the manufacturer's instructions. After being stripped, the membranes were reprobed
167 with anti-actin monoclonal antibody (Chemicon, Temecula, CA). Immunoblots were
168 quantified by densitometry using the Bio-Rad Gel Doc 2000.

169 2.4. Coimmunoprecipitation

170 For coimmunoprecipitation, primary antibody and normal rabbit serum were
171 coupled to protein A or G Dynabeads (Invitrogen) and then incubated with nuclear
172 extracts prepared from rat pup brain or ESCs. The lysate-beads/antibody conjugate
173 mixtures were incubated overnight at 4°C with gentle circular rocking.
174 Immunoprecipitated proteins were eluted from the beads using a magnetic particle
175 concentrator and then subjected to SDS-PAGE and immunoblotting. CREB1 (catalog
176 number 05-767; Upstate), Id2 (catalog number sc-489; Santa Cruz Biotechnology)
177 and E47 (catalog number sc-763; Santa Cruz Biotechnology) antibodies were used for
178 coimmunoprecipitation.

179 2.5. EMSA

180 Electrophoretic mobility shift assay (EMSA) was performed as previously
181 reported [27]. Each 20 µl of reaction mixture contained 10–25 µg of nuclear extract,
182 10 mM HEPES (pH 7.9), 50 mM KCl, 2 mM MgCl₂, 4% Ficoll, 1 mM EDTA, 1 mM
183 DTT, 0.5 µg of poly (dI-dC), and 1–3 pmol of a [γ -³²P]ATP -labeled double-stranded
184 oligonucleotide probe containing the CRE consensus sequence (underlined),
185 5' GGCTGTAGAATTTGACGTCATCACTCATTACACTGA-3' (top strand). After
186 15 min of incubation at room temperature, the assay mixture was loaded onto a 4%
187 polyacrylamide gel containing 0.25 TBE (25 mM Tris-borate, 1 mM EDTA) as the
188 running buffer and electrophoresed at 180 V for 4-5 hr at 4°C. Free probe was run off
189 the bottom of gels to facilitate the clear identification of high molecular weight
190 shifted bands.

191 In some binding reactions, the extracts were preincubated for 10-15 min at room
192 temperature with 1-2 µl of one of the following reagents: CREB1 (catalog number
193 05-767; Upstate), p-CREB1 (Ser¹³³) (catalog number 06-519; Upstate) CREB
194 (catalog number 9197; Cell Signaling Technologies), p-CREB (Ser¹³³) (catalog
195 number 9198; Cell Signaling Technologies), anti-Cyclin D1 (catalog number 556470,

196 BD Biosciences), normal rabbit serum (Bio-Rad Laboratories) and Id1 (C20X)
197 (catalog number sc-488X), Id2 (C20X) (catalog number sc-489X), Id3 (C20X)
198 (catalog number sc-490X), Id4 (L20X) (catalog number sc-491X), E12 (H208X)
199 (catalog number sc-762X), E47 (N-649X) (catalog number sc-763X), ATF3 (C19X)
200 (catalog number sc-188X), ATF4 (also designated CREB-2) (C20X) (catalog number
201 sc-200X), control IgGs (all from Santa Cruz Biotechnology).

202 From 50 to 300-fold molar excess of CRE or non CRE-containing OCT-1 (top strand
203 5' - TGTCGAAATGCAAATCACTAGAA -3') cold oligonucleotide competitor (Oct1
204 box is underlined) was included in some binding reaction mixtures.

205 2.6. Chromatin immunoprecipitation (ChIP) assays

206 Undifferentiated and 24h EPA (10 mM) treated ESCs were cultured as described
207 above. ChIP assays were performed as previously described [28] with minor
208 modifications. Briefly, formaldehyde (Fisher Scientific, Pittsburgh, PA) was added to
209 the culture medium to a final concentration of 1%. For each immunoprecipitation, a
210 total of 2×10^6 of ESCs untreated or treated with EPA were cross-linked and sonicated.
211 Sheared chromatin samples were immunoprecipitated overnight at 4°C with
212 antibodies specific for anti-CREB1 (3 µg; catalog number 05-767, Upstate) or an
213 irrelevant anti-cyclin B1 antibody (3 µg; catalog number sc-752, Santa Cruz
214 Biotechnology). The final DNA pellets were resuspended in ultra-pure H₂O (Life
215 Technologies) and subjected to PCR. Samples representing 0.02% of total chromatin
216 (input) were included in the PCR analysis. A genomic region of 364 bp containing a
217 non canonical CRE binding site in the mouse myelin proteolipid protein (PLP)
218 promoter (GenBank: X66421.1) was amplified by PCR using specific primers
219 flanking the non canonical CRE site (forward)(957 /977 bp) 5'
220 TTTTGAGCCTGGTCACACACAG3' and reverse (1301 /1321 bp)
221 5'GGATTCAAGAACCCCTCCAT3' and PCR conditions: 94°C for 5min; 40 cycles
222 of (95 °C for 60s; 58°C for 60s; 72°C for 90s) and 72°C for 5min. PCR products
223 were analysed by Southern blot analysis and hybridized with an internal ³²P-labeled

oligonucleotide probe containing the putative CRE-like sequence (underlined) in the mouse myelin promoter (1239/1268 bp):

5'CATGACTTTACATGCTCAGACCCAGGTATG3'. The Southern blot analysis and hybridization was performed as previously described [29]. ChIP experiments were repeated three times with similar results.

2.7. Building of structural model

We used the CREB1 basic leucine zipper domain (residues 285-339) and the helix-loop-helix domains of ID2 (residues 30-88) and E47 (residues 543-602) to build a model for the interaction of CREB1 and ID2:E47 heterodimer. The basic leucine zipper domain is crucial for CREB1 DNA binding, dimerization and interactions with transcriptional coactivators (e.g. CRT2) [30]. The HLH domain of both ID2 and E47 is essential for heterodimer recognition [31, 32]. Starting from the crystal structure of the [E47:NeuroD1]₂ tetramer (PDB ID: 2QL2) (Fig. 6A), we replaced by overlay one [E47:NeuroD1] unit by a [CREB]₂:DNA unit (PDB ID: 1DH3); and substituted NeuroD1 by ID2 (PDB ID: 4AYA) in the other [E47:NeuroD1] unit using the program PyMol (The PyMOLGraphics System 2002, DeLano Scientific, Palo Alto, CA, USA [<http://www.pymol.org>]).

After visual inspection of this initial model, we noted a small cleft between the CREB1 homodimer and the ID2:E47 unit. We tested therefore if the size of the contact surface (interface) could be increased by a slight relative rotation of the CREB1 homodimer and the ID2:E47 unit around the two axes indicated in Fig. 6A. Using the program CHARMM [33], we performed a 2-dimensional scan that monitored the size of the interface when the two rotational angles are varied from -60° to +60° in steps of 15°. Initial input files for CHARMM were generated with the CHARMM-GUI [34]. The size of the interface between the ID2:E47 unit and the CREB1 homodimer was calculated as follows:

$$I = \{ A([\text{CREB}]_2) + A(\text{ID2:E47}) - A([\text{CREB1}]_2:\text{ID2:E47}) \} / 2, \quad (\text{Eq. 1})$$

where $A[X]$ stands for the surface area of complex X. The surface areas were calculated with the program CHARMM (command “COOR SURF”) using the atomic radii from all-atom force field c36 and a solvent probe radius of 1.6 Å. Supplementary Table 1 lists the size of the interface as a function of the two scanned angles. The function is rather peaked with a clear maximum for (-30°, -15°). The negative sign indicates a rotation in left-handed direction. Through these relative rotations the size of the interface could be increased by 10% from 922 to 1012 Å². To judge if a given relative rotation would lead to steric clashes, we also determined the minimal distance between the backbone Calpha atoms of the CREB1 homodimer and the ID2:E47 unit for each combination of rotational angles (see last column in Supplementary Table 1). We consider a minimal distance of less than 3.5 Å as steric clash [35, 36]. The rotation (-30°, -15°) slightly increases the minimal distance from 4.2 to 4.9 Å. Thus no steric clash is observed for the rotation with maximal interface size.

Finally, to investigate if an alternative binding mode (where E47 faces the CREB1 dimer instead of ID2) is also probable, we performed docking studies. We docked the ID2:E47 complex (ligand) to the CREB1 homodimer (receptor) using the ZDOCK server (<http://zdock.umassmed.edu>) with the software version 3.0.2. [37] Residues 285-295 of CREB1 were blocked because they form the DNA binding site. For the 10 top-ranked ZDOCK predictions we calculated the contribution of ID2 and E47 to the buried surface area:

$$B_{\text{ID/E47}} = A_{\text{ID/E47}}(\text{ID2:E47}) - A_{\text{ID/E47}}([\text{CREB1}]_2:\text{ID2:E47}) \quad (\text{Eq. 2})$$

with $A_Y[X]$ the contribution of subunit Y to the surface area of complex X. Using the program CHARMM, we first added hydrogen atoms to a given predicted structure with the command HBUILD and then calculated the difference in surface area between the dimer ID2:E47 and the multimer $[\text{CREB1}]_2:\text{ID2:E47}$. The calculation of

the surface area is detailed above. The command “SCALAR WMAIN STAT” permits the extraction of the contribution of two subunits ID2 and E47 to the buried surface area. Note that the sum of these two contributions is slightly different from the value of the interface area because the former (Eq. 2) considers only contributions from ID2 and E47 whereas the latter (Eq. 1) is an average over the contributions of ID2, E47 and the CREB1 homodimer.

2.8. Molecular dynamics simulations

The structure of the tetramer [CREB1]₂:ID2:E47 bound to DNA was simulated with the CHARMM all-atom force field c36 [38] using the program ACEMD [39] with GPU-acceleration (Nvidia GTX). Standard parameters were used if not specified. The liquid phase was mimicked with 46788 explicit TIP3P water molecules and (charge-neutralizing) counter-ions (CaCl, 0.15 M) in a cubic box with an initial side length of 112 Å. Periodic boundary conditions were applied. The cutoff for short-range nonbonded interactions was 9 Å; long-range electrostatic interactions were treated with Particle-Mesh Ewald summation with a grid spacing of 0.875 Å. The equation of motion was integrated with a time step of 4 fs with constrained hydrogen bonds and a scaling factor of 4 for ACEMD’s hydrogen masses redistribution. The temperature of the system was set to 310 K (Langevin thermostat). During the first 25 ns, the molecular dynamics was carried out at constant temperature and pressure (Berendsen barostat) to equilibrate the volume. From the last 5 ns of this equilibration phase we determined an average side-length of 113.8 Å for the cubic simulation box. The remaining 500 ns of molecular dynamics were carried out at constant volume and temperature. Snapshots were saved every 1 ns.

2.9. Statistical Analysis

Statistical analysis for biochemical studies was carried out using the Student’s t-test, with $P < 0.05$ defined as statistically significant.

304 To quantify the amount of variation in the contribution of subunits ID2 and E47 to
305 the buried surface area, we calculated the uncorrected sample standard deviation of
306 the set of docking predictions.

307

308 **3. RESULTS**

309

310 *3.1. p-CREB1 levels and CREB1 occupancy on the PLP promoter in ESCs after EPA* 311 *treatment*

312 Our previous results in the rat pup brain indicate that EPA treatment induces a
313 significant decrease in p-CREB1 both in the cortex and in the cerebellum 1h post-
314 injection, whereas no significant difference was found at 16 h post-injection [15]. We
315 extended this analysis to mESCs, a well-established *in vitro* model of early
316 development for the identification of EPA-derived signaling molecules with
317 therapeutic potentials [40-42]. Initially, we measured the levels of CREB1 protein at
318 different time points of EPA treatment. Our results show that p-CREB1 significantly
319 increases 3 h after EPA treatment compared to LIF control sample (Fig. 1A, B).
320 Afterwards, p-CREB1 decreased from 6h until 24 h, whereas the levels of total-
321 CREB1 (t-CREB1) protein were relatively constant from 1h to 24 h after EPA
322 treatment (Fig. 1 A, C). In conclusion EPA up-modulates p-CREB1 at early times of
323 ESCs treatment with a kinetic that differs from that reported in rat pup brains [15].

Fig. 1. p-CREB1 levels and CRE-DNA binding activity in mESCs and rat pup brains after EPA treatment. (A) Western blot analysis of t-CREB1 and p-CREB1 in EPA treated ESCs at different time points. (B, C) The levels of p-CREB (B) and t-CREB (C) were analysed by densitometric scanning and reported in the histogram after normalization with β-actin. Results are mean ± SEM of four independent experiments. *P < 0.05 vs. control. (D) ChIP analysis of CREB1 occupancy on the PLP proximal promoter in EPA treated ESCs. Cross-linked chromatin fragments from LIF-treated (lanes 3, 6) or EPA+LIF treated (lanes 2,4,5,7) ESCs were incubated with anti-CREB1 antibodies (lanes 2, 3) or irrelevant anti-cyclin B1 antibodies (lane 4). Immunoprecipitated DNA was analyzed by PCR using primers specific for the mouse myelin PLP promoter. Samples representing 0,02% of total chromatin were included in the PCR analysis (lanes 5-7). (E to F) Supershift analysis with anti-t-CREB1 and anti-p-CREB1 antibodies in untreated or EPA treated nuclear extracts from the cortex (E), medulla and cerebellum (F, G). Nuclear extracts from the cortex were used as positive control (F, G, lane 8). Asterisks indicate the supershifted complexes. NS, are nonspecific bands; NRS, normal rabbit serum.

CREB1 is an important mediator regulating the expression of myelin-specific genes. Interestingly, a CRE binding site is present in the promoter region of the genes encoding for both myelin PLP and myelin oligodendrocyte glycoprotein [43-45]. In addition, the level of PLP mRNAs increased in nearly all brain regions of EPA

344 treated pups [15]. A CRE-like binding sequence 5'-TGACTTCA-3' (instead of the
345 consensus CRE binding site 5'-TGACGTCA-3') is located within the distal footprint
346 FP330 of the rat PLP promoter [43]. In mice, a non-canonical CRE-binding sequence
347 5'-TGACTTTA-3' (6/8 identical to the canonical CRE) is present at the orthologous
348 CRE site of the rat PLP promoter (see Materials and Methods for details).

349 To determine whether CREB1 binds to the non-canonical CRE-binding sequence in
350 the PLP promoter, we initially performed ChIP assays in cross-linked chromatin from
351 ESCs untreated or treated with EPA for 24 h using anti-CREB1 antibodies or the
352 unrelated anti-cyclin B1 antibodies used as negative control (Fig.1D). The
353 immunoprecipitates were amplified by PCR using primers flanking the non-canonical
354 CRE-binding sequence in the PLP promoter region and hybridized to the internal
355 ³²P-labeled oligonucleotide PLP probe containing the non-canonical CRE site as
356 described in Materials and Methods. Our results showed that CREB1 is recruited on
357 PLP target gene after 24h EPA treatment in ESCs but not in the control sample (Fig.
358 1D, lanes 2 and 3 respectively). This result confirms that EPA treatment induces the
359 CREB1 occupancy at a non-canonical CRE site in the PLP promoter.

360

361 *3.2. The CRE-DNA binding activity in different brain regions of pups after EPA* 362 *treatment*

363 To characterize the molecular mechanism of CREB1 transcription function in
364 vivo, we analyzed the CRE-DNA binding activity in different brain regions of pups
365 intracerebroventricularly treated or untreated with EPA. We performed EMSA
366 analysis using a ³²P-labeled double-stranded oligonucleotide probe containing a
367 canonical CRE consensus sequence. According to our EMSA analysis of nuclear
368 protein extracts from the cortex, the CRE DNA-binding activity is composed of
369 several protein complexes including two high molecular weight complexes (complex
370 I and II), three intermediate molecular weight complexes (CRE1, CRE2 and CRE3),
371 and low molecular weight nonspecific bands (Fig. 1E). One of the most distinct

nonspecific bands is indicated by NS (Fig. 1E). Sequence specificity of binding was verified by the addition of 50-fold and 300-fold molar excess of cold competitor containing the canonical CRE box element (Supplementary Fig. S1, lanes 2 and 3) or with a non-CRE oligonucleotide containing OCT-1 sequence (Supplementary Fig. S1, lane 4). Similar shifts were also observed using medulla and cerebellum nuclear extracts (Fig. 1 F and G) indicating the critical role of each one of the CRE-specific DNA-binding complexes in the rat pup brain. Interestingly, the CRE1 is the most abundant intermediate molecular weight complex in all brain regions (Fig. 1E, F and G). On the other hand, the CRE3 complex is mainly present in the cortex (Fig. 1 E). Furthermore, the CRE1 and CRE2 complexes are sharply supershifted in the untreated or EPA treated cortex (Fig. 1E, lanes 3 and 7), medulla (Fig. 1F, lanes 2 and 4), and cerebellum (Fig. 1G, lanes 6 and 7) by incubation with an anti t-CREB1 antibody, but not with a normal rabbit serum (Fig. 1E, lanes 2 and 6). Similar results of supershift assays were obtained with an anti-p-CREB1 antibody in the untreated or EPA treated cortex (Fig. 1E, lanes 4 and 8), medulla (Fig. 1F, lanes 2 and 4), and cerebellum (Fig. 1G, lanes 6 and 7) to show that the CRE1 and CRE2 complexes contain an active p-CREB1 protein. Unfortunately, the presence of p-CREB1 was not assessable in the complex I due to an intense supershift band of the anti-p-CREB1 that colocalized within complex I binding region on the autoradiogram masking this complex. Therefore, in different brain regions of pups, at least CRE1 and CRE2 complexes contain canonical p-CREB1 (Ser133) proteins. Probably, the CRE1, CRE2 and CRE3 complexes are generated by dimerization of different splice variants of CREB1 gene as the slower migrating CREB1- α isoform and the faster migrating CREB1 Δ isoform [46] or by ATF1/CREB1 heterodimerization [47]. However, CREB1 interactions with other as yet unidentified novel partners cannot be excluded. Intriguingly, we found similar shift and supershifts of CRE-DNA binding complexes in untreated or 1h EPA-treated samples of nuclear extracts from different rat pup brain regions (Fig. 1 E, F, G).

3.3. The effect of EPA on the CRE-DNA binding activity in mESCs after LIF withdrawal and RA-induced differentiation

With the aim to better characterize changes in the CRE DNA-binding complex during multi-protein complex formation in early development, we extended our investigation to EPA treated mESCs that provide a suitable in vitro model for neural-glia differentiation [48]. As shown in Fig. 2A, incubation of a ³²P-labeled double-stranded oligonucleotide probe containing a CRE-box consensus sequence with LIF-treated mESC extracts generated specific protein-DNA complexes. In particular, complex I and CRE1, CRE2 and CRE3 complexes, were specifically competed by the addition of 50-fold, 150-fold and 300-fold molar excess of cold competitor oligonucleotide containing the canonical CRE sequence (Fig. 2A, lanes 2-5). The higher and lower-mobility complexes were unspecific bands (Fig. 2A, the NS1 and the NS2 band, respectively). In the LIF-treated ESC extracts (Fig. 2A, lane 2), the complex II present in the rat pup brain was not detected and the CRE-1 complex was less abundant compared to the CRE2 and CRE3 complexes. Interestingly, the CRE2 and CRE3 complexes were present in larger quantity in the LIF-treated ESCs when compared to the cortex of rat pup brain (Fig. 2A, lanes 1 and 2). Moreover, the CRE1 complex was more abundant in the cortex than in the undifferentiated ESCs (Fig. 2A, lanes 1 and 2). The complex I, CRE1 and CRE2 complexes were supershifted by incubation with anti-p-CREB1 antibody (Fig. 2B, lane 4) while they were not affected by normal rabbit serum (Fig. 2B, lane 2). In addition, the complex1, CRE1, CRE2 and CRE3 complexes were sharply decreased by incubation with anti-t-CREB1 (Fig. 2B, lane 3). As a general comment to our EMSA data, we believe that the disappearance or decrease of a shifted band without causing a supershift can support the identification of the protein of interest when used with the proper controls (e.g. normal rabbit serum). Therefore, our results strongly suggest that p-CREB1 and apparently t-CREB1 are present in the complex I as well as the CRE1, CRE2 and CRE3 complexes in LIF-treated ESCs.

Fig. 2. The effect of EPA on the CRE-DNA binding activity in ESCs after LIF withdrawal and RA-induced differentiation. (A) Competition EMSA with 50 to 300-fold molar excesses of cold CRE wild-type oligonucleotides in LIF-treated ESCs. (B, C) Supershift analysis with anti-t-CREB1 and anti-p-CREB1 antibodies in LIF-treated ESCs (B) or EPA treated ESCs after LIF withdrawal (C). (D, E) Changes in the profile of CRE DNA-binding activity in ESCs after LIF withdrawal and RA-induced differentiation. EMSA analysis was performed with anti-E12 antibodies (D) and anti-ID2 antibodies (E) in the corresponding EPA plus RA treated samples. Asterisks indicate the supershifted complexes (B, C, E). Dots denote distinct bands

437 that are decreased in signal intensity (D). NS1 and NS2 are nonspecific bands; NRS, normal rabbit serum.

438

439 We next investigated the CRE-DNA binding activity in ESCs treated with EPA
440 for 24h after LIF withdrawal. Intriguingly, the high molecular weight complex I and
441 the CRE1 band present in the LIF-treated ESCs, were barely detected in extracts from
442 ESCs treated with EPA for 24h after LIF withdrawal (Fig. 2C, lane 1). On the other
443 hand, the CRE2 and CRE3 complexes were both present in the EPA treated ESCs
444 after LIF withdrawal (Fig. 2C, lane 1). Furthermore, nuclear extracts from EPA
445 treated ESCs were incubated with anti-t-CREB1 and anti-p-CREB1 antibody (Fig.
446 2C, lanes 3 and 4). The anti-p-CREB1 antibody generates a sharp supershift band of
447 the CRE1 and CRE2 complex (see the dashed line with asterisk in Fig. 2C, lane 4). In
448 addition, the CRE1 and CRE2 complexes were decreased by incubation with anti-t-
449 CREB1 antibodies (Fig. 2C, lanes 3 and 4), while they were not affected by normal
450 rabbit serum (Fig. 2C, lane 2). The downmodulation of complex I and CRE1 complex
451 after LIF withdrawal suggests a cross-talk between the LIF/STAT3 and the EPA-
452 PPAR/RXR-CREB signaling pathway occurring during ESC self-renewal.

453 Remarkably, RA-induced differentiation of mESCs has been previously used as a
454 cellular model for studying neural commitment and specification [48–50]. Taking
455 this into account, we investigated the CRE DNA-binding complexes induced by RA
456 in early differentiating mESCs. Outstandingly, the CRE DNA-binding complexes
457 were rapidly changed by RA treatment of mESCs showing a pattern of shift bands
458 very similar to the observed shifted bands in pup brain tissues (Fig. 2, compare lane 1
459 in panel A with lane 2 in panel D). More precisely, in the mESCs induced to
460 differentiate by RA treatment for 24h, the complex I and II were successfully
461 reformed and the CRE-1 band was up-modulated when compared to the pattern of
462 CRE-DNA binding complexes observed in the mESCs after LIF withdrawal (Fig. 2C,
463 lanes 1 and 2). In the EPA treated mESCs after 24 h of RA-induced differentiation,

464 complex I and complex II were mainly up-modulated according to the time of EPA
465 treatment (1h, 3h and 6h), reaching the highest intensity at 6h after treatment (Fig.
466 2E, lanes 2, 4 and 6).

467

468 *3.4. Changes in the level of ID proteins in the rat pup brain and mESCs after EPA* 469 *treatment*

470 To determine whether EPA affects ID2 transcription factor activity, the level of
471 ID2 protein was investigated in untreated and EPA treated rat pup brains at 1h, 6h
472 and 24 h. We found that at 1h, the ID2 protein is highly expressed in untreated
473 nuclear extracts from the cortex, less abundant in the cerebellum, and far less
474 expressed in the medulla (Fig 3A). Interestingly, western blot analysis (Fig. 3A) of
475 pup brain nuclear extracts prepared after 1h EPA treatment showed an increase of
476 ID2 protein expression in the cortex, cerebellum and medulla. More precisely, the
477 ID2 protein increased (32%) in the medulla and in the cerebellum (18%) as well as in
478 the cortex (18%) at 1h after EPA treatment (Fig. 3A). Modulation of ID2 levels may
479 represents an early event caused by EPA treatment in the rat pup brain. On the other
480 hand, the ID2 protein decreased in the medulla by 28% and in the cortex by 25%
481 after a 6h EPA treatment (Fig. 3B). Moreover, we observed a more significant
482 decrease of ID2 in the medulla (49%) and in the cortex (24%) after a 24 h EPA
483 treatment (Fig. 3C). In the cerebellum, no significant differences were found in the
484 ID2 level after 6h and 24 h EPA treatment compared to the control samples (Fig. 3B
485 and C). The early ID2 activation in the pup brain regions was coincident with the
486 decrease of the p-CREB1 activity in the cortex and cerebellum after 1 hr of EPA
487 treatment [15].

Fig. 3. Changes in the level of ID proteins in the rat pup brain and mESCs after EPA treatment. (A, B, C) Western blot analysis of ID2 protein in the medulla (lanes 1,2), cerebellum (lanes 3,4) and cortex (lanes 5,6), untreated or treated with EPA after 1h (A), 6h (B) and 24h (C). (D) Western blot analysis of ID1 protein in rat pup brains untreated or treated with EPA after 6h. (E to H) Western blot analysis of ID1 (E), ID2 (F), ID3 (G) and ID4 (H) proteins in mESCs after EPA treatments at different time points. The blots were subsequently re-probed with anti-β-actin antibodies to monitor equal loading of the samples. ID1, ID2, ID3 and ID4 levels were analyzed in the rat pup brain and mESCs by densitometric scanning and reported in the histogram after normalization to the β-actin. Values are expressed as mean ± SEM of three independent experiments. *P<0.005 vs. control.

We also investigated the expression levels of the ID1 protein in untreated and EPA treated pups. Interestingly, the only relevant difference of ID1 protein level compared

501 to ID2 modulation after EPA treatment, was a very sharp decrease (92%) of ID1 in
502 the medulla after 6h EPA treatment (Fig. 3D). However, no significant difference of
503 ID1 levels was detected in the cerebellum and in the cortex after 6h EPA treatment
504 (Fig. 3D). The ID1 downregulation by EPA is an interesting finding, which should
505 form the basis for future studies. During brain development, EPA might accelerates
506 the myelinogenesis process in the medulla possibly according to a temporal-spatial
507 pattern of myelination, an hypothesis supported also by findings reported in [15].

508 Importantly, it has been well established that ID proteins are involved in the
509 BMP/SMAD pathway that plays a key role in ESC self-renewal [51]. To better
510 evaluate the EPA effect on ID proteins activity, the levels of ID1, ID2, ID3 and ID4
511 were investigated at different time points in EPA treated ESCs. Our results showed
512 that ID1 protein expression increased almost four fold after 3h of EPA treatment with
513 respect to the LIF treated ESCs used as control (Fig. 3E). In addition, we observed a
514 significant decrease (88%) of ID1 expression level after 24h of EPA treatment. On
515 the other hand, ID2 proteins showed a slight decrease from 1h up to 6 h after EPA
516 treatment with respect to the control LIF ESCs (Fig. 3F). Interestingly, ID2 increased
517 significantly (1.7 fold) over basal LIF level at 24 h following EPA treatment,
518 suggesting a delayed ID2 activation. The expression of ID3 proteins after EPA
519 treatment was similar to that of ID1 protein, reaching a peak at 3h when compared to
520 the LIF treated ESC levels (Fig. 3G). Finally, ID4 protein expression decreased
521 progressively up to 24h after EPA treatment of undifferentiated ESCs (Fig. 3H).

522 Altogether, our results show only modest changes in the expression levels of ID2
523 after EPA-treatment in the medulla, cerebellum and cortex of neonatal rats. In
524 contrast to the brain tissues, EPA treatment of undifferentiated mESCs had a more
525 pronounced effect on IDs expression levels. Probably in LIF-treated mESCs, EPA
526 cooperate with LIF/STAT3 pathway to sustain mESC self-renewal through the
527 induction of IDs (particularly ID1 and ID3 early expression), thus blocking neural
528 lineage committment [51]. However, the upregulation of ID2, which is delayed with
529 respect to ID1 and ID3, could be the consequence of a tight control of ID2 expression

530 levels in LIF-treated mESCs. Indeed, elevated ID2 expression deregulates neural
 531 stem cells self-renewal in mice [52]. In conclusion, EPA-treatment affects diversely
 532 the ID protein expression levels in mESCs and pup rat brain, suggesting a G-protein
 533 dependent or -independent mechanism for EPA signaling pathway.

534

535 *3.5. CREB1 and ID family members interact in the rat pup brain and in ESCs treated*
 536 *with EPA plus RA*

537 Since the results of EMSA analysis suggest the presence of high molecular weight
 538 CRE DNA-binding complexes in pup neural extracts, we explored the “in vivo”
 539 biochemical interaction between CREB1 and ID2 proteins in rat pup brains. Nuclear
 540 extracts were treated or not with 1h EPA and immunoprecipitated with anti-ID2
 541 antibodies or normal rabbit serum as control. The resulting immunoprecipitates were
 542 immunoblotted using an antibody specific for the CREB1 protein (Fig. 4A).

543
 544

Fig. 4. CREB1 and ID family members interact in the rat pup brain. (A, B) CREB1 is complexed with ID2 protein in the medulla, cerebellum and cortex of the rat pup brain: reciprocal IP and WB analysis. Nuclear extracts from the medulla, cerebellum and cortex untreated or treated for 1h with EPA were used as positive controls (lanes 1 and 2 in each panel). (C to E) EMSA analysis of CRE DNA-binding activity of nuclear extracts from medulla, cerebellum and cortex in rat pups treated with EPA at 1h or untreated after incubation with an anti-ID2 serum (C, lanes 2,4,6,8,10), an anti-ID3 serum (D, lanes 2,4,6,7,8,11) and with an anti-ID1 serum (E, lanes 2,4,6,7,9). Supershifted complexes are marked by asterisks (*). NS, are nonspecific bands.

The CREB1 protein was immunoprecipitated in nuclear extracts from medulla, cerebellum and cortex with an anti-ID2 serum (Fig. 4A, lanes 3 and 4 in each panel) but not with the normal rabbit serum (Fig. 4A, lanes 5 and 6 in each panel). In the reciprocal experiments, nuclear extracts from the medulla, cerebellum, and cortex treated or untreated with 1h EPA were immunoprecipitated with anti-CREB1 antibodies, which co-precipitates only a single band, as revealed by western blot analysis with anti-ID2 antibody (Fig. 4B, lanes 3 and 4 in each panel). This band was absent in the normal rabbit serum immunoprecipitates (Fig. 4B, lanes 5 and 6 in each panel). Taken together, the coimmunoprecipitation and western blot experiments indicate a biochemical interaction between CREB1 and ID2 proteins, thus suggesting the presence of an ID2/CREB1 complex in the rat pup brain.

Based on our coimmunoprecipitation results, we performed supershift analysis with anti-ID2 antibody to elucidate the composition of the two high molecular weight CRE DNA-binding complexes in the rat pup brain. EMSA analysis of the CRE-DNA binding activity showed that only complex I was sharply supershifted when an anti-ID2 antibody was added to the reaction mixture of untreated or EPA treated medulla (Fig. 4C, lane 2), cerebellum (Fig. 4C, lanes 4 and 6), and cortex (Fig. 4C, lanes 8 and 10), but not with the normal rabbit serum used as control (Fig. 4C, lane 9). Therefore, a high molecular weight ID2:CRE-DNA protein complex is present in both untreated or 1h EPA treated brain extracts.

Subsequently, we investigated whether CRE-DNA binding complexes interact with other ID family proteins. EMSA analysis of CRE DNA-binding activity of

575 nuclear extracts from rat pup medulla, cerebellum, and cortex untreated or treated
576 with EPA for 1 h were incubated with anti-ID3 or anti-ID1-specific antiserum. We
577 observed that the addition of an anti-ID3 antibody caused a decrease in the intensity
578 of complex I and II, which was more evident in the medulla (Fig. 4 D, lanes 2 or 4)
579 compared to the cerebellum and cortex (Fig. 4D, lanes 6, 7 and 8, 11 respectively).
580 Similarly, the anti-ID1 antiserum caused a specific decrease in the intensity of the
581 complex I and II in medulla (Fig. 4E, lanes 2 and 4), cerebellum (Fig. 4E, lanes 6 and
582 7), and cortex (Fig. 4E, lane 9). Taken together, these data indicate that ID2, and
583 probably ID1 and ID3 proteins, can interact with high molecular weight CRE-DNA
584 binding complexes in all pup brain regions.

585 Finally, we attempted to further define the protein components of the two high
586 molecular weight CRE DNA-binding complexes that were reformed within 24 h after
587 RA differentiation of ESCs. For this purpose, we performed supershift analysis with
588 an anti-ID2 antibody in EPA plus 24h RA-treated ES cells at different time points.
589 Similar to the EMSA experiments obtained with rat pup brain, we observed that
590 complex I in EPA plus RA-treated ES cell, was sharply supershifted when an anti-
591 ID2 antibody was added to the reaction mixture (Fig. 2E, lanes 3, 5 and 7) but not
592 with the normal rabbit serum used as control (Fig. 2E, lane 8). It is remarkable that
593 the intensity of the complex I supershift caused by the anti-ID2 antibody is related to
594 the length of EPA treatment in the RA differentiating ESCs. We speculate that this
595 dosage dependent kinetic could reflect the induction of EPA treatment in stimulating
596 the binding of ID2 protein into the CRE-DNA binding complex I during early stages
597 of neural commitment of m ESCs. Taking into account the presence of p-CREB1 or
598 t-CREB1 in the complex I of ESCs (Fig. 2B, lanes 3 and 4), these observations
599 strongly suggest that an ID2:CREB1-DNA binding complex was efficiently reformed
600 and up-modulated by EPA treatment during the early stages of RA induced ESC
601 differentiation.

602

603 *3.6. E47 protein expression and CREB1:E2A interaction in RA differentiating ESCs*
604 *and in the rat pup brain after EPA treatment*

605 Although the E2A gene has been regarded as ubiquitously expressed [21],
606 variations in E12 and E47 protein expression among different tissues has been
607 observed [53]. To determine whether EPA affects E2A transcription factor activity,
608 the level of E47 protein was investigated in ESCs treated with RA after LIF
609 withdrawal. Interestingly, western blot analysis of nuclear extracts from RA
610 differentiating ESCs showed an increase (2.8 fold) of E47 protein expression after 24
611 h of EPA treatment compared to the control LIF sample (Fig. 5A and B). This result
612 is consistent with the hypothesis that EPA can modulate E2A proteins in
613 differentiating ESCs. Subsequently, we investigated whether CRE DNA-binding
614 complexes can directly interact with the E12/E47 protein members during RA-
615 induced neural differentiation of mESCs. In our EMSA experiments, we found that
616 the anti-E12 antiserum sharply decreased the intensity of complex I, complex II and
617 complex CRE3 in nuclear extracts from ESCs treated with RA for 24h plus 3h EPA
618 (Fig. 2D, lane 5), while they were not affected by normal rabbit serum used as control
619 (Fig. 2D, lane 4). Therefore, the E12 antibody may affect the intrinsic DNA binding
620 ability of complex I, complex II and complex CRE3 without causing a supershift.
621 These results suggest that E2A plays a role in the early stages of neural induction of
622 ESCs generating functionally varied E2A:CRE DNA-binding complexes.

Fig. 5. E47 protein expression and CREB1:E2A interaction after EPA treatment. (A,B) E47 protein expression in RA differentiating ESCs after EPA treatment at different time points. The levels of E47 protein were analysed by densitometric scanning and reported in the histogram after normalization to β-actin. Values are expressed as mean ± SEM of three independent experiments. *P< 0.05 vs. control. (C, D) E47 is complexed with CREB1 protein in the cortex of the rat pup brain: reciprocal IP and WB analysis. (E) EMSA assay of CRE-DNA binding activity of nuclear extracts from cortex after incubation with an anti-E12 antibody. Extracts from ESCs treated with LIF were used as control (lane 1). Dots denote distinct bands that are decreased in signal intensity by the anti-E12 antibody (lane 4). (F) EMSA analysis of CRE DNA-binding activity of nuclear extracts from medulla, cerebellum and cortex in rat pups untreated (lanes 1,2,5,6,9,10) or treated (lanes 3,4,7,8,11) with EPA after incubation with anti-E12 or anti-E47 antibodies. Supershifted complexes are marked by asterisk (lanes 2,4,6). NS, are nonspecific bands.

Furthermore, we investigated the “in vivo” interactions between CREB1 and E12/E47 proteins in the rat pup brain. Nuclear extracts from cortex were treated or untreated with EPA for 1h and immunoprecipitated with anti-CREB1 antibodies or normal rabbit serum used as control. The resulting immunoprecipitates were immunoblotted using an antibody specific for E47 protein (Fig. 5C). The E47 protein was immunoprecipitated with anti-CREB1 antibodies (Fig. 5C, lanes 3 and 4) but not with the normal rabbit serum used as control (Fig. 5C, lanes 5 and 6). In the reciprocal immunoprecipitation experiments with anti-E47 antibodies (Fig. 5D), only a single immunoprecipitated band was detected by western blot analysis with anti-CREB1 antibody (Fig. 5D, lanes 3 and 4). In conclusion, our coimmunoprecipitation experiments indicate the presence of an E2A:CREB1 complex in the rat pup brain.

646 Finally, we performed supershift experiments with anti-E2A antibodies in the rat
647 pup brain. Briefly, the anti-E12 antiserum caused a specific supershift of both
648 complexes I and II in the medulla (Fig. 5F, lanes 2 and 4) and the cerebellum (Fig.
649 5F, lane 6). In addition, the anti-E12 and the anti-E47 antiserum significantly and
650 specifically decreased the bands related to complex I and complex II in the cortex
651 (Fig. 5E and 5F). Interestingly, the E12 antibody induced a decrease of the CRE2
652 band in the cortex, where this isoform is expressed (Fig. 5E, lane 4). Taken together,
653 our data suggest that E12 and E47 can interact with different CRE DNA-binding
654 complex in specific region of the rat pup brain to form stable dimeric (CREB:E2A),
655 trimeric (CREB:ID:E2A) or tetrameric ($[\text{CREB}]_2\text{:ID:E2A}$) naturally assembled
656 complexes on a CRE consensus site.

657

658 *3.7. Structural model of the $[\text{CREB1}]_2\text{:ID2:E47}$ tetramer bound to the CRE site of* 659 *DNA*

660 Focusing on our hypothesis that ID2, E47 and CREB1 are found in a single
661 complex when bound to the CRE-site (see experiments above), we aimed to build a
662 structural model for this complex. This model is based on the CREB1 basic leucine
663 zipper domain and the HLH domain of ID2 and E47 (as described in Materials and
664 Methods). Crystal structures of the corresponding domains have been solved [32, 54,
665 55]. The stoichiometry and the 3D structural arrangement of the domains in the
666 tetrameric complex are, however, elusive. The helix-loop-helix motifs of ID2 and
667 E47 are known to form homo- and hetero-dimers [32, 54]. EMSA experiments
668 performed using purified E47 and ID2 proteins showed a dose response effect, which
669 is indicative of a direct interaction between these two proteins [32]. Under
670 physiological conditions ID2 represses E47 by forming heterodimers [32, 56].
671 Therefore, we favor a model with E47:ID2 heterodimer. CREB1 homodimerizes

when bound to the CRE-site of DNA [55], but it can also form heterodimers by direct interaction with bHLH proteins as suggested by Skinner and coworkers [57]. From in vitro binding assays, a recent report [30] suggested that CREB homodimer can be stabilized in a tetrameric complex. Therefore, we propose that CREB1 homodimer (bound to the CRE-site of DNA) interacts with E47:ID2 heterodimer. An indication of how these two dimeric units could be arranged in a tetrameric structure comes from the crystal structure of the tetramer of [E47: NeuroD1]₂ (see Fig. 6A, right) [54]. Using this tetramer as template, we obtained an initial model through superimposition with other crystal structures (see Material and Methods). First, NeuroD1 was substituted by ID2 in one of the two NeuroD1:E47 units. NeuroD1 and ID2 feature a very similar three-dimensional HLH arrangement so that the NeuroD1-ID2 exchange leads to a matching interface with E47. Second, by replacing the other NeuroD1:E47 unit with a CREB1 homodimer (Fig. 6A, left) we obtained a multimer that showed a complementary contact surface between the CREB1 homodimer and the ID2:E47 unit with an interface area of 922 Å². There was, however, a small cleft between these two dimers. Slight rotation (see Material and Methods) of the CREB1 homodimer and the ID2:E47 unit relative to each other (as indicated in Fig. 6A) maximizes the size of the interface (1012 Å²) and leads to the structure shown in Fig. 6B. The structural model is available upon request. A short molecular dynamics simulation (500 ns) indicates that this structure is a (meta)-stable conformation (Fig. 6C).

Fig. 6. Structural model and molecular dynamics of the [CREB1]₂:ID2:E47 tetramer bound to DNA. (A) Crystal structure of the CREB dimer (left, PDB ID: 1DH3), and tetramer structure of NeuroD1 (dark grey) and E47 (light grey) (right, 2QL2). (B) It is possible to overlay the CREB dimer on one of the NeuroD1:E47 units and thereby generate a matching binding interface with the other NeuroD1:E47 unit. Replacing NeuroD1 by ID2 yields the structure displayed in the figure. (C) The root-mean-square deviation of the C α , C β -atoms is shown for the complex structure of figure 6B during a molecular dynamics trajectory of 500 ns at temperature of 310 K. Using the initial structure as reference (t=0 ns, open circles), the formation of a plateau is observed at about 70 ns. The remaining trajectory shows a constant and lower RMSD with respect to a reference structure picked at 73 ns (filled squares). (D) Snapshot structure picked at 73 ns of the molecular dynamics trajectory. (For reasons of clarity, terminal and highly flexible nucleotides are not shown). An overlay of all calcium positions sampled during the trajectory reveals the most likely calcium binding sites (blue cloud). (E, F) Two sites are found at the interface of ID2 and CREB1 where glutamic acids from both monomers contribute to the binding.

710 In the model of Fig. 6B the ID2 subunit is the primary interactor with the CREB1
711 homodimer. In principle, one can hypothesize an alternative arrangement, where the
712 E47 unit faces the CREB1 dimer. Docking studies (see Material and Methods)
713 indicate, however, that the CREB1 homodimer preferentially interacts with the ID2
714 subunit: the mean contribution of ID2 to the buried surface area (when averaged over
715 the ten top-ranked docking predictions) is $734 \pm 157 \text{ \AA}^2$. The contribution of E47 is
716 three times smaller ($230 \pm 134 \text{ \AA}^2$). In the model of Fig. 6B these contributions are
717 673 and $309 \text{ \AA}^2$ for ID2 and E47, respectively, in agreement with the docking results.
718 Therefore, the alternative binding mode where E47 faces the CREB1 homodimer was
719 not taken into further consideration.

720 It has been proposed that rigidification of the ID2 loop is necessary for heterodimer
721 formation (e.g., with E47) [32]. In our tetrameric model, the ID2 loop (residues 48-
722 63) forms multiple intermolecular hydrogen bonds with CREB1. Thus, binding of
723 ID2 to $[\text{CREB1}]_2$ confines the ID2 loop into a specific, rigid conformation that may
724 favour the ID2:E47 heterodimer formation.

725 Distinct calcium (Ca^{2+})-binding sites can be identified for the $[\text{CREB1}]_2$: ID2: E47
726 tetramer (Fig. 6D). Remarkably, the most pronounced sites are found in the binding
727 interface between ID2:E47 and $[\text{CREB1}]_2$. Glutamic acids from ID2 (Glu63), E47
728 (Glu596) and CREB1 (Glu312; Glu327) contribute to the binding of Ca^{2+} (Fig. 6E
729 and F). There is another Ca^{2+} -binding site close to the ID2-loop (Glu48). When
730 bound to Glu48 the calcium ion also weakly interacts (5% of sampled molecular
731 dynamics-snapshots) with the side chain of the central residue of the ID2-loop
732 (Gln55) (Supplementary Fig. 2A). This may further rigidify the ID2-loop. This
733 observation supports previous results that Ca^{2+} -binding to the ID2- loop is important
734 for heterodimer formation [32].

735 Furthermore, the simulation revealed several Ca^{2+} -binding sites involving acid amino
736 acids of CREB1 (e.g., Glu292 and Glu295) and phosphate groups of the DNA,
737 (Supplementary Fig. 2B). It is known that divalent cations (i.e. Ca^{2+} ; Mg^{2+}) enhance

738 significantly (more than 25 times) the binding of CREB1 to DNA [55]. Thus, the
739 stability of the proposed tetrameric protein complex and its binding to the DNA is
740 potentially influenced by the Ca^{2+} concentration. For visualization purposes an
741 additional movie (Video S1) of the identified Ca^{2+} binding sites in the tetrameric
742 complex, is included in the supplementary materials.

743

744 **4. DISCUSSION**

745

746 *4.1. EPA modulates the interaction between the HLH:bHLH proteins ID and E2A* 747 *and the CREB/ATF family member CREB1*

748 The purpose of this study was to elucidate the molecular mechanism by which
749 EPA may exert its beneficial effects in neurological diseases. The current study
750 reveals that EPA modulates the exquisite interplay of interaction of the HLH:bHLH
751 proteins ID and E2A and the CREB/ATF family member CREB1, thereby delivering
752 mechanistic insights into specific neural differentiation program. Due to the
753 importance of HLH:bHLH and CREB1 transcription factors in key developmental
754 events, this association may be at the basis of general mechanisms that regulate
755 multiprotein complex formation in other differentiation processes.

756 Here, we show the ability of EPA to up-modulate p-CREB1 at early times of
757 treatment in ESCs and to induce the CREB1 occupancy at a non-canonical CRE site
758 in the myelin PLP promoter. These data suggest that an EPA-CREB signaling
759 pathway activates PLP transcription. However, p-CREB1 alone is not sufficient to
760 activate target gene transcription. To clarify this regulatory aspect, EPA treatment
761 might provide a novel level of specificity of CREB1 target gene activation by
762 stabilizing the CREB1:HLH:bHLH complex formation within the promoter region of
763 distinct glial or neural genes involved in the coordination of myelin biogenesis. This
764 protein complex assembly was suggested by our coimmunoprecipitation and EMSA

765 analysis of rat pup brain and RA differentiating ESCs after EPA treatment, together
766 with our previous data on the expression of myelin proteins [15]. More specifically,
767 we provide evidence for the natural assembly of CREB1, ID2, and E47 on a CRE
768 consensus site. On the basis of the crystal structures of the CREB basic leucine
769 zipper-CRE complex [55], ID2 homodimers [32] and the E47/ Neuro D1 [54], we
770 could establish a structural model for this assembly (with a 2:1:1 stoichiometry of
771 CREB1, ID2 and E47) that yields a stable molecular dynamics trajectory.

772

773 *4.2. Biological relevance of the interaction between CREB1 and ID2*

774 An important aspect for examining the interaction between CREB1 and ID2 is
775 related to the key role played by ID2 in neurodevelopmental disorders that raised our
776 interest in the investigation of the EPA-CREB-ID2 axis. Even though ID1 and ID3
777 appear more dynamically regulated than ID2 in mESCs after EPA treatment, only the
778 ID2-antibody yielded a distinct supershift of the multimer formed by ID2 with
779 CREB1 (Fig. 2E). Indeed, ID2 inhibits oligodendroglia differentiation [58] which is
780 required for remyelination, and might have a role in multiple sclerosis, a disease
781 characterized by axonal demyelination [59, 60].

782 We noted that medulla, cerebellum and cortex of neonatal rats show only very
783 modest changes in expression levels of ID2 after EPA-treatment. In contrast in LIF-
784 treated mouse ESCs, EPA-treatment displays a much more pronounced effect on ID
785 protein levels; the upregulation of ID2 is, however, delayed with respect to ID1 and
786 ID3. In the same way, in the rat pup brain, we observed no effect at 1h of EPA
787 treatment in the profile of CRE-DNA binding activity and particularly of Complex I
788 containing CREB1 and ID2 proteins. In contrast, in the EPA treated mESCs after 24
789 h of RA-induced differentiation, both complexes I and II were up-modulated
790 according to the time of EPA treatment, reaching the highest intensity at the end of
791 the experiments (6h after treatment, Fig. 2E). This time-dependent response can be
792 seen as an indirect evidence for EPA-induced assembly of ID2 and CREB1 into the

793 high-molecular weight complex I during early stages of neural commitment of
794 mESCs.

795

796 *4.3. How we can explain this evidence regarding the difference in EPA treatment*
797 *between mouse and rat?*

798 In this respect, recent studies underline the complexity of the mechanism
799 regulating gene expression by dietary ω -3 PUFA [16, 61]. The effect of EPA in
800 neurogenesis may need the availability of cell surface ω -3 PUFA receptors, specific
801 fatty acid binding proteins and other regulatory transcriptional proteins (including
802 PPARs, RXR and RA receptors) that are crucial for the induction of the signal
803 transduction processes. Recent data suggest that GPR40 (also known as
804 FFA1/FFAR1), a seven-transmembrane domain receptor, can bind PUFA to induce
805 Ca^{2+} mobilization which phosphorylates CREB1 promoting CREB-mediated gene
806 transcription in neural cells [61, 63]. Expression of GPR40 has been confirmed in
807 various areas of the monkey and human central nervous system [63]. Interestingly,
808 GPR40 induced pCREB1 by activating a mitogen activated protein
809 kinase/extracellular signal-regulated kinase cascade for neural differentiation and
810 synaptic plasticity [16]. Therefore, PUFA, GPR40 and pCREB1 appear involved in
811 the same signaling pathways promoting adult neurogenesis in primates [64].

812 According to previously reported data [16], the rat pup brain lacks the cell surface
813 GPR40 receptor and this might cause a temporal delay of EPA effects ($> 1\text{h}$). Our
814 previous results in the rat pup brain showed that EPA stimulates the expression of
815 myelin PLP and myelin oligodendrocyte glycoprotein transcripts from day 1 post-
816 injection in the medulla and cerebellum, whereas in the cortex EPA effects on PLP
817 and oligodendrocyte glycoprotein transcripts are evident only at day 3 post-injection
818 [15]. Indeed, a significant expression of GPR40 in primary culture of mouse neurons
819 [65] and brain [66] were recently reported. Probably, the GPR40 receptor on the
820 extracellular membrane of mESCs favoured the conduction of EPA induced signals

821 to the nucleus for CREB1 phosphorylation at early time points. In addition, RA-
822 induced differentiation of mESC may involve PPAR/RXR nuclear receptor complex
823 that triggers the EPA-GPR40-CREB signaling pathway. Thus, the mouse may
824 represent a more reliable model to address the role of the PUFA-GPR40-CREB
825 signaling pathway during early neural development in humans. More importantly, the
826 EPA-induced Ca^{2+} mobilization [63] stabilizes the CREB1, ID2 and E47 interactions
827 and triggers the tetrameric protein complex formation to modulate target neural gene
828 expression. Altogether, our data support the important role of EPA in promoting
829 CREB1 binding to DNA, which allows its interaction with ID2 proteins linked to E47.

830

831 *4.4. CREB1 sequestration model of ID2-E47 heterodimers after EPA treatment in*
832 *murine neural cell differentiation.*

833 From our analysis, the molecular function of CREB1 can be to dynamically
834 regulate the location-specific assembly or disassembly of bHLH:HLH proteins
835 complex on neural/glial target genes. CREB1 monomers bind DNA sequentially to
836 CRE-binding sequence according to an assembly pathway as previously described
837 [67]. During neural specification, CREB1 homodimers sequester inactive (non-DNA
838 binding) ID:E2A heterodimers in an active CRE DNA-binding complex (Fig. 7).
839 Therefore, in our CREB1 sequestration model, DNA-bound CREB1 homodimers
840 relocate ID2:E47 containing complexes and fine-tune their activities through
841 potential interaction with associated cofactors/chromatin remodelers (Fig. 7).

842

843 **Fig. 7.** CREB1 sequestration model of ID2-E47 heterodimers after EPA treatment in murine neural cell
 844 differentiation. CREB1 monomers bind DNA sequentially to the non-canonical CRE-binding sequence (5'-
 845 TGACTTTA-3') present in the mouse myelin PLP promoter according to an assembly pathway as previously
 846 described [67]. ID2 exists as homodimers in its native state [32]. After EPA addition, the increase of Ca^{2+}
 847 favours the ID2-E47 heterodimerization [32] and enhances CREB1 dimerization and DNA-binding affinity
 848 to CRE box sequence carrying degenerated semi-palindrome on the PLP promoter [55]. During neural
 849 specification, CREB1 homodimers sequester ID2:E47 heterodimers (this work) generating an active

850 [CREB1]₂:ID2:E47 tetrameric complex that initiate transcription through potential interactions with
851 associated cofactors. See Discussion for details.

852

853 The proposed sequestration model supports the one-to-many binding mechanism of
854 CREB1, whereby CREB- sequestering proteins and associated cofactors/chromatin
855 remodelers (i.e. the repressor element-1 silencing transcription factor (REST) and the
856 corepressor CoREST) can regulate neural lineage specific CREB1-target genes [68].
857 In addition, E47 binds the histone methyltransferase LSD1 to activate neural genes
858 containing CRE sites [69]. This model can also explain how a limited number of E
859 proteins binding to CREB1 homodimers generates functionally varied complexes
860 conferring diversity to neural cells.

861 Our structural tetrameric model provide evidence that Ca²⁺ can modify the CRE
862 DNA-binding affinity of the complex I increasing the residence time of the
863 [CREB1]₂:ID2:E47 tetrameric complex on DNA. In so doing, a CRE box sequence
864 carrying a degenerated semi-palindrome (Fig. 7) may be sufficient for specific
865 genomic target recognition [30]. Therefore, an additional level of
866 promoters/enhancers specific binding is achieved by the [CREB1]₂:ID2:E47
867 tetrameric complex, having physiological relevance. Furthermore, ID2:E47
868 heterodimer stability is also influenced by Ca²⁺ [32], thus variation in Ca²⁺ levels may
869 allow the release of ID2 and the E47 from the tetrameric complex (Fig. 6E and F).
870 Subsequently, ID2 and E47 proteins into different DNA-bound complexes or
871 sequestering cofactors can activate or repress specific target genes thereby triggering
872 a cascade of regulatory events controlled by CREB1.

873

874 *4.5. Emerging roles for HLH:bHLH DNA-binding specificity driven by CREB/ATF*
875 *family members in spermatogenesis, myogenesis, leukemogenesis and neurogenesis*

876 Consistent with our findings, an interaction between CREB family members and
877 bHLH has been reported in Sertoli cells and myogenic precursors where
878 ATF4/CREB2 and scleraxis heterodimerize, thus integrating the transcriptional
879 signaling pathways [57]. Interestingly, a CREB1: Lyl-1 complex is present in acute
880 myeloid leukemia and alters the expression of CREB1 target genes [70].
881 Furthermore, a CREB1: Lyl-1 complex is assembled on the Stathmin promoter at a
882 CRE site [71]. Therefore, similar models of HLH:bHLH DNA-binding specificity
883 driven by CREB/ATF family members are emerging in spermatogenesis, myogenesis
884 leukemogenesis and neurogenesis. Thus, HLH:bHLH bound protein partners are
885 directed to lineage specific CREB target genes. Importantly, integrative genomic
886 analysis of CREB [72] shows that transcription factors from multiple families (i.e.
887 PPAR-alpha, glucocorticoid receptor (NR3C1), CCAAT/enhancer-binding protein
888 beta and forkhead box protein A2) influence the direction of the CREB regulatory
889 response to the feeding/ fasting metabolic switch in the liver, suggesting a more
890 widespread cooperation between CREB1 and transcriptional regulators on individual
891 regulatory elements at CREB target genes.

892

893 **5. CONCLUSIONS**

894 Overall, our results strongly support a role of EPA as an attractive molecule for
895 the prevention of neurodegenerative diseases. The ability of CREB [73] and ID
896 proteins [74-76] to function as central “hubs” for the coordination of multiple
897 pathways controlling the timing of neural stem cell differentiation during
898 development may share light on the symbiotic relationship of the axon and myelin
899 sheath in the central nervous system. As strongly suggested by recent findings [77],
900 this interaction could play a key role in the pathogenesis of multiple sclerosis. Future
901 studies on the EPA-CREB axis should reveal new molecular targets for multiple
902 sclerosis treatment.

903

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911 L.V. supervised the project, performed cell culture of embryonic stem cells, and
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913 analysis. M.S. performed the molecular dynamics simulations. S.S. and C.N.
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915 We declare that we have no conflicts of interest with the contents of this article.

916

917 **Appedix A. Supplementary data**

918

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Appendix A. SUPPLEMENTARY DATA.

Supplemental Figure S1

Supplementary Fig. S1. EMSA analysis of CRE DNA-binding activity of nuclear extracts from cortex in rat pups treated with EPA for 1 h. Sequence specificity of binding was verified by the addition of 50-fold and 300-fold molar excess of CRE cold competitor oligonucleotides (lanes 2 and 3) or in the presence of 300-fold molar excess of the non-CRE containing OCT-1 oligonucleotides, as negative control (lane 4). Lanes 5 and 6, positive and negative controls with or without nuclear extracts. NS, are nonspecific bands.

Supplemental Figure S2.

Supplementary Fig. S2. Distinct Ca^{2+} binding sites identified in the $[\text{CREB1}]_2$: ID2: E47 tetramer. (A) Ca^{2+} binding sites close to the ID2-loop involving acid amino acids Glu48 and Glu55, may further rigidify the ID2-loop. (B) Ca^{2+} binding sites involving amino acids of CREB1 (Glu292 and Glu299) and phosphate groups of the DNA.

Supplemental Table 1: Optimization of the interface area between the ID2:E47 unit and the CREB1 homodimer for rotations around the axes of Fig. 6A.

Rotation angle for left axis (deg)	Rotation angle for right axis (deg)	Size of interface (Å ²)	Minimal Cα- Cα distance (Å)	Rotation angle for left axis (deg)	Rotation angle for right axis (deg)	Size of interface (Å ²)	Minimal Cα- Cα distance (Å)
-60	-60	820	1,3	0	0	922	4,2
-60	-45	807	2,7	0	15	890	3,7
-60	-30	871	4,3	0	30	803	0,7
-60	-15	945	4,0	0	45	777	1,6
-60	0	964	3,1	0	60	765	0,8
-60	15	940	2,2	15	-60	785	2,2
-60	30	807	1,7	15	-45	769	2,8
-60	45	751	1,6	15	-30	820	2,9
-60	60	759	1,7	15	-15	855	4,1
-45	-60	811	2,2	15	0	885	3,7
-45	-45	789	4,0	15	15	848	4,2
-45	-30	876	5,3	15	30	833	3,0
-45	-15	1000	4,2	15	45	740	0,5
-45	0	998	3,5	15	60	697	2,2
-45	15	941	2,8	30	-60	796	1,1
-45	30	819	1,7	30	-45	749	2,8
-45	45	740	1,9	30	-30	790	3,0
-45	60	778	1,3	30	-15	796	4,0
-30	-60	839	3,3	30	0	806	2,5
-30	-45	842	4,2	30	15	759	4,5
-30	-30	927	5,8	30	30	834	3,8
-30	-15	1012	4,9	30	45	682	1,7
-30	0	965	4,5	30	60	596	1,3
-30	15	896	3,4	45	-60	804	1,2
-30	30	796	2,5	45	-45	747	2,6
-30	45	734	0,5	45	-30	761	2,1
-30	60	797	1,8	45	-15	756	4,2
-15	-60	841	3,0	45	0	734	2,7
-15	-45	863	3,4	45	15	678	4,0
-15	-30	928	4,9	45	30	761	5,1
-15	-15	986	5,3	45	45	644	3,1
-15	0	935	4,8	45	60	538	3,2
-15	15	870	3,5	60	-60	817	2,4
-15	30	781	1,8	60	-45	757	2,0
-15	45	770	1,1	60	-30	766	2,2
-15	60	787	1,7	60	-15	759	4,3
0	-60	809	2,2	60	0	716	3,9
0	-45	824	3,5	60	15	666	3,0
0	-30	852	3,7	60	30	634	4,9
0	-15	943	4,4	60	45	606	4,5
0	0	922	4,2	60	60	583	4,2

Legend of Supplementary Table 1: The size of the interface between the ID2:47 unit and the CREB1 homodimer is listed as function of the angles of rotation. A negative sign indicates left-handed rotation. The starting point of the optimization (0°,0°) and the entry with maximal interface size (-30°, -15°) are marked by frames. Furthermore, the minimal distance between the backbone Cα atoms of the CREB1 dimer and the ID2:E47 complex is given. Combinations of rotation angles that do not lead to a steric clash are highlighted in grey.

.261 **Legend of Supplementary Video 1.** A movie showing distinct Ca^{2+} binding sites
.262 identified in the [CREB1]₂: ID2: E47 tetramer. An overlay of all calcium positions
.263 sampled during a 500 ns molecular dynamics simulation reveals the most likely
.264 calcium binding sites (blue cloud). The color code is as follows: CREB1: magenta;
.265 ID2: gray; E47: white; DNA: black; calcium ions: blue dots.

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