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CCAAT/enhancer binding protein δ is a transcriptional repressor of α-synuclein

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ABSTRACT

α-Synuclein is the main component of Lewy bodies, the intracellular protein aggregates representing the histological hallmark of Parkinson's disease. Elevated α-synuclein levels and mutations in SNCA gene are associated with increased risk for Parkinson's disease. Despite this, little is known about the molecular mechanisms regulating SNCA transcription. CCAAT/enhancer binding protein (C/EBP) β and δ are b-zip transcription factors that play distinct roles in neurons and glial cells. C/EBPβ overexpression increases SNCA expression in neuroblastoma cells and putative C/EBPβ and δ binding sites are present in the SNCA genomic region suggesting that these proteins could regulate SNCA transcription. Based on these premises, the goal of this study was to determine if C/EBPβ and δ regulate the expression of SNCA. We first observed that α-synuclein CNS expression was not affected by C/EBPβ deficiency but it was markedly increased in C/EBPδ-deficient mice. This prompted us to characterize further the role of C/EBPδ in SNCA transcription. C/EBPδ absence led to the in vivo increase of α-synuclein in all brain regions analyzed, both at mRNA and protein level, and in primary neuronal cultures. In agreement with this, CEBPD overexpression in neuroblastoma cells and in primary neuronal cultures markedly reduced SNCA expression. ChIP experiments demonstrated C/EBPδ binding to the SNCA genomic region of mice and humans and luciferase experiments showed decreased expression of a reporter gene attributable to C/EBPδ binding to the SNCA promoter. Finally, decreased CEBPD expression was observed in the substantia nigra and in iPSC-derived dopaminergic neurons from Parkinson patients resulting in a significant negative correlation between SNCA and CEBPD levels. This study points to C/EBPδ as an important repressor of SNCA transcription and suggests that reduced C/EBPδ neuronal levels could be a pathogenic factor in Parkinson's disease and other synucleinopathies and C/EBPδ activity a potential pharmacological target for these neurological disorders.

INTRODUCTION

α-synuclein plays a central role in the pathogenesis of Parkinson's disease (PD). This protein is the main component of Lewy bodies, the intracellular inclusions that are a hallmark of PD, and various single point mutations in the α-synuclein gene (*SNCA*) cause early-onset familial forms of the disease [1, 2, 3]. Several lines of evidence support that increased α-synuclein levels contribute to PD pathogenesis. Thus, copy number variations in the *SNCA* gene cause familial PD and a correlation exists between α-synuclein load and the severity of the PD phenotype [4, 5, 6, 7, 8] (see however [9, 10]); polymorphisms in *SNCA* regulatory regions promoter that enhance α-synuclein expression are associated with increased PD risk [11, 12, 13, 14] or with differential PD age-at-onset[15]; overexpression of α-synuclein in animals induces nigrostriatal degeneration [16, 17, 18] and α-synuclein species contained in PD-derived Lewy bodies are pathogenic having the capacity to initiate a PD-like pathological process [19]. Targeting *SNCA* expression is therefore a promising strategy for the design of disease-modifying therapies in PD [20].

Surprisingly little is known about the transcriptional regulation of the *SNCA* gene (for recent revision see Piper et al., 2018[21]). The best characterized regulator of *SNCA* transcription is the transcription factor zinc finger and SCAN domain containing 21 (ZSCAN21) which binds to an intron 1 site both in the rodent and human *SNCA* gene and it can activate or repress *SNCA* transcription [22, 23, 24]. Other transcription factors that regulate *SNCA* are GATA2, which activates *SNCA* transcription by binding to an intronic site [23, 25], ZNF219, which can function both as a transcriptional repressor and activator by binding to a site at the 5'-proximal promoter region [22], p53, which promotes *SNCA* transcription in SH-SY5Y cells by binding to a specific site located 970bp upstream of the transcription start site (TSS) [26], EMX2/NKX6-1 which represses *SNCA* transcription by binding to an intron 4 enhancer [27] and PARP1 which negatively regulates *SNCA* transcription by binding to a polymorphic microsatellite region, called NACP-Rep1, associated with increased PD risk and located far upstream, approximately 9Kb, from the TSS [28].

The transcription factor CCAAT/enhancer binding protein β (C/EBPβ) is also a candidate to regulate *SNCA* transcription. C/EBPβ binds to intron 4 of the *SNCA* gene

in human PC12 cells and in rat brain [29]. However, overexpression of C/EBPβ induces *SNCA* expression in neuroblastoma cells [30] whereas *Snca* mRNA levels are moderately upregulated in the brains of C/EBPβ deficient mice [29]. Further studies are needed to clarify the functional effects of C/EBPβ on *SNCA* transcription.

C/EBPβ is a basic-leucine zipper transcription factor of the C/EBP family that participates in memory formation and synaptic plasticity in neurons and in the regulation of the pro-inflammatory program in astrocytes and microglia [31]. This dual role of C/EBPβ is shared among C/EBP proteins only by C/EBPδ, which is the closest to C/EBPβ both phylogenetically and functionally [31]. The goal of the present study was to analyze the involvement of C/EBPβ and C/EBPδ in the regulation of SNCA expression. We first analyzed α-synuclein in the brains of C/EBPβ- and C/EBPδ-deficient mice. Our findings of a marked increase in *Snca* expression in C/EBPδ- but not in C/EBPβ-deficient CNS prompted us to focus on the role for C/EBPδ in SNCA expression. This study provides various independent findings indicating that the transcription factor C/EBPδ is a potent novel repressor of *SNCA* transcription.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the Guidelines of the European Union Council (86/609/EU) and Spanish Government (BOE 67/8509-12), and approved by the Ethic and Scientific Committees of the University of Barcelona and registered at the "Departament d'Agricultura, Ramaderia, Pesca i Alimentació de la Generalitat de Catalunya". Mice were maintained under regulated light and temperature conditions at the specific pathogen-free animal facilities of the School of Medicine, University of Barcelona. All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used. C/EBPβ and C/EBPδ deficient mice on a C57BL/6 background, kindly provided by E Sterneck (Center for Cancer Research, National Cancer Institute, Frederick, MD, U.S.A.) were genotyped as described previously by [32] and [33], respectively.

Mixed Glial Cultures

Mixed glial cultures were prepared from P0-P3 mice as described previously [34]. Briefly, cortical glial cells were seeded at a density of 3.0 x 10^5 cells/mL and cultured at 37°C in humidified 5% CO_2. Medium was replaced every 5–7 days. After 21 days in vitro, glial cells were processed for protein and mRNA extraction.

Primary cortical neuronal cultures

Primary cortical neuronal cultures were prepared from C57BL/6 mice at embryonic day 16 as described previously [35]. Briefly, cells were seeded at a density of 8×10^5 cells/mL in 48-well culture plates coated with poly-D-lysine (Sigma-Aldrich) and cultured at 37°C in humidified 5% CO_2–95% air. Neuronal cultures were used at 5 days in vitro and neurons were processed for protein and mRNA extraction.

Primary cerebellar granular neuron cultures

Cerebellar granular neurons were prepared using a modification of described procedures [36]. Briefly, Cerebella from P6-8 mice were removed, cut into small pieces of approximately 1 mm and digested with 1% trypsin and 1 mg/mL DNase (Sigma-Aldrich) in PBS at 37°C for 15 min. After that, the tissue was triturated using pipettes to obtain a single cell suspension and centrifuged (1000 rpm) at room temperature for 3 min. Cells were then resuspended in Neurobasal medium (Invitrogen) containing B-27 serum-free supplement. Cerebellar granular neurons were purified by Percoll gradient centrifugations and resuspended in the above described medium. Cells were electroporated in suspension (10 µg of DNA per 3×10^6 cells) using a Microporator MP-100 (Digital Bio, Seoul, Korea) according to the manufacturer's instructions, with a single pulse of 1700 V for 20 ms. Electroporated cells were diluted at a density of 2×10^5 cells/mL in Neurobasal medium (Invitrogen) containing B-27 serum-free supplement, 0.15% D-glucose, 2 mM L-glutamine, 20 mM KCl, 100 U/mL penicillin and 100 µg/mL streptomycin and plated on cell culture plates (24 wells) coated with poly-L-Lysine plus laminin. Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Culture medium was changed 2 days after seeding by partial medium replacement. After 2 days in vitro 500 nM all-*trans* retinoic acid, RA (Sigma-Aldrich), was added in cell plates to induce neuronal differentiation. Cerebellar granular

neurons were used 5 days after in vitro. Cells were lysed and processed for protein and RNA extraction or fixed in 2 or 4 % PFA for ChIP or immunocytochemistry, respectively, and conditioned media was collected for ELISA.

SH-SY5Y cell cultures

SH-SY5Y neuroblastoma cells were obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in Dulbecco's modified Eagle medium/Ham's F-12 medium, DMEM/F-12 (Sigma–Aldrich), supplemented with 15% fetal bovine serum, FBS (Sigma–Aldrich), glutamine (Sigma–Aldrich), penicillin/streptomycin (Sigma–Aldrich) and non-essential amino acids (Sigma–Aldrich) and maintained at 37 °C in humidified 5 % CO_2–95 % air. Medium was replaced every 5 days. Cells were electroporated in suspension (10 µg of DNA per $2{,}5\times10^6$ cells) using a Microporator MP-100 (Digital Bio, Seoul, Korea) according to the manufacturer's instructions, with a single pulse of 1700 V for 20 ms. Electroporated cells were plated on cell culture plates (24 wells) coated with 10 µg/mL laminin at a density of 2.5×10^5 cells/mL in the in the above described medium. Cells were maintained in a humidified incubator at 37 °C in a 5 % CO_2 atmosphere. To induce SH-SY5Y differentiation, cells were incubated with 10 µM RA in DMEM/F-12 containing 3% FBS for 72 hours. Cells were used at 90 % confluence and processed for protein and mRNA extraction. Conditioned media were used for ELISA techniques and some cell plates were fixed in 2 and 4 % paraformaldehyde-PBS and processed for ChIP or immunocytochemistry, respectively.

C17.2 cell cultures

C17.2 mouse cerebellar neuron cell line was a kind gift from Dr. Evan Snyder. Cells were cultured in Dulbecco's modified Eagle Medium supplemented with 10% FBS, 5% Glutamine and 5% Penicillin/Streptomycin and maintained at 37 °C and 5% CO_2. Cells were used at 80% confluence and were free from mycoplasma.

Samples from PD patients and generation of iPSC-derived DAn

We used mature induced pluripotent stem cells (iPSC)-derived dopaminergic neurons (DAn) previously generated from skin fibroblasts form PD patients and healthy controls. Patient and cell line characterization of the samples used here [37, 38] or the reprogramming and differentiation protocols [39] are described into detail elsewhere.

Briefly, we used samples from leucine rich repeat kinase 2 (LRRK2)-associated PD patients carrying the G2019S mutation (L2PD, n = 4) and sporadic PD patients lacking PD family history and mutations in known PD genes (sPD, n = 6), as well as samples from healthy controls without neurological disease history (controls, n = 4). Primary cultures of fibroblasts were reprogrammed to iPSC using retroviral delivery of OCT4, KLF4, and SOX2. Resulting iPSC were differentiated to ventral midbrain dopaminergic neurons using the lentiviral delivery of the ventromedial midbrain DAn determinant LMX1A together with DAn patterning factors and co-cultured with mouse PA6 feeding cells. The percentage of iPSC□derived DAn was ~30 % [37, 38]. Mature iPSC-derived DAn cells were characterized and used for gene expression analysis. As cellular control of iPSC-derived DAn, iPSC-derived neural cultures not-enriched-in-DAn were generated from a subset of representative PD patients (n = 6) and healthy subjects (n = 3) as described [37].

DNA constructs

Mouse and human C/EBPδ cDNA constructs (kindly gift by Dr Knut Steffensen, Karolinska Institute, Sweden, and by Drs. Karin Milde-Langosch and Birgit Gellersen, respectively) were electroporated to cerebellar granular neurons and SH-SY5Y cells, respectively. To test the efficient delivery of C/EBPδ into granular or SH-SY5Y cells, pcDNA3-EGFP (Addgene) was used.

Total protein extraction

Cell cultures were gently washed with pre-chilled phosphate buffer saline (PBS) and lysed in pre-chilled RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich) using a cell scraper. Cell lysates were transfered to 1,5mL tubs on ice for 15 minutes and sonicated three times for two seconds. After 5 minutes on ice, sonicated cells were centrifuged (at 13,000 x g for 5 minutes at 4 °C) and supernatants collected and stored at -80 °C. For mouse samples, total protein extracts were obtained by tissue (10 mg) homogenization in 1 mL pre-chilled RIPA buffer with protease inhibitor cocktail using a hand-held Polytron homogenizer. After 10 minutes at 4°C, the homogenates were centrifuged (at 13,000 x g for 10 minutes at 4°C) and supernatants collected and stored at -80 °C. For cell and tissue lysates, protein quantification was determined by the Bradford assay (Bio-Rad).

Western blot

Western blots were performed as previously described [40]. Briefly, 30 μg of protein extract were subjected to 12% SDS polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) which were incubated with primary (rabbit anti-C/EBPδ, Rockland Immunochemicals Inc.; mouse or rabbit anti-α-synuclein, Santa Cruz Biotechnology Inc.; rabbit anti-α-synuclein antibody, Cell Signaling Technology) and secondary (goat anti-mouse HRP or goat anti-rabbit HRP) antibodies. Finally, membranes were developed with ECL-Plus (Amersham) and images were obtained using a VersaDoc System camera (Bio-Rad Laboratories, Hercules, CA, USA). Data were expressed as the ratio between the band intensity of the protein of interest and that of the loading control (β-actin).

Human samples

Postmortem human brain samples used in this study were supplied by the Neurological Tissue Bank of the Biobanc-Hospital Clínic-IDIBAPS (Barcelona, Spain) in accordance with the Helsinki Declaration, Convention of the Council of Europe on Human Rights and Biomedicine and Ethical Committee of the University of Barcelona. Substantia nigra (used in qRT-PCR experiments) and frontal cortex (used in ChIP experiments) samples were obtained from non-neurological controls (n = 10; 5 women and 5 men; age, 78.1 ± 10.7 y; postmortem delay, 12:05 ± 6:16h) and patients with a diagnosis of Parkinson's disease, PD (n = 21; 5 women and 16 men; age, 78.3 ± 9.2 y; postmortem delay, 11:28 ± 4:42h). The samples from patients with PD corresponded to areas with 4-6 stages of Lewy body disease in according to the classification described by Braak et al. [41]. For protein and mRNA extractions, frozen tissue blocks were used.

ELISA

Total-α-synuclein was determined in conditioned media of granular and SH-SY5Y cells using ELISA technique described previously by [42] with minor modifications. Briefly, 96-well ELISA plates were coated overnight at 4°C with anti-α-synuclein antibody (1 μg/mL of mouse 211 antibody (Santa Cruz Biotechnology Inc.) for SH-SY5Y cells and 1 μg/mL of goat n-19 antibody (Santa Cruz Biotechnology Inc.) in 200 mM $NaHCO_3$, pH=9.6. After several washes in PBS-Tween and blocking for 2 h in the same buffer

solution with 2% BSA, 100 µl/well of conditioned medium or standard α-synuclein were added and incubated for 3h at 37 °C. Conditioned medium or standard were removed, wells washed in PBS-Tween and plates were incubated for 2 h at 37 °C with rabbit anti-α-synuclein antibody (1:1000, FL140, Santa Cruz Biotechnology Inc.) in blocking buffer. After several washes, wells were incubated for 1h at room temperature with a secondary HRP-conjugated anti-rabbit antibody (1:2000) in blocking buffer. After several washes, plates were developed in the dark with 100 µl of TMB for 25 minutes at room temperature, and stopped the developer with 100 µl of 0.3M H_2SO_4. Absorbance was read at 450 nm and results were expressed in ng/mL.

Immunocytochemistry and immunohistochemistry

Free-floating sections for mice sample and cell cultures were processed for immunohistochemistry or immunocytochemistry as previously described [33]. Briefly, the sections were washed in PBS, and the endogenous peroxidase activity was inactivated with 2% H_2O_2 in PBS. Then, the sections were permeabilized with PBS-0.5% and incubated in blocking solution (0.2 M glycine, lysine 0.2 M, 10% FBS, 0.5% triton on PBS) for 1h, and then were incubated with the primary antibody (rabbit anti-C/EBPδ, Rockland Immunochemicals Inc., and mouse or goat anti-α-synuclein, Santa Cruz Biotechnology Inc.) in the same blocking solution for at least 24h at 4 °C with gentle agitation. After that, the sections were rinsed in PBS-0.5% triton and incubated with the appropriate secondary antibody (biotinylated or fluorescent secondary antibodies). For sections, after several washes, were incubated with ExtrAvidin-HRP and developed with 0.05% diaminobenzidine in 0.1 M PB and 0.01% H_2O_2 for 10 minutes. After washes in PBS, sections were mounted on gelatinized slides and covered with Mowiol medium. For immunofluorescence the cells were washes in PBS and observed in fluorescence microscope with the adequate filters. Sections and cells were photographed in an NIKON Eclipse 901 microscope/Nikon digital sight camera, using a 10× and 20× objective lens.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cell cultures with RNA Miniprep kit (Roche Diagnostics) and from frozen tissue samples using the Trizol method (Tri®Reagent, Sigma-Aldrich) One microgram of RNA was reverse transcribed with random primers using

Transcriptor Reverse Transcriptase (Roche Diagnostics). Then, cDNA was diluted 1/10 (human substantia nigra samples) and 1/30 (mouse samples) to perform qRT-PCR with IQ SYBRGREEN SuperMix (Bio-Rad Laboratories) as previously described [40]. The primers (Integrated DNA technologies) used to amplify mouse or human mRNAs are shown in Table 1. Relative gene expression values were calculated with the comparative Ct or ΔΔCt method [43] using CFX 2.1 software (Bio-Rad Laboratories).

Quantitative chromatin immunoprecipitation (qChIP)

MatInspector and JASPAR were used to identify the proximal C/EBP consensus sequence in the analyzed promoters. The sequences for each amplified locus are indicated in Table 2. qChIP was performed as previously described [33]. ChIP samples from human frontal cortex were analyzed with qPCR using SYBR green (Bio-Rad Laboratories). Samples were run for 40 cycles (95°C for 30 s, 62°C for 1 min, 72°C for 30 s). For C17.2 cells ChIP assay was performed as previously described [44]. Briefly, cells were lysed and chromatin from crosslinked cells was sonicated. Chromatin was incubated with 5 μg of C/EBPδ (600-401-A61, Rockland) in RIPA buffer (50 mM Tris − HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na3VO4, 0.5 μg/μl aprotinin, 10 μg/μl leupeptin) adding 20 μl of Magna ChIP Protein A magnetic beads (Millipore). Samples were incubated in rotation overnight at 4 ºC. Beads were washed with low-salt buffer, highsalt buffer, LiCl buffer and TE buffer. Subsequent elution and purification of the immunoprecipitated DNA-proteins complexes was performed using the IPure kit (Diagenode) according to manufacturer's protocol. Samples were analyzed by qPCR. Primer sequences used for qPCR of *SNCA* promoter genomic regions are listed in Table 2.

Luciferase experiments

Luciferase vector was obtained by cloning a specific region containing 2000bp upstream of the murine SNCA TSS (NM1042451.2) into a pGL3 vector (Promega). Primers for the selected gene were designed by adding MluI and BgIII target sequences at 5′ and 3′, respectively. The primers used for amplification were: SNCA promoter Forward 5′ - CTAGAAGGAGAGAAGTCGATAGTG- 3′, SNCA promoter Reverse 5′ - GGAGCACATTCCCCCGGATGGAAG- 3′. Amplification of SNCA promoter

sequence was done by PCR using genomic DNA and cloning the PCR products into a pGL3 vector. Human embryonic kidney 293T cells (ATCC) were co-transfected with a CMV-βGal vector, a luciferase pGL3 vector containing or not the -2000bp region of the murine SNCA promoter, and a shRNA control or shRNA targetting C/EBPδ (Sigma-Aldrich). Lipofectamine2000 (Invitrogen) was used as transfection agent. β-galactosidase and luciferase assays were performed 48 h after transfection. β-galactosidase activity was detected using ONPG (Sigma-Aldrich) and read at 405 nm wavelength. Luciferase assays (Luciferase Assay System; Promega) were performed following manufacturer's instructions. Luciferase/β-galactosidase ratio was calculated and expressed as arbitrary units (RLU: relative light unit).

Data presentation and statistical analysis

Statistical analyses were performed using one-way ANOVA and the Newman-Keuls post-hoc test or two-way ANOVA when comparing three or more experimental groups. Student's t-tests were performed when two experimental groups were compared. The correlated expressions between *SNCA* and C/EBPβ or C/EBPδ genes were measured by Pearson correlation coefficients. Statistical analyses were performed using GraphPad Prism 4.02 (GraphPad Software, Inc., La Jolla, USA). All results are presented as mean ± SD values, unless otherwise stated. Values of $p<0.05$ were considered statistically significant.

RESULTS

Snca expression is markedly upregulated in C/EBPδ-deficient mouse brain

We first analyzed *Snca* expression in the cerebral cortex of C/EBPβ- and C/EBPδ-deficient mice. Whereas *Snca* mRNA levels were not affected by C/EBPβ deficiency (Fig 1a), a marked increase (35,7 fold increase; $p<0,0001$) was observed in C/EBPδ-deficient mouse cortex (Fig 1b). This increase was also observed at the protein level by western blot (4,5 fold increase; $p<0,0001$; Fig 1c), not only in cerebral cortex but also in other brain regions such as striatum or hippocampus (Fig 1d). A strong band with an apparent molecular weight of 14-15 kDa, corresponding to monomeric α-synuclein was

observed. Immunohistochemical staining with anti-α-synuclein antibodies showed a marked increase of α-synuclein immunoreactivity in several brain regions from C/EBPδ-deficient mice (Fig 1e). The widespread and punctate α-synuclein immunostaining in these samples suggests neuropil localization and it is compatible with the reported predominant localization of α-synuclein in presynaptic nerve terminals [45]. Interestingly, α-synuclein immunostaining in neuronal soma was observed in some regions such as substantia nigra and hippocampus (Fig 1e). To further study the cellular localization of up-regulated α-synuclein in the CNS of C/EBPδ-deficient mouse we analyzed *Snca* expression in primary glial and neuronal cultures from wild type and C/EBPδ-deficient mice. In primary mixed glial cultures, mainly composed of astrocytes and microglia, *Snca* expression was barely detectable and did not differ between wild-type and C/EBPδ-deficient samples (data not shown). In contrast, in primary cortical neuron cultures α-synuclein levels were upregulated in C/EBPδ-deficient samples (Fig 1f). These findings clearly show that the absence of the transcription factor C/EBPδ strongly correlates with the upregulation of *Snca* expression in the CNS, predominantly, if not exclusively, in neurons and suggest that C/EBPδ may act as an SNCA transcriptional repressor in neurons.

Overexpression of C/EBPδ decreases neuronal *SNCA* expression

In order to support the hypothesis that C/EBPδ represses SNCA transcription we analyzed the effects of C/EBPδ overexpression in *SNCA* levels in neurons. For this study we selected primary mouse cerebellar granular neurons because of their very low C/EBPδ mRNA levels and moderate *Snca* mRNA levels (data not shown). These cultures were transfected with a pcDNA3 vector containing a copy of the murine Cebpd gene under a constitutive promoter. A robust increase in C/EBPδ mRNA and protein levels was observed 48h after transfection (Fig 2a, b). C/EBPδ overexpression led to a marked decrease in *Snca* mRNA (50,3% decrease; p=0,0003; Fig 2c) and protein levels (Fig 2d). Downregulation of α-synuclein protein levels was confirmed by immunocytochemistry (Fig 2e). Electroporation efficiencies were high in cerebellar granular neuronal cultures: 88.1% with pcDNA and 82.3% with pcDNA-Cebpd (Fig 2f). ELISA experiments revealed that C/EBPδ overexpression led also to increased α-synuclein levels in the conditioned medium of primary cerebellar neuronal cultures (Fig 2g). To extend these findings into human cells, we overexpressed human CEBPD in SH-SY5Y neuroblastoma cells. Transfection of pcDNA3 vector containing human

C/EBPδ gene induced a robust increase in C/EBPδ expression in retinoid acid-differentiated SH-SY5Y cells (Fig 3a) which was accompanied by a decrease in *SNCA* expression both at mRNA (35,1% decrease; p=0.0028; Fig 3b) and protein levels (Fig 3c-d).

C/EBPδ binding to *Snca* gene in mouse neurons

The increased *SNCA* expression in the absence of C/EBPδ together with the decreased *SNCA* expression when C/EBPδ is overexpressed strongly suggest that C/EBPδ, a transcription factor itself, acts as a direct repressor of *SNCA* transcription. Since these effects could also be indirect, we performed chromatin immunoprecipitation (ChIP) experiments to analyze the possible recruitment of C/EBPδ to *SNCA* genomic regulatory regions in neurons. We identified six putative C/EBPδ binding sites, named δ1-δ6 (Table 2), located in the 8 Kb region upstream of the canonical TSS in the mouse *Snca* gene (Fig 4a). ChIP experiments in C17.2 mouse neuronal cells revealed significant binding of C/EBPδ to five such regions (Fig 4b) strongly suggesting a direct effect of C/EBPδ on *Snca* transcription in mouse neurons.

C/EBPδ binding to *SNCA* gene in PD brain

Since the regulation of *SNCA* transcription by C/EBPδ could be relevant in PD pathogenesis we next analyzed C/EBPδ expression and binding to *SNCA* genomic region in human substantia nigra samples from PD patients and non-neurological controls. To study the binding of C/EBPδ to *SNCA* genomic regulatory regions, 14 putative C/EBPδ binding sites located in the promoter of human *SNCA* gene were selected and named boxes 1-14 (see Table 2 for sequences and coordinates). C/EBPδ binding to these regions was analyzed by chromatin immunoprecipitation. Significant binding was observed in amplified regions comprising boxes 1, 13 and 14 (Fig 4d). Interestingly, C/EBPδ binding to these regions was clearly decreased in samples from PD brains when compared to controls (Fig 4d). In addition, we also studied the C/EBPδ binding sites in introns 2 and 4 of the *SNCA* gene. Four putative C/EBPδ binding sites located in intron 2 and 14 in intron 4 were selected (see Table 2 for sequences and coordinates). Significant C/EBPδ binding to regions comprising boxes 2 and 3 of intron 2 and boxes 1, 2, 8, 9 and 14 of intron 4 was detected in control brains (Fig 4e and 4f), and the C/EBPδ binding to these regions was clearly decreased in samples from PD

brains (Fig 4e and 4f). Moreover, qRT-PCR experiments revealed that C/EBPδ mRNA levels were reduced in PD samples when compared to controls (44,0% decrease; p=0,0037; Fig 4e). Altogether, these results strongly suggest that C/EBPδ regulates the expression of the *SNCA* gene in basal conditions and alterations in *SNCA* gene regulation by C/EBPδ may be involved in the pathogenesis of Parkinson's disease.

Repressor effect of C/EBPδ on SNCA transcriptional activity in 293T cells

The functional effect of C/EBPδ on SNCA transcription was further investigated by luciferase experiments. In 293T cells expression of a luciferase reporter gene under a constitutive SV40 promoter was strongly reduced by insertion of a DNA sequence corresponding to the 2000bp region of the proximal promoter of the murine SNCA gene (Fig 5a) indicating the presence of strong repressor elements in this sequence. Note that this sequence harbors the δ6 element identified in figure 4a. Transfection of 293T cells with shRNA targeting C/EBPδ resulted in a marked decrease in C/EBPδ protein levels (Fig 5b) and significantly reversed the inhibitory effect of the SNCA 2000bp sequence on luciferase reporter expression (Fig 5a). These data suggest that C/EBPδ is a repressor of SNCA transcription by binding to sequences on the proximal 2000bp region of murine SNCA promoter, probably to the δ6 element located 1660bp upstream of the TSS.

Inverse correlation of *SNCA* and *C/EBPδ* expression in human iPSC-derived dopaminergic neurons

Finally, we analyzed the expression of C/EBPδ and *SNCA* in iPSC-derived neurons from PD patients and controls. As it is described in methods, two protocols were used to differentiate iPSC into dopaminergic or non-dopaminergic neurons. In iPSC-derived dopaminergic neurons from PD patients *SNCA* mRNA levels were upregulated (4,8 fold increase; p=0,0006; Fig 6a) and C/EBPδ mRNA levels were downregulated (69,2% decrease; p<0,0001; Fig 6b) whereas C/EBPβ mRNA levels were unchanged (Fig 6c). This resulted in a significant inverse correlation between *SNCA* and C/EBPδ mRNA levels (r= -0,7641; p=0,0024; Fig 6d) and not between *SNCA* and C/EBPβ (Fig 6e). Intriguingly, when the same experiments were performed in iPSC-derived non-dopaminergic neurons no significant changes in *SNCA*, C/EBPδ or C/EBPβ mRNA levels were observed (Fig 6f-h).

DISCUSSION

This study shows that the absence of the transcription factor C/EBPδ leads to a marked upregulation of *SNCA* expression in mouse CNS and primary neuronal cultures whereas exogenous expression of C/EBPδ in neurons downregulates α-synuclein levels. ChIP experiments show C/EBPδ binding to *SNCA* genomic regions in mouse cerebellar neurons and also in post-mortem substantia nigra from PD patients. Combined luciferase and shRNA experiments show that C/EBPδ has a repressive effect on transcription driven by a SNCA promoter region. Reduced *CEBPD* expression was observed in PD post-mortem brain and in dopaminergic neurons derived from PD iPSC. Consistently, in iPSC-derived dopaminergic neurons the expressions of *CEBPD* and *SNCA* were inversely correlated. Our study identifies the transcription factor C/EBPδ as a novel repressor of *SNCA* and suggests that a deficit of C/EBPδ leading to enhanced expression of SNCA could be relevant in PD pathogenesis.

Increased levels of α-synuclein are a risk factor for PD [4, 5, 6, 7, 8, 11, 12, 13, 14]. Transcriptional regulation of *SNCA* gene is one of the main layers of regulation of α-synuclein levels together with CpG methylation, histone modifications, miRNAs and α-synuclein post-translational modifications (reviewed by [46]). To our knowledge, eight transcription factors have been shown to date to regulate *SNCA* transcription. GATA2, p53 and C/EBPβ promote *SNCA* transcription; PARP1, EMX2 and NKX6/1 repress it and ZSCAN21 and ZFN210 play a dual role (see Introduction for references). In this context our study shows for the first time that C/EBPδ is a potent repressor of *SNCA* transcription in neurons. Most previous studies show that C/EBPδ is predominantly an activator of gene transcription [33, 47, 48]. There are however examples of genes transcriptionally repressed by C/EBPδ such as THBS1 in astrocytes [49], prolactin in pituitary prolactinoma cells [50] or ABCA1 in macrophages [51]. *SNCA* is to our knowledge the first gene shown to be transcriptionally repressed by C/EBPδ in neurons. A possible mechanism for C/EBPδ repression involves the recruitment of mSin3 and HDAC1 [52]

suggesting a potential involvement of epigenetic mechanisms in the regulation of SNCA by C/EBPδ.

It has been suggested that the epigenetic and transcriptomic alterations previously found in iPSC-derived dopaminergic neurons are related with the deficit of a network of transcription factors relevant to PD [37]. In addition, developmental deficits of key transcription factors related with the differentiation of iPSC-derived dopaminergic neurons such as Lmx1b have been associated to PD pathology [53]. The findings presented here on C/EBPδ support the hypothesis of a downregulation in a subset of transcription factors in PD, and link specifically C/EBPδ with a key molecule in PD pathogenesis such as SNCA. This is in keeping with the hypothesis that in spite of SNCA duplications and triplications being rare causes of familial PD, more modest but significant increases in α-synuclein expression might be common and mechanistically relevant in sporadic PD.

C/EBPδ is expressed in the CNS both by neurons and glial cells [31]. The increased levels of α-synuclein seen in the mouse CNS in the absence of C/EBPδ could a priori be neuronal, glial or both. In C/EBPδ-deficient primary glial cultures we did not observe upregulation of *Snca* expression suggesting that C/EBPδ, despite being expressed by astrocytes and microglia[33, 54], is not an endogenous repressor of *Snca* transcription in these cells. In contrast, C/EBPδ-deficient neuronal cultures showed increased α-synuclein levels. Also, the immunohistochemistry of α-synuclein upregulation in several brain regions of C/EBPδ-deficient mice suggested a neuronal localization. This finding strongly points to C/EBPδ as a constitutive repressor of neuronal *Snca* transcription. An important question here is whether this repression occurs in all neurons or in specific neuronal subsets. In situ hybridization histochemistry and immunohistochemistry studies have shown that C/EBPδ is not expressed by all neurons[33, 55, 56] but the nature of the C/EBPδ-expressing cells has not been defined. Similarly, α-synuclein is expressed at very different levels in different neuronal populations[57, 58]. It is therefore possible that the relative expression of C/EBPδ is an important factor at determining α-synuclein levels in different neuronal populations. A quantitative determination of α-synuclein and C/EBPδ in individual neurons would be important to clarify this question.

The observation that C/EBPδ represses *SNCA* transcription in neurons suggests that any factor, be it genetic, metabolic or exogenous, causing decreased levels or activity of C/EBPδ in specific neurons could constitute a risk factor for PD by increasing α-synuclein levels in vulnerable neurons. It is therefore important to understand how C/EBPδ expression and activity are regulated in neurons. Unlike the regulation of C/EBPδ in astrocytes and microglia which has been studied in depth, particularly in response to proinflammatory stimuli that cause C/EBPδ up-regulation [54, 59], there are no reports to our knowledge of factors regulating C/EBPδ expression in neurons, the only exception being the increased C/EBPδ levels in specific neurons in learning paradigms[56, 60]. Interestingly, C/EBPδ levels in the mouse CNS are markedly downregulated in aging [61] a risk factor for PD (see however [62]). There are data suggesting that increased C/EBPδ levels in the CNS could be beneficial. Thus, hypoxic[63] or hyperbaric oxygen preconditioning[64], which are neuroprotective against subsequent brain ischemia, cause the upregulation of C/EBPδ in the CNS. This has led to the hypothesis of a neuroprotective role for C/EBPδ [64]. Our findings suggest that this putative neuroprotective role of elevated C/EBPδ levels in the CNS could be mediated, at least in part, by maintaining low/physiological α-synuclein levels in neurons. If true, drugs promoting C/EBPδ activation could be of interest. These drugs should be neuronal-specific since astroglial and microglial C/EBPδ activation is potentially harmful by inducing a proinflammatory response[31].

We have observed that C/EBPδ levels and C/EBPδ binding to *SNCA* genomic regions are decreased in PD brain samples. It is unlikely that these decreases are due to neuronal loss because they occur in various brain regions, including frontal cortex or hippocampus where no overt neuronal death occurs in PD. Since C/EBPδ is upregulated in neuroinflammation [31], astroglial and microglial C/EBPδ levels are likely to be increased in PD brain which would imply that the decrease of C/EBPδ in the neuronal compartment is in fact stronger than the one we report here. Such a decrease in C/EBPδ levels in PD could cause the upregulation of α-synuclein in neurons and participate in pathogenesis. However, caution is needed when interpreting data from post-mortem PD samples because these are obtained at a very advanced stage of the disease and because of potential confounding factors such as post-mortem delay and agonic state. In iPSC-derived dopaminergic neuron-like cells from PD patients, C/EBPδ and α-synuclein levels are decreased and increased, respectively, when compared to cells from healthy

subjects. This results in a strongly significant negative correlation between both parameters. It is important to note that these cells are derived from skin biopsies and therefore post-mortem delay, agonic state or terminal disease stage are not confounding factors. These data strongly suggest that the decrease in neuronal C/EBPδ expression is PD-linked and supports a role for this transcription factor in PD pathogenesis.

In summary, this study provides evidence that the transcription factor C/EBPδ is a negative regulator of *SNCA* transcription in neurons. Besides, the inverse association between the expression of SNCA and C/EBPδ in iPSC-derived dopaminergic neuron-like cells from PD patients suggests that deficient expression of C/EBPδ may participate in PD pathogenesis by increasing α-synuclein levels. Knowledge of the molecular pathways involved in the regulation of C/EBPδ activity in neurons may define pharmacological strategies to modulate the levels of α-synuclein which could have an impact in the progression of PD and other synucleinopathies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Expression of α-synuclein in Cebpb-/- and Cebpd-/- mice. (a) No significant change in cerebral cortex *Snca* mRNA levels ($p = 0.8402$) is observed between Cebpb+/+ and Cebpb-/- mice. (b) *Snca* mRNA expression increases 35,7 fold ($p < 0.0001$) in the cerebral cortex of Cebpd-/- mice when compared with Cebpd+/+ mice. (c) The 15 kDa α-synuclein protein increases 4,5 fold ($p < 0.0001$) in the cerebral cortex of Cebpd-/- mice when compared with Cebpd+/+ mice. (d) A 15 kDa band of α-synuclein protein is increased significantly in Cebpd-/- mice in different brain areas: cerebral cortex ($p = 0.0002$), striatum ($p = 0.0002$) and hippocampus ($p = 0.0009$). (e) Increased α-synuclein immunostaining is observed in several brain areas (cortex, striatum and hippocampus) of Cebpd-/- mice when compared with Cebpd+/+ mice. Many substantia nigra (S. Nigra) cells are intensely immunostained in Cebpd-/- mice. Magnification bar 300μm, 100μm in inset. (f) A significant increase in the 15 kDa band of α-synuclein protein is observed in primary cortical neuron cultures prepared from Cebpd-/- mice ($p = 0.0036$). *Snca* mRNA is evaluated in the mouse cerebral cortex by qRT-PCR using *Rn18s* as housekeeping gene. α-Synuclein protein is assessed in mouse brain areas and neuronal cultures by Western Blot using β-actin as the normalizing protein. Mice used in the in vivo experiments were of 2 months of age. In d white and black bars correspond to Cebpd+/+ and Cebpd-/- respectively and bars shows mean ± SD of n=3 (d) animals. ** $p < 0.01$ and *** $p < 0.001$, using Student's t-test.

Figure 2. Overexpression of Cebpd gene in mouse cerebellar granular cultures. (a) A significant increase in Cebpd mRNA expression is observed in pcDNA3-Cebpd granular cultures when compared with pcDNA3 cerebellar granular cultures ($p < 0.0001$). (b) C/EBPδ protein increases 12,2 fold ($p < 0.0001$) in pcDNA3-Cebpd cerebellar granular cultures. (c) A significant decrease in *Snca* mRNA expression is observed in pcDNA3-Cebpd cerebellar granular cultures when compared with pcDNA3 cerebellar granular cultures ($p = 0.0003$). (d) The 15 kDa band of α-synuclein protein is decreased in pcDNA3-Cebpd cerebellar granular cultures ($p = 0.0062$). White and black bars correspond to pcDNA3 and pcDNA3-Cebpd, respectively. Bars show means ± SD; n=6 independent experiments. (e) Immunofluorescence shows a clear decrease in α-synuclein immunoreactivity in pcDNA3-Cebpd cerebellar granular cultures.

Magnification bar, 100µm. (f) Electroporation efficiencies of pcDNA3 and pcDNA3-Cebpd were 88,1% and 82,3%, respectively. (G) A significant decrease in α-synuclein levels ($p < 0.0001$) in the conditioned media of pcDNA3-Cebpd cerebellar granular cultures when compared with pcDNA3 cerebellar granular cultures was determined by ELISA. Cebpd and *Snca* mRNAs are evaluated in cerebellar granular cultures by qRT-PCR using *Hprt* and *Rn18s* as housekeeping genes. C/EBPδ and α-synuclein protein are assessed in cerebellar granular cultures by Western Blot using β-actin as the normalizing protein. ** $p < 0.01$ and *** $p < 0.001$, using Student's t-test.

Figure 3. Overexpression of CEBPD gene in human SH-SY5Y cultures. (a) A significant increase in CEBPD mRNA expression (7,6 fold) is observed in SH-SY5Y cultures transfected with pcDNA3-CEBPD when compared with pcDNA3 ($p < 0.0001$). (b) A significant decrease in *SNCA* mRNA is detected ($p = 0.0028$) in pcDNA3-CEBPD transfected cultures. (c) A significant decrease in α-synuclein protein levels ($p = 0.0098$) in the conditioned media of SH-SY5Y cultures transfected with pcDNA3-CEBPD was determined by ELISA. (d) The 15 kDa band of α-synuclein protein is decreased in the pcDNA3-CEBPD SH-SY5Y cultures ($p = 0.0002$). In this bargraph bars show means ± SD of n= 6 independent experiments and white and black bars correspond to pcDNA3 and pcDNA3-CEBPD, respectively. *CEBPD* and *SNCA* mRNAs are evaluated in SH-SY5Y cultures by qRT-PCR using *ACTB* and *HPRT1* as the housekeeping genes. α-Synuclein protein is evaluated in SH-SY5Y cultures by Western Blot using β-actin as normalizing protein. ** $p < 0.01$ and *** $p < 0.001$, using Student's t-test.

Figure 4. C/EBPδ qChIP of mouse *Snca* and human *SNCA* genomic regulatory regions. (a) Schematic representation of the mouse *Snca* promoter showing the localization of six possible binding sites (named δ1, δ2, δ3, δ4, δ5 and δ6) for the transcription factor C/EBPδ in the *Snca* promoter region. (b) C/EBPδ protein binding to several *Snca* promoter boxes in granular neurons using qChIP: δ1 ($p = 0.0223$), δ2 ($p = 0.0236$), δ3 ($p = 0.0045$), δ4 ($p = 0.0371$) and δ6 ($p = 0.0372$). (c) Schematic representation of the human *SNCA* genomic regions showing that the transcription factor C/EBPδ has fourteen possible binding sites in the *SNCA* promoter (named δ1, δ2-4, δ5, δ6-7, δ8-10, δ11-12 and δ14), four possible binding sites in the *SNCA* intron 2 (named δ1, δ2-3 and δ4), and fourteen possible binding sites in the *SNCA* intron 4 (named δ1-2, δ3-4, δ5-7, δ8-9, δ10-12, δ13 and δ14). (d) C/EBPδ protein binding to two

SNCA promoter boxes in human cerebral cortex using qChIP: δ1 ($p < 0.0001$) and δ14 ($p < 0.05$). A significant decrease in C/EBPδ binding to human *SNCA* promoter boxes δ1 ($p = 0.0018$), δ13 ($p = 0.0026$) and δ14 ($p < 0.0001$), by qChIP was observed in cerebral cortex samples from PD patients (n=8) vs non-neurological controls (n=8). (e) C/EBPδ protein binding to *SNCA* intron 2 boxes in human cerebral cortex using qChIP: δ2-3 ($p < 0.0001$). A significant decrease in C/EBPδ binding to human *SNCA* intron 2, boxes δ2-3 ($p = 0.0004$) by qChIP is observed in cerebral cortex samples from PD patients (n=7) vs non-neurological controls (n=6). (f) C/EBPδ protein binding to *SNCA* intron 4 boxes in human cerebral cortex using qChIP: δ1-2 ($p < 0.01$), δ8-9 ($p < 0.01$) and δ14 ($p < 0.001$). A significant decrease in C/EBPδ binding to human *SNCA* intron 4 boxes δ1-2 ($p = 0.0001$), δ8-9 ($p = 0.0015$) and δ14 ($p < 0.0001$) by qChIP is observed in cerebral cortex samples from PD patients (n=8) vs non-neurological controls (n=8). (f) Expression of CEBPD mRNA in substantia nigra samples from non-neurological controls (n = 9) and PD (n = 21) patients. A significant downregulation in CEBPD mRNA ($p = 0.0037$) is observed in PD samples when compared with control samples. Bars show mean ± SD of 7 independent experiments with granular neuronal cultures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs respective no Ab, and $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs respective control, using Student's t-test.

Figure 5. Luciferase assays. (A) 293T cells were transfected with an empty pGL3 vector (Ø) or with a vector harboring 2000bp of the murine SNCA promoter (pGL3-PromSNCA 2000bp). Cells were cotransfected with shRNA control or shRNA for C/EBPδ and the relative expression of luciferase reporter gene was measured. Bars show mean ± SD of three independent experiments. *$p<0.05$ and **$p<0.01$ vs respective pGL3- Ø; $^{\#\#}$ $p<0.01$ vs respective shCtrl; Student's t-test. (B) C/EBPδ protein levels were determined by western blot in 293T cells transfected with shRNA control or shRNA for C/EBPδ. Tubulin was used as loading control.

Figure 6. *SNCA* and CEBPD gene in iPSC-derived dopaminergic neurons and non-dopaminergic neurons from healthy subjects (n = 4) and PD (n = 9) patients. (a) A significant increase in *SNCA* mRNA is detected ($p = 0.0006$) in iPSC-derived dopaminergic neurons from PD patients when compared with controls. (b) A significant decrease in CEBPD mRNA expression is observed in iPSC-derived dopaminergic neurons from PD patients ($p< 0.0001$) when compared with controls. (c) No significant

differences in CEBPB mRNA levels (p = 0.5879) are observed in iPSC-derived dopaminergic neurons from control vs PD patients. (d) A negative correlation is observed between the mRNA expression of CEBPD and *SNCA* in iPSC-derived dopaminergic neurons. (e) No significant correlation is observed between the mRNA levels of CEBPB and *SNCA* in the iPSC-derived dopaminergic neurons. In contrast, in non-dopaminergic neurons derived from iPSC no significant differences between controls and PD samples are observed in the mRNA levels of *SNCA* (f), CEBPD (g) and CEBPB (h). *CEBPB, CEBPD* and *SNCA* mRNAs are evaluated in iPSC-derived neurons by qRT-PCR using *ACTB* and *GADPH* as the housekeeping genes. *** $p < 0.001$ using Student's t-test; ** $p < 0.01$ using Pearson's correlation.

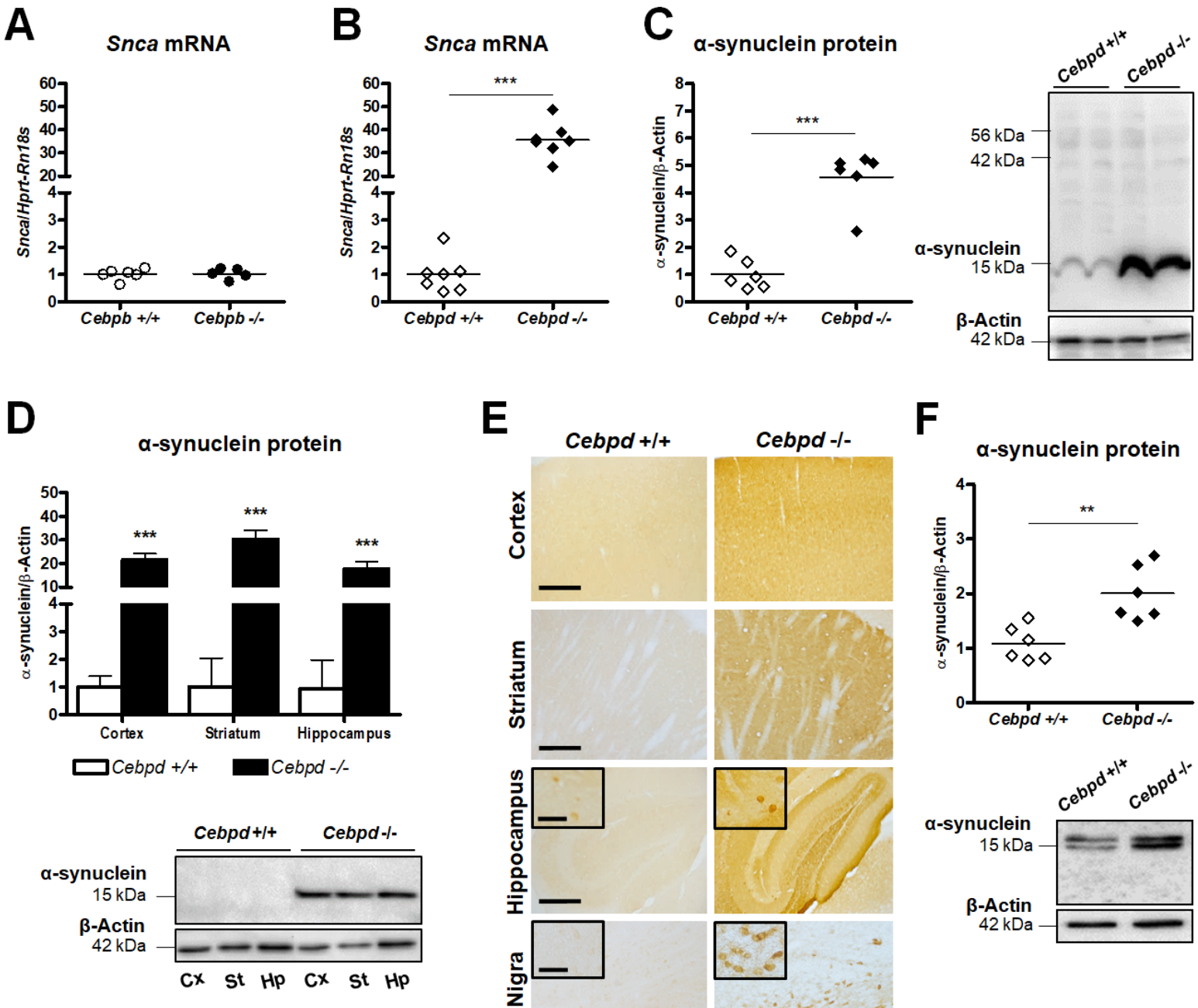
TABLE 1: Primers used for qRT-PCR

Target gene	Accession number	Forward primer	Reverse primer
Human			
ACTB	NM_001101.3	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
CEBPB	NM_005194.3	GCGCGAGCGCAACAACATC	TGCTTGAACAAGTTCCGCAG
CEBPD	NM_005195.3	CCATGTACGACGACGAGAG	TGTGATTGCTGTTGAAGAGG
GAPDH	NM_001289746.1	GAAGGTGAAGGTCGGAGTCA	GTTAAAAGCAGCCCTGGTGA
HPRT1	NM_000194.2	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT
RPS18	NM_022551.2	GATGGGCGGCGGAAAAT	CTTGTACTGGCGTGGATTCTGC
SNCA	NM_000345.3	GAATTCATTAGCCATGGATGTA	TGCTCCCTCCACTGTCTTCTG
Mouse			
Cebpd	NM_007679.4	CTCCACGACTCCTGCCATGT	GAAGAGGTCGGCGAAGAGTTC
Hprt	NM_013556.2	ATCATTATGCCGAGGATTTGG	GCAAAGAACTTATAGCCCCC
Rn18s	NR_003278.3	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Snca	NM_001042451.2	TGTACAGTGTGTTTCAAAGTCTTCC	GAAGCCACAACAATATCCACAGC

TABLE 2: Primers used for qChIP on *SNCA* genomic regulatory regions

	Box (distance to TSS*)	Forward primer	Reverse primer
Mouse *Snca*	Promoter		
	Cebpδ1 (-7249bp)	ACCTCTTGGTCCAGGCTTCT	TGGGGTTTGGAGGAATGTAA
	Cebpδ2 (-6456bp)	GGACCAGAATGGTTGGTCAT	GCCAAAGCAATTGTCCATGT
	Cebpδ3 (-5304bp)	GGCAATCGTTTCAATGCTACT	AAAAGGAGCATGTCCAGCAG
	Cebpδ4 (-4018bp)	TATTGGCAGCTTCTCTGCTG	CATGAGACATGTGCTGCTGA
	Cebpδ5 (-2358bp)	GGCCAGACTGTGTGACTGAA	GGTACTGGTCAGGGTGTTGC
	Cebpδ6 (-1660bp)	CAGGCCTGGCAAAACATTAT	AGGCTTCAGCCTTTTCCTTC
Human *SNCA*	Promoter		
	CEBPδbox1 (-4109bp)	GTATTTCTTGGGCTCATTAATGTG	CTTGGAATCTTGCATGCCTT
	CEBPδboxes2-4 (-3812bp; -3788bp; -3691bp)	GGCTTTCTCTACTCTACCCAC	CTTGTGTTCACAACAAATACGG
	CEBPδbox5 (-3084bp)	CTTGAGCAGCTTTCCCTCC	GGATGTTTACTGTGTTCAAGGC
	CEBPδboxes6-7 (-2920bp; -2860bp)	GGTAGTAAGAAATCATCTCCCCC	TCTTGATTTCCATTGTCACATCC
	CEBPδboxes8-10 (-2602bp; -2562bp; -2515bp)	TGGAGCTCAGTTTCTCTGTC	AAAAGTTTGGAATCATGTTAAACG
	CEBPδboxes11-12 (-2446bp;-2327bp)	CCAGAAATAATTCTCTCACATTGG	CTAGTTGTAAAGAGGCACAATGG
	CEBPδbox1 (-2008bp)	GTGGGTTACACTAATGCATGG	CCCTAGGTGTAAATTACACTGC
	CEBPδbox1 (-1279bp)	GCTTCCTGTTCCTTGTGGT	CGCAAGAATCAGACAAAGC
	Intron 2		
	CEBPδbox1 (+1745)	TGGAAATCTGAGGACAAACGGA	TGCATCTCATCAAAGTTCACAACA
	CEBPδbox2,3 (+2396, +2582)	TTTGCAGACATAGACGGAGCA	CCTAAAGTTCCACCTTGGGGT
	CEBPδbox4 (+3154)	ACATCACAGGGGCATATCAAAGTC	AGGCCAAGGAGGGAGTTGTG
	Intron 4		
	CEBPδboxes1-2 (+35366bp; +35533bp)	ACAATTGGCCTCAAGAATTGA	AAAGAGTGACAAGTTAGTGGAA
	CEBPδboxes3-4 (+35797bp; +35921bp)	TTGGAATTTTGCTTTTCTGTAAATAC	CGCTGTTGCCATCCTAAAG
	CEBPδboxes5-7 (+36393bp; +36413bp; +36438bp)	TCTACAATGCACAGGACATC	GAATTGTACTGACTACACCACG
	CEBPδboxes8-9 (+36662bp; +36762bp)	AAATTTAGCCTGGAGTGAGTAAT	CCTATTACATTCTTGCCCATGT
	CEBPδboxes10-12 (+38132bp; +38172bp; +38196bp)	AGACACATGTTGCTATCAAGC	CAACTTATTCAAAATGTTATAGTTTCTAC
	CEBPδbox13 (+38345bp)	ATTTGTTAAATGTGGTGGCTAGA	CCATGGGCCACGGGTTA
	CEBPδbox14 (+38560bp)	CAGCTATCATGAGTGTTAGTGTA	TCCAGTCTTTTGGCTTCCC

* TSS of variant NM000345.3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Cortex
Striatum
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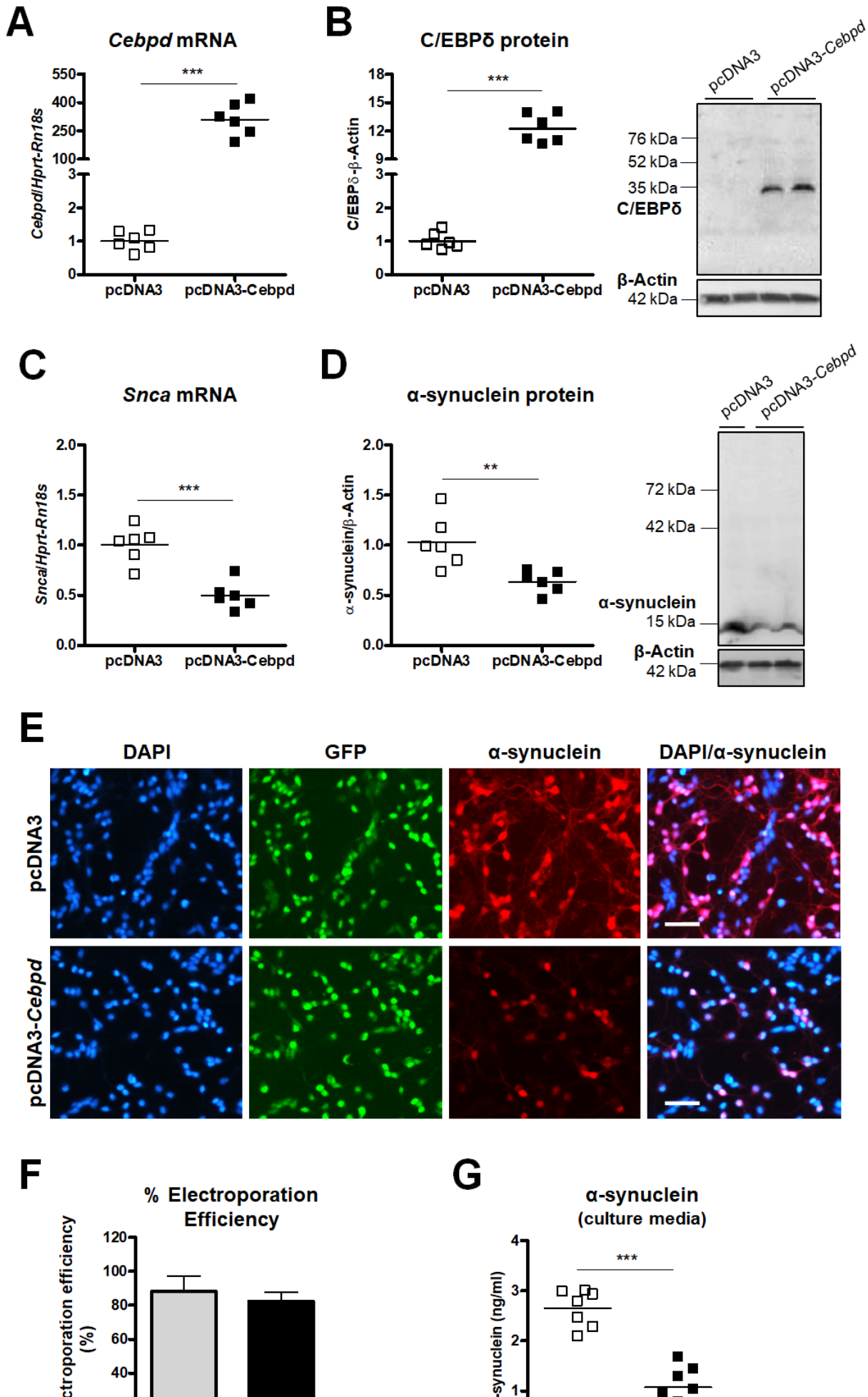
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35 kDa
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42 kDa
C
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42 kDa
α-synuclein
15 kDa
E
DAPI
GFP
α-synuclein
DAPI/α-synuclein
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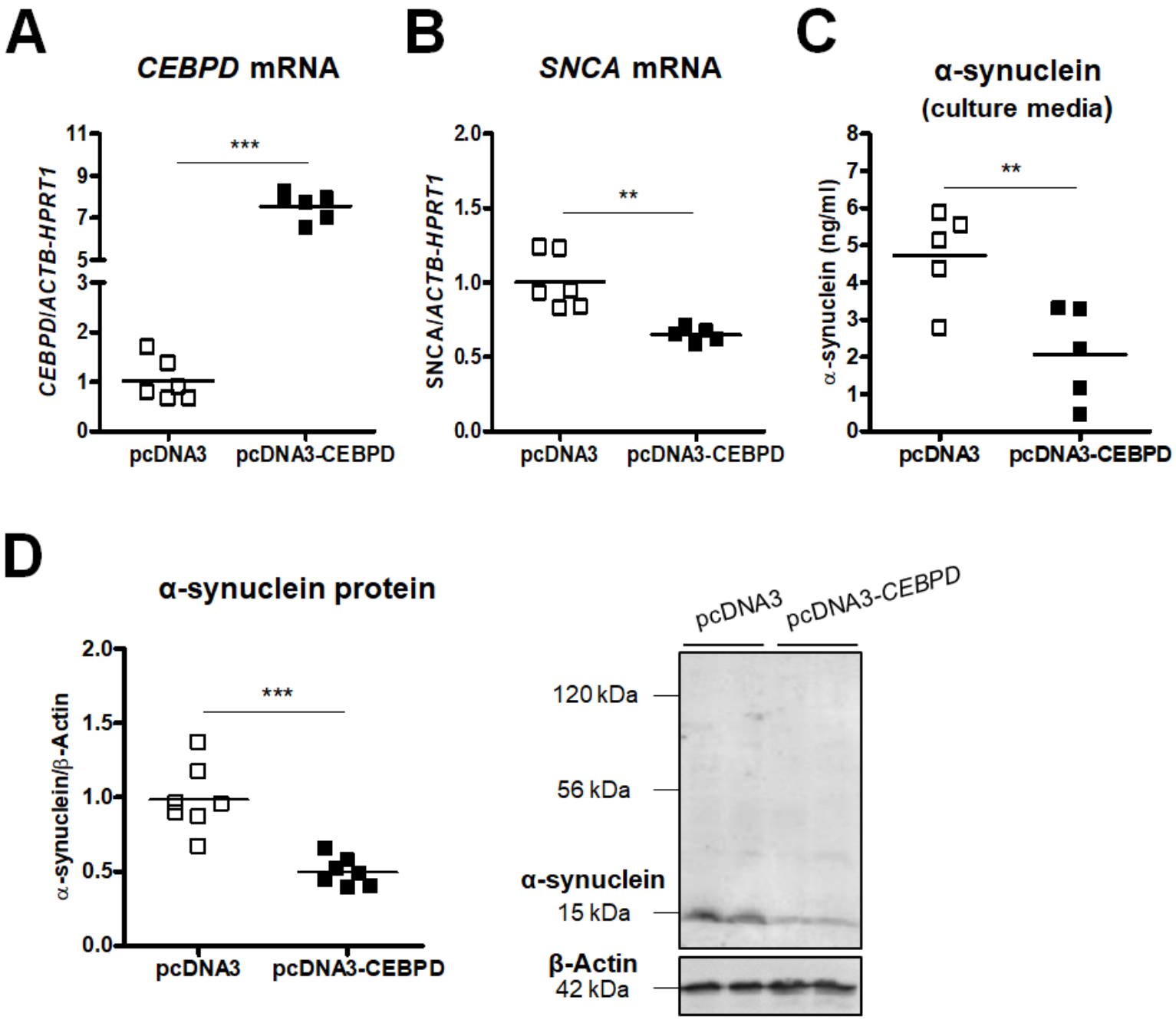
% Electroporation Efficiency
GFP electroporation efficiency (%)
G
α-synuclein (culture media)
α-synuclein (ng/ml)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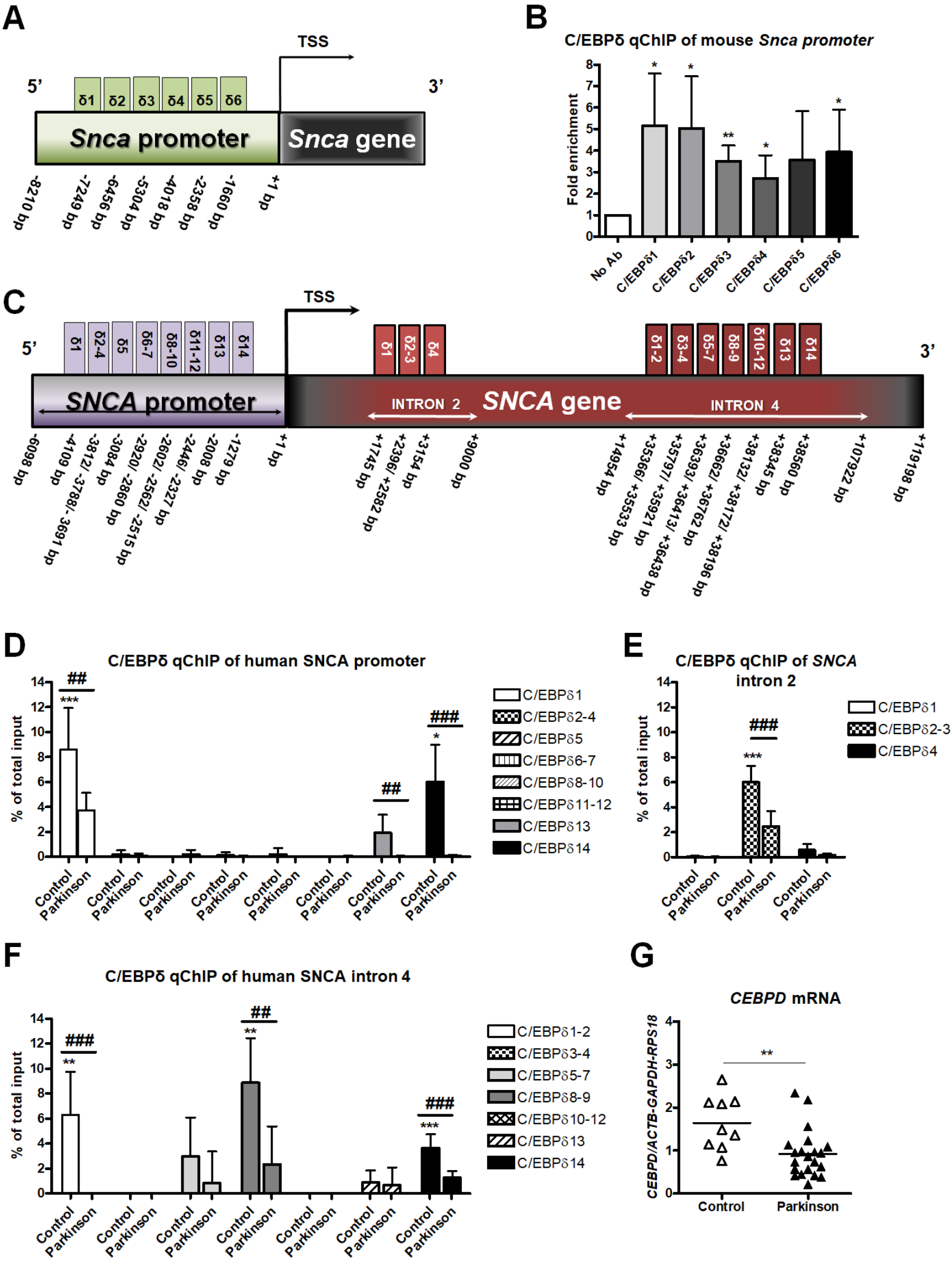
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+1 bp
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Fold enrichment
No Ab
C/EBPδ1
C/EBPδ2
C/EBPδ3
C/EBPδ4
C/EBPδ5
C/EBPδ6
C
TSS
5'
3'
δ1
δ2-4
δ5
δ6-7
δ8-10
δ11-12
δ13
δ14
δ1
δ2-3
δ4
δ1-2
δ3-4
δ5-7
δ8-9
δ10-12
δ13
δ14
SNCA promoter
INTRON 2
SNCA gene
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% of total input
Control
Parkinson
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C/EBPδ2-4
C/EBPδ5
C/EBPδ6-7
C/EBPδ8-10
C/EBPδ11-12
C/EBPδ13
C/EBPδ14
E
C/EBPδ qChIP of SNCA intron 2
% of total input
C/EBPδ1
C/EBPδ2-3
C/EBPδ4
F
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% of total input
C/EBPδ1-2
C/EBPδ3-4
C/EBPδ5-7
C/EBPδ8-9
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Control
Parkinson

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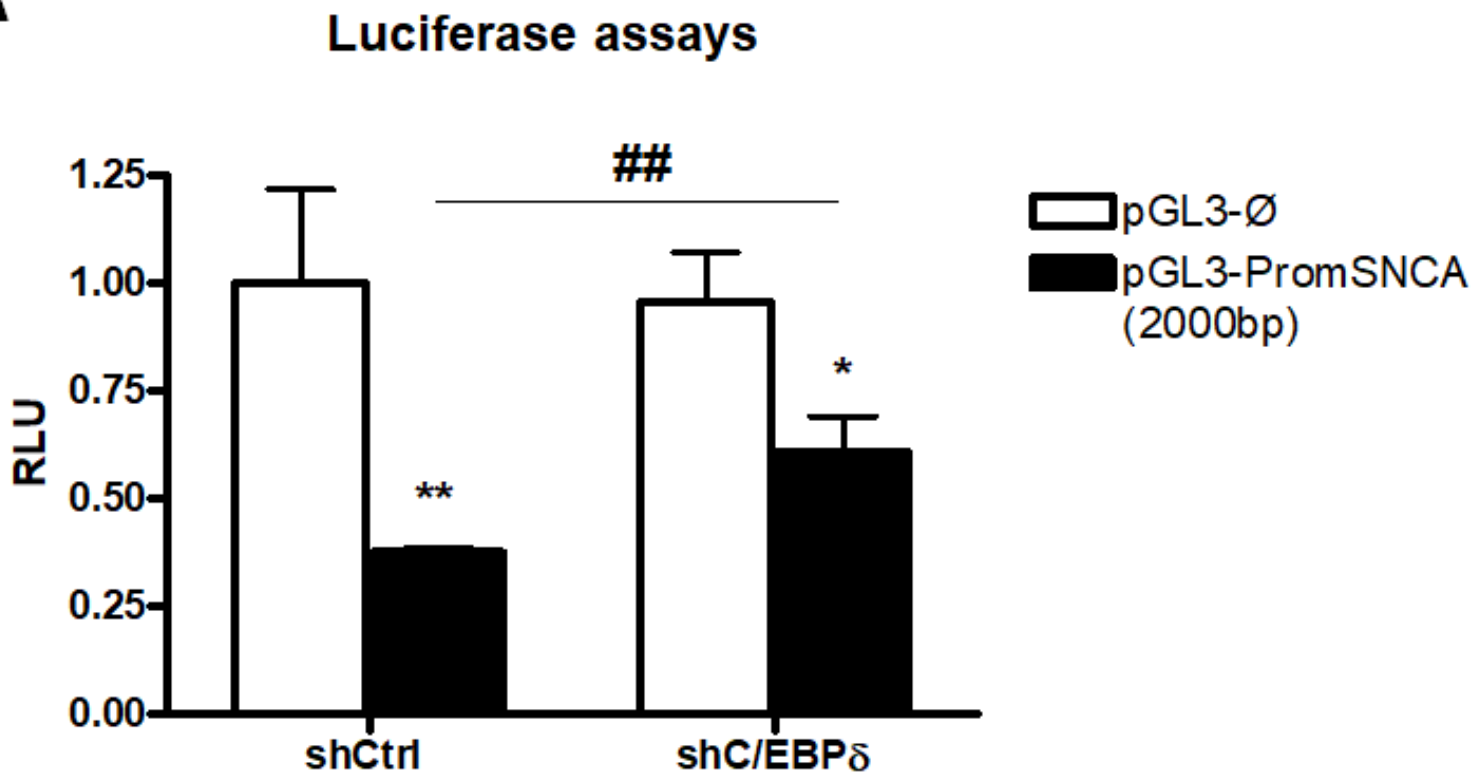
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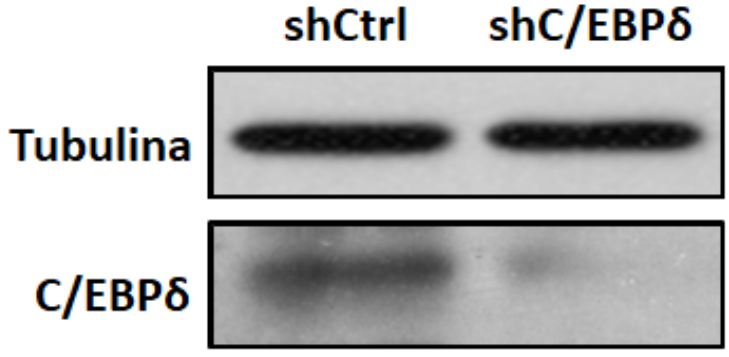
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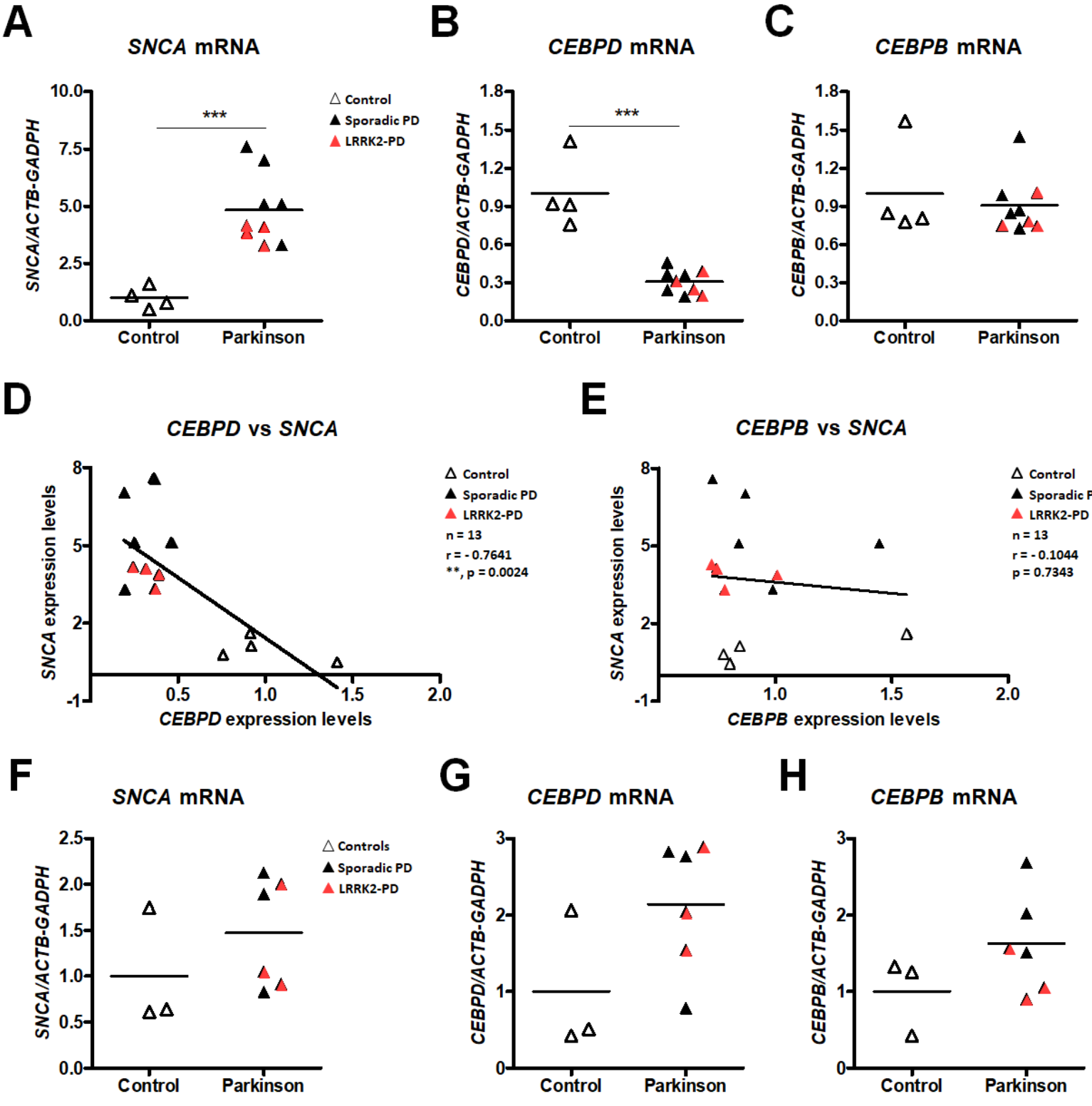
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Control
Parkinson
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Control
Parkinson
D
CEBPD vs SNCA
SNCA expression levels
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5
2
-1
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1.0
1.5
2.0
CEBPD expression levels
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1.5
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CEBPB expression levels
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SNCA/ACTB-GADPH
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2.0
1.5
1.0
0.5
0.0
Controls
Sporadic PD
LRRK2-PD
Control
Parkinson
G
CEBPD mRNA
CEBPD/ACTB-GADPH
3
2
1
0
Control
Parkinson
H
CEBPB mRNA
CEBPB/ACTB-GADPH
3
2
1
0
Control
Parkinson