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Effect of donepezil on the expression and responsiveness to LPS of *CHRNA7* and *CHRFAM7A* in macrophages: A possible link to the cholinergic anti-inflammatory pathway

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ABSTRACT

The α7 nicotinic acetylcholine receptor (CHRNA7) modulates the inflammatory response by activating the cholinergic anti-inflammatory pathway. CHRFAM7A, the human-restricted duplicated form of CHRNA7, has a negative effect on the functioning of α7 receptors, suggesting that *CHRFAM7A* expression regulation may be a key step in the modulation of inflammation in the human setting. The analysis of the *CHRFAM7A* gene's regulatory region reveals some of the mechanisms driving its expression and responsiveness to LPS in human immune cell models. Moreover, given the immunomodulatory potential of donepezil we show that it differently modulates *CHRFAM7A* and *CHRNA7* responsiveness to LPS, thus contributing to its therapeutic potential.

1. Introduction

Inflammation is a vital host response to infection and tissue damage, and plays a major role in host defence, tissue remodelling and repair, and the restoration of tissue homeostasis. The α7 nicotinic acetylcholine receptor (α7 nAChR, *CHRNA7*) is a homo-pentameric ligand-gated ion channel that is pivotally involved in synapse function, neuroprotection, neuronal survival, and the resolution of systemic inflammation (Sinkus et al., 2015). Its expression and activation in inflammatory cells (peripheral monocytes, macrophages, microglia) attenuates the release of pro-inflammatory cytokines, including TNF alpha, IL-1β, IL-6, an effect known as the cholinergic anti-inflammatory pathway (CAIP) (Hoover, 2017; Tracey, 2002; Wang et al., 2003). This makes α7 nAChR a suitable target for the pharmacological treatment of disorders characterised by systemic inflammation in which alterations in its expression and function have been reported (Bacchelli et al., 2015; Burghaus et al., 2000; Conejero-Goldberg et al., 2008; Gotti and Clementi, 2004; Ramos et al., 2016; Yang et al., 2015), including sepsis, diabetes, osteoarthritis, inflammatory bowel disease, stroke, traumatic brain injury, and neurodegenerative disease, including Alzheimer's disease (Conejero-Goldberg et al., 2008; Confaloni et al., 2016; Dineley et al., 2015; Egea et al., 2015; Han et al., 2017; Hoover, 2017; Quik et al., 2015).

Recent studies indicate that, in addition to their major function of choline esterase inhibition, acetylcholinesterase (AChE) inhibitors (donepezil, galantamine, huperzine A, physostigmine), which are widely used for the symptomatic treatment of Alzheimer's disease and other dementias, have neuroprotective properties mediated *via* α7 nAChRs in preclinical models (Akaike et al., 2010; Arias et al., 2005; Wang et al., 2008). These inhibitors have also been shown to significantly modulate innate immunity (Pavlov et al., 2009; Pohanka, 2014; Reale et al., 2004), possibly as a result of the increased availability of acetylcholine activating the cholinergic anti-inflammatory pathway (Pavlov et al., 2009; Pohanka, 2014). Moreover, accumulating evidence strongly suggests that donepezil, a selective non-competitive inhibitor of AChE, directly affects cellular functions *via* a mechanism

Abbreviations: nAChR, nicotinic acetylcholine receptor; CNS, central nervous system; AChE, acetyl choline esterase; LPS, Lypopolysaccharide; TNF alpha, Tumor necrosis factor-alpha; IL-1β, interleukin-1β; IL-6, interleukin 6; RACE, rapid amplification of cDNA ends; TSS, transcription start site; DRB, 5, 6-dichloro-1-b-D-ribofuranosylbenzimidazole; CAIP, cholinergic anti-inflammatory pathway; DPZ, donepezil

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that is independent of its acetylcholinesterase inhibition (Akasofu et al., 2008; Takada-Takatori et al., 2008). In particular, Hwang *et al* have reported that its anti-inflammatory effects on microglia cell lines occurs in the absence of AChE activity (Hwang et al., 2010), and suggested that donepezil directly stimulates α7 nAChR although they cannot rule out that still unknown pathways may be involved. Moreover, single nucleotide polymorphisms (SNPs) that may lead to altered expression and/or function of α7 nAChR have been proved to play an important role in therapeutic response to AChE inhibitors in Alzheimer's disease (Braga et al., 2015; Clarelli et al., 2016; Russo et al., 2017; Weng et al., 2013).

The human *CHRNA7* gene underwent a recombination event that gave rise to the human-restricted *CHRFAM7A* gene, located at Chr.15q13.3, as the product of the partial duplication and fusion of *CHRNA7*'s exons 5–10 with the novel exon D (whose provenance is unknown), and exons C, B and A, belonging to the *ULK4* gene (Gault et al., 1998; Sinkus et al., 2015). The *CHRFAM7A* gene is particularly expressed in the central nervous system (CNS) and immune system, and is translated into two protein products obtained from alternative splicing (Benfante et al., 2011). The CHRFAM7A protein lacks the N-terminal domain of the CHRNA7 subunit, including the signal peptide and the acetylcholine-binding domain, but the overall structural analogies between the two subunits may indicate their association. In fact, the CHRFAM7A subunit is capable of assembling with CHRNA7 subunits and exerting a dominant negative effect on α7 nAChR function (Araud et al., 2011; Wang et al., 2014), suggesting a role in affecting CNS processes, such as cognition and memory, and mental health (Bacchelli et al., 2015; De Luca et al., 2006; Gault et al., 2003; Kunii et al., 2015; Neri et al., 2012; Ramos et al., 2016; Sinkus et al., 2015; Yasui et al., 2011) and in the pathogenesis of neurodegenerative diseases. It is known that CHRFAM7A is the mainly expressed α7 subunit in leukocytes and other non-neuronal tissues (Benfante et al., 2011; Costantini et al., 2015a; Costantini et al., 2015b; Dang et al., 2015) and that in these cells it can regulate the anti-inflammatory effects of α7 nAChR activation (Araud et al., 2011; de Lucas-Cerrillo et al., 2011).

Little is known about the expression and functional mechanisms of CHRFAM7A in inflammatory diseases, although a number of recent studies have identified alterations in *CHRFAM7A* expression in inflammatory bowel disease and colon cancer (Baird et al., 2016; Costantini et al., 2015a; Costantini et al., 2015b; Dang et al., 2015; Ramos et al., 2016). Interestingly, we have shown that acute stimulation of human primary monocytes and macrophages with LPS, a wall component of Gram Negative bacteria able to trigger acute inflammation, down-regulates CHRFAM7A expression at mRNA and protein levels by a mechanism driven by NF-κB in a way that is paralleled by the up-regulation of *CHRNA7* mRNA (Benfante et al., 2011; Khan et al., 2012), which suggests a regulatory role in activating the cholinergic anti-inflammatory pathway. The regulation of *CHRFAM7A* expression may be a key step in the modulation of inflammation, as recently described (Maldifassi et al., 2018), but the mechanisms driving the transcriptional regulation of *CHRFAM7A* gene in human immune tissues are largely unknown. The identification and detailed analysis of the *CHRFAM7A* gene's regulatory region, shown here, reveals some of the complex mechanisms driving its expression and responsiveness to pro-inflammatory stimuli in a human immune cell model.

Given the anti-inflammatory potential of the acetylcholinesterase inhibitor donepezil, the little current knowledge about its mechanism of action, and the role of CHRFAM7A in the regulation of CHRNA7 function in humans, we investigated donepezil effect on the expression profile of *CHRFAM7A* and *CHRNA7* in human immune cell models (the promonocytic THP-1 cell line and primary macrophages) and found the unexpected up-regulation of both the *CHRFAM7A* and *CHRNA7* genes, suggesting that the immunomodulatory potential of the drug may be exerted by regulating the activation of cholinergic anti-inflammatory pathway through the modulation of the expression of *α7* nAChR and *CHRFAM7A* at transcriptional level.

Given the great therapeutic potential of donepezil, we consider that the results provided could contribute to a better characterization of its pharmacological activity.

2. Materials and methods

2.1. Cell lines and treatments

The human acute monocytic leukaemia cell line THP-1 was cultured in RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) at 37 °C in the presence of 5% CO_2. The cells were maintained at a recommended density of between 2×10^5 and 10^6 cells/mL, and seeded at a density of 3×10^5 cells/mL on the day before all treatments. Each experiment was performed using cells at low passage.

The primary monocytes and macrophages were obtained as previously described (Benfante et al., 2011). Briefly, blood $CD14^+$ monocytes were obtained from the buffy coats of healthy donors using Ficoll-Hypaque (Ficoll, Biochrome) and Percoll (Amersham Pharmacia Biotech) gradient centrifugation. The macrophages were obtained from monocyte differentiation after six days of culturing under standard conditions in the presence of 100 ng/mL macrophage colony-stimulating factor (M-CSF).

Lypopolysaccharide (LPS from *Escherichia coli* 055:B5 strain; Sigma-Aldrich) was used at a final concentration of 1 μg/mL, for the times indicated in the figure legends. The cholinesterase inhibitors donepezil (Sigma-Aldrich) and galantamine hydrobromide (Space) were used as described in the figure legends. The transcription blocker 5, 6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB 75 μM; Sigma-Aldrich) was used for the times indicated in the figure legend. (−)-Nicotine hydrogen tartrate salt (Sigma-Aldrich) was used at 100 nM for three hours.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted using the RNeasy Mini kit and accompanying QIAshredder (Qiagen) in accordance with the manufacturer's instructions. Briefly, a maximum of 9×10^6 cells were collected by means of centrifugation, and lysed with 600 μL of buffer RLT plus β-mercaptoethanol (10 μL/mL of buffer RLT). The lysate was homogenised using a QIAshredder column centrifuged for two minutes at maximum speed. To avoid DNA contamination, the samples were on-column incubated with DNase I for 15 min, and the RNA eluted using 50 μL of RNase-free water.

The amount of eluted total RNA was determined by means of spectrophotometry at 260 nm, and its purity was evaluated using the 260/280 ratio. One microgram per sample was reversed transcribed using the SuperScript™ IV (Life Technologies) in accordance with the manufacturer's instructions.

2.3. 5′ RACE

The *CHRFAM7A* mRNA 5′ terminus was determined using the *FirstChoice RLM-RACE* kit (AMBION) in accordance with the manufacturer's instructions. Briefly, the total RNA of the THP-1 cells, primary monocytes and macrophages was treated with the calf intestinal alkaline phosphatase (CIP) at 37 °C for one hour in order to remove the 5′-phosphate group from the ribosomal RNA, tRNA, degraded mRNA fragment and genomic DNA, and ligated to a 45 bp long adapter by means of T4 RNA ligase after TAP-mediated mRNA decapping. The RNA was collected by means of centrifugation at 14,000 rpm for 20 min at 4 °C; the pellet was washed with 0.5 mL of 70% ethanol and re-suspended in RNase-free water. The adapter-ligated mRNA was reverse transcribed using *SuperScript III First-Strand Synthesis System RT-PCR* (Life Technologies) and random hexamers, and the cDNA was amplified

using nested PCR with *GoTaq Flexi* (Promega) in accordance with the manufacturer's instructions: an initial denaturation step at 95 °C for three minutes, followed by 40 cycles of 30″ denaturation at 95 °C, 45"annealing at 60 °C, 80″ elongation at 72 °C and one 5-min cycle of elongation at 72 °C. The primers used for the outer PCR reaction were: *forward outer* primer 5′ – GCT GAT GGC GAT GAA TGA ACA CTG - 3′; reverse primer *#ex5*, 5′ – GTT AGT GTG GAA TGT GGC GTC AAA GC - 3′. The primers for the inner PCR reaction were: *forward inner* primer 5′- CGC GGA TCC GAA CAC TGC GTT TTG CTG GCT TTG ATG - 3′; reverse primers *#exA* 5′- GCA GTT TGC AGC TAT CCA CAA AAT GC - 3′.

2.4. Quantitative real-time PCR

The gene expression analyses were made using a quantitative real-time PCR assay, a Quant Studio 5 Thermocycler (Applied Biosystems) and Quant Studio 5 software. The target sequences were amplified from 50 ng of cDNA in the presence of TaqMan® Gene Expression Master Mix (Life Technologies). The TaqMan® primer and probe assays were human *CHRNA7* (ID #Hs01063373_m1), human *CHRFAM7A* (ID #Hs04189909_m1) and the endogenous control *GADPH* (ID #Hs99999905_m1), and *ACTB* (ID#Hs01060665_g1). The $2^{-\Delta\Delta CT}$ method was used to calculate the results, thus allowing the normalisation of each sample to the endogenous control, and comparisons with the calibrator for each experiment (set to a value of 1) where indicated.

2.5. Plasmid vectors

All of the reporter constructs were obtained by sub-cloning fragments of the human *CHRFAM7A* gene 5′-flanking region into the pGL4 basic plasmid (pGL4.11, Promega) as described below.

CHRFAM7A −2122/−184: The genomic region including exon D (from −2122 to −155 with respect to the ATG codon in exon B) was obtained by means of PCR amplification of THP-1 genomic DNA using an *Expand High Fidelity Kit* (Roche), in the presence of a forward (5′- ATG ACA CCA ACC ATG AGG TCC CA -3′) and reverse primer (5′- CTT AAT GTT GCG GTG GGG CG -3′). The fragment was cloned into the PCRII vector (Life Technologies) in accordance with the manufacturer's instructions to generate the *CHRFAM7A* −2122/−155_pCR II vector. The fragment containing the putative promoter region and exon D (from −2122 to −184 with respect to the ATG codon in exon B) was cut off by digestion with *Eco* RV and *Sac* II, blunted using T4 polymerase (NEB), and cloned into the pGL4.11 vector linearized with *Eco* RV.

CHRFAM7A −2015/−184: Region −2122/−2015 was deleted by digesting the *CHRFAM7A* -2122/−184 with Eco RV and *Avr* II, followed by a fill-in with the Klenow fragment (NEB) and re-ligation of the plasmid vector.

CHRFAM7A −1819/−184: Region −2122/−1819 was deleted from the *CHRFAM7A* -2122/−184_pGL4.11 vector by means of Eco RV and PflM I digestion, followed by end blunting using T4 polymerase and re-ligation of the plasmid vector.

CHRFAM7A −1459/−184: The −1459/−735 region was cut out from vector *CHRFAM7A* -2122/−184 by means of *Nhe* I and *Nru* I digestion. The fragment was filled in with the Klenow enzyme and re-cloned into the *CHRFAM7A* -2122/−184_pGL4.11 vector digested with Eco RV and Nru I.

CHRFAM7A −1163/−184 _pGL4.11: The *CHRFAM7A* −2122/−184 construct was digested with *Spe* I and *Pvu* II, and the obtained fragment was filled in with the Klenow fragment and then cloned into the pGL4.11 vector digested with Eco RV and Pvu II.

CHRFAM7A −735/−184: *CHRFAM7A* -2122/−184 was digested with Nru I and Hind III and re-cloned into the pGL4.11 vector digested with Eco RV and Hind III.

CHRFAM7A −557/−184: *CHRFAM7A* −2122/−184 was digested with Eco RV and *Eco* NI, followed by re-ligation of the plasmid vector.

CHRFAM7A −2122/−557: *CHRFAM7A* −2122/−184 was digested with Hind III and Eco NI, and the 1570 bp fragment blunted by means of Klenow treatment was cloned into the pGL4.11 vector linearized with Eco RV.

CHRFAM7A −4280/−184: In order to generate this construct, region −4280/−1207 was amplified by means of PCR using the primers: #-forward 5′- AGA CTG ATG CTC AGC CCC TT 3′ and #reverse 5′ - CCA GAC ACA TCT AAC CTA CCA AG 3′, and subcloned into the pCRII vector (Invitrogen). The cloned fragment was then cut out by means of Eco RV and PflM I digestion, and inserted into *CHRFAM7A* −2122/−184_pGL4.11 digested with the same enzymes.

2.6. Transient transfection and luciferase assay

THP-1 cells were transfected by means of a lipid-based method (DREAMFECT Gold, Li Star Fish). The day before transfection, 1×10^6 cells were plated in 2 mL complete RPMI medium in a 6-well plate. On the day of transfection, 50 µL of plain RPMI medium were mixed with 2 µg of plasmid DNA (Mix 1) and both *Firefly*- and *Renilla*-containing constructs (pGL4.74, Promega) in a 1:1 M ratio; in another tube 50 µL of plain RPMI medium were mixed with 8 µL of DREAMFECT gold (Mix 2) in order to have a 1:4 DNA lipid ratio. Mix 2 was gently added to Mix 1, and the mixture was left for 20 min at room temperature in order to allow the formation of lipid/DNA complexes, which were added dropwise to the cells. All of the transfections were performed in duplicate, and each construct was tested in at least three independent experiments using different batches of plasmid preparation.

Five hours after transfection, the cell medium was changed and on the following day, the cells were treated with LPS 1 µg/mL for six hours.

The activity of the *CHRFAM7A* constructs was evaluated using a dual-luciferase reporter assay system (Promega) and a GloMAx Discovery luminometer (Promega).

2.7. TNF-α ELISA

TNFα release by macrophages was quantified using the human Tumor Necrosis Factor-α ELISA kit (InVitrogen), according to the manufacturer's protocol. Cells are incubated for three hours with 20 µM donepezil, followed by drug removal and incubation for other three hours with 1 µg/mL LPS in the absence or presence of 100 nM nicotine. 1 mL medium sample was taken and snap-frozen in liquid nitrogen, followed by storage at −80 °C until sample analysis. The medium samples were diluted in the incubation buffer before analysis, and hundred microliters of medium sample were analysed in duplicate on a streptavidin coated microtiter plate (MTP), together with standards of known TNFα concentration for a standard curve. The manufacturer's protocol was followed using the biotin-labelled capture antibody and the peroxidase-conjugated detection antibody, which was developed using the tetramethylbenzidine (TMB) substrate. The colour produced was measured in the Glomax Discovery (Promega) reader at 450 nm and the sample TNFα concentration was determined by means of comparison with the standard curve.

2.8. Total cell extract and western blot analysis

Total cell extracts were prepared as described (Benfante et al., 2011) with minor modifications. 50 µg of total primary macrophage cell extracts were size-fractionated on a 10% SDS-PAGE mini gel and transferred to a nitrocellulose membrane (GE Healthcare). The primary antibodies used were α7 (Benfante et al., 2011) or mouse anti-actin (Sigma-Aldrich Co. Cat. n. A 4700). The secondary antibodies were goat-anti-rabbit (for α7) or goat-anti-mouse (for actin) conjugated with horseradish peroxidase (Pierce Biotechnology Inc., Rockford, IL). The signals were developed using Westar Supernova or Westar EtaC Ultra

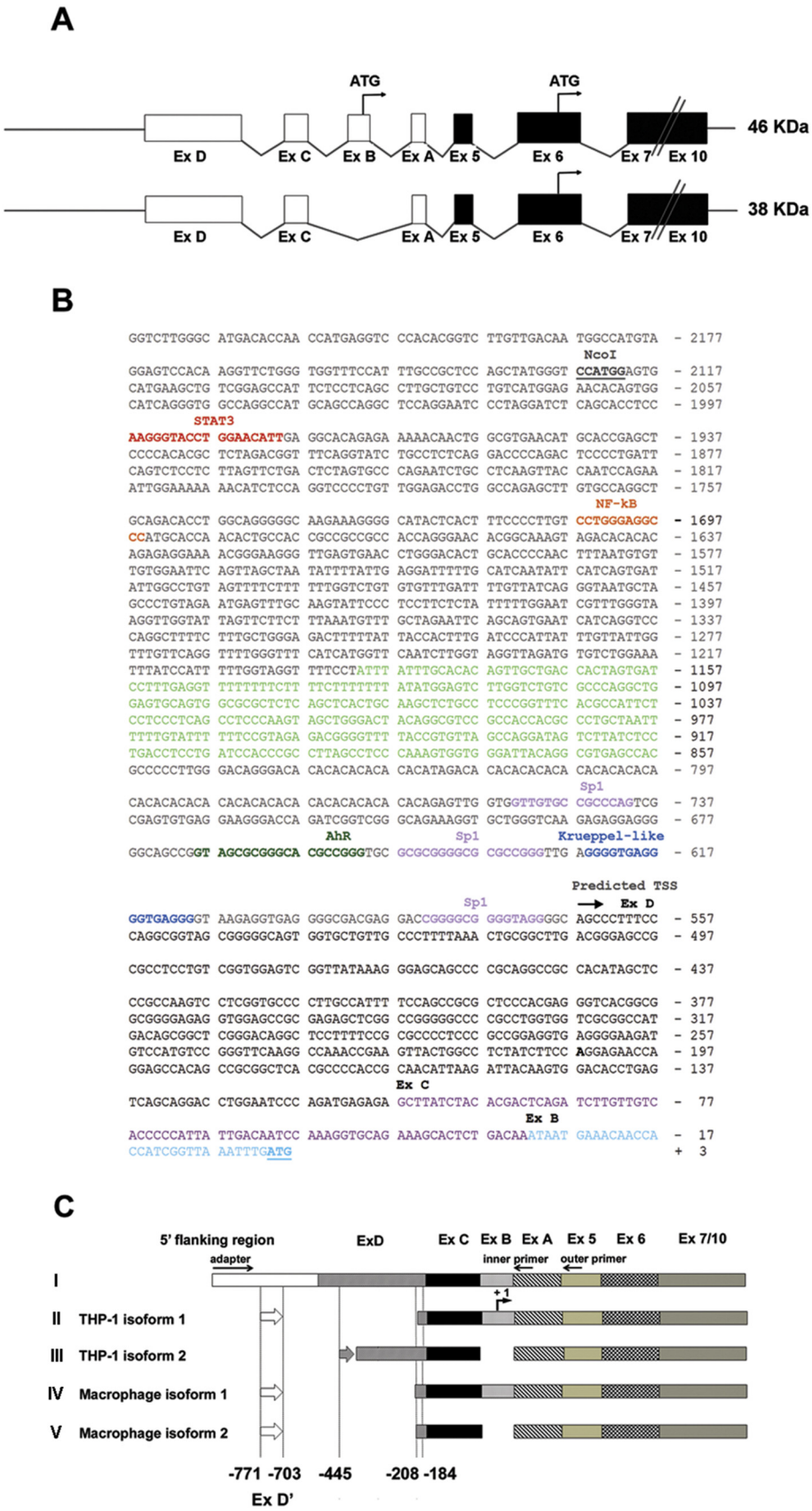

Fig. 1. The *CHRFAM7A* gene and identification of the transcription start site. A) Schematic representation of the two *CHRFAM7A* gene spliced isoforms. Top: isoform 1 is characterised by the presence of exon D to exon 10. Two Open Reading Frames (ORFs) are predicted with the ATG codon respectively located in exon B and exon 6. Isoform 1 translated from the ATG in exon B originates the longest protein (about 46 KDa), which is characterised by a 27 amino acids N-terminal domain encoded by exons B and A, whereas translation from the ATG in exon 6 generates the shortest protein (about 35 KDa), which lacks the N-terminal domain. Bottom: isoform 2 is characterised by a skipped exon B and is translated from the ATG in exon 6, which only gives rise to the shortest protein. B) MatInspector Software (http://www.Genomatix.de) bioinformatics analysis of a region of about 2000 bp upstream of exon B of the *CHRFAM7A* gene and showing Ex B, Ex C, Ex D and about 1500 bp of the genomic region upstream of Ex D. The numbering does not take into account the length of intron 1 (between Ex D and Ex C: 6264 bp) or intron 2 (between Ex C and Ex B: 3480 bp). The analysis reveals the presence of a number of consensus sequences for transcription factors involved in immune cell differentiation and maturation. In particular, it highlights the presence of an NF-κB consensus sequence (located −1706/−1635 bp from the ATG in exon B) and an AhR and STAT3 consensus sequence. The EST analysis predicted the start of transcription −566 bp from the ATG in exon B (black arrow), in correspondence with the first nucleotide of exon D. The University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu) highlights an *Alu* sequence (green) in *CHRFAM7A* 5′ flanking the region encompassing −1190 bp/−857 bp with respect to the ATG codon in exon B. C) 5′ RACE analyses. I) The 5′RACE analyses of the *CHRFAM7A* gene in the THP-1 cell line and human macrophages were performed using a specific outer primer (an oligonucleotide complementary to the *CHRFAM7A* exon 5) and an inner primer complementary to exon A. II) and III) The analysis of the THP-1 cell line identified two TSS: one at −771 bp with respect to the ATG in the exon B (II), the other at −445 bp (III). IV and V) A single TSS located at −771 bp was identified in both primary human macrophage isoforms. +1 indicates the ATG codon in exon B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

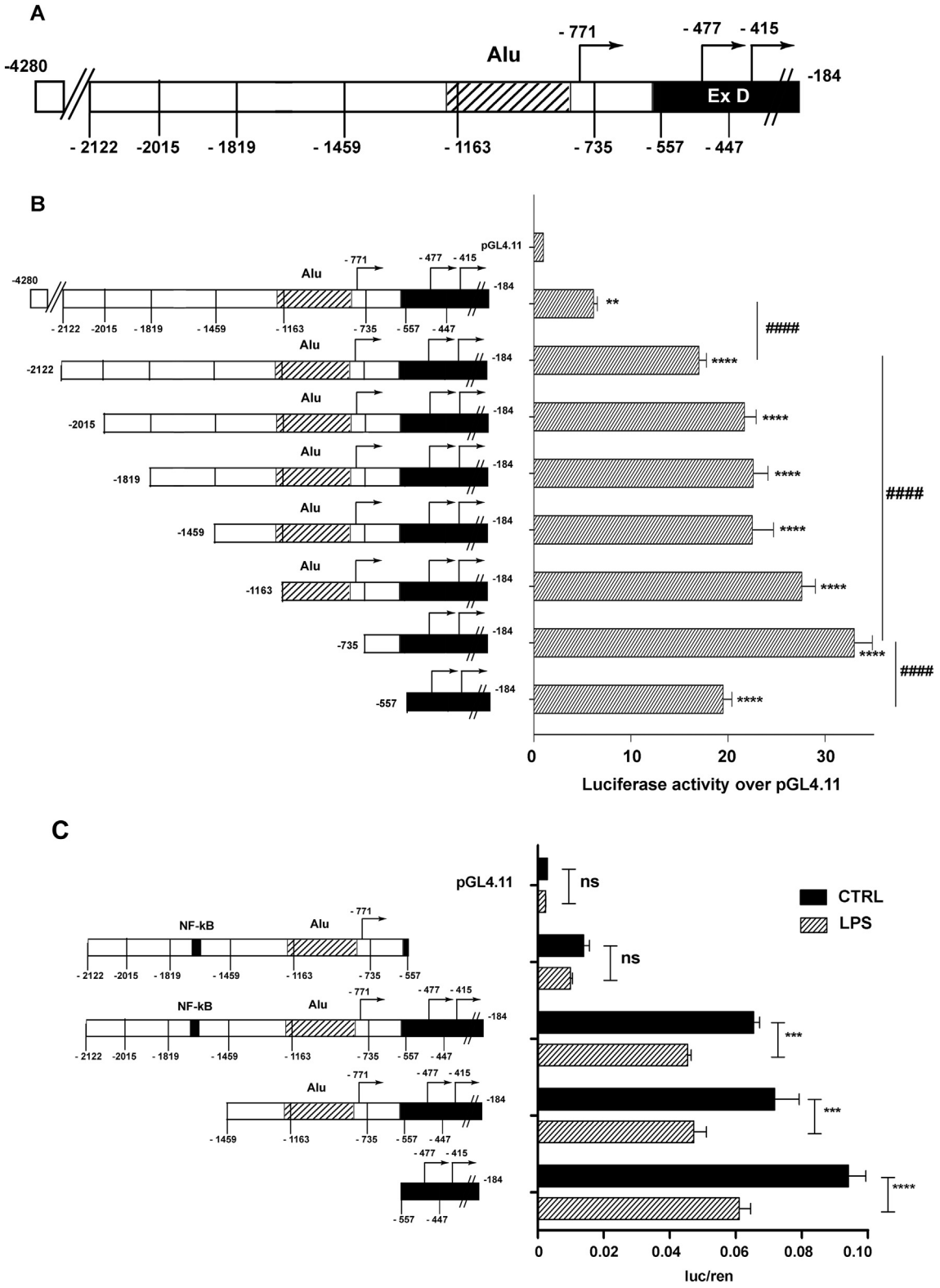

Fig. 2. Functional analysis of the *CHRFAM7A* 5′ flanking region. A) Schematic representation of the *CHRFAM7A* 5′flanking region. The arrows indicate the TSS identified by the 5′ RACE analysis, and the striped box the *Alu* sequence. The numbers below indicate the ends of the deleted constructs used for the functional analysis. B) Functional analyses of the *CHRFAM7A* 5′flanking region. Left: schematic representation of the constructs transiently transfected in THP-1 cells. Right: luciferase assay. The results are expressed as fold increases over the promoter-less vector, pGL4.11, and are the mean values ± standard error of four independent transfections performed in triplicate. The data were analysed using one-way ANOVA followed by Tukey's test and GraphPad Prism 5 software (GraphPad Software, Inc.); $^{**}p < .01$ and $^{****}p < .0001$ in comparison with pGL4.11. $^{\#\#\#\#}p < .0001$. C) Mapping of the LPS responsive region. THP-1 cells were transiently transfected with the indicated constructs and a luciferase assay was carried out after six hours of LPS treatment. The results are expressed as the mean values ± standard error of each construct in at least three independent experiments. The data were analysed using one-way ANOVA followed by Tukey's test and GraphPad Prism 5 software (GraphPad Software, Inc.); $^{***}p < .001$; $^{****}p < .0001$. ns, not significant.

(Cyanagen, Bologna, Italy), and densitometrically quantified by ChemiDoc™ Imaging System (Bio-Rad) at different time exposures.

3. Results

3.1. Identification of the CHRFAM7A regulatory region

CHRNA7 and its duplicated gene *CHRFAM7A* are located 1.6 Mb apart on Chr.15q13-q14, thus making it unlikely that the two genes are controlled by the same regulatory region. This assumption is strengthened by the fact that in human monocyte-derived macrophages pro-inflammatory stimuli (*e.g.* LPS) up-regulate *CHRNA7* expression and have an opposite effect on *CHRFAM7A* (Benfante et al., 2011).

CHRFAM7A is encoded by a ten-exon gene (a hybrid of exons D-A and exons 5–10 of CHRNA7) whose transcription gives rise to two alternative spliced mRNAs, one with and one without exon B (http://www.ensembl.org; isoform 1 ID: ENST00000299847.6 and isoform 2 ID: ENST00000397827.7). These two transcripts contain an open reading frame due to the presence of two ATG codons (one in exon B of isoform 1, and one in exon 6 of isoform 2), thus giving rise to two proteins of respectively 46.22 kDa and of 35.48 kDa (Fig. 1A) (Benfante et al., 2011). An EST analysis of the *CHRFAM7A* gene predicted a transcription start site (TSS) located 566 bp upstream of the ATG codon in exon B, thus defining a length of 410 bp for exon D and a 5′-UTR specifying region of 566 bp (in the case of the mRNA encoding isoform 1) or 919 bp (in the case of isoform 2).

An *in silico* analysis of a region spanning 2000 bp of the *CHRFAM7A* 5′-flanking region using the MatInspector database (http://www.genomatix.de) identified a number of sites for housekeeping and tissue-specific transcription factors (Sp1, AhR, Kruppel-like), including NF-kB, which is known to play a crucial role in the negative regulation of the *CHRFAM7A* gene in response to LPS (Fig. 1B) (Benfante et al., 2011). The presence of consensus sequences of known transcription factors suggested that this region was a good candidate driver of *CHRFAM7A* gene expression. The *in silico* sequence analysis also allowed us to map an *AluY* sequence located between 560 and 260 bp upstream of the putative transcriptional start site (−1190/−857 with respect to the ATG codon in exon B; green in Fig. 1B); this typically 300 bp long sequence is highly repeated in the human genome, and is associated with both positive and negative regulatory effects on gene expression (Bakshi et al., 2016; Bouttier et al., 2016; Deininger, 2011; Ebihara et al., 2002) as has been demonstrated in the case of nicotinic subunit genes among others (Ebihara et al., 2002; Fornasari et al., 1997).

3.2. CHRFAM7A transcription starts from multiple TSS in primary human macrophages and the THP-1 cell line

In order to map and functionally characterise the *CHRFAM7A* gene regulatory region, total RNA extracted from THP-1 cells and primary cultures of human macrophages was analysed using 5′-RACE. THP-1 cells do not express the *CHRNA7* gene (Benfante et al., 2011), whereas primary macrophages express both the *CHRNA7* and *CHRFAM7A* genes, which share 99% sequence homology in the region encompassing exons 5–10. Two transcripts were identified in the THP-1 cell line: one missing the region between −208 and −703, with a transcription start site mapping 771 bp upstream of the ATG codon in exon B (referred as +1) and corresponding to isoform 1 as it retained exon B (Fig. 1C, lane II); the other starting 120 bp downstream of the site predicted by the EST analysis (Fig. 1C, lane III), and 445 bp upstream of the ATG codon in exon B and corresponding to isoform 2, as exon B is not present (Fig. 1C, lane III).

As expected, the main *CHRFAM7A* transcript in primary monocyte-derived macrophages corresponded to that starting at −771 (Ex D') and missing region −703/−208 of exon D. Both isoforms 1 and 2 were detected (Fig. 1C, lanes IV and V).

3.3. Functional analysis of the CHRFAM7A regulatory sequence

5′ RACE experiments identified alternative transcripts characterised by different transcription start sites and the presence/absence of some regions of exons D and exon B. The region containing the promoter was identified on the basis of these data, which led us to hypothesise that the regulatory sequence was located in the 5′ flanking region of the identified mRNA. In order to test this hypothesis, we generated the *CHRFAM7A* −2122/−184_pGL4.11 construct containing a 2100 base long fragment retaining the TSS identified in macrophages (located at −771 bp), the TSS identified in the THP-1 cell line (located at −445 bp) and lacking most of the putative 5′ UTR-specifying region (from −184 to +1), and tested its activity by means of transient transfection in the THP-1 cell line (Fig. 2A). A luciferase assay showed that this construct had 17 times greater promoter activity than the empty pGL4.11 vector (Fig. 2B). Interestingly, construct −4280/−184 (which retains a longer 5′flanking region of the *CHRFAM7A* gene) had significant reduced activity in THP-1 cells than the −2122/−184 construct (6.16-fold ± 0.39 over pGL4.11 *vs* 17-fold ± 0.78 over pGL4.11), thus indicating the presence of negative regulatory elements. Deletion analysis showed that a construct lacking the *AluY* sequence (−735/−184_pGL4.11) has significantly increased activity in comparison with the −2122/−184 construct (33-fold ± 1.86), thus indicating the presence of a number of strong positive elements (sufficient to drive robust *CHRFAM7A* expression in the monocytic cell line) and confirming that the *Alu* sequence is a negative modulator of *CHRFAM7A* expression. Finally, deletion of the region encompassing the −771 TSS (−735/−557) significantly decreased the activity of the −557/−184 construct 1.5 times in comparison with the −735/−184 construct, although the fact that its activity was still comparable with that of the full-length construct (−2122/−184) indicated that the −557/−184 fragment contains the *CHRFAM7A* minimal promoter.

3.4. Identification of the LPS-responsive region

We have recently shown that immune cells respond to a pro-inflammatory stimulus (*i.e.* LPS) by rapidly and transiently down-regulating *CHRFAM7A* expression by means of a transcriptional mechanism dependent on NF-κB, which is in apparent contrast with the up-regulation of the *CHRNA7* gene in order to activate the cholinergic anti-inflammatory pathway (Benfante et al., 2011).

Our preliminary data showed that the down-regulation of the *CHRFAM7A* gene after LPS challenge was accompanied by a decrease in the acetylation of the *CHRFAM7A* promoter as verified by means of chromatin immunoprecipitation with specific anti-acetylated histone 4 antibodies (data not shown). Moreover, a MatInspector *in silico* analysis has predicted the presence of putative NF- κB binding sites in the *CHRFAM7A* promoter. Consequently, we used the deleted promoter constructs shown in Fig. 2B (in particular the full-length construct −2122/−184, the −1459/−184 construct lacking one of the predicted NF-κB sequences and the shortest −557/−184 construct corresponding to the minimal *CHRFAM7A* promoter region) to map the region bearing the LPS-responsive element. All of the LPS-treated constructs showed significantly less activity (~35%) than the untreated sample, thus indicating that the LPS-responsive element is located in the −557/−184 region (Fig. 2C).

In order to confirm this finding, we generated a complementary deletion construct carrying only the promoter region from −2122 to −557, which is predicted not to respond to LPS if the NF-κB responsive sequence is located between −557 and −184. Consistent with this, although the −2122/−557 construct was less active than the full-length construct, it was not significantly down-regulated after the LPS challenge, thus confirming that *CHRFAM7A* negative regulation by LPS challenge is driven by a regulatory element located in the −557/−184 region.

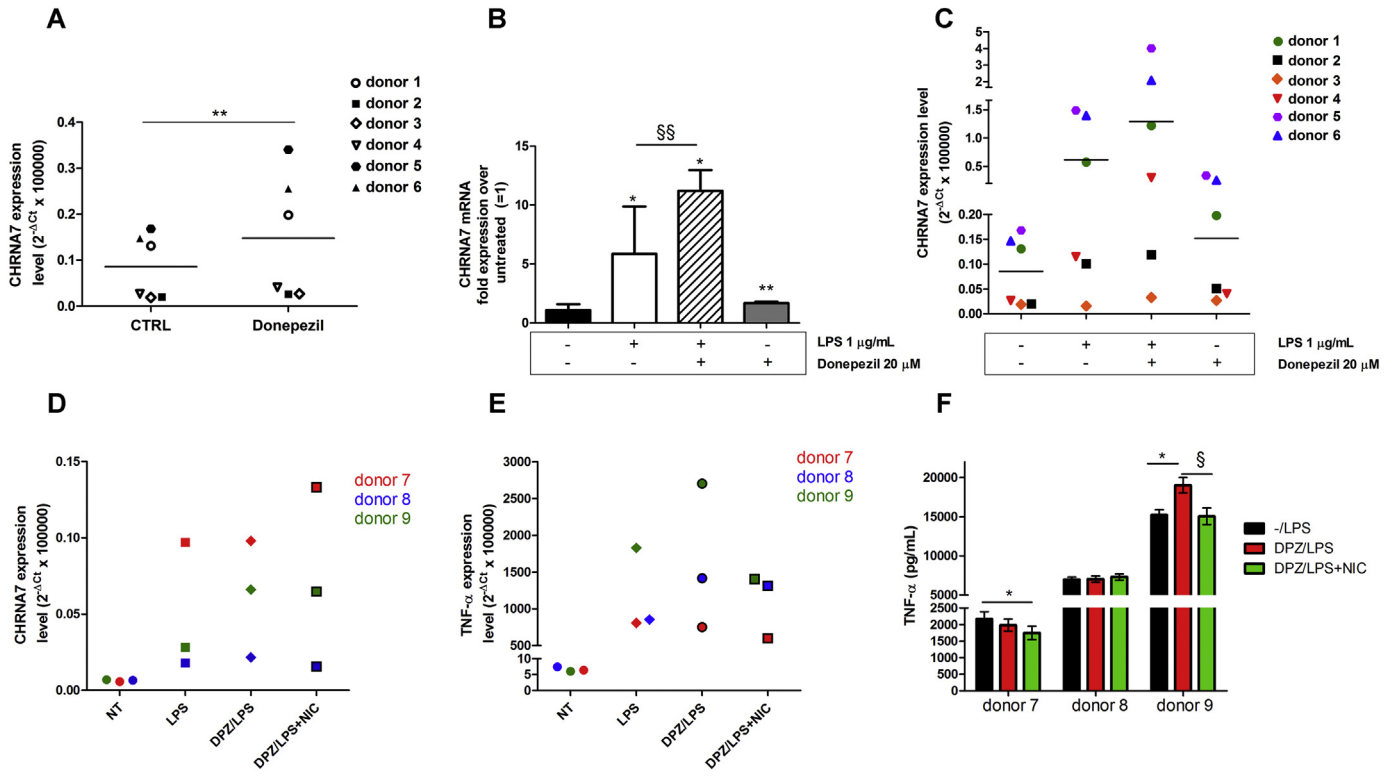

Fig. 3. Effect of donepezil on *CHRNA7* and *CHRFAM7A* expression in human macrophages. A) Donepezil treatment (20 μM, three hours) of human macrophages up-regulated *CHRNA7* transcript. The data were obtained by means of quantitative real-time PCR of *CHRNA7* mRNA level normalised to the endogenous standard *ACTB*, and are expressed as $2^{-\Delta Ct}$ x 10^5. Each data point represents one donor, and the black lines correspond to the mean value. The statistical analysis was made using a paired *t*-test on transformed values (Y = log(Y)); a *p* value of $< .05$ was considered significant. B) *CHRNA7* mRNA level is expressed as fold differences from the untreated sample (black bars) ± SD calculated using the $2^{-\Delta\Delta Ct}$ method, and are the mean values of independent experiments involving macrophages from six (B) donors. The statistical analysis was made using a paired Student's *t*-test; $^{*}p < .05$ and $^{**}p < .01$ in comparison with the untreated sample (black bar); $^{\S\S}p < .01$ between LPS- and LPS + donepezil-treated samples (striped bar *vs* white bar). C) *CHRNA7* expression shown as normalised values with respect to the endogenous *ACTB* control and expressed as $2^{-\Delta Ct}$ x 10^5. Each data point represents one donor in order to show between-sample variability: the black lines correspond to the mean values. D) and E) *CHRNA7* and *TNF-α* mRNA expression level upon donepezil pre-treatment (20 μM, three hours) followed by LPS or LPS + nicotine of macrophages from three independent donors is determined by means of qPCR and analysed as described in A. F) Photometric enzyme-linked immunosorbent assay (ELISA) measuring TNF-α protein released from macrophages from three donors. TNF-α protein content was analysed in duplicate in a 100-μL sample of medium. The colour produced from the development of the peroxidase-conjugated detection antibody was measured at 450 nm, and TNF-α concentration was determined by comparison with a standard curve. The absolute values of TNF-α (pg/ mL) are shown as mean values ± SE. $^{*}p < .05$ and $^{\S}p < .05$ in comparison with LPS treated sample (black bars) and DPZ/LPS (red bar), respectively (two-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Donepezil treatment up-regulates CHRNA7 and CHRFAM7A expression in human macrophages and THP-1 cells

Recent findings indicate that, in addition to its major function as an acetylcholinesterase (AChE) inhibitor, donepezil can significantly modulate innate immunity by acting as an anti-inflammatory compound (Pohanka, 2014; Reale et al., 2014; Reale et al., 2004), an effect that is thought to be due to the activation of the cholinergic anti-inflammatory pathway as a result of the increased availability of acetylcholine (Pavlov et al., 2009; Pohanka, 2014).

For this reason, we treated monocyte-derived macrophages from healthy human donors that expressed both the *CHRNA7* and *CHRFAM7A* genes with donepezil 20 μM for three hours, and quantified mRNA levels by means of qPCR. There was a statistically significant up-regulation of *CHRNA7* expression (Fig. 3A) in the donepezil-treated samples taken from all of the donors, which suggests that donepezil activates the cholinergic pathway. Similar results were obtained when the macrophages were treated with LPS for three hours (Fig. 3B, white bar *vs* black bar), as previously described (Benfante et al., 2011; Khan et al., 2012). It is worth noting that the response to LPS varied as, unlike the other donors, donor 3 showed no significant induction of *CHRNA7* transcripts (Fig. 3C).

These data suggested that donepezil and an inflammatory stimulus may cooperate in the activation of the cholinergic anti-inflammatory pathway and, to investigate this, we pre-treated human monocyte-macrophages with 1 μg/mL LPS for one hour in order to activate the pathway, and then introduced a 3-h donepezil challenge. Fig. 3B shows that, after LPS pre-treatment, donepezil further increased *CHRNA7* expression in comparison with the untreated and LPS-treated samples (striped bar *vs* black and white bars) thus indicating a synergy with the LPS response that may potentiate the cholinergic anti-inflammatory pathway.

In order to verify this, we pre-treated macrophages from three different donors with 20 μM donepezil, to increase CHRNA7 expression, and after washing out the drug, we stimulated the cells with LPS, in the presence or absence of nicotine. As shown in Fig. 3D, we confirm that LPS alone increases *CHRNA7* expression, with a high variability between donors, paralleled by an increase in TNF-α mRNA and protein release (Fig. 3E and F, respectively). Donepezil pre-treatment further increases alpha7 expression in one out of three donors (Fig. 3D, DPZ/LPS *vs* LPS, green), which results in variable changes in TNF-α secretion, according to donors, upon a 3-h LPS challenge (Fig. 3F, red bars). In the presence of 100 nM nicotine, we observed a significative reduction in TNF-α release in two out of three donors (Fig. 3F, green bars), in particular in donor 7 that showed a further increased in *CHRNA7* expression (Fig. 3D, DPZ/LPS + NIC), thus suggesting that donepezil by

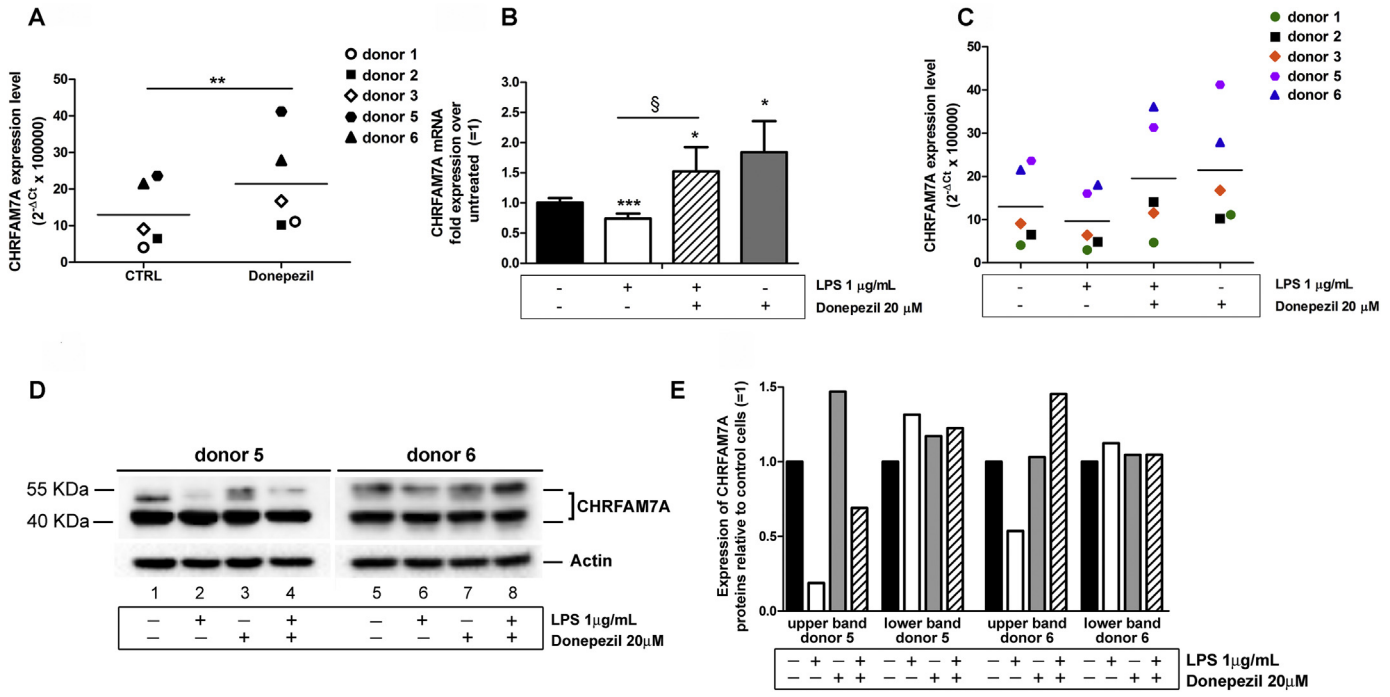

Fig. 4. Effect of donepezil on *CHRFAM7A* expression in human macrophages.
A) Donepezil treatment (20 µM, three hours) of human macrophages up-regulated *CHRFAM7A* transcript. The data were obtained by means of quantitative real-time PCR of *CHRFAM7A* mRNA level normalised to the endogenous standard *ACTB*, and are expressed as $2^{-\Delta Ct}$ x 10^5. Each data point represents one donor, and the black lines correspond to the mean value. The statistical analysis was made using a paired *t*-test on transformed values (Y = log(Y)); a p value of < -.05 was considered significant. B) *CHRFAM7A* mRNA levels are expressed as fold differences from the untreated sample (black bars) ± SD calculated using the $2^{-\Delta\Delta Ct}$ method, and are the mean values of independent experiments involving macrophages from five donors (E). The statistical analysis was made using a paired Student's *t*-test; *p < .05 and ***p < .001 in comparison with the untreated sample (black bar); §p < .05 between LPS- and LPS + donepezil-treated samples (striped bar *vs* white bar). C) *CHRFAM7A* expression shown as normalised values with respect to the endogenous *ACTB* control and expressed as $2^{-\Delta Ct}$ x 10^5. Each data point represents one donor in order to show between-sample variability: the black lines correspond to the mean values. D) Western blot analysis. 50 µg of total protein extracts from primary macrophages of two donors (donor 5 and 6) untreated (lanes 1 and 5) or stimulated for 4 h with LPS 1 µg/mL (lanes 2 and 6), or 3 h with donepezil 20 µM (lanes 3 and 7), or 1 h LPS followed by 3-h donepezil challenge (lanes 4 and 8), were size-fractionated by means of SDS-PAGE and transferred to a nitrocellulose membrane. The blot shows the expression of CHRFAM7A isoforms. The signals were normalised to that of actin. E) Densitometric analysis of the western blot in D. The two CHRFAM7A isoforms (46 kDa, upper band; 36 kDa, lower band) in the two donors have been quantified separately. The data are the values in relation to untreated macrophages (lane 1 and 5), set to a value of 1.

inducing alpha7 expression can potentiate the cholinergic anti-inflammatory pathway.

As we have previously reported that LPS induces an anti-inflammatory response by decreasing *CHRFAM7A* and increasing *CHRNA7* expression (Benfante et al., 2011), we hypothesised that donepezil would decrease *CHRFAM7A* expression further. Unexpectedly, and like that of *CHRNA7, CHRFAM7A* mRNA expression was up-regulated after three hours treatment with 20 µM donepezil in all cases (Fig. 4A) except in donor 4, who did not express the *CHRFAM7A* gene.

The observed increase in *CHRFAM7A* mRNA suggested that donepezil may counteract the down-regulation of *CHRFAM7A* expression following LPS treatment. As previously described (Benfante et al., 2011), LPS alone significantly down-regulates *CHRFAM7A* expression (Fig. 4B, white bar *vs* black bar), but this was counteracted when donepezil was added one hour after LPS pre-treatment (Fig. 4B, striped bar *vs* white bar) as *CHRFAM7A* expression was restored to levels observed in the untreated sample (Fig. 4B, striped bar *vs* black bar). These effects were confirmed also at protein level in primary macrophages of two different donors (Fig. 4D and see 4E for quantification). In particular, we detected the two bands corresponding to the alternative isoforms of CHRFAM7A proteins, with the shorter isoform being more highly expressed. As already reported (Benfante et al., 2011), LPS down-regulated only the longer 46 kDa isoform (Fig. 4D, lanes 2 and 6). Donepezil increases the expression of the longer isoform in donor 5 (Fig. 4D, lane 3) but not in donor 6 (Fig. 4D, lane 7). However it counteracts the LPS effect in both donors (Fig. 4D, lanes 4 and 8), with a further increase in donor 6 (Fig. 4E), in parallel to what observed at the mRNA level (Fig. 4C). These data, although presented in only two independent donors, support the findings that variations at mRNA level are predictive of variations at protein level, and that response to donepezil may vary between donors. Indeed, a similar response to donepezil observed in donor 6, at the mRNA level, in the presence or absence of LPS was also observed in donor 2 (Fig. 4C). It is worth mentioning that despite the increased level of *CHRNA7* mRNA, we did not detect the band corresponding to the CHRNA7 receptor, probably due to the low expression of this receptor, below the assay limit, similarly to our previously published results (Benfante et al., 2011).

The effect of donepezil on *CHRFAM7A* expression was confirmed in THP-1 cells, which do not express *CHRNA7* and represent a validated *in vitro* model for predicting monocyte behaviour (Benfante et al., 2011). The THP-1 cells were pre-treated with 1 µg/mL LPS for two hours, followed by an additional two hours in the presence of 30 µM donepezil. As expected, LPS alone significantly down-regulated the *CHRFAM7A* transcript (Fig. 5A, grey bar *vs* white bar), donepezil alone significantly up-regulated it (Fig. 5A, hatched grey bar *vs* white bar), and the combination of both significantly up-regulated *CHRFAM7A* mRNA in comparison with the control (Fig. 5A, black bar *vs* white bar), although the increase was significantly less than that induced by the donepezil treatment alone (Fig. 5A, black bar *vs* hatched grey bar).

Finally, in order to investigate whether the effect of donepezil is common to other AChE inhibitors, we treated THP-1 cells with increasing concentrations of galantamine, which failed to affect

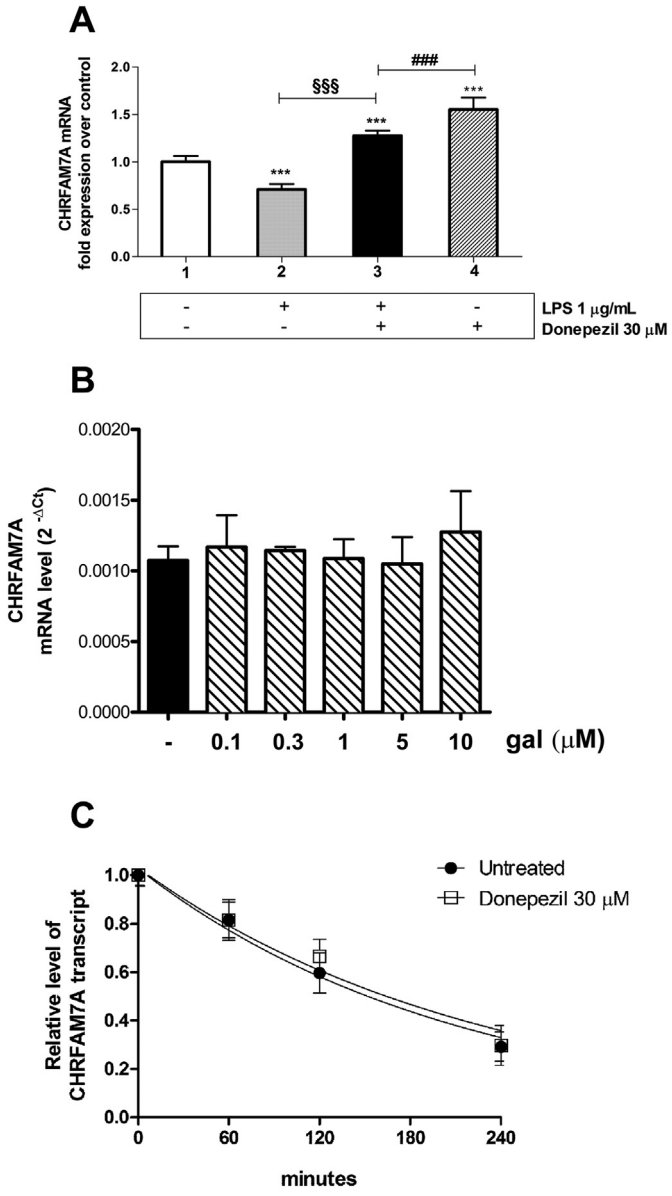

Fig. 5. Effect of donepezil on *CHRFAM7A* in the THP1 cell line. A) Donepezil up-regulates *CHRFAM7A* expression in the THP-1 cell line. THP-1 cells pre-treated for four hours with 1 µg/mL LPS (grey bar) showed the statistically significant down-regulation of the *CHRFAM7A* transcript in comparison with the untreated sample (grey bar *vs* white bar). Two hours of donepezil treatment up-regulates *CHRFAM7A* expression in comparison with sham-treated cells (hatched bar *vs* white bar), whereas two hours pre-treatment with 1 µg/mL LPS followed by two hours treatment with 30 µM donepezil (black bar) showed that donepezil counteracts the down-regulation of the *CHRFAM7A* transcript induced by LPS (black bar *vs* grey bar), leading to its statistically significant up-regulation in comparison with the control sample (white bar). The results are normalised to the endogenous standard *GAPDH*, are expressed as fold changes in expression over the untreated sample (white bar) ± SD, were calculated using the $2^{-\Delta\Delta Ct}$ method, and are the mean values of three independent experiments. The statistical analysis was made using one-way ANOVA followed by Tukey's test; ***$p < .001$ in comparison with control (white bar); ###$p < .001$ in comparison with the cells treated with donepezil alone (black bar *vs* hatched grey bar); §§§$p < .001$in comparison with the cells treated with LPS alone (black bar *vs* grey bar). B) Galantamine does not affect *CHRFAM7A* expression. The THP-1 cells were treated with increasing concentrations of galantamine for six hours and mRNA was quantified by means of qRT-PCR. The results are expressed as fold changes in expression over the untreated sample (black bar) ± SD, are the mean values of three independent experiments, and were calculated using the $2^{-\Delta\Delta Ct}$ method. The statistical analysis was made using one-way ANOVA followed by Tukey's test. There was no significant change at any tested galantamine concentration. C) Transcription arrest analysis. THP-1 cells were treated for three hours with 30 µM donepezil followed by one, two and four hours of treatment with the RNA Pol II inhibitor DRB. There was no significant difference in *CHRFAM7A* mRNA levels between the untreated (black circles) and donepezil-treated samples (white squares) at any time point. The results are expressed as fold changes in expression over time 0 (set as 1) ± SD, and are the mean values of three independent experiments. The statistical analysis was made using one-way ANOVA followed by Tukey's test; p values of $< .05$ were considered significant.

CHRFAM7A expression and thus confirmed that the effect of donepezil on the α7 duplicated isoform is exclusive (Fig. 5B).

We conclude that donepezil is capable of counteracting *CHRFAM7A* down-regulation due to a pro-inflammatory stimulus (*e.g.* LPS), which suggests that its up-regulation may be involved in resolving inflammatory responses, and that the up-regulation of *CHRFAM7A* does not depend on *CHRNA7* expression.

3.6. Donepezil up-regulates CHRFAM7A expression by means of a transcriptional mechanism

We investigated whether the increase in *CHRFAM7A* mRNA was due to increased transcription or a post-transcription mechanism (*i.e.* increased mRNA stability). To this end, we pre-treated THP-1 cells with 30 µM donepezil for three hours, and then quantitative analysed mRNA stability over time in the presence of the RNA Pol II inhibitor DRB. In the absence of donepezil, the half-life of the *CHRFAM7A* transcript was approximately 120 min (Fig. 5C), as previously reported (Benfante et al., 2011). Donepezil pre-treatment did not affect *CHRFAM7A* mRNA stability (Fig. 5C, white squares *vs* black dots), thus indicating that the increase in *CHRFAM7A* transcript level is not due to increased mRNA stability, but likely to an increase in the rate of gene transcription.

4. Discussion

Ever since its discovery, the *CHRFAM7A* gene has been considered a candidate gene for various neurodegenerative, psychiatric and inflammatory disorders (Costantini et al., 2015a; Sinkus et al., 2015) but, although numerous studies have tried to investigate the effect of the *CHRFAM7A* genotype on the risk of developing such diseases (De Luca et al., 2006; Neri et al., 2012; Severance et al., 2009; Swaminathan et al., 2012), there is still little or no evidence that its protein product is a possible therapeutic target (see Sinkus et al., 2015 and references therein). However, interest continues to thrive because of its responsiveness to pro-inflammatory stimuli (Benfante et al., 2011; de Lucas-Cerrillo et al., 2011; Ramos et al., 2016) and its putative role as the dominant negative regulator of the α7 nicotinic receptor (Wang et al., 2014).

The findings of our study provide new insights into *CHRFAM7A* as a candidate pharmacological target by showing that donepezil increases its expression in immune cells (THP-1 cells and human macrophages), both at mRNA and protein level, thus highlighting its possible role in controlling and modulating the cholinergic anti-inflammatory pathway and/or modulating CHRNA7 function. Interestingly, the effect on CHRFAM7A protein seems to be limited to the 46 kDa isoform. However, we cannot exclude the possibility that the shorter isoform has a different stability, thus making it difficult to measure any change in our experimental settings.

It has been reported that donepezil induces the up-regulation of CHRNA7 protein by means of post-translational mechanisms mediated by the activation of the PI3K and MAPK signalling pathways (Akaike et al., 2010; Takada-Takatori et al., 2008; Tyagi et al., 2010). In human macrophages, we found that donepezil induces *CHRNA7* mainly at transcriptional level, and that this effect is synergistically influenced by LPS treatment. Unexpectedly, we also found that donepezil has effects on both α7 isoforms. It can be hypothesised that, as it is an anti-

inflammatory drug, donepezil is more likely to down-regulate the *CHRFAM7A* transcript, thus inducing an increase in the number of functional membrane α7 nAChRs and potentiating the cholinergic anti-inflammatory pathway, as has been observed in PBMCs taken from donepezil-treated patients with Alzheimer's disease (Conti et al., 2016), and as we have shown in macrophages from healthy donors, upon activation with nicotine. The parallel increase in *CHRFAM7A* may be due to a compensatory effect aimed at restorating homeostasis after an anti-inflammatory stimulus by counteracting the LPS-induced down-regulation of *CHRFAM7A* expression, which suggests that the up-regulation of *CHRFAM7A* expression may be a key step in modulating inflammatory responses. We are aware that the concentration of donepezil used in our experiments is higher than the therapeutic concentration; a longer time course experiment up to 24 h with lower concentration of donepezil (3 μM; data not shown) in THP-1 cells showed that *CHRFAM7A* expression started to increase at 24 h, thus suggesting that the higher concentration enhances the effect that it may be observed under chronic treatment at lower concentration (Kume et al., 2005; Takada-Takatori et al., 2008). In conclusion, these data may reinforce the assumption that the presence of this duplicated isoform in humans, but not in rodents, is an important means of ensuring a constant CHRNA7:CHRFAM7A ratio, and that an altered ratio may have a detrimental effect on neuronal survival, the development of neuropsychiatric disorders, the risk of lung cancer (Bordas et al., 2017; De Luca et al., 2006; Kunii et al., 2015; Ramos et al., 2016), and, as it has been recently shown, in human sespis (Maldifassi et al., 2018).

Little is known about how *CHRFAM7A* is regulated at different levels and how its transcription responds to changing cell environments under developmental, physiological or pathological conditions. *CHRFAM7A* is the predominantly expressed subunit in macrophages (1000 times more expressed than *CHRNA7*), whereas SH-SY5Y neuronal cells and human brain areas specimens express about three times more *CHRNA7* than *CHRFAM7A* (data not shown). Previously published studies found a weaker regulatory region located upstream of the ATG start codon in intron 2 of the annotated sequence (http://www.ensembl.org; isoform 1 ID: ENST00000299847.6) that was identified as the *CHRFAM7A* promoter (Costantini et al., 2015b; Dang et al., 2015), but the authors did not consider the extended 5′UTR region predicted for the *CHRFAM7A* gene by the Ensembl Human (GRCh38.p10) annotations, which includes untranslated exons (exons D, C and a part of B) separated by long intronic sequences and predicts a gene promoter region ~10 kb upstream.

Our study characterises a 1.5 kb *CHRFAM7A* promoter region and a transcriptional start site at position −771 (upstream of the predicted exon D) and shows that its expression is controlled by both negative and positive regulatory elements, including an *AluY* sequence whose effect on gene expression can be modulated by variations in DNA methylation level (Bakshi et al., 2016). In particular, our 5′RACE experiments showed that part of the genomic region is retained in the mRNA (−771/−703; now called exon D') and that part of exon D (−703/−208) is excluded by the 5′UTR of *CHRFAM7A* mRNA (like that identified in macrophages and isoform 1 in THP-1 cells), thus indicating that some of the strong positive elements driving the expression of the *CHRFAM7A* gene in the −557/−184 region have an intronic location. As part of this region is retained in the transcript encoding isoform 2 in THP-1 cells (TSS at −445 bp) we hypothesise that it may be involved in regulating the expression of this transcript, an aspect that deserves further investigation.

Of note, the gene is present in one or two copies in > 95% of the population (Sinkus et al., 2015). Its absence in a small percentage of subjects may represent a later deletion of the primary duplication event due to further recombination(s) or an ancestral sequence that did not undergo *CHRNA7* duplication. Some people carry a 2 bp deletion polymorphism in exon 6, which is associated with schizophrenia and bipolar disorders and is in linkage disequilibrium with an inversion of the *CHRFAM7A* gene and in the same orientation as the gene encoding the conventional *CHRNA7* subunit (Flomen et al., 2008). This is in line with our observation that the level of *CHRFAM7A* expression varies between donors. It has recently been shown that different *CHRNA7* genotypes alter the clinical response to donepezil treatment, as *α7* up-regulation is higher in the presence of particular *CHRNA7* SNPs in the lymphocytes of patients with AD (Russo et al., 2017). Our findings support this hypothesis, as we observed that the responses of both *CHRNA7* and *CHRFAM7A* to donepezil varied widely in the absence or the presence of LPS, and reinforce the assumption that responses to inhibitor-based treatments may be predicted by the patient's genotype.

Controversial results suggest that donepezil may exert its anti-inflammatory effects *via* both AChE dependent and independent mechanisms as cell lines devoid of AChE also respond to donepezil. Some reports indicate that CHRNA7 receptors facilitate the activation of intracellular signalling pathway (Takada-Takatori et al., 2008) or a mechanism mediated by the direct binding of donepezil to α7 receptors (Akaike et al., 2010), whereas others exclude the involvement of α7 receptors (and consequently the activation of the cholinergic anti-inflammatory pathway) as they observed that the presence of α7 nAChR antagonists, in cell models stimulated with LPS, does not block the reduction of pro-inflammatory cytokine levels (Arikawa et al., 2016; Hwang et al., 2010). However, the finding that THP-1 cells completely lack *CHRNA7* expression but still retain slight but detectable AChE activity (Thullbery et al., 2005) seems to rule out the involvement of α7 receptors in mediating the anti-inflammatory effect of donepezil in this cell line. On the other hand, as an anti-inflammatory effect was observed in primary macrophages expressing both the *CHRNA7* and *CHRFAM7A* genes, it can be speculated that donepezil might bind both α7 and CHRFAM7A at still unidentified sites, as has been shown in the case of galantamine (Ludwig et al., 2010), or the effect may be mediated by means of the direct inhibition of the AChE or other still unknown targets.

It is important to note that donepezil reduces the LPS-dependent NF-κB nuclear translocation (Arikawa et al., 2016; Hwang et al., 2010), which suggests that in our cell models, where down-regulation after LPS treatment has been shown to be controlled by a transcriptional mechanisms involving NF-κB (Benfante et al., 2011), the observed up-regulation of *CHRFAM7A* may be mediated by the inhibition of NF-κB binding to the mapped LPS responsive region (−557/−184) of the *CHRFAM7A* gene promoter. Although we do not have any indication as to which subunits of NF-κB family proteins mediate the LPS-induced down-regulation of *CHRFAM7A* gene (the classical RelA:p50 heterodimer generally thought to promote gene transcription, or the p50:p50 homodimer generally considered as an inhibitor of transcription), the observation that LPS decreases the acetylation of the *CHRFAM7A* gene promoter supports the hypothesis that it may be the p50:p50 homodimer, which is capable of binding promoter sequences and repressing transcription by recruiting HDAC1 or preventing the binding of the canonical NF-κB heterodimer (Elsharkawy et al., 2010).

As several reports (Hoover, 2017 and references therein) showed that α7 nAChR activation reduces the release of pro-inflammatory cytokines by inhibiting NF-κB activity, these support our findings that donepezil can indeed potentiate the cholinergic anti-inflammatory pathway by a synergistic mechanism that may involve also pathways alternative to that of NF-κB (Hoover, 2017; Maldifassi et al., 2014; Sun et al., 2013). This aspect deserves further investigation.

5. Conclusions

To conclude, the possibility that CHRFAM7A plays a regulatory role in the localization and function of the conventional CHRNA7 receptors, and may thus interfere with a correct response to pro-inflammatory stimuli, makes it a candidate regulator of the cholinergic anti-inflammatory pathway in humans. It would be interesting to investigate whether chronic inflammatory diseases are associated with alteration in the regulation of this mechanism due to the absence of the gene or

defects in the regulation of its transcription. This would make CHRFAM7A a new pharmacological target for the development of therapies involving α7, especially because the use of agonists is limited by the occurrence of side effects.

Moreover, given the therapeutic potential of donepezil and the fact that little is currently known about its mechanism of action, we believe that our findings can contribute to improving the characterization of its pharmacological activity.

Competing interests

The author(s) declare no competing interests.

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