Donepezil modulates the endogenous immune response: implications for Alzheimer’s disease

Elisa Conti¹, Lucio Tremolizzo¹,²*, Marta Elena Santarone¹, Marco Tironi¹, Isabella Radice¹, Chiara Paola Zoa¹, Angelo Aliprandi³, Andrea Salmaggi³, Roberto Dominici⁴, Marco Casati⁵, Ildebrando Appollonio¹,² and Carlo Ferrarese¹,²

¹Laboratory of Neurobiology, School of Medicine and Milan Center for Neuroscience, University of Milano-Bicocca, Monza, Italy
²Neurology, San Gerardo Hospital, Monza, Italy
³Neurology, Manzoni Hospital, Lecco, Italy
⁴Laboratory of Chemical and Clinical Analyses, Magenta Hospital, Italy
⁵Laboratory of Chemical and Clinical Analyses, San Gerardo Hospital, Monza, Italy

Objective Donepezil (DNPZ) is a drug commonly used for Alzheimer’s disease (AD) that may favour a T helper 2 phenotype leading to increased naturally occurring auto-antibodies (NAb) against beta-amyloid (Aβ). We hypothesized the involvement of the cholinergic receptors [α7-nicotinic acetylcholine receptor (α7nAChR)] expressed on peripheral blood mononuclear cells (PBMC).

Methods Fifty patients with mild-to-moderate AD, DNPZ treated (DNPZ+, n = 25) or not (DNPZ−, n = 25), and 25 matched controls were enrolled and PBMC extracted for both in vitro cultures, and real-time polymerase chain reaction and chromatin immunoprecipitation assay. Plasma samples were also obtained for Aβ and NAb determination.

Results Donepezil increased in vitro the expression of the transcription factor GATA binding protein 3 (GATA3) through α7nAChR, because prevented by the specific antagonist methyllycaconitine. Ex vivo PBMC α7nAChR mRNA expression was increased in both AD groups, while GATA3 expression was not. A significant increase in the GATA3/interleukin 5 promoter association was found in DNPZ+ patients. Finally, DNPZ+ patients showed both significantly higher plasma levels of anti-Aβ NAb with respect to DNPZ− patients and Aβ 1-42 with respect to normal controls.

Conclusions Donepezil might modulate a T helper 2 bias via α7nAChR leading to increased expression of NAb. Further studies on the role of the modulation of the immune response against Aβ may pave the way to innovative therapeutic strategies for AD. Copyright © 2016 John Wiley & Sons, Ltd.

KEY WORDS—beta-amyloid; donepezil; Alzheimer’s disease; GATA3; nicotinic acetylcholine receptors; peripheral blood mononuclear cells

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia for which effective treatments, able to stop progression, are still lacking. A selective cholinergic denervation of the cerebral cortex, in particular at the hippocampal level, has been previously demonstrated and is well-characterized in AD brains (Bartus et al., 1982). Acetylcholinesterase inhibitors (AChEI), albeit exerting demonstrated effects on cognition and neuropsychiatric symptoms as well as on daily living activities, cannot efficiently delay the progression of the underlying pathology, displaying, therefore more properly, a symptomatic effect. However, AChEI might improve AD symptoms through the regulation of APP processing via different pathways; based on these premises, some authors put forward the hypothesis that the clinical efficacy of AChEI could be linked to a putative neuroprotective effect, supposedly leading to a reduction in toxic beta-amyloid (Aβ) fibrils, that is, a real anti-pathogenic effect (Kimura et al., 2005; Camps et al., 2008; Easton et al., 2013). Interestingly, the inhibition of AChE also regulates the immune system response; indeed, the inflammatory phenomenon is involved in the pathogenesis of AD, where the chronic accumulation of Aβ leads to activation of microglial cells, which surround senile plaques in order to phagocyte and remove the fibrillogenic peptide (Kim and Joh, 2006). Microglia are ontogenetically and functionally related to their systemic counterparts of the mononuclear phagocytic system, and peripheral monocytes represent

*Correspondence to: L. Tremolizzo, University of Milano-Bicocca, building U8, via Cadore 48, 20090 Monza, MI, Italy. Tel: +39-02.6448.8128; Fax: +39-02.6448.8108 E-mail: lucio.tremolizzo@unimib.it

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the major source for brain macrophages (Gate et al., 2010).

Furthermore, peripheral lymphomonocytes (PBMC) have an independent cholinergic system and synthesize and release acetylcholine, which in turn acts as immuno-modulator on both nicotinic and muscarinic receptors on other immune system cells (Kawashima and Fujii, 2003; De Rosa et al., 2005; Feuerbach et al., 2005).

Reale and colleagues (Reale et al., 2006) already demonstrated that AChEI reduce the production of T helper (Th)1 inflammatory cytokine and activate the Th2 response, increasing the expression of interleukin (IL)4 in lymphocytes from AD patients. IL4 rapidly induces GATA binding protein 3 (GATA3), a transcription factor involved in the switch of naïve Th to the Th2 line. Moreover, GATA3 can transactivate the promoter region of IL5, a typical Th2 cytokine (Elson et al., 1995; Zhang et al., 1997; Murphy and Reiner, 2002). The switch to the Th2 phenotype stimulates B lymphocytes, increasing the antibody-mediated immune response. This is quite interesting, because, recently, many studies focused on the importance of Aβ-targeted immune therapy for AD. The formation of peripheral Aβ-antibodies complexes could favour brain Aβ clearance through the blood-brain barrier thereby reducing Aβ deposition in plaques, and supporting a potential protective role for the naturally circulating auto-antibodies (NAb) against Aβ, found in AD patients as well as healthy individuals (Du et al., 2001; Weksler et al., 2002; Nath et al., 2003; Baril et al., 2004; Brettschneider et al., 2005). We have previously shown a cross-sectional association between chronic AChEI treatment in AD and normal levels of anti-Aβ NAb in plasma, whereas they were significantly lower in untreated AD patients (Conti et al., 2010). Because the hypothesis that a defective anti-Aβ NAb production might contribute to the reduced control over aggregation and spreading of the pathology in AD has already been put forward (Britschgi et al., 2009; Conti et al., 2010), gaining knowledge on the mechanisms regulating this key process might lead to define novel targets for AD immunotherapetics. The present study, accordingly, evaluated if donepezil (DNPZ) effects on GATA3-driven Th2 phenotypic differentiation were associated to an increased production of anti-Aβ NAb. Moreover, because DNPZ directly interacts with the α7-nicotinic acetylcholine receptor (α7nAChR) (Akaiake et al., 2010; Shen et al., 2010) expressed by PBMC, we also investigated this receptor in mediating the drug-induced immuno-modulatory response.

SUBJECTS AND METHODS

Subjects, peripheral blood mononuclear cells preparation and apolipoprotein E genotyping

The present study was approved by the ethical Committee of the San Gerardo Hospital (Monza, Italy). After informed consent, 25 AD patients receiving DNPZ+ (10 mg o.d. since at least 6 months), 25 AD patients not receiving AChEI (DNPZ−) and 25 healthy controls were recruited. AD specialists diagnosed probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association criteria (McKhann et al., 1984), and alternative diagnoses were excluded by brain imaging and an extensive neuropsychological test battery. Healthy controls had no personal or family history of neurological or psychiatric disorders; lack of cognitive impairment was established by a clinical interview, including a Mini-Mental State Examination (MMSE) score >26. Individuals with recent infections or surgery or under anti-inflammatory, corticosteroid or immuno-suppressive drug treatments were excluded. Clinical and demographic data are shown in Table 1. Blood samples (10 ml) were collected in dipotassium ethylene-diaminetetraacetic acid (4.08 mM final concentration) after overnight fasting, to obtain plasma or peripheral blood mononuclear cells (PBMC). To analyze apolipoprotein E (ApoE) genotype, total DNA was extracted from peripheral blood using a commercial DNA extraction kit (Qiagen, Venlo, Netherlands), and DNA amplification was performed using specific primers as previously reported (Conti et al., 2015).

Table 1. Demographic and clinical data of the study sample

<table>
<thead>
<tr>
<th>Enrolled subjects</th>
<th>AD DNPZ+</th>
<th>AD DNPZ−</th>
<th>CTRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Gender, M (%)</td>
<td>10 (40)</td>
<td>9 (36)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Age, years</td>
<td>74.5 ± 8.7</td>
<td>75.7 ± 4.7</td>
<td>71.3 ± 4.2</td>
</tr>
<tr>
<td>MMSE, score</td>
<td>18.5 ± 4.5**</td>
<td>15.4 ± 7.8**</td>
<td>29.1 ± 1.1</td>
</tr>
<tr>
<td>Disease duration, months</td>
<td>22.0 ± 10.2*</td>
<td>33.8 ± 22.2</td>
<td>N/A</td>
</tr>
<tr>
<td>ApoE ε4 allele carriers, n (%)</td>
<td>10 (40)</td>
<td>13 (52)</td>
<td>2 (12)**</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD (range).

AChEI, acetylcholinesterase inhibitors; AD, Alzheimer’s disease; ApoE, apolipoprotein E; CTRL, controls; DNPZ, donepezil; MMSE, Mini-Mental State Examination; SD, standard deviation.

1DNPZ 10 mg o.d. since at least 6 months (17 ± 12 months).

*p = 0.03 vs. AD without AChEI.

**p < 0.05 vs. CTRL.

***χ² 11.64, p < 0.003.
Cell stimulation assays

In five subjects out of the 25 enrolled healthy controls, we obtained further 20 ml of peripheral blood that was collected in heparinized tubes and immediately processed. PBMC were isolated by density-gradient centrifugation by Lympholyte (Cedarlane, Burlington, Canada). Pellet from 5 ml blood was resuspended in culture medium (RPMI, Basel, Switzerland) added with 1% penicillin/streptomycin, 1% Glutamine and 10% of fetal calf serum. PBMC cultures (1 × 10^7 cells) were stimulated with DNPZ hydrochloride monohydrate (Sigma-Aldrich, Saint Louis, MO, USA) at different concentrations (25, 50 and 100 ng/ml), which magnitude was chosen based on correspondence to clinical practice (Rogers et al., 1998; Apostolou et al., 2007; Patel et al., 2008), with or without the α7nAchR antagonist methyllycaconitine citrate salt (MLA) 0.5 μM or with MLA 0.5 μM alone. Moreover, to induce cell activation, 5 μg/ml phytohemagglutinin was added to each culture. Cells were incubated at 37 °C and 5% CO2 atm for 7 days, changing medium each 2 days (Onodera et al., 2010; Navarro-Partida et al., 2012). At the end of incubation, samples were centrifuged at 700 g for 10 min at room temperature, and PBMC collected and stored at −80 °C until mRNA extraction. The same concentrations of DNPZ, MLA and phytohemagglutinin were used also for preliminary experiments with whole-blood samples (5 ml) for 24 h and with extracted PBMC (1 × 10^7 cells) for 2 days.

Transcription factor and receptor expression by real-time polymerase chain reaction

Total RNA was extracted from lymphocytes using the RNeasy Mini kit (Qiagen), according to the manufacturer instructions. cDNA was synthesized from 500 ng of RNA using the SuperScriptVILO cDNA Synthesis Kit (Invitrogen, by Life Technologies Carlsbad, CA, USA) at the following conditions: 10 min at 25 °C and 60 min at 42 °C. The reaction was terminated at 85 °C for 5 min and tubes chilled on ice. cDNAs were stored at −20 °C until quantitative polymerase chain reaction (qPCR) assay. Primers for transcription factors GAT3 and T-bet and for α and β1-42 antibodies plasma levels were determined by a ChIP assay kit according to the manufacturer’s protocol (EZ-Magna ChIP G, Millipore, Billerica, MA, USA) as previously described (Tremolizzo et al., 2014). Briefly, PBMC (2 × 10^6) obtained from AD patients treated or not with DNPZ and from controls were treated with formaldehyde 1%. After cell lysis, cross-linked chromatin was sonicated and then immunoprecipitated with anti GATA3 antibody (Santa Cruz biotechnology, Inc, Dallas, TX, USA). Protein/DNA complexes were reverted, then ChIP-enriched DNA samples were quantified by real-time PCR and the data were expressed as the percentage of input. The primer pairs used to amplify the IL5 promoter were as follows: forward GGGTCTC ACTATATTGTCCAGGCT; reverse TGTGTGCAA ATACTCCCGATAGC. PCR condition was 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 30 s.

Determination of beta-amyloid 1-42 and anti-beta-amyloid 1-42 antibodies plasma levels by enzyme-linked immunosorbent assay

Plasma was obtained after centrifugation of whole blood (3700 g, 20 min) and stored at −80 °C until assay. The concentration of anti-Aβ 1-42 antibodies in plasma was determined by enzyme-linked immunosorbent assay, as previously described (Conti et al., 2010). Aβ 1-42 plasma levels were determined by a commercially available ELISA kit (Millipore). Total Immunoglobulin G (IgG) plasma content was evaluated by an automated immunoturbidimetric analysis on Modular P analyzer (Roche Diagnostics Basel, Switzerland).

Statistical analyses

Data are shown as mean ± standard error. Statistical analysis was performed with GRAPHPAD PRISM, version 4.00 program (La Jolla, CA, USA). Either,
unpaired sample, or repeated measures analysis of variance, or Kruskal–Wallis tests were used as appropriate and followed by the opportune post hoc tests when analyzing more than two groups of values. Differences between two groups were computed by the Student’s t-test. Correlation was computed by the Pearson’s r-test and differences between ApoE ε4 carriers by the $\chi^2$ test.

RESULTS

**GATA binding protein 3 mRNA levels in cultured peripheral blood mononuclear cells treated with donepezil and methyllycaconitine**

Preliminary experiments were performed on whole-blood samples incubated for 24h with DNPZ 100 ng/ml, demonstrating a mild increase of GATA3 mRNA expression (~20%, $p=0.03$ repeated-measures one-tailed Student’s t-test). Looking for a gain in magnitude and reproducibility of this effect, we decided to increase the duration of incubation and to decrease the influence of external factors by in vitro treating for 7 days PBMC isolated from whole-blood samples. DNPZ was able to induce in these conditions an increase of ~60% in GATA3 mRNA expression with respect to vehicle-treated PBMC. MLA was able to prevent this increase at the concentration of 0.5 μM (Figure 1).

**α7 nicotinic receptor and GATA binding protein 3 mRNA levels in ex vivo peripheral blood mononuclear cells**

Alzheimer’s disease patients, regardless of treatment, showed a significant 3-fold increase in $\alpha7$nAChR mRNA levels with respect to controls (Figure 2). To assess if the putative DNPZ-induced stimulation of the $\alpha7$nAChR could induce the intracellular cascade leading to shift toward the Th2 line, we analyzed GATA3 mRNA levels. However, we did not find any difference in GATA3 mRNA levels in PBMC from the recruited subjects (1.12±0.11 vs. 1.45±0.15 vs. 1.17±0.14 RQ, CTRL vs. DNPZ− vs. DNPZ+, respectively, not significant). Clinical and demographic data (Table 1) did not influence $\alpha7$nAChR and GATA3 results.

**Interleukin 5 promoter association to GATA binding protein 3**

We next addressed if a differential chromatin remodelling in the IL5 promoter could result in a modified accessibility of the transcription factor GATA3. For this purpose, we semi-quantified by ChIP assay the degree of association between IL5 promoter and GATA3 in DNA obtained from a subgroup ($n=5$ each) of subjects. Albeit values displayed a significant overall degree of dispersion, DNPZ+ had a significant (about 5-fold to 7-fold) increase of GATA3 binding to the IL5 promoter with respect to both DNPZ− patients and controls (Figure 3) suggesting that a Th2 shift might be eventually operative in DNPZ+ patients.

**Anti-beta-amyloid 1-42 natural auto-antibodies and beta-amyloid 1-42 plasma levels**

DNPZ+ patients showed significant higher levels (+30%) of anti-Aβ 1-42 NAb with respect to the DNPZ− group. NAb levels in control subjects resulted similar to those of DNPZ+ and also significantly increased with respect to DNPZ− patients (+48%, Figure 4A). Furthermore, plasma total IgG were assessed in the same subjects without showing differences among the three groups (943.6±25.2 vs. 921.4...
± 43.8 vs. 986.9 ± 42.9 mg/dl for CTRL vs. DNPZ− vs. DNPZ+, respectively).

At the same time, AD patients displayed overall an about 2-fold increase of Aβ 1-42 plasma levels with respect to healthy controls (32.6 ± 4.1 pg/ml vs. 17.5 ± 2.9, respectively, p < 0.03). Furthermore, dichotomizing patients, DNPZ+ showed a significant more than 2-fold increase in Aβ 1-42 plasma levels with respect to control subjects (Figure 4B). Only for the group of DNPZ+ patients a positive correlation was shown between Aβ 1-42 plasma levels and the duration of disease (r = 0.73, p < 0.0005) besides a correlation with the duration of DNPZ treatment (r = 0.77, p < 0.005). Finally, a positive correlation between plasma Aβ 1-42 and anti-Aβ 1-42 NAb was found only for DNPZ− patients (r = 0.50, p = 0.001). Finally, the ApoE genotype was not related to any of the investigated parameters.

DISCUSSION

In this study, we show that DNPZ increases GATA3 relative expression in cultured PBMC of healthy controls via the activation of the α7nAChR. This result is in line with previous studies showing that nicotine activation of the α7nAChR increased GATA3 expression, regulating a shift toward the Th2 lineage (Nizri et al., 2008, 2009). In addition, our results suggest that α7nAChR mRNA is upregulated in PBMC obtained from AD patients, regardless of DNPZ treatment, offering a potential substrate for magnifying the action of the drug. α7nAChR is already known to be highly expressed in brain regions relevant to cognitive and memory functions (Vallés et al., 2014). Notably, increased α7nAChR protein expression in PBMC has already been proposed as a diagnostic marker for AD (Chu et al., 2005).

To confirm our initial hypotheses of a DNPZ mediated endogenous immune response against Aβ, we directly evaluated GATA3 mRNA levels and its ability to induce the expression of Th2 typical cytokines (IL5) in PBMC from both treated and untreated patients. Initially, our study did not demonstrate a difference in GATA3 mRNA levels between DNPZ+ and DNPZ− AD patients or controls.
This result might be somewhat explained by the ‘inflammaging’ phenomenon, a slight chronic inflammation observed during ageing characterized by the reduction in T naïve lymphocytes (Fagiolo et al., 1993). Moreover, we have to consider that one usual major exclusion criterion for therapy with AChEI (DNPZ−) may be the comorbidity with severe asthma or severe chronic obstructive pulmonary disease. These conditions are associated to a reduced Th1/Th2 ratio and increased GATA3 levels with respect to healthy controls (Dong et al., 2006). On the contrary, healthy controls cells incubated ex vivo responded with a clear cut induction of GATA3 expression. As a potential caveat, however, we should remark that our in vitro results were obtained at a DNPZ concentration of 100 ng/ml, roughly corresponding to a single oral dose of 23 mg that has been hypothesized to offer potential additional benefits with respect to the canonic 10 mg o.d. per os dose (Ferris et al., 2011). Even if more studies are necessary to support this hypothesis, we regard as intriguing the possibility that this simple dose increase might shift DNPZ action from symptomatic to anti-inflammatory phenomena.

According to our assumptions, GATA3 drives this phenotypic switch via chromatin remodelling of the IL4, IL5 and IL13 genes; in particular GATA3 directly binds to the IL5 promoter and to a DNase I hypersensitive site II within the intron 2 of the IL4 gene (Yagi et al., 2011). There is some evidence that GATA3 deletion in established Th2 cells results in a modest reduction of IL4 production but in a strong reduction of IL5 and IL13 expression, suggesting that the binding of GATA3 to IL5 and IL13 promoters can, more strongly and directly, influence their transcription (Zhu et al., 2004). For this reason, we decided to evaluate by ChIP assay the hypothesis that a modified GATA3 accessibility to the IL5 promoter could be operative in DNPZ+ patients. As a matter of fact, these patients showed a significant larger GATA3 binding to the IL5 promoter with respect to both controls and DNPZ− patients. Previously, Klein-Hessling and colleagues demonstrated that Th2 cells possess high level of intracellular cyclic adenosine monophosphate that mediates the acetylation of histone H3 within the IL5 promoter region and the consequent binding of GATA3 and NFAT to the promoter (Klein-Hessling et al., 2008). An activation of adenylate cyclase with increase in cyclic adenosine monophosphate could be due to an intracellular influx of calcium ions, mediated by the α7nAChR (Oshikawa et al., 2003). These data all together suggest that DNPZ, through its interaction with the α7nAChR (Akaike et al., 2010), may eventually increase IL5 production, implying a putative phenotypic remodelling toward the Th2 line. Future experiments will include the assessment of IL5 levels in plasma obtained from our patients.

Finally, we confirmed again (Conti et al., 2010) the association between chronic treatment with DNPZ and the restoration of plasma NAb levels against Aβ to control values. This increase was specific for anti-Aβ 1-42 NAb levels, because total IgG content was similar for all the recruited subjects. Anti-Aβ NAb may play a potential role in Aβ-clearance, and we, therefore, wondered if DNPZ treatment might influence Aβ plasma levels. We report an increase in Aβ 1-42 plasma levels in DNPZ+ AD patients with respect to controls that surely need future validation and further reflections. Although a relationship between AChEI activity and Aβ metabolism within the central nervous system is documented (Boncristiano et al., 2002; Pákáski and Kálumn, 2008; Dong et al., 2009; Kim et al., 2014), little is known about their interactions at the peripheral level (Sobow et al., 2007, 2009). Our finding of an increase in Aβ 1-42 plasma levels in DNPZ+ patients supports a role for the drug in favouring this peptide mobilization from deposits within the affected brain areas and a consecutive brain-to-plasma efflux via the so-called ‘peripheral sink effect’ (DeMattos et al., 2001).

Finally, these results should be compared with those of several studies that tried, with conflicting evidence, to define if plasma Aβ levels or the variation in the Aβ 42/40 ratio might be possible biomarkers for the diagnosis of AD (Mehta et al., 2000; Fukimoto et al., 2003; Van Oijen et al., 2006; Graff-Radford et al., 2007; Pesaresi et al., 2007; Mayeux and Schupf, 2011). Possibly, our study may suggest the hypothesis that the analysis of plasma Aβ 1-42 levels could be tested as one possible biomarker of the efficacy of DNPZ treatment. Further longitudinal studies aimed at verifying the relationship between Aβ plasma level, clinical response to DNPZ and disease progression could confirm this suggestion.

In conclusion, our results support the existence of an endogenous immune response against toxic Aβ, possibly compromised in ageing and contributing to AD onset (Britschgi et al., 2009). This hypothesis bolsters the potential of anti-Aβ immunotherapy strategies, in particular favouring intravenous immunoglobulin, because a full repertoire of polyclonal antibodies is available in these preparations. Given that intravenous immunoglobulin recognize multiple sites on conformational Aβ epitopes, their use might be preferred compared with monoclonal antibodies, which target single Aβ species (Loeffler, 2013). We propose that
the modulation of the cholinergic pathway in immune cells fuelling the endogenous response against Aβ toxic species might be an interesting alternative solution for the problem of designing effective disease-modifying strategies in AD.

CONFLICT OF INTEREST
The authors have declared no conflict of interest.

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REFERENCES


