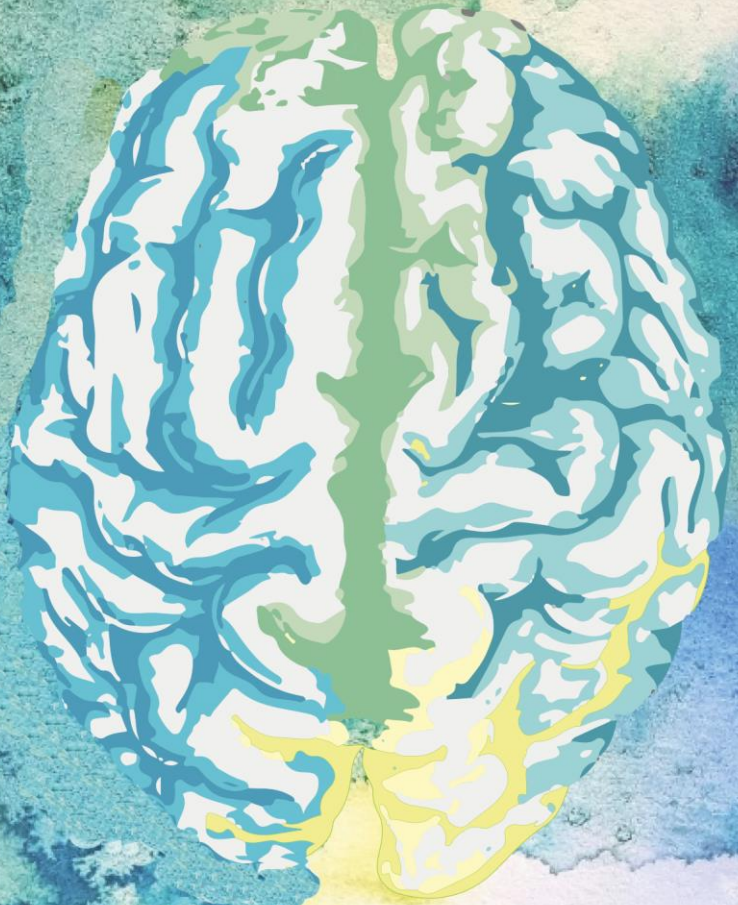
Doctoral Thesis

Bilingualism and dementia: neural mechanisms and implications for cognitive reserve



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Bilingualism and dementia: neural mechanisms and implications for cognitive reserve

Memòria presentada per Lidón Marin Marin per a optar al grau de doctora per la Universitat Jaume I

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Autors: Víctor Costumero*, Lidon Marin-Marin*, Marco Calabria, Vicente Belloch, Joaquín Escudero, Miguel Baquero, Mireia Hernandez, Juan Ruiz de Miras, Albert Costa, Maria-Antònia Parcet, César Ávila. *Víctor Costumero and Lidon Marin Marin contributed equally to this work.

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Autors: Lidón Marin-Marin*, María-Ángeles Palomar-García, Anna Miró-Padilla, Jesús Adrián-Ventura, Naiara Aguirre, Esteban Villar-Rodríguez, Victor Costumero

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Structural but not functional connectivity differences within default mode network indicate conversion to dementia (Under review)

Autors: Lidón Marin-Marin*, Anna Miró-Padilla, Victor Costumero

Activity in Memory Brain Networks During Encoding Differentiates Mild Cognitive Impairment Converters from Non-Converters

Autors: Naiara Aguirre*, Victor Costumero, Lidón Marin-Marin, Joaquín Escudero, Vicente Belloch, Maria-Antònia Parcet, César Ávila

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LIST OF ABBREVIATIONS

AAL: automatic anatomical labelling

ACC: anterior cingulate cortex

ACH: Adaptive Control Hypothesis

AD: Alzheimer's Disease

AoA: age of acquisition

AxD: axial diffusivity

Aβ: β amyloid

BNT: Boston Naming test

CAT12: Computational Anatomy Toolbox

CDR: Clinical Dementia Rating

CR: cognitive reserve

CSF: cerebrospinal fluid

DARTEL: Diffeomorphic Anatomic Registration Through Exponentiated Lie algebra

DMN: default mode network

DRM: Dynamic Restructuring Model

DTI: diffusion tensor imaging

ECN: executive control network

EFA: exploratory factor analysis

FA: fractional anisotropy

fALFF: fractional amplitude of low-frequency fluctuations

FAQ: functional activities questionnaire

FC: functional connectivity

FD: framewise displacement

FDG PET: fludeoxyglucose–positron emission tomography

FSL: FMRIB Software Library

FWE: family-wise error

FWHM: full-width at half-maximum

GAMMs: generalized additive mixed models

GLM: general linear model

GM: grey matter

HC: healthy controls

ICA: independent component analysis

IFG: inferior frontal gyrus

KMO: Kaiser-Meyer-Olin

LPBA40: LONI Probabilistic Brain Atlas

LSBQ: Language and Social Background Questionnaire

L2: second language

MCI: Mild Cognitive Impairment

MCI-c: MCI converters

MCI-nc: MCI non-converters

MD: mean diffusivity

MEG: magnetoencephalography

MMSE: Mini Mental State Examination

MNI: Montreal Neurological Institute

MRI: magnetic resonance imaging

MTL: medial temporal lobe

NIA-AA: National Institute on Aging—Alzheimer's Association

NPS: neuropsychological

PCA: principal-components analysis

PCC: posterior cingulate cortex

PET: positron emission tomography

PH: parahippocampal

pSTG: posterior parts of the superior temporal gyrus

RBM: region-based morphometry

RD: radial diffusivity

ReHo: regional homogeneity

ROI: region of interest

SD: Standard Deviation

TFCE: threshold-free cluster enhancement

TIV: total intracranial volume

UF: uncinate fasciculus

WAIS-III: Wechsler adult intelligence scale-III

WLA: word list acquisition

WLR: word list recall

WM: white matter

WMS III: Wechsler memory scale-III

GENERAL INTRODUCTION

1. Dementia, Alzheimer's disease and Mild Cognitive Impairment

Alzheimer's disease (AD) is a progressive neurodegenerative disease that constitutes the most common cause of dementia, accounting for up to 80% of the cases (Alzheimer's Association, 2021). It was first described by Aloysius Alzheimer more than a hundred years ago, when he reported the post-mortem brain analysis of a 51-year old woman, whose unusual case draw the attention of clinicians (Alzheimer, 1907; for an English translation, see Stelzmann, Norman Schnitzlein, & Reed Murtagh, 1995). She was disoriented in time and space, presented inadequate behaviors as a consequence of her disorientation, and suffered from repeated episodes of ravings and hallucinations. Crucially, she showed a serious memory impairment. The post-mortem microscopic analysis of her brain revealed an abnormal accumulation of bundles of fibrils in cells, which Alzheimer described as "a parallel process to the deposition of a pathological metabolic substance in the neuron whose closer examination is still pending." At the end of his report, Alzheimer alerted of an increase in similar cases, and advocated for exhaustive neural and clinical examinations in order to determine the characteristics of the illness with more detail.

More than a century after this initial description, it is estimated that 44 million people around the world live with AD (Lane, Hardy, & Schott, 2018). With an incidence in elderly population that varies greatly between countries (2-17% in Europe and the United States of America), there is a prevalence of 3-7% of cases in population older than 60 years, causing substantial caregiver burden and economic costs to the healthcare system (Takizawa, Thompson, Van Walsem, Faure, & Maier, 2015). For instance, in Spain, the principal caregiver dedicates 39 to 73 hours per week to looking after the AD patient, which is more than the maximum number of working hours legally allowed in this country.

Mild Cognitive Impairment (MCI) as a term was introduced in literature as early as in 1988 (Reisberg et al., 1988), and represents an intermediate stage from normal cognitive function to dementia where symptoms have already appeared but do not interfere yet with normal functioning in activities of daily living (Roberts & Knopman, 2013). The overall prevalence of MCI in population over the age of 60 years is estimated to be around 12-18%. Crucially, annual rates of conversion from MCI to AD are around 8-15% (Petersen, 2016; Petersen et al., 2001), which makes the construct useful for clinicians to identify individuals at higher risk of progression to dementia and plan preventive interventions (Jongsiriyanyong & Limpawattana, 2018). Moreover, since not all MCI patients convert to AD, following their evolution and identifying risk factors that could mark progression to AD has also become especially relevant for researchers (Yaffe, Petersen, Lindquist, Kramer, & Miller, 2006).

1.1. Clinical diagnose

The most common symptoms of dementia resemble Alzheimer's description in his first report. Criteria used for the clinical diagnose of general dementia are cognitive or behavioral symptoms of *at least two* of the following domains: memory (impaired ability to acquire and recall recently learned information), executive function (judgment and handling of complex tasks), visuospatial abilities, language and personality (McKhann et al., 2011). These alterations must interfere with the patient's previous ability to function at their usual activities, represent a significative decline from prior levels of functioning and not be related to a delirium or major psychiatric disorder.

Alternatively, the following diagnostic guidelines are proposed for MCI: patients must present a subjective cognitive complaint in one or more cognitive domains, confirmed by objective evidence of worse performance than expected for the patient's age and educational background, but present essentially normal functioning in daily activities and not fulfill the criteria for dementia (Albert et al., 2011; Petersen et al., 2009). Importantly, impairment in episodic memory is typically found in MCI patients who will later convert to dementia (Albert et al., 2011; Yaffe et al., 2006). That is why amnestic MCI is the focus of numerous investigations trying to elucidate the characteristics of progression to dementia (Alderson et al., 2017; Cha et al., 2013; Ferreira, Diniz, Forlenza, Busatto, & Zanetti, 2011; Sorg et al., 2007; Yu, Lam, & Lee, 2017).

1.2. NIA-AA's research framework biomarkers

Until recently, the definitive confirmation that dementia was caused by AD could only be performed after a post-mortem neuropathological examination (Ball et al., 1997), so the terminology *probable* AD dementia was used when diagnosing living patients (McKhann et al., 2011). However, the more recent National Institute on Aging—Alzheimer's Association (NIA-AA) research framework proposes a diagnose based on biomarkers that can be performed *in vivo* (Jack et al., 2018). This focuses on defining the disease as a biological construct, still associated to a cognitive performance continuum (cognitively unimpaired, MCI and dementia), but based on the presence of three types of biomarkers: deposition of β amyloid (Aβ), pathologic tau and neurodegeneration. In this framework, both Aβ and tau biomarkers are required to diagnose if an individual is in the Alzheimer's continuum, but neurodegenerative biomarkers and cognitive symptoms are only used to stage severity. It is worth noting that Aβ and tau biomarkers have not been easily available for clinical or research environments until very recently, which is why the authors of the NIA-AA's research framework also affirm that "it is premature and inappropriate to use this research framework in general medical practice" and "this research framework should not be used to restrict alternative approaches to hypothesis testing that do not use biomarkers" (Jack et al., 2018). In fact, the studies included in this thesis are also affected by this limitation: data was

obtained before the publication of this research framework and Aβ and tau biomarkers were not accessible for our studies at the time.

The focus on Aβ and tau biomarkers goes in line with previous evidence showing that the first brain abnormalities observed in AD are decreased levels of Aβ42, the long form of Aβ, in the cerebrospinal fluid (CSF), detected as early as 25 years before the onset of the disease, followed by increased Aβ deposition as measured by positron emission tomography (PET), elevated tau levels in the CSF, and followed by cognitive decline, brain atrophy and hypometabolism (Gallardo & Holtzman, 2019). Figure 1 shows a hypothetical temporal ordering of neuropathological processes in the course of MCI and AD (Petersen et al., 2009). The *amyloid cascade hypothesis*, which affirms that Aβ deposition is the causal factor that initiates AD pathology due to its neurotoxicity (Hardy & Higgins, 1992), was the most accepted model until recently reformulated, due to new evidence suggesting that Aβ might be necessary but not sufficient in order to develop synaptic and neuronal loss (Gallardo & Holtzman, 2019). This evidence comes mainly from studies showing that individuals can carry substantial amyloid burdens in their brains without presenting any clinical symptoms of dementia, and the failure of clinical trials to improve cognitive impairment when targeting Aβ (Gallardo & Holtzman, 2019; Herrup, 2015). Other hypothesis explaining the pathogenesis of AD focus on mitochondrial dysfunction (Moreira, Carvalho, Zhu, Smith, & Perry, 2010), disturbance of the cerebrovascular system (Bu, Liu, & Kanekiyo, 2013), and infectious agents in the brain (Itzhaki et al., 2016). Reformulations of the amyloid hypothesis have also appeared, shifting the causal role to the systemic age-related decrease in the efficiency of the clearance of Aβ, and proposing the accumulation of Aβ only above a critical threshold as a neurotoxic factor (Paroni, Bisceglia, & Seripa, 2019). Regardless of the multiple proposals for its pathogenesis, it is widely accepted that AD is a dual proteinopathy that needs both abnormal Aβ and tau accumulation to appear and develop (Gallardo & Holtzman, 2019; Jack et al., 2018).

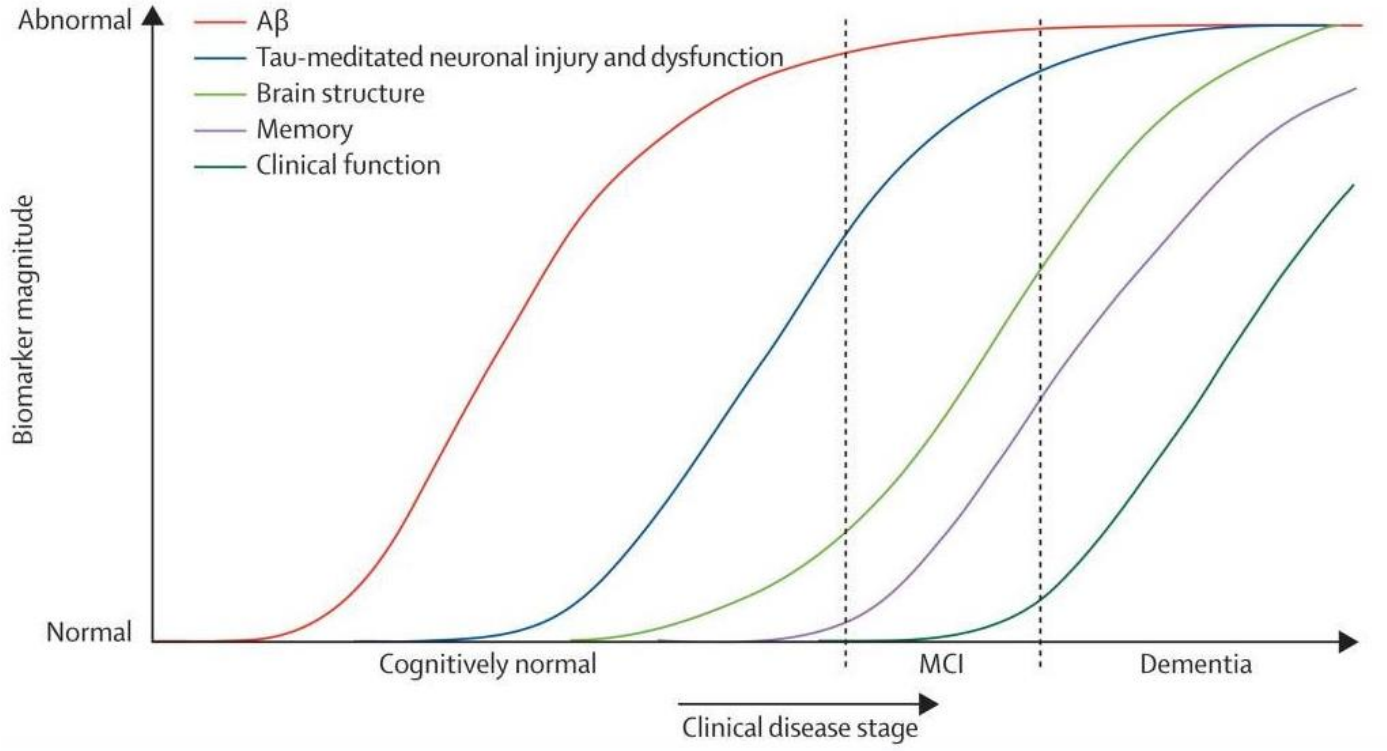

Figure 1. Hypothesized ordering of the dynamic biomarkers of the pathological cascade along the AD continuum, extracted from (Jack et al., 2010). CSF, cerebrospinal fluid; FDG PET, fludeoxyglucose 18–positron emission tomography.

1.3.Neuroimaging pathology

Although neuroimaging biomarkers are not considered specific of AD and, accordingly, are not necessarily required for its diagnose, they are still included in the NIA-AA research framework, since their combination with Aβ and pathologic tau biomarkers increases the power of prediction of subsequent cognitive decline (Jack et al., 2018). The most consistent magnetic resonance imaging (MRI) finding in AD is hippocampal atrophy (Bayram, Caldwell, & Banks, 2018; Clerx, Visser, Verhey, & Aalten, 2012), although abnormalities in other regions, such as the parahippocampus and posterior cingulate gyrus/precuneus, have also been consistently identified (Wang et al., 2015). Evidence has shown that MCI individuals present atrophy in similar brain regions, with levels that fall between normal aging and AD patients (Chandra, Dervenoulas, & Politis, 2018; Yang et al., 2012), in agreement with Braak's staging (Braak & Braak, 1995).

Regarding integrity of the structural connections between brain regions, cingulum abnormalities have been reported since long in AD (Clerx et al., 2012), extending to the limbic network in fibers such as the

parahippocampal cingulum and the fornix, but also to the uncinate and inferior fronto-occipital fasciculi (Acosta-Cabronero & Nestor, 2014; Qin, Guo, McClure, & Mu, 2020). In contrast to this widespread pattern of WM disintegration, amnestic MCI patients present reliable alterations only in the fornix, uncinate fasciculus (UF) and parahippocampal (PH) cingulum (Yu et al., 2017), and in thalamic projection fibres to posterior cingulate cortex/precuneus and lateral inferior frontal parietal lobe (Alderson et al., 2017). The fact that not only the medial temporal lobe (MTL), traditionally associated to episodic memory functions, but also limbic regions show atrophy and/or damaged connections in the AD continuum, suggests that a wider mnemonic network is probably vulnerable to degeneration in this disease (Aggleton, Pralus, Nelson, & Hornberger, 2016).

Along the same lines, the regions that show atrophy and structural disconnection in the AD continuum are part of the default mode network (DMN) (Mohan et al., 2016), which also shows a spatial overlap with Aβ accumulation (Mormino et al., 2011; see Figure 2), and decreases in resting-state connectivity between its regions in AD and MCI when compared to controls (Alderson et al., 2017; Badhwar et al., 2017; Binnewijzend et al., 2012; Cha et al., 2013; Kim et al., 2016; Sorg et al., 2007). The definition of the DMN emanated from resting-state investigations that found joint activation – also called functional connectivity (FC) - of a subset of regions when individuals were awake and alert, but not focused on an external task (Buckner, Andrews-Hanna, & Schacter, 2008). These regions typically include the posterior cingulate gyrus/precuneus, medial prefrontal cortex, inferior parietal lobules, lateral temporal cortices, and hippocampus (Mohan et al., 2016). After years of research, the DMN has been shown to be active in different internally oriented cognitive tasks such as daydreaming, conceiving the perspectives of others, retrieving autobiographical memory, and future envisioning (Buckner et al., 2008), but it has also been recently proposed as a dynamic integrator of internal and external information over long timescales, which distances the network from its exclusively ‘intrinsic’ role (Yeshurun, Nguyen, & Hasson, 2021).

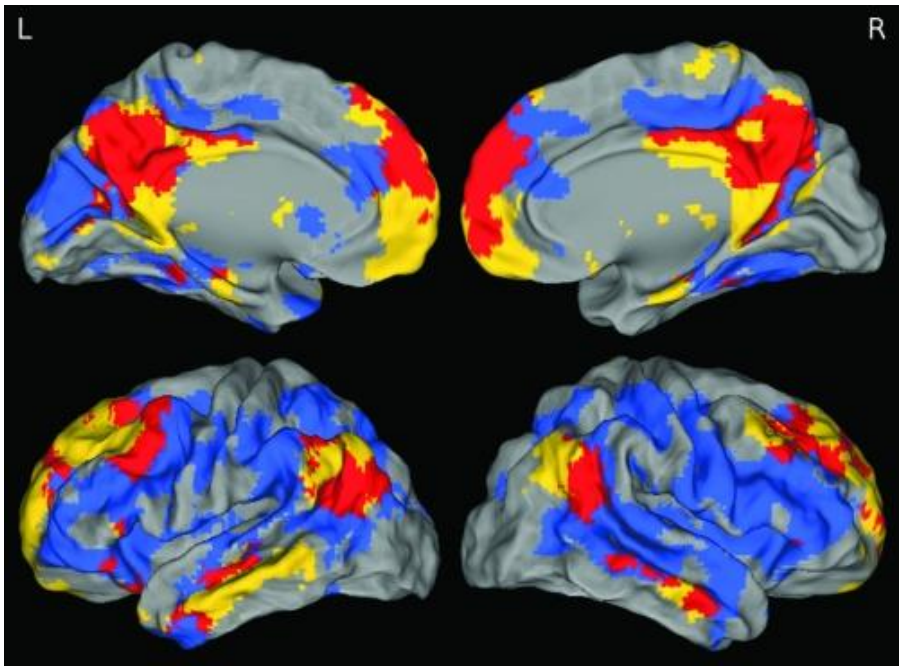

Figure 2. Overlap between Aβ deposition and DMN, extracted from (Mormino et al., 2011)**.** In yellow, one sample t-test of DMN best-fit components; in blue, 2-sample t-test comparing subjects with high and low Aβ; and in red, their overlap.

Multiple proposals tried to find an explanation for the relationship between AD and the DMN. The "metabolism hypothesis" suggests that the continuous high baseline activity of the DMN increases the cascade that leads to dementia pathology (Buckner et al., 2005). Conversely, a recent modification of the disconnection model proposes that abnormalities in AD are especially present in the DMN because the regions first affected by Aβ and tau pathology, although presenting neural dysfunction, continue to communicate with other areas of their network in a disrupted way, progressively causing general dysfunction in the network (Brier, Thomas, & Ances, 2014). Finally, other hypotheses emphasize that the DMN, among other multimodal networks, is associated with multiple cognitive functions and supports the integration of information, being especially vulnerable to early and fast spread of pathology for that reason (Mišić et al., 2015)

1.4.Progression from MCI to AD

In order to improve the prognostic precision of the MCI diagnose, finding neural biomarkers that could signal conversion to AD has become crucial for investigators (DeCarli et al., 2007; Ferreira et al., 2011). A recent multicenter study that looked at GM volumes in AD-related regions of interest found that reductions in the hippocampus, basal forebrain, parieto-temporal cortices, cingulate gyrus and occipital areas indicated increased conversion risk (Brueggen et al., 2015). However, a previous meta-analysis of voxel-based whole-brain morphometry studies found only one significant cluster of GM volumetric reduction in amnestic MCI patients who converted to AD, located in the left hippocampus and parahippocampal gyrus (Ferreira et al., 2011). In line with those results, a study that aimed to test the usefulness of structural MRI for prediction of conversion found the highest discrimination power for bilateral hippocampus and left entorhinal cortex (Nesteruk et al., 2015). WM fiber integrity in multiple fibers, such as left hippocampus and cingulate (Marcos Dolado et al., 2019), as well as bilateral corticospinal, right hippocampal cingulum, right inferior fronto-occipital, left inferior longitudinal, right superior longitudinal and left uncinate fasciculi (Stone, Ryman, Hartman, Wertz, & Vakhtin, 2021), has also been described as a significant predictor of progression. Finally, a recent investigation that used structural and resting-state MRI for classification of MCI converters and non-converters, obtained six optimal features that improved the accuracy of classification: cortical thickness of left superior temporal gyrus, cortical area of right inferior temporal gyrus, and different connectivity measures of left precuneus, right dorsolateral prefrontal cortex and left occipital (Hojjati, Ebrahimzadeh, Khazaee, & Babajani-Feremi, 2018). Evidence gathered from all these studies suggests that differences in GM atrophy in the temporal lobe and hippocampus may be solid biomarkers of conversion to AD, but evidence on WM and FC biomarkers is still scarce or conflicting.

2. Brain and cognitive reserve in dementia

The concepts of brain reserve and cognitive reserve come from the observation that some patients present a mismatch between their neural pathology and the amount of cognitive impairment expected as a consequence of that damage (Stern, 2002). For instance, cases of cognitively normal elderly that did not show the clinical features of AD but presented neocortical Aβ plaques have been described since long (Robert Katzman et al., 1988). In those cases, it was hypothesized that they were more resilient to AD neuropathology due to possessing larger brains than the average. This is the idea of brain reserve – individuals with increased brain volume or synaptic density need to lose more synapses in order to reach a critical threshold and manifest the disease symptoms (R. Katzman, 1993; Stern, 2002; see Figure 3).

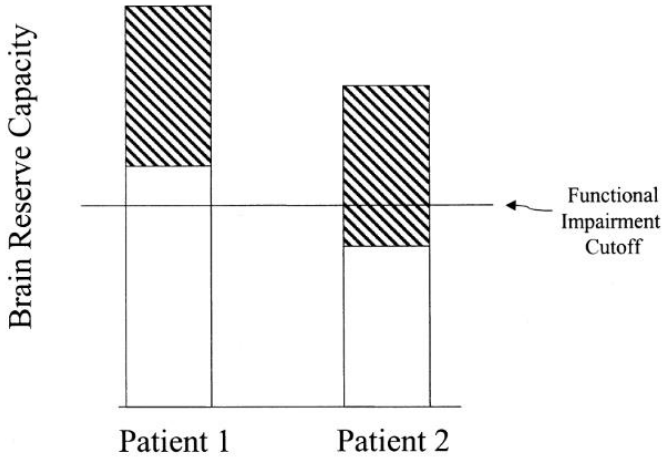

Figure 3. Visual representation of the threshold or brain reserve model, extracted from (Stern, 2002). A brain lesion in Patient 2, which has less brain reserve, exceeds the threshold of brain damage sufficient to produce a functional impairment, while Patient 1 remains unaffected.

However, this is not the only mechanism by which patients can present brain damage while maintaining cognitive functioning. Since the original concept of brain reserve is believed to be quantitative and passive – a patient with a large brain is simply expected to tolerate more pathology - the idea of cognitive reserve emerges as its active counterpart, focusing on brain function and networks rather than brain size, and suggesting that the brain can also endure pathology by using alternative cognitive processing approaches or compensatory mechanisms (Stern, 2012). Thus, brain reserve implies differences in the amount of available neural basis, whereas cognitive reserve is more related to differences at the level of brain networks (Stern, 2009). Moreover, cognitive reserve can take two forms: neural reserve and neural compensation. Neural reserve refers to differences in resilience of brain networks used for the cognitive function of interest and affected by pathology, by means of efficiency, capacity or flexibility of these networks, whereas neural compensation designates the use of alternative regions or networks, not

typically used by healthy individuals for that cognitive function, in order to compensate for pathology (Steffener & Stern, 2012; Stern, 2012).

Previous studies have provided evidence for the existence of both mechanisms. For instance, a study showed that healthy elderly compared to young participants needed higher increases in network activation when faced with higher demands of an encoding task, which was interpreted as an exemplification of how age can limit the efficiency of a network (Zarahn, Rakitin, Abela, Flynn, & Stern, 2007). Moreover, elderly participants presented activation in a network that was not used by the younger group during the retention phase of the task, and this activation correlated negatively with overall task performance. This was interpreted as a mechanism of neural compensation – as the primary network gets affected and loses functionality, the additional network is recruited so that the task can still be performed, but the individuals who need to resort to this mechanism will have a worse performance (Zarahn et al., 2007). In line with these findings, a recent study investigating healthy older participants performing a face-name memory task found that GM volume in regions of the three most commonly activated components during the task - visual, left executive and attention network – was negatively correlated to the amount of networks they recruited for the task (Ji et al., 2018). This was interpreted as a compensatory mechanism, by which the engagement of additional networks would happen in compensation for reduced GM volume in the three core networks for the task.

Brain and cognitive reserve have been hypothesized to emerge from experience-related modifications in the structure and function of the brain (Barulli & Stern, 2013). Multiple life experiences have been proposed as possible factors that contribute to neuroplasticity and might build cognitive and brain reserve, such as education level (Coffey, Saxton, Ratcliff, Bryan, & Lucke, 1999), adult-life work complexity (Fratiglioni & Wang, 2007; Gaser & Schlaug, 2003), participation in lifelong complex cognitive activities (Landau et al., 2012; Reed et al., 2011; Sattler, Toro, Schönknecht, & Schröder, 2012; Valenzuela, Sachdev, Wen, Chen, & Brodaty, 2008) and, crucially, bilingualism (Anderson, Hawrylewicz, & Grundy, 2020).

3. Bilingualism

Bilingualism is generally defined as the experience of using two languages in everyday life (Schweizer, Ware, Fischer, Craik, & Bialystok, 2012). In language research, bilinguals had been traditionally considered an atypical group (Kroll, Dussias, Bogulski, & Valdes Kroff, 2011), but this has been recently reconceptualized, due to evidence showing that more than 20% of the population in the U.S.A. and Canada, and more than 50% in Europe, normally use more than one language (Bialystok, Craik, & Luk, 2012). It is well-established that in fluent bilinguals, both languages are jointly activated and available, so they compete for selection when one of them is being used (Bialystok, 2009; Guo & Peng, 2006; Marian & Spivey, 2003). Thus, it has been hypothesized that bilingual speakers have to deal with higher cognitive control demands than monolinguals (Bialystok, 2009; DeLuca, Segaert, Mazaheri, & Krott, 2020; Tao, Wang, Zhu, & Cai, 2021).

The Adaptive Control Hypothesis (Green & Abutalebi, 2013) posits the existence of eight cognitive control processes implicated in bilingual language production - goal maintenance, interference control, salient cue detection, selective response inhibition, task disengagement and engagement, and opportunistic planning -, which would be recruited differentially depending on the interactional context in which the individual makes use of their languages: single language contexts where languages are used separately in different environments, dual-language contexts where both languages are used together in the same environment but separately with different speakers, and dense code-switching contexts where speakers use both languages at the same time alternating them during the interaction. However, bilingual experience does not only vary in the amount of mixing between languages in specific contexts, or in length of exposure. Living in an environment where both languages are used in a naturalistic manner could have different effects on learning a second language within a society where it is not naturalistically used – such as for classroom learners of foreign languages – or after immigration to another country, where full immersion usually happens (Kroll et al., 2011). For instance, the terms active and passive bilingual emerged as a way of describing the experiences of bilinguals who are exposed to the same environment where two languages are spoken, but still use languages differently – passive bilinguals only actively speak one of them, while active bilinguals speak both (Calabria et al., 2020). Thus, a recent model encompassing all previous evidence – the Unified Bilingual Experience Trajectory model - highlighted a variety of relevant bilingual experience factors that would produce different neurocognitive adaptations: intensity and diversity of use, switching, proficiency, and duration of bilingual experience (DeLuca et al., 2020).

3.1. Neural basis

It has been hypothesized that the higher cognitive control demands that bilingual speakers have to deal with results in specific consequences for brain structure and function (Bialystok, 2009; DeLuca et al., 2020; Tao et al., 2021). However, evidence regarding the specific brain regions affected by bilingual experience and the direction of these changes continues to be mixed (Tao et al., 2021).

The Adaptive Control Hypothesis proposes a brain network for language control and speech, composed by inferior frontal, parietal, anterior cingulate, motor and premotor cortices, thalamus, caudate, putamen, cerebellum and insula (Green & Abutalebi, 2013). These regions have been previously related to processes that are relevant for bilingual language use. For instance, anterior cingulate and premotor cortices are related to conflict monitoring and initiating speech during language switching, parietal cortex is more related to maintenance of task representations, and prefrontal cortex is relevant for cognitive control, selecting a target stimulus and controlling interference. Subcortical regions also play relevant roles for bilingual language use – the putamen is related to articulation, the caudate nucleus to switching between languages, the thalamus to selection of relevant lexical and semantic representations, and the cerebellum to morphosyntactic processing (for a thorough description, see Abutalebi & Green, 2016). These regions are also hypothesized to be differentially implicated by bilingual experience depending on the interactional context: while dense code-switching contexts would affect more the cerebellum and left inferior frontal cortex, dual or multiple language interactional contexts would engage bilateral inferior frontal, anterior cingulate and parietal cortices, caudate, putamen and the thalamus (Green & Abutalebi, 2013).

Other authors that focus on the temporal component of the bilingual experience propose dynamic patterns of brain changes as a function of length of exposure to bilingualism (Grundy, Anderson, & Bialystok, 2017; Pliatsikas, 2020). Specifically, the Bilingual Anterior to Posterior and Subcortical Shift (BAPSS, Grundy et al., 2017) and the Dynamic Restructuring Model (DRM, Pliatsikas, 2020) propose initial tissue volume increases in frontosubcortical circuits, that would be followed by reductions in volume and lower functional recruitment of frontal executive regions, as well as greater recruitment and further expansions of posterior and subcortical areas. Finally, the Unified Bilingual Experience Trajectory Model proposes two types of bilingual neurocognitive adaptations as a function of bilingualism: increases in recruitment of cortical regions due to increases in executive control demands, and renormalization of cortical regions and increased recruitment of subcortical and posterior structures due to increases in efficiency (DeLuca et al., 2020). They also propose specific interactions between the factors, such as a faster appearance of efficiency mechanisms with increased intensity and diversity of use of both languages.

3.2.Reserve factor against dementia

Bilingualism has been proposed as an experience-based factor that protects against dementia due to evidence showing that bilinguals present the first symptoms of the disease around 5 years later than monolinguals (Alladi et al., 2013; Bialystok, Craik, & Freedman, 2007; Calabria et al., 2020; Craik, Bialystok, & Freedman, 2010; Perani et al., 2017; Woumans et al., 2015; see Figure 4 for visual representation). This delay has been recently shown to be independent of socioeconomic status, education, and publication bias (Anderson et al., 2020).

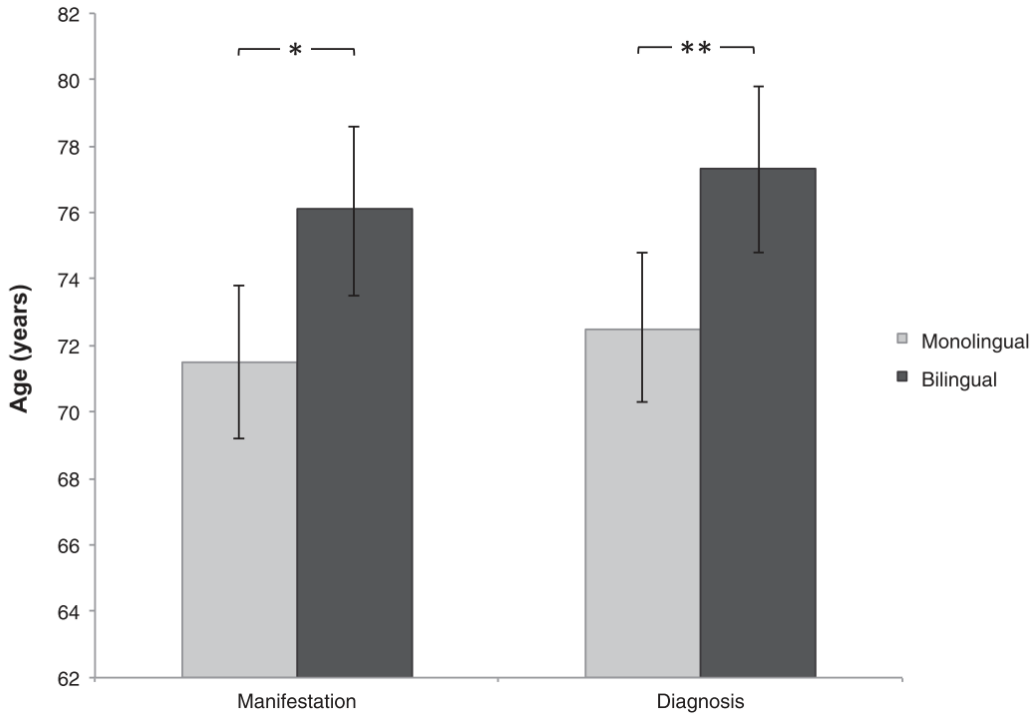

Figure 4. Age of manifestation and onset of AD, from (Woumans et al., 2015).

Supporting the protective effects of bilingualism against dementia, early bilingual experience has been associated to lower levels of tau in CSF (Estanga et al., 2017). Neuroimaging investigations have also found that bilingual and multilingual patients with AD show greater amounts of brain atrophy or lower tissue density in AD-related areas than monolinguals, but comparable cognitive impairment levels (Duncan et al., 2018; Schweizer et al., 2012). This has been interpreted as a protective effect – bilinguals would require more atrophy in order to present the same level of cognitive impairment. Similar findings have also emerged in metabolism studies, where bilinguals with AD showed more severe hypometabolism, but increased metabolic connectivity in the DMN, executive and language networks

than monolingual patients at the same cognitive level, which was interpreted as a result of neural reserve and compensation due to bilingualism (Perani et al., 2017; Sala et al., 2021).

EXPERIMENTAL SECTION

Research justification

We have reviewed solid evidence supporting the protective role of bilingualism against dementia, such as the consistently delayed onset of the disease in bilinguals (Alladi et al., 2013; Bialystok et al., 2007; Calabria et al., 2020; Craik et al., 2010; Perani et al., 2017; Woumans et al., 2015). However, the neural basis of this effect is still unknown. Bilingual experiences are hypothesized to produce neuroplastic changes in executive control and attention circuits (Perani & Abutalebi, 2015; Perani et al., 2017), as well as in regions belonging to other brain circuits such as the DMN or language network (Kousaie, Chai, Sander, & Klein, 2017; Sala et al., 2021). These changes could be the underlying mechanism by which bilingualism exerts a protective effect against dementia, producing neural reserve in AD-related regions, and neural compensation in alternative brain areas and circuits. Nevertheless, more research is needed in order to confirm this. For instance, studies investigating the effects of bilingualism on the integrity of brain structural connections in dementia patients are lacking – they can only be found in healthy elderly participants (Grady, Luk, Craik, & Bialystok, 2015; Luk, Bialystok, Craik, & Grady, 2011). Differences in brain connectivity between bilinguals and monolinguals with dementia have been studied, but only using metabolic connectivity, a measure that does not capture the temporal synchronization between brain regions (Perani et al., 2017). Moreover, most studies used a cross-sectional perspective, which has been suggested to be especially vulnerable to the influence of cohort effects (Nyberg, Lövdén, Riklund, Lindenberger, & Bäckman, 2012). Finally, the neural effects of bilingualism in prodromal dementia stages (MCI), crucial for AD prevention, have been scarcely investigated (Bialystok, Craik, Binns, Ossher, & Freedman, 2014; Calabria et al., 2020; Duncan et al., 2018). Thus, during the development of this doctoral thesis, we were interested in filling these gaps by studying the effects of bilingualism in a prodromal dementia stage, MCI, and its longitudinal development across time. To do so, we investigated the differences between bilinguals and monolinguals with MCI in parenchymal volumes cross-sectionally and longitudinally (Study 1). We also investigated differences between bilinguals and monolinguals with MCI in brain circuits by using WM and FC measures (Studies 2 and 3).

Regarding the characteristics of the neuroplasticity produced by bilingualism in the healthy brain, conflicting evidence continues to emerge (Tao et al., 2021). This is probably due to sample differences between studies, as well as the use of methods that only look at linear relationships between variables and categorical approaches – dividing the sample in "bilingual" and "monolingual" groups –, which may miss the variability and dynamicity of bilingual experience (García-Pentón, García, Costello, Duñabeitia, & Carreiras, 2015; Grundy et al., 2017). Thus, we also considered relevant to improve our

methods by learning how to move beyond the categorical approach used in our first studies. In order to achieve this, we collaborated with an international research group that had experience in treating bilingualism as a continuous variable that is composed by multiple experience-based factors (Deluca, Rothman, Bialystok, & Pliatsikas, 2019), and using non-linear methods (Pliatsikas et al., 2020), which resulted in a joint work on the dynamic effects of bilingualism in cortical and subcortical volumes in a young healthy sample (Study 4).

Finally, evidence on the features that could mark progression from MCI to AD is still conflicting, especially regarding brain networks and the connections between its nodes (Hojjati et al., 2018; Marcos Dolado et al., 2019; Stone et al., 2021). GM atrophy in the temporal lobe and hippocampus seem to be consistent biomarkers of conversion to AD (Brueggen et al., 2015; Ferreira et al., 2011; Nesteruk et al., 2015), but similar agreement on structural and functional connectivity markers (WM integrity and FC measures) is lacking. Therefore, in Study 5 and 6, we investigated differences in brain structure and function (during resting-state and a memory-encoding task) of the DMN and MTL networks between healthy controls, AD, MCI patients that converted to AD during a 2-year follow-up, and MCI patients that remained stable and did not convert during that time.

Objectives and hypotheses

The general aim of this thesis is to study the neural basis of bilingualism and dementia, and its possible implications on cognitive reserve. In order to do so, we explored behavioral data and brain structural and functional images obtained with MRI. We analyzed the data focusing on these general and specific objectives:

1. To study the neural effects of bilingualism on a prodromal dementia stage: MCI.

    1.1 To investigate cross-sectional and longitudinal differences between bilinguals and monolinguals with MCI in parenchymal volume.

    1.2 To investigate differences between bilinguals and monolinguals with MCI in WM integrity of the fornix, UF and PH cingulum.

    1.3 To investigate differences between bilinguals and monolinguals with MCI in resting-state FC between regions within the ECN, DMN and language network, and in and in the synchronization and amplitude of regional spontaneous activity.

2. To investigate the neural characteristics of the AD continuum and the MRI markers of conversion from MCI to AD.

    2.1 To determine the contribution of brain alterations in the DMN to conversion from MCI to AD.

    2.2 To investigate the effects of AD on the activity of brain networks associated to precuneus and MTL during an encoding task.

3. To investigate the non-linear effects of bilingualism on GM structure in a sample of healthy bilinguals.

Based on previous evidence and our objectives, we formulated the hypotheses that can be found in each one of our articles after the Introduction section. These studies have been published or submitted to international peer-reviewed journals in their original format.

4. Studies

Study 1: A cross-sectional and longitudinal study on the protective effect of bilingualism against dementia using brain atrophy and cognitive measures.

Study 2: Effects of bilingualism on white matter atrophy in Mild Cognitive Impairment: a diffusion tensor imaging study.

Study 3: Bilingualism's Effects on Resting-State Functional Connectivity in Mild Cognitive Impairment.

Study 4: Dynamic effects of immersive bilingualism on cortical and subcortical grey matter volumes.

Study 5: Structural but not functional connectivity differences within default mode network indicate conversion to dementia.

Study 6: Activity in Memory Brain Networks During Encoding Differentiates Mild Cognitive Impairment Converters from Non-Converters.

Study 1

A cross-sectional and longitudinal study on the protective effect of bilingualism against dementia using brain atrophy and cognitive measures

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Abstract

Evidence from previous studies suggests that bilingualism contributes to cognitive reserve because bilinguals manifest the first symptoms of Alzheimer's disease (AD) up to five years later than monolinguals. Other cross-sectional studies demonstrate that bilinguals show greater amounts of brain atrophy and hypometabolism than monolinguals, despite sharing the same diagnosis and suffering from the same symptoms. However, these studies may be biased by possible preexisting between-group differences. In this study, we used global parenchymal measures of atrophy and cognitive tests to investigate the protective effect of bilingualism against dementia cross-sectionally and prospectively, using a sample of bilinguals and monolinguals in the same clinical stage and matched on sociodemographic variables. Our results suggest that the two groups did not differ on their cognitive status at baseline, but bilinguals had less parenchymal volume than monolinguals, especially in areas related to brain atrophy in dementia. In addition, a longitudinal prospective analysis revealed that monolinguals lost more parenchyma and had more cognitive decline than bilinguals in a mean follow-up period of 7 months. These results provide the first prospective evidence that bilingualism may act as a neuroprotective factor against dementia and could be considered a factor in cognitive reserve.

Keywords: bilingualism, cognitive reserve, Alzheimer's disease, Mild Cognitive Impairment, brain atrophy, region-based morphometry

Introduction

The continuous use of two languages has been shown to be among the different social, physical, mental, and leisure activities (Fratiglioni et al., 2004) that can promote cognitive reserve (CR) (Bialystok et al., 2012; Freedman et al., 2014; Guzmán-Vélez & Tranel, 2015). CR refers to individual differences in clinical resilience to brain pathology as a result of differences in neural efficiency and/or the use of a compensatory neural network (Steffener & Stern, 2012; Stern, 2002). This relationship between CR and bilingualism has often been related to the supposed cognitive benefits of having to manage two languages –a phenomenon, however, that is currently under dispute (Abutalebi & Green, 2016; Kroll & Bialystok, 2013; Perani & Abutalebi, 2015a).

Most of the evidence supporting the potential effect of bilingualism on CR comes from studies with brain-damaged patients with cognitive decline or dementia (Alzheimer's disease (AD), single-domain amnestic Mild Cognitive Impairment (MCI) patients, or other patients with dementias, such as the vascular type). In this regard, the most consistent finding is that the onset of the clinical symptoms associated with the

disease is significantly delayed in bilinguals, compared to monolinguals—a delay of about 4-5 years (Suvarna Alladi et al., 2013; Bialystok et al., 2007; Craik et al., 2010a; Freedman et al., 2014; Ossher et al., 2013; Perani et al., 2017; Perani & Abutalebi, 2015a; Woumans et al., 2015). Moreover, protective effects of bilingualism against age-related cognitive decline have been found independently of baseline cognitive ability (childhood intelligence), therefore dismissing reverse causality – the fact that childhood differences such as intelligence could lead to bilingualism instead of bilingualism leading to cognitive differences (Bak et al., 2014). However, these results have not always been replicated (Calvo et al., 2016; Guzmán-Vélez & Tranel, 2015).

The delay in the onset of dementia in bilinguals has been proposed as evidence of the contribution of bilingualism to CR (Perani & Abutalebi, 2015a). Two brain mechanisms are suggested to explain the increased CR: neural reserve and neural compensation. Neural reserve addresses the idea that CR could be associated with individual differences in the resilience of pre-existing cognitive networks (Perani & Abutalebi, 2015a; Stern, 2012). In this regard, evidence from neuroanatomic studies with healthy older participants suggests that bilingualism promotes grey matter volume and white matter integrity as a result of using two languages (Abutalebi, Guidi, et al., 2015; Anderson, Grundy, et al., 2018a; Luk et al., 2011). Neural compensation appears when cognitive function is maintained in the presence of brain atrophy due to better utilization of alternative networks (Barulli & Stern, 2013; Perani & Abutalebi, 2015a; Stern, 2012). It has been suggested that neural compensation promotes brain reserve; that is, it increases brain size in specific areas that allow more plasticity to overcome pathology and neurological insult (Barulli & Stern, 2013).

This study takes this perspective and investigates brain atrophy in bilingual and monolingual individuals with MCI. Therefore, if bilingualism promotes neural reserve and compensation, bilinguals might be expected to tolerate greater amounts of neuropathology or atrophy once the disease is manifested. This prediction is based on neuroimaging studies showing that, at the same cognitive level, bilinguals have more brain pathology than monolinguals. The first study to investigate the CR effects of bilingualism showed that bilingual patients suffering from AD exhibited much greater brain atrophy than monolinguals in regions associated with the pathology, such as the left middle temporal lobes (Schweizer et al., 2012). Because the two language groups showed the same degree of cognitive decline, the interpretation is that bilinguals would tolerate greater amounts of neuropathology or atrophy before the disease is manifested. Consistent with this interpretation, bilinguals with AD also showed greater levels of hypometabolism in left parietal, temporal and frontal areas than monolinguals (Perani et al., 2017). However, it is worth noting that the opposite results have been reported in MCI patients (Duncan et al., 2018).

The second objective of the study is to prospectively investigate the atrophy rate in MCI participants. Although the number of studies is limited, there is evidence suggesting that bilingualism promotes neural

compensation. On the one hand, studies in healthy older individuals and patients with Alzheimer's disease have shown increased functional connectivity in bilinguals compared to monolinguals (Grady et al., n.d.; Perani et al., 2017). On the other hand, evidence from studies investigating structural differences in healthy older participants suggests that bilingualism contributes to brain reserve, by showing that monolinguals present more extended age-related brain atrophy than bilinguals in diverse areas of the frontal, parietal and temporal lobules, and that these differences are associated with cognitive performance on different kinds of tasks (Abutalebi et al., 2014; Borsa et al., 2018; L. Li et al., 2017; Olsen et al., 2015). Together, these results support the view that bilingualism contributes to neural compensation and brain reserve during all the stages of neurodegeneration. However, this interpretation is limited by the fact that all these findings stem from cross-sectional designs that could be biased by cohort effects (Nyberg et al., 2012).

In the present study, we adopt both a cross-sectional and a longitudinal perspective to determine the role of bilingualism in brain atrophy and cognitive decline. Based on the reviewed literature, we hypothesize the following: (1) MCI bilinguals matched with MCI monolin- guals on a cognitive level, and sociodemographic factors would transversally present greater brain atrophy; (2) MCI bilinguals would show less atrophy and cognitive decline than monolinguals longitudinally. By combining the cross-sectional and longitudinal data, we attempt to provide a plausible explanation for the nature and origin of the bilingual delay in the onset of dementia.

Methods

Participants

Ninety-nine MCI individuals were included in this study (49 women; mean age=73.9±5.8). All of them were born in Spain and recruited from dementia units of the Valencian community public healthcare system, and they met the following inclusion criteria: (1) subjective memory complaints (self-reported or confirmed by an informant); (2) objective memory impairment assessed with the logical memory subtest II of the Wechsler memory scale-III (WMS-III; Wechsler, 1997a); (3) essentially intact activities in daily living; (4) no evidence of dementia; and (5) a CDR score of 0.5. Exclusion criteria were having the following: (1) other nervous system diseases, such as a brain tumor, cerebrovascular disease, encephalitis, or epilepsy, or meeting the criteria for dementia; (2) a Geriatric Depression Scale (Martínez et al., 2002; Yesavage et al., 1982) score > 6; (3) visible cerebral abnormalities reported by a radiologist with experience in magnetic resonance images, such as leukoaraiosis and infarction; and (4) a current psychiatric disorder or use of psychoactive medication.

Language group formation

All the participants resided permanently in the Spanish region of Valencia. During a clinical interview, language history was assessed using a short interview with the patient and some relatives. We asked for relevant information about three issues: (1) Age of acquisition of Catalan and Spanish; (2) self-rating of language proficiency, including their speaking and comprehension; and (3) language use based on the frequency with which they currently speak each of the two languages. Participants who reported Catalan as their mother tongue and Spanish as a second language were considered bilinguals (n=39), whereas those who only spoke Spanish were considered monolinguals (n=60). All the bilinguals learned Catalan at home before schooling and used it in their daily life, but they also spoke Spanish frequently. In the area where this study was made, a 60% of population only uses Spanish to speak, whereas a 38% uses Spanish and Catalan (see survey Knowledge and social use of Valencian language, 2010: http://www.ceice.gva.es/va/web/dgplgm/enquestes). Because these two Romance languages are similar, the same survey showed that a 90% of Spanish monolinguals understands Catalan. This percentage reached 100% in our sample. This information was obtained from the interview when monolinguals self-reported an acceptable or good comprehension of Catalan, but poor or null fluency. Thus, an important consideration in our study is that, for simplicity purposes, we use the term *monolingual* throughout our work to refer to individuals in our sample who only speak Spanish. However, within a more comprehensive categorization, these participants could be referred to as *passive bilinguals*, since Spanish speakers who permanently reside in the Valencian region do not speak Catalan but are usually able to understand it. Finally, it is worth mentioning that our bilingual group did not include immigrant individuals. This rules out possible between-group differences in life conditions and cultural background, given that bilinguals and monolinguals in the Valencian region share the same sociocultural context and environment (e.g., neighborhood, school system, workplace).

Participants were invited to undergo a second magnetic resonance imaging (MRI) and neuropsychological evaluation, which took place between 6 and 9 months after the baseline acquisition. Fifty-nine participants (43 monolinguals and 16 bilinguals) performed this second evaluation. These 2 samples significantly differed on sex ($\chi^2 = 6.68$; $p = 0.01$) and were close to differing significantly on age ($t(57) = -1.36$; $p = 0.09$). These differences limited the interpretation of longitudinal analyses, given that any possible result could simply reflect baseline differences on these variables. Therefore, we used sequential matching (Anderson et al., 2018; Gold et al., 2013) in to order to select a subsample of 16 monolinguals from the pool of 43 that match on sex (12 men and 4 women), and show the minimal differences in age ($t(30)=-0.57$; $p > 0.1$) and years of schooling ($t(30) = -0.36$; $p > 0.1$) compared to the bilingual sample. The mean time between the first and the second MRI session in the selected sample of 32 participants was 6.91 ± 1.3 months. In the subsequent sections, all analyses referred to as cross-sectional involve the whole sample of 99 participants, whereas the analyses referred to as longitudinal involve the subsample of 32 participants. Longitudinal analysis with the whole unmatched sample of 59 participants yielded similar

differences to those reported in the "Results" section. Furthermore, there were no significant differences between the whole sample and the follow-up sample in demographic variables, parenchymal volume, or any of the neuropsychological variables (all $p > 0.1$).

Neuropsychological assessment

All participants underwent a structured clinical interview and a neuropsychological assessment, which included a short form of the Boston naming test (Serrano et al., 2001a), a Word List Acquisition and Recall test, two Fluency tests (semantic and phonetic), a remote memory test, and the clock-drawing test (Cacho et al., 1996b). The results of these tests were transformed into z-scores and averaged, in order to obtain a composite measure of the Global Cognitive Level. Participants also completed the MMSE (Folstein et al., 1975; Lobo et al., 2002) and the Functional Activities Questionnaire (FAQ; Pfeffer et al., 1982). Statistics for neuropsychological tests are reported in Table 1. In addition to the memory impairment, 96% of the participants (94.9% of bilinguals and 96.7% of monolinguals) showed impairment in another cognitive domain, thus meeting the criteria for multiple-domain amnestic MCI.

MRI Acquisition

MRI data acquisition was performed on a 3T MRI scanner (Siemens Magnetom Trio, Erlangen, Germany) using a 12-channel head coil. Participants were placed inside the scanner in the supine position, and their heads were immobilized with cushions. Whole-brain 3-D images were collected using sagittal T1-weighted images (MP-RAGE sequence, 176 slices, 256x256 matrix, TR=2300 ms, TE=2.98 ms, flip angle=9º, spatial resolution 1x1x1 mm).

Image preprocessing and statistical analyses

Global tissue differences

All analyses were performed with CAT12 (Computational Anatomy Toolbox; C. Gaser, Jena University Hospital, Jena, Germany; http://dbm.neuro.uni-jena.de/cat/) as implemented in SPM12 (Statistical Parametric Mapping 12; Wellcome Trust Centre for Neuroimaging, University College, London, UK; http://www.fil.ion.ucl.ac.uk/spm/) and SPSS 25 (IBM Corp.). Before data processing, the first quality check was conducted to detect images affected by important inhomogeneity or movement artefacts. To study global tissue differences, individual volumes of grey matter (GM), white matter, and cerebrospinal fluid were estimated after applying the standard segmentation procedure implemented in CAT12. Then, the brain parenchyma volume for each participant was obtained from the sum of the absolute volumes of gray matter and white matter. Between-group comparisons were carried out by means of ANCOVA models. For cross-sectional analyses, the model included global parenchymal volumes as dependent variable and bilingualism as independent variable. The possible confound of total intracranial volume

(TIV) was included as covariate of no interest. For the study of longitudinal differences, the model included the parenchymal volumes for the first and second MRI scan as dependent variable, time as within-subject factor, and bilingualism as between-subject factor. The possible confound of time between scans (in months) and the differences in TIV estimations between the first and second MRI scan were included as covariates of no interest. Planned comparisons were evaluated to test the hypothesis of higher atrophy in bilinguals in the cross-sectional analysis and the hypothesis of a higher rate of atrophy in monolinguals in the longitudinal analysis, using a significance level threshold of $p < 0.05$.

Region-based morphometry

In order to study region-specific volumetric differences, region of interest (ROI) analysis implemented in CAT12 was performed. In this analysis, also called region-based morphometry (RBM), an anatomical atlas is transformed into native subject space, and the sum of the local GM inside the atlas' pre-defined ROIs is estimated. The LONI Probabilistic Brain Atlas (LPBA40; Shattuck et al., 2008) was used as a reference atlas. In this atlas, the whole brain is divided into 56 parcels comprising both cortical and subcortical areas. Statistical analyses were similar to the ones used to investigate global tissue differences. For cross-sectional analyses, ANCOVA models were performed, including ROI volumes as dependent variable, bilingualism as independent variable, and TIV as confound. For longitudinal analyses, ANCOVA models were carried out, including ROI volumes for the first and second MRI scan as dependent variable, time as within-subject factor, and bilingualism as between-subject factor. Again, the time between scans (in months) and differences in TIV estimations were included as confounds. Then, planned comparisons were conducted to test the hypothesis of higher atrophy in bilinguals in the cross-sectional analysis and a higher rate of atrophy in monolinguals in the longitudinal analysis, using a significance level threshold of $p<0.05$ false discovery rating (FDR) corrected.

Results

Cross-sectional Sociodemographic and Neuropsychological Results

We used t-tests or chi-squares to compare these variables (see Table 1 and figure 1a). The results confirmed that there were no significant between-group differences between the bilingual and monolingual groups in age, gender, years of schooling, and Global Cognitive Level. Importantly, the two groups did not differ in their performance on the Boston Naming Test in Spanish or in the analyses of the other neuropsychological tests. This pattern of results confirmed that the two groups presented a similar cognitive status at the time of the first MRI scan.

Table 1. Sociodemographic and neuropsychological variables for monolingual and bilingual MCI patients

	Monolinguals (N = 60)[a]	Bilinguals (N = 39)[a]	Statistical differences	p value
Gender	M/F = 26/34	M/F = 24/15	$\chi^2 = 3.13$	0.08
Age	73.58 (5.76)	74.26 (5.78)	$t = -0.56$	0.57
Years of schooling	8.62 (3.45)	8.33 (2.43)	$t = 0.47$	0.63
Cognitive level	– 0.05 (0.6)	0.07 (0.64)	$t = -1.00$	0.32
MMSE	26.95 (2.63)	27.23 (2.18)	– 0.55	0.58
FAQ	3.3 (2.58)	3.82 (2.48)	– 0.97	0.32
Boston	9.33 (1.45)	9.77 (1.31)	– 1.52	0.13
Phonetic fluency	8.37 (2.14)	8.51 (2.62)	– 0.30	0.76
Semantic fluency	10.63 (2.47)	10.74 (2.19)	– 0.23	0.82
WLA	9.03 (2.88)	9.79 (2.78)	– 1.30	0.20
WLR	1.07 (0.86)	1.10 (0.91)	– 0.20	0.84
Remote memory	9.18 (1.46)	9.49 (1.23)	– 1.08	0.28
Clock-drawing	7.14 (1.80)	7.00 (1.41)	0.39	0.69

N sample size, *M/F* males/females, χ^2 chi-squared test, *t* *t*-value for two-sample *t* test, *MMSE* Mini-Mental State Examination, *FAQ* Functional Activities Questionnaire, *WLA* word list acquisition, *WLR* word list recall

[a]Mean and standard deviation (in parentheses) are shown for quantitative variables

Cross-sectional MRI results

In agreement with our hypothesis, the study of global differences in brain parenchyma volume showed reduced brain volume ($t_{(96)}$ = 3; p = 0.002) in bilinguals, compared to monolinguals (see Figure 1b). We ran an RBM analysis to locate where these differences were more prominent (see Figure 1c). This analysis showed that bilinguals, compared to monolinguals, present significantly lower volume in the right supramarginal gyrus ($t_{(96)}$=3.48; p=0.021 FDR corrected) and the left lingual gyrus ($t_{(96)}$=3.12; p=0.034 FDR corrected). The opposite comparison (bilinguals > monolinguals) did not show any significant differences, even when using a lower threshold of p < 0.05 uncorrected.

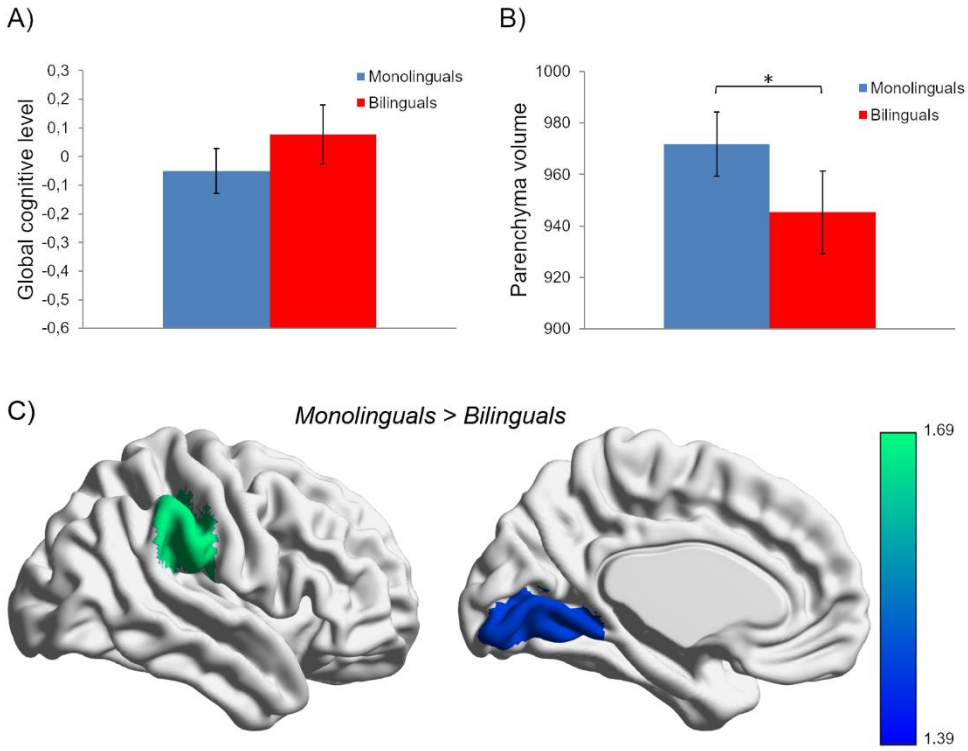

Figure 1. Cross-sectional results; a) Mean and standard error bars for Global Cognitive Level measure; b) Mean and standard error bars for parenchyma volume (cm^3). *significant differences at a threshold of p<0.05; c) Region-based morphometry results. Figure shows the brain regions with grey matter volume reduction in bilinguals compared to monolinguals. The color bar represents the log-scale p-value FDR corrected applicable to the image.

Longitudinal Neuropsychological Results

Two participants (one bilingual and one monolingual) did not complete the second neuropsychological evaluation and were excluded from this analysis. Similar to the brain atrophy analyses, we used an ANCOVA model to study the hypothesis of higher cognitive decline in monolinguals than in bilinguals. Thus, the global cognitive level for the first and second evaluations was included as dependent variable, time as within-subject factor, bilingualism as between-subject factor, and time between explorations (in months) as covariate of no interest. Planned comparisons revealed a faster global cognitive decline in monolinguals compared to bilinguals ($t_{(27)}$ = 2.50: p = 0.009; see Figure 2a). Post hoc analyses investigating this effect showed that the global cognitive decline was significant in monolinguals (t(14) = 4.02; p < 0.001), but not in bilinguals (t(14) = 0.85; p = 0.205). The same analyses for individual tests showed a significantly faster decline in the monolingual group as compared to the bilingual group on the phonetic fluency test, that is, a similar pattern to the one observed in the global cognitive level (see Table 2).

Longitudinal MRI Results

As expected, planned comparisons to test the hypothesis of a higher rate of atrophy in monolinguals than bilinguals revealed a significant interaction between the time and language groups ($t_{(28)}$ = 2.02; p = 0.027), reflecting a slower parenchymal volume loss in bilinguals compared to monolinguals across the time points (see Figure 2b). The study of region-specific longitudinal differences by means of RBM analyses did not show any significant differences at a threshold of p<0.05 FDR corrected in any ROI. However, using a more liberal threshold of p<0.05 uncorrected, we found differences in the right cingulate gyrus ($t_{(28)}$ = 2.46; p = 0.01), right putamen ($t_{(28)}$ = 2.16; p = 0.019), right caudate ($t_{(28)}$ = 1.74; p = 0.046), right hippocampus ($t_{(28)}$ = 1.94; p = 0.031), and left fusiform gyrus ($t_{(28)}$ = 1.93; p = 0.032). All these regions showed a similar pattern of faster volume reduction over time in monolinguals compared to bilinguals. Of note, these uncorrected results are reported for descriptive purposes to provide information about the areas within the parenchyma where the differences are more pronounced in our data, and they should not be used to draw inferences.

Table 2. Neuropsychological results for the subgroups of monolinguals and bilinguals included in the longitudinal study

	Monolinguals		Bilinguals		Group effects	Time effects	Interaction effects
	Time 1	Time 2	Time 1	Time 2	*F*-score[1]	*F*-score[2]	*F*-score[3]
MMSE	26.8 (2.73)	25.7 (3.02)	27.7 (2.13)	27.1 (3.02)	1.46	5.87*	0.68
FAQ	3.80 (2.96)	5.40 (4.47)	3.07 (1.49)	5.40 (2.92)	0.15	9.15*	0.42
Boston	9.53 (1.68)	8.87 (2.36)	9.93 (1.03)	9.40 (1.12)	0.70	7.74*	0.09
Phonetic fluency	14.0 (8.34)	6.73 (2.79)	11.2 (3.73)	9.13 (1.81)	0.02	14.74*	4.19*
Semantic fluency	21.4 (16.3)	10.0 (3.05)	13.6 (5.41)	9.60 (1.55)	2.56	15.92*	3.45
WLA	10.0 (3.09)	9.73 (3.31)	8.47 (1.36)	9.13 (2.03)	1.74	0.75	0.90
WLR	0.87 (0.83)	0.53 (0.64)	1.00 (0.65)	1.13 (0.52)	3.39	0.49	2.51
Remote memory	8.80 (2.01)	9.13 (1.64)	9.47 (1.13)	9.93 (1.03)	2.71	1.54	0.06
Clock-drawing	7.50 (2.28)	6.29 (2.46)	7.40 (1.30)	6.73 (1.03)	0.08	9.39	0.73

Cells in the table indicate the groups' means (standard deviation in parenthesis) for each neuropsychological test at time 1 and time 2. Two participants (1 bilingual and 1 monolingual) did not complete the neuropsychological evaluation at time 2. Thus, the table shows statistics excluding these participants (N = 30; 15 bilinguals and 15 monolinguals)

MMSE Mini-Mental State Examination, *FAQ* Functional Activities Questionnaire, *WLA* word list acquisition, *WLR* word list recall

*$p < 0.05$ uncorrected

[1]The main effect of bilingualism estimated by means of a two-way mixed ANOVA, including time as within-subject factor and bilingualism as between-subject factor

[2]The main effect of time estimated by means of a two-way mixed ANOVA, including time as within-subject factor and bilingualism as between-subject factor

[3]The interaction effect between time and bilingualism estimated by means of an ANCOVA model, including time as within-subject factor, bilingualism as between- subject factor, and time between explorations (in months) as covariate of no interest

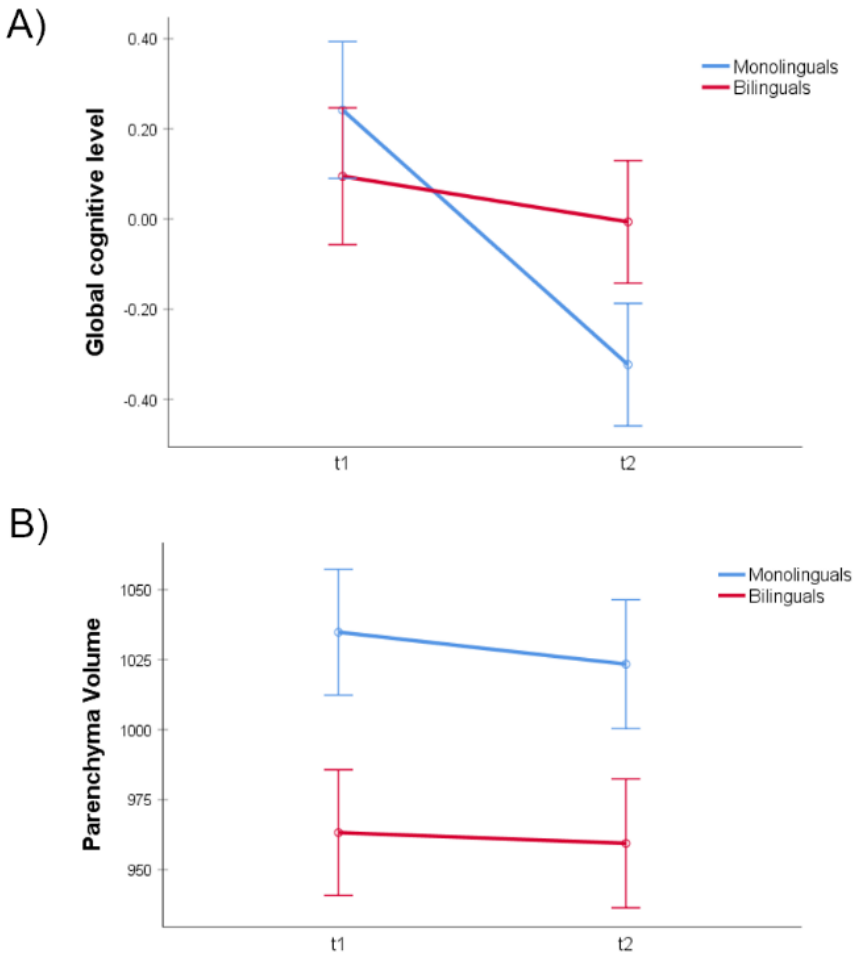

Figure 2. Longitudinal results; a) Mean and standard error bars for Global Cognitive Level measures at the time of the first and second neuropsychological evaluations; b) Mean and standard error bars for parenchyma volumes (cm^3) at the time of the first and second scans.

Discussion

We investigated the neural bases of the putative protective effect of bilingualism against dementia by comparing the brain atrophy of bilinguals and monolinguals suffering from MCI. To this end, we selected two groups of monolingual and bilingual MCI patients with similar sociodemographic characteristics and education levels, living in the same area in the city of Valencia. The cross-sectional analysis showed that MCI bilinguals showed a greater amount of brain atrophy than MCI monolinguals, but no differences in global cognitive level or age. In the present study, we also took prospective longitudinal measures to shed light on atrophy rates in both groups. In agreement with our hypotheses, monolinguals showed higher brain atrophy rates and more cognitive decline than bilinguals in a 7-month period. Specifically, in this period, monolinguals, but not bilinguals, showed significant brain atrophy and cognitive decline. Together, our results suggest that the active use of two languages throughout life not only promotes CR, but also brain reserve, providing a neural-based frame- work that could explain why bilinguals, compared to monolinguals, show a delay in the onset of dementia.

The results of the present study are consistent with previous cross-sectional studies showing that bilinguals require a greater amount of neuropathology in the brain to manifest the same cognitive status level. Specifically, one study using positron emission tomography (PET) in patients with AD showed that bilinguals had lowered hypometabolism, especially in the temporo-parietal cortex (Perani et al., 2017), whereas another study using Computed Tomography showed more GM atrophy in bilingual AD patients than in monolingual patients (Schweizer et al., 2012). Our cross-sectional analysis is consistent with all these results, but it presents some additional features that should be specifically discussed. First, our study is the first to demonstrate this neuroprotection in MCI patients. This condition is a preclinical form of dementia that preserves the ability to perform daily life activities. Our clinical sample was mostly composed of multi-domain amnestic MCI patients, and the results showed no age differences between the language groups because bilinguals were only 7 months older than monolinguals. These results were consistent with a previous report showing age differences in single-domain, but not multi-domain, MCI patients (Ossher et al., 2013). In that study, the authors proposed the possible coexistence of vascular risk factors as a possible explanation for the lack of age effects in this group, but this is unlikely in our sample because patients with vascular problems identified in the MRI were removed from the sample, and because bilingualism also protects against deterioration in stroke patients (Suvarna Alladi et al., 2016). Thus, our data are the first to show increased CR in MCI, that is, higher atrophy in MCI bilinguals at the same cognitive level as MCI monolinguals. Second, we have demonstrated the effect of bilingualism on brain atrophy in the absence of relevant between-group differences on factors such as education, age, or the environment (i.e. same city of residence) as obtained in previous studies (Perani et al., 2017; Schweizer et al., 2012). It is important that the two groups did not differ on their cognitive

status, as assessed by a neuropsychological profile. Thus, the results obtained in the present study strengthen the interpretation of a bilingual advantage because we controlled for the potential cofounding factors. According to recent proposals (Perani & Abutalebi, 2015a), differences may arise from a better capacity of bilinguals to functionally compensate for the greater loss of brain parenchyma. This increased neural compensation would arise from the continuous use of two languages, which entails a stronger use of certain brain areas involved in language control and executive functions (Perani et al., 2017). The fact that our monolingual individuals could be better categorized as *passive bilinguals* favors this interpretation, given that the observed differences would arise not from the knowledge of the second language per se but from the frequency of use.

RBM analysis revealed that the between-group significant differences showed in our cross-sectional study were mainly located in the lingual and the supramarginal gyrus. These two regions have been reported to be affected by AD in functional and structural meta-analyses (Schwindt & Black, 2009; Yang et al., 2012). Specific alterations in MCI individuals has been shown in the lingual gyrus during tasks involving episodic memory (Terry et al., 2015). Moreover, differences in the supramarginal gyrus has been shown when comparing anatomical likelihood estimate maps of MCI converters and non-converters in a meta-analysis integrating results from different neuroimaging modalities (Schroeter et al., 2009). The right supramarginal area was also involved in previous morphometric studies in bilingualism. For instance, increased GM volume in this region has been related to better proficiency in the second language and the number of non-native languages spoken (Mechelli et al., 2004a). Furthermore, healthy older bilinguals showed higher white matter integrity than monolinguals in the superior longitudinal fasciculus (Anderson, Grundy, et al., 2018a; Luk et al., 2011), that is, the white matter tract that connects the supramarginal gyrus to frontal and temporal regions. All these results may suggest that brain areas related to the use of language would increase their efficiency in bilinguals and, in turn, compensate for the effects of AD neuropathology.

One of the main objectives of this study was to investigate how bilingualism impacts the course of dementia. For this reason, we retested a subsample of patients who did not differ at baseline on any of the cognitive or sociodemographic variables (age, education, and gender). Our longitudinal results suggest that monolinguals have a faster rate of cognitive decline and brain atrophy than bilinguals. On the neuropsychological measures, both groups showed a significant cognitive decline on the MMSE, FAQ, phonetic and semantic fluency, Boston naming test, clock-drawing test, and the overall measure in the seven-month period. Crucially, monolinguals showed a greater decline than bilinguals on the overall cognitive measure. This result coincides with previous findings showing a heterogeneous pattern of cognitive decline that does not focus on any specific domain (Dicks et al., 2018; Johnson et al., 2012). In this regard, it is noteworthy that most of the patients who participated in this study were multiple-

domain amnestic. Importantly, monolinguals also suffered a higher rate of brain atrophy than bilinguals in the seven-month period. These results coincide with findings from cross-sectional studies in healthy older individuals showing increased age-related brain atrophy in monolinguals compared to bilinguals (Abutalebi et al., 2014; Borsa et al., 2018; L. Li et al., 2017; Olsen et al., 2015). In this regard, the results of our study agree with these findings and suggest that the neuroprotective effect of bilingualism is also maintained during early stages of dementia. A possible neural mechanism driving this effect was proposed by Barulli and Stern, who suggested that neural compensation may increase brain reserve by promoting neuroplasticity (Barulli & Stern, 2013). Thus, the functional compensation required to maintain performance will eventually lead to changes in the brain itself. RBM analyses investigating the specific brain areas with a higher atrophy rate in monolinguals than bilinguals did not show significant results at the pre-established threshold ($p<0.05$ FDR corrected). However, uncorrected results suggest that the main differences found in the parenchymal analyses were located in areas related to language or executive control, such as the cingulate gyrus and the striatum (Abutalebi & Green, 2007), and in crucial areas in dementia, such as the hippocampus. Although speculative, we suggest that the continuous use of two languages in bilinguals may help to preserve the brain areas involved in controlling two languages and, potentially, executive control (Calabria et al., 2018). This neuroprotection would have a compensatory effect on the manifestation of cognitive symptoms of MCI and dementia (Perani et al., 2017).

This study has several limitations that should be considered. First, the sample of our study was composed by MCI individuals only. MCI cohorts are heterogeneous groups with variable rates of conversion to dementia. Therefore, cautious interpretation is required while extending the results to protective effects of bilingualism on dementia. Second, due to participants' drop out, only 59.6% of the initial sample performed the longitudinal recording. Furthermore, this subsample was unbalanced. Therefore, the longitudinal results were based on a relatively small sample of 32 individuals. However, the results obtained when comparing the unbalanced sample of 16 bilinguals and 43 bilinguals yielded similar results. Future studies with higher sample sizes may provide further differences not detected in our study. Third, the following up period could be considered short for a long-term disease such as AD. Therefore, our longitudinal analysis could be considered the first one to provide empirical evidence about sort term patterns of atrophy and cognitive decline in bilinguals and monolinguals with MCI. However, further studies investigating longitudinal changes in these populations with longer temporal windows are necessary. Fourth, the bilinguals in this study spoke Spanish and Catalan, which could be considered two similar languages. This could be a limitation in many studies of bilingualism; however, the protective influence of bilingualism on dementia has been demonstrated in different contexts (e.g., English-Spanish, English-Polish, English-Yiddish, Telugu-Hindi, Dutch- French). Furthermore, for the specific purposes of this study, this similarity might be a strength. It has been proposed that the

relationship between cognitive reserve and bilingualism is due to the additional demands on the control system in bilinguals to effectively manage the two languages (Abutalebi & Green, 2016; Bialystok, 2011; Perani & Abutalebi, 2015b). The positive findings in our study suggest that the contribution of bilingualism to CR is effective even in bilinguals speaking similar languages, where the cognitive demands on the control system might be considered lower than in dissimilar languages. In fact, in our study, we compared active and passive bilinguals; therefore, our results suggest that the contribution of bilingualism to cognitive reserve is related to the active use of the two languages and not just their comprehension. The same conclusions could be drawn from other studies (Woumans et al., 2015).

Conclusions

As a general conclusion, in this study, we found that bilinguals with the same cognitive level, age, years of schooling, sociocultural origin, and disease severity (MCI) as monolinguals showed lower parenchymal volume, especially in areas related to bilingualism and those previously described as affected in AD. Furthermore, monolinguals have prospectively shown a higher atrophy rate and greater cognitive decline than bilinguals over time. Together, our results suggest that bilingualism promotes both CR and brain reserve. The combination of these two factors may provide a neural framework to explain the nature and origin of the bilingual advantage in the delay of dementia.

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Study 2

Effects of bilingualism on white matter atrophy in Mild Cognitive Impairment: a diffusion tensor imaging study

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Abstract

Background: Previous investigations show that bilinguals exhibit the first symptoms of dementia 4-5 years later than monolinguals. Therefore, bilingualism has been proposed as a cognitive reserve mechanism. Recent studies have advanced towards an understanding of the brain mechanisms underlying bilingualism's protection against dementia, but none of them deals with white matter (WM) diffusion.

Methods: In this study, we investigated this topic by measuring WM integrity in a sample of 35 bilinguals and 53 passive bilinguals with Mild Cognitive Impairment (MCI).

Results: We found no significant differences between the groups in cognitive level, education, age, or sex. However, bilinguals showed higher mean diffusivity (MD) in the fornix, but higher fractional anisotropy, lower MD, axial diffusivity, and radial diffusivity (RD) in the parahippocampal cingulum, and lower RD in the right uncinate fasciculus. We also found significant correlations between WM integrity in the left parahippocampal cingulum and the Boston Naming Test in passive bilinguals.

Conclusions: These results suggest that bilingualism contributes to a differential pattern of WM disintegration due to MCI in fibers related to bilingualism and memory.

Introduction

Bilingualism has been proposed as one of the factors that contribute to cognitive reserve (CR)(Guzmán-Vélez & Tranel, 2015). This proposal is based both on the fact that the first symptoms of dementia appear about 4-5 years later in bilinguals than in monolinguals(S. Alladi et al., 2013; Bialystok et al., 2007; Craik et al., 2010a) and on previous studies investigating brain atrophy(Duncan et al., 2018; Schweizer et al., 2012) and hypometabolism(Perani et al., 2017). Previous investigations showed that, in healthy young samples, bilingualism increases white matter (WM) integrity in bilateral language-related pathways(Ghazal Mohades et al., 2015; Hämäläinen et al., 2017; Pliatsikas et al., 2015). However, there is no evidence about the effects of bilingualism on WM integrity in dementia.

To date, the only evidence about the effects of bilingualism on CR in dementia using WM measures comes from studies in healthy elderly samples. Regarding WM volume, Olsen et al. found greater frontal lobe WM in older bilinguals compared to monolinguals(Olsen et al., 2015). Focusing on diffusion tensor imaging (DTI) scalars, another investigation found higher fractional anisotropy (FA) – higher WM integrity – in the corpus callosum in elderly bilinguals compared to monolinguals(Luk et al., 2011). A subsequent study with a similar sample found greater axial diffusivity (AxD) in bilinguals compared to monolinguals in the left superior longitudinal fasciculus, a fiber connecting areas relevant to the language network, which was interpreted by the authors as enhancing WM integrity in bilinguals(Anderson, Grundy, et al., 2018b). However, opposite results have also been found. Thus, a previous study using a sample of older adults found lower FA and higher mean diffusivity (MD) (i.e. lower WM integrity) in bilinguals compared to monolinguals in a number of tracts, including the corpus callosum and the fornix, which form part of the brain's memory circuitry(Gold et al., 2013).

To the best of our knowledge, differences in WM between bilinguals and monolinguals with dementia have not yet been studied. Without taking bilingualism into consideration, alterations in WM in amnestic Mild Cognitive Impairment (MCI) have been previously defined as mainly affecting the fornix, uncinate fasciculus (UF), and parahippocampal (PH) cingulum(Yu et al., 2017), that is, brain tracts related to episodic memory. The PH cingulum and the fornix have been identified as relevant components of the hippocampal-diencephalic-cingulate networks, which are considered essential for normal episodic memory(Bubb et al., 2017b). Moreover, the UF connects the lateral orbitofrontal cortex and the anterior temporal lobes bidirectionally, and it also plays a relevant role in episodic memory(Von Der Heide et al., 2013).

Thus, the objective of our study was to investigate the proposed contribution of bilingualism to CR by comparing

WM atrophy in active and passive bilingual MCI patients. Based on previous studies showing that bilinguals with Alzheimer's Disease (AD) present more neuropathology than AD monolinguals in areas related to this disease(Perani et al., 2017; Schweizer et al., 2012), bilinguals were expected to show lower levels of WM integrity than passive bilinguals in the tracts affected in MCI, that is, in the fornix, UF, and PH cingulum(Yu et al., 2017).

Methods

Participants

Eighty-eight patients with MCI were selected for this study (43 women, mean age=73.10±5.764), recruited from dementia units in the Valencian Community public healthcare system (see *Appendix S1* for diagnosis and inclusion criteria).

All the participants were asked about their use of languages. They spoke Catalan and Spanish (n=35) or only Spanish (n=53) as their native languages. We will refer to these groups as bilinguals (active) and passive bilinguals, respectively. It is worth mentioning that, because Spanish speakers who permanently reside in the Valencian region do not speak Catalan but are usually able to understand it, we refer to them as *passive bilinguals* rather than *monolinguals*. All the bilinguals had learned Catalan during childhood at home. In the area of Valencia, bilinguals and passive bilinguals share the same everyday life environments, such as neighborhood, school system, and workplace, with no significant differences apart from the fact that they speak only Spanish or Catalan and Spanish. None of the groups included immigrant individuals. Sample descriptive statistics are reported in Table 1.

All the participants were informed of the nature of the research and provided written informed consent prior to their participation in the study. All the study procedures were approved by the Deontological Comission of University Jaume I and conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Neuropsychological assessment

All the participants underwent a structured clinical interview and a neuropsychological assessment (see *Supplementary methods* for information on the scales included). Descriptive statistics and the results of a two-sample *t* test for each scale are reported in Table 1.

DTI analyses

Information about image acquisition and processing is reported in *Appendix S1*. For each individual, we estimated the following diffusion measures: FA, MD, AxD and radial diffusivity (RD).

Between-group comparisons, including sex, age, and MMSE scores as covariates, were conducted. The threshold-free cluster enhancement (TFCE) statistic was estimated, and statistical significance was determined using permutation-based nonparametric inference (5000 permutations per analysis) at a threshold of $P < 0.05$ family-wise error (FWE) corrected. The analyses were restricted to a mask that included the fornix, PH cingulum, and UF as tracts of interest, based on a recent meta-analysis showing that MCI patients present microstructural WM abnormalities in these regions(Yu et al., 2017). We also performed whole-brain analyses using the mean FA skeleton as a mask.

Correlation with neuropsychological variables

For each cluster where we observed significant differences between the groups, we investigated its relationship with each of the neuropsychological variables by means of partial correlations, including age and sex as covariates, in each group separately. Statistically significant thresholds were set at $p<0.05$, corrected using the Bonferroni method.

Results

Differences in neuropsychological variables

Our analyses showed no significant differences in the performance on any of the neuropsychological tests between bilinguals and passive bilinguals with MCI, or on age ($t=-0.543$; $p>0.1$), the proportion of men and women ($\chi^2=3.19$; $p>0.1$), or years of schooling ($t=0.564$; $p>0.1$) (Table 1).

Table 1. Socio-demographic and neuropsychological measures for passive bilinguals and (active) bilinguals.

	Monolinguals (N=53)		Bilinguals (N=35)		Statistical differences	p
Sex	M/F = 23/30		M/F = 22/13		$\chi^2 = 3.195$	.074
	M	SD	M	SD	t	p
Age	72.83	5.65	73.51	5.99	-.543	.589
Years of schooling	8.79	3.64	8.40	2.37	.564	.575
MMSE	27.26	2.49	27.31	2.07	-.099	.922
FAQ	3.26	2.81	4.29	3.30	-1.558	.123
Boston	9.38	1.46	9.74	1.31	-1.197	.235
Phonetic fluency			8.66	2.22	-.475	.636
Semantic fluency	10.70	2.63	10.74	2.15	-.084	.933
Delayed recall	1.06	.86	1.14	.94	-.442	.660
Immediate recall	8.94	3.00	9.60	2.56	-1.06	.291
Remote memory	9.26	1.443	9.60	.976	1.208	.231
Clock-drawing test	7.17	1.85	7.00	1.45	.464	.644

MMSE = Mini Mental State Examination; FAQ = Functional Activities Questionnaire; N = sample size; p = p-value regarding statistical significance; M/F = males/females; M = Mean; SD = Standard Deviation; χ^2 = Chi-squared test; t = t-value for Two-sample t-Test.

Differences in DTI

Using the mask that included the fornix, PH cingulum, and UF, we found that bilinguals exhibited higher MD in the fornix than passive bilinguals ($p<0.05$, FWE corrected, Fig 1). They also showed higher FA and lower MD, AxD, and RD in the PH cingulum bilaterally ($p<0.05$, FWE corrected, Fig 2), and lower RD in the right UF ($p<0.05$, FWE corrected, Fig 3). Exploratory whole brain analyses did not show any significant differences.

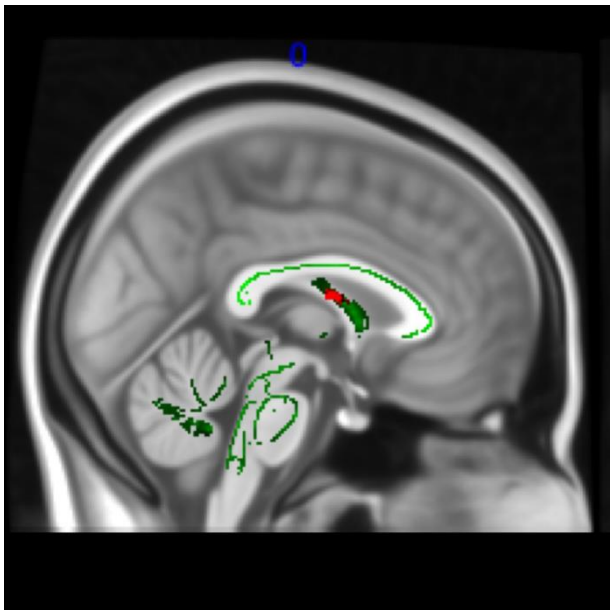

Figure 1. Differences in MD in the fornix between bilinguals and passive bilinguals. In green, skeleton tracts, and in red, tracts with significantly higher MD in bilinguals compared to passive bilinguals.

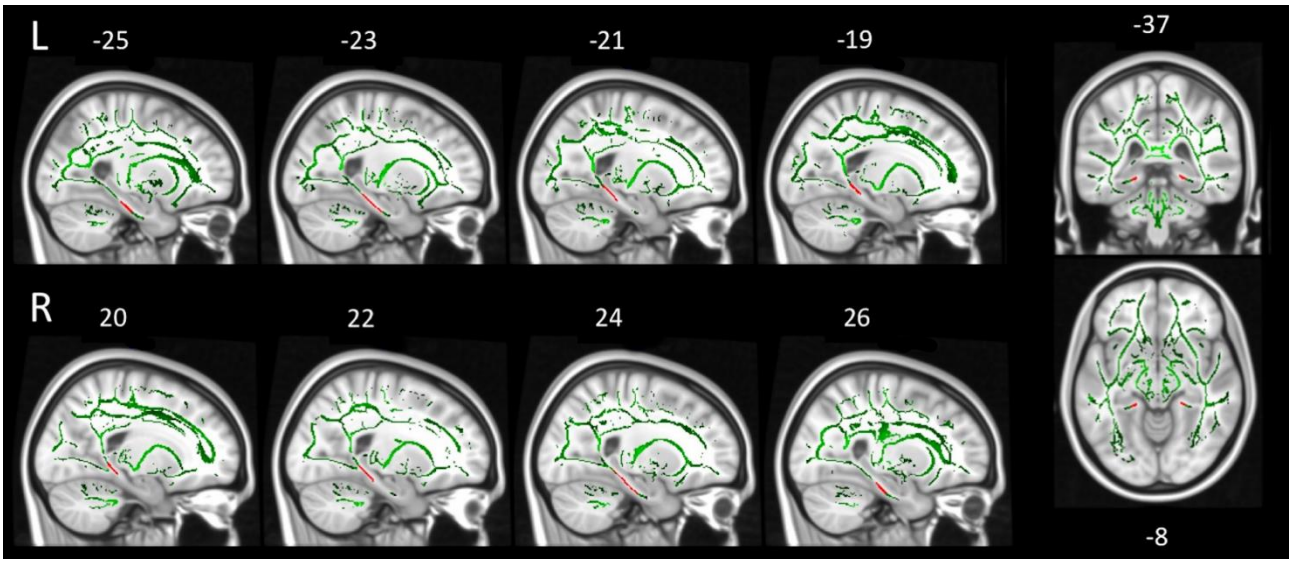

Figure 2. Differences in RD in the PH cingulum. In green, skeleton tracts, and in red, tracts with significantly higher RD in passive bilinguals compared to bilinguals.

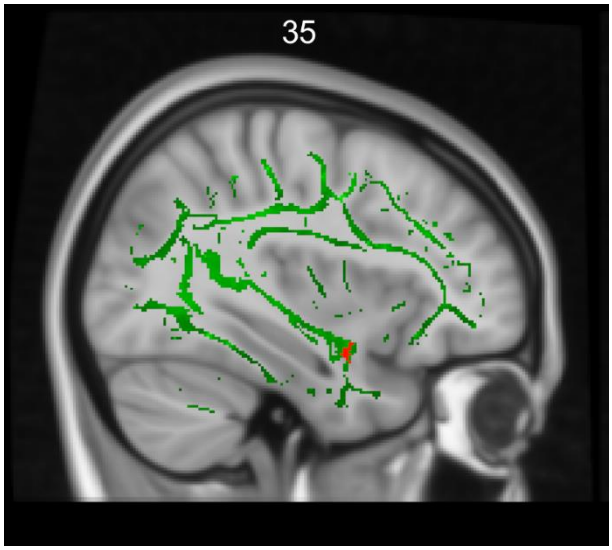

Figure 3. Differences in RD in the right UF. In green, skeleton tracts, and in red, tracts with significantly higher RD in passive bilinguals compared to bilinguals.

Correlation between DTI and neuropsychological variables

In the passive bilingual group, we found a significant correlation between the performance on the Boston naming test (BNT) and RD levels (R=-0.425, p=0.014 FWE corrected) in the left PH cingulum. In the bilingual group, we found no significant correlations with the pre-established FWE corrected threshold. However, taking into account uncorrected results in this group, we found an association between RD levels in the left PH cingulum and immediate recall (R=-0.413, p=0.017 uncorrected), similar to the results reported in previous studies investigating WM integrity in MCI participants(C. Metzler-Baddeley et al., 2012; Claudia Metzler-Baddeley et al., 2012). No other significant results were found for any of the other neuropsychological variables.

Discussion

In this study, the differences in WM diffusion between bilinguals and passive bilinguals with MCI who shared similar sociodemographic characteristics, with no significant differences in cognitive status, years of schooling, age, or the proportion of men and women, were investigated. However, bilinguals showed lower WM integrity in the fornix and higher in the PH cingulum and UF. Furthermore, WM integrity in the PH cingulum was positively correlated with performance on the BNT in the passive bilingual group. These results suggest that the active use of two languages leads to a differential pattern of WM disintegration in MCI.

The fornix and the PH cingulum are part of the hippocampal-diencephalic-cingulate networks for memory and emotion(Bubb et al., 2017b), initially proposed in the work by Papez(Papez, 1995). The fornix is a fiber bundle situated in the midline of the brain, originating from the hippocampus and terminating at the septal nuclei, nucleus accumbens, and hypothalamus(Oishi & Lyketsos, 2014). Damage to the fornix can cause memory impairment(Aggleton et al., 2000; Bubb et al., 2017b; Ibrahim et al., 2009), its WM integrity is altered in MCI(Yu et al., 2017), and it has recently been identified as a useful target for deep brain stimulation in dementias(Mao et al., 2018; Xu & Ponce, 2018). Regarding the PH cingulum, it is a subdivision located in a temporal position and extending caudally, connecting the posterior cingulate cortex, visual areas from the occipital lobe, and parietal areas such as the intraparietal cortex with the medial temporal lobe(Jones et al., 2013). Its function is more related to familiarity-based memory, that is, to the automatic feeling triggered by contextual cues that an item has been recently experienced(C. Metzler-Baddeley et al., 2012). The latter connections seem especially relevant in relation to our study because previous investigations have linked bilingualism to increased gray matter volume(Abutalebi, Canini, et al., 2015; Mechelli et al., 2004c) and cortical thickness(Duncan et al., 2018) in inferior parietal areas. Finally, the UF connects the frontal orbital cortex and the temporal lobe(Von Der Heide et al., 2013). Due to the nature of these connections, it has also been proposed as a relevant fiber for memory, with DTI studies supporting its role in verbal and auditory memory(Von Der Heide et al., 2013).

The fornix, UF, and PH cingulum are all impaired in MCI(Yu et al., 2017). The fornix and PH cingulum have been found to play different roles in its clinical manifestation(C. Metzler-Baddeley et al., 2012; Claudia Metzler-Baddeley et al., 2012). Thus, a study comparing healthy controls and patients with MCI found higher atrophy in the fornix and PH cingulum in patients(Claudia Metzler-Baddeley et al., 2012). Importantly, this study showed differential associations between performance on a free recall test and fornix and PH cingulum pathology: in controls, free recall correlated with fornix WM integrity, whereas in patients, the strongest relationship was found with the PH cingulum, a result that was replicated(Ray et al., 2015). This was interpreted as a sequential compensatory mechanism through which, in the presence of an impairment of episodic memory functions in the fornix, the PH cingulum may later support memory performance using familiarity processing resources as an alternative route(Claudia Metzler-Baddeley et al., 2012; Ray et al., 2015). Based on this previous literature, a possible explanation for the similarities between the performance of our groups on neuropsychological testing might be a compensatory mechanism: due to a more compromised fornix in bilinguals, memory processes might be supported by the PH cingulum, which acquires special relevance as a compensatory route. Thus, although our

initial hypothesis was to find lower WM integrity in all tracts of interest in bilinguals, our results might still be interpreted as a contribution of bilingualism to CR, considering that bilinguals use familiarity as a compensatory mechanism of episodic memory deficits that depend on the fornix. This compensatory use has been associated to a delay in the manifestation of AD(De Simone et al., 2019). Supporting this interpretation, we found a relationship between RD in the left PH cingulum and immediate recall performance only in bilinguals. However, this result did not reach significance when correcting for multiple comparisons, and so it should be interpreted with caution. Alternatively, some of our findings might be explained by neuroplasticity mechanisms induced by bilingual experience. The PH cingulum connects with inferior parietal areas that are particularly relevant in bilingualism(Abutalebi, Canini, et al., 2015; Mechelli et al., 2004c). In young samples, bilingualism increases WM integrity in fibers related to language and adjacent to the PH cingulum, such as the inferior fronto-occipital fasciculus or the direct segment of the arcuate fasciculus(Ghazal Mohades et al., 2015; Hämäläinen et al., 2017; Pliatsikas et al., 2015). Additionally, previous studies showed higher FA in the UF in healthy younger bilinguals(Pliatsikas et al., 2015) and greater frontal lobe WM in older bilinguals(Olsen et al., 2015), compared to monolinguals. Crucially, there is also evidence that experiences during adulthood might lead to changes in the WM microstructure(Fields, 2008), such as cognitive training increasing WM integrity(Lövdén et al., 2010). Thus, the higher WM integrity in the PH cingulum and UF might be associated with the long-term active use of two languages in bilinguals, and might contribute to the lack of cognitive differences between groups. Future studies should determine if the observed differences in PH cingulum and UF precede MCI, are a consequence of fornix pathology or are a combination of both effects.

Finally, our results may also be relevant in understanding evidence found in previous studies investigating bilingualism. Firstly, a previous study also found lower WM integrity in the fornix in healthy older bilinguals compared to monolinguals(Gold et al., 2013). These results were contrary to the evidence found in previous studies with elderly samples(Luk et al., 2011), and so they were interpreted by the authors as a possible higher incidence of preclinical AD in their sample of elderly bilingual participants. Our study confirms that MCI bilingual patients show lower WM integrity in the fornix than MCI monolinguals, thus validating this interpretation. Secondly, a correlation was found between performance on the BNT and WM integrity in the left PH cingulum in the monolingual group. This result might be of interest given the existing literature showing a relationship between performance on the BNT in healthy elderly individuals and GM volume in left temporal areas(Obler et al., 2010; Zhang et al., 2013) and FA in temporal fibers(Obler et al., 2010). Previous studies also found better

performance in monolinguals compared to bilinguals on the BNT(P. M. Roberts et al., 2002; Sheppard et al., 2016). Therefore, our results might suggest that passive bilingual brains make use of the PH cingulum in the neural mechanisms involved in naming processes.

In conclusion, our results show that, in a sample of patients with MCI with similar sociodemographic characteristics and cognitive levels, bilinguals exhibit lower WM integrity than passive bilinguals in the fornix, and higher WM integrity in the PH cingulum and UF, suggesting that bilingualism results in a differential pattern of MCI-related WM disintegration. Together, our results add knowledge about the neural mechanisms through which bilingualism may protect against dementia.

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Conflicts of interest

The authors declare no financial or other conflicts of interest.

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Appendix S1. Supplementary methods

1. Criteria for diagnosis and inclusion

MCI diagnoses were performed by experienced neurologists and based on clinical criteria. These criteria were: (1) memory complaints, self-reported or confirmed by another informant; (2) objective memory impairment assessed with the logical memory subtest II from the Wechsler memory scale-III (WMS-III) (Wechsler, 1997); (3) essentially intact activities of daily living; (4) no evidence of dementia; and (5) a score of 0.5 on the Clinical Dementia Rating (CDR) (Morris, 1997). Criteria that implied exclusion from the study were the following: (1) suffering from other nervous system diseases such as a brain tumor, cerebrovascular disease, encephalitis, or epilepsy, or meeting the criteria for dementia; (2) a score higher than 6 on the Geriatric Depression Scale (Martínez et al., 2002; Yesavage et al., 1982); (3) visible abnormalities in magnetic resonance images, such as leukoaraiosis and infarction, reported by an experienced radiologist; and (4) suffering from a current psychiatric disorder or using psychoactive medication.

2. Neuropsychological assessment

The neuropsychological assessment included a short form of the Boston Naming test (BNT) (Serrano et al., 2001a), a Word List Acquisition and Recall test (to evaluate immediate and delayed recall), two Fluency tests (semantic and phonetic), a remote memory test, and the clock-drawing test (Cacho et al., 1996b). Participants also completed the Mini Mental State Examination (MMSE) (Folstein et al., 1975; Lobo et al., 2002) and the Functional Activities Questionnaire (FAQ) (Pfeffer et al., 1982).

3. Image acquisition

MRI data acquisition was performed on a 3 T MRI scanner (Siemens Magnetom Trio, Erlangen, Germany) using a 12-channel head coil. Participants were placed inside the scanner in a supine position, and their heads were immobilized with cushions. Axial diffusion tensor images (DTI) were acquired with an echo-planar imaging sequence (EPI) with 20 gradient directions. The scan parameters used were

the following: TR = 10300 ms, TE = 104 ms, b0/b = 0/1000s/mm^2, FOV = 256 mm, matrix = 128x128, flip angle = 90º, number of slices = 70, slice thickness = 2 mm, gap = 0mm.

4. Image processing and statistical analysis

All DTI data were processed using the FMRIB Software Library (FSL) (Smith et al., 2004). First, diffusion weighted images were corrected for eddy current distortions using *eddycorrect*. Brain extraction and deletion of non-brain tissue were performed using *bet* (Brain Extraction Tool) (Smith, 2002). Then, *dtifit* was applied to extract FA and MD, also including AxD and RD.

Voxelwise statistical analysis of FA, MD, AxD, and RD data was carried out using Tract-Based Spatial Statistics (TBSS) (Smith et al., 2006). This included non-linear registration of all FA individual images to a common space, using the tool FNIRT (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FNIRT). Next, a mean FA skeleton was created and thinned in order to represent the center of all tracts common to the subjects. Finally, each subject's aligned FA data were projected onto this skeleton. The same process was used for MD, AxD, and RD data. The resulting data were fed into voxelwise cross-subject statistics. The tracts of interest used in cross-subject statistics were extracted from the JHU ICBM-DTI-81 white-matter label atlas (Mori & Crain, 2005).

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Study 3

Bilingualism's Effects on Resting-State Functional Connectivity in Mild Cognitive Impairment

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Abstract

Background: Bilingualism is considered a cognitive reserve (CR) factor, due to the delay in the onset of dementia in bilinguals compared to monolinguals. Two neural mechanisms have been suggested to underlie CR: neural reserve and neural compensation. However, it is still unclear how bilingualism contributes to these mechanisms.

Methods: In this study, we used cognitive tests, functional connectivity (FC), regional homogeneity, and fractional amplitude of low-frequency fluctuation (fALFF) measures to study resting-state brain patterns in a sample of bilingual and monolingual subjects with mild cognitive impairment (MCI).

Results: We found no significant differences between the groups in age, sex, education, or cognitive level, but bilinguals showed higher FC than monolinguals between the posterior part of the superior temporal gyrus and the precuneus, positively correlated with Mini-Mental State Examination (MMSE) scores, and higher fALFF in the thalamus bilaterally.

Conclusions: Our results suggest that bilingualism may act as a CR factor that protects against dementia through neural compensation.

Impact statement

Recent investigations suggest that neural compensation is one of the cognitive reserve mechanisms underlying the protection of bilingualism against dementia. Although brain changes in functional connectivity have been proposed as evidence of this mechanism, no study has directly used functional connectivity to study neural compensation in bilingualism. Our findings show that MCI bilinguals manifest higher resting state functional connectivity than monolinguals between the language network and the precuneus, supporting the involvement of neural compensation in the protection of bilingualism against dementia. Moreover, we found bilingualism effects in the spontaneous activity of the thalamus, a region related to atrophy in dementia.

Keywords: bilingualism; mild cognitive impairment; resting-state; functional connectivity; cognitive reserve; fMRI

INTRODUCTION

Cognitive reserve (CR) refers to the mechanisms underlying the discrepancy between a person's level of brain pathology and his/her cognitive performance, which would be expected to match this pathology. Bilingualism has been proposed as one of the experience-based factors that contribute to CR, based on previous evidence showing that bilinguals exhibit the clinical manifestations of dementia four to five years later than monolinguals (Woumans et al., 2015).

In order to investigate the neural mechanisms of this supposed protective effect of bilingualism against dementia, some neuroimaging research has focused on patients with this condition. Thus, a first study found that bilinguals with the same cognitive level as monolinguals had more brain atrophy indicative of pathology in specific temporal areas that are normally used to distinguish dementia patients from controls (Schweizer et al., 2012). Similar results have been found in subjects suffering from mild cognitive impairment (MCI) (Costumero et al., 2020; Marin-Marin et al., 2019). In a recent investigation, bilinguals showed reduced parenchymal volume and gray matter (GM) volume in areas related to atrophy in dementia (Costumero et al., 2020). Crucially, this study also found longitudinal differences: during a seven-month follow-up, monolinguals lost more parenchyma and had more cognitive deterioration than bilinguals. Regarding white matter (WM) disintegration, bilinguals and monolinguals with MCI showed different patterns of atrophy in a diffusion tensor imaging study. Bilinguals showed higher WM integrity in the parahippocampal cingulum and uncinate fasciculus, but lower integrity in the fornix, all of which are fibers associated with language and memory (Marin-Marin et al., 2019).

When investigating the neural basis of CR, two brain mechanisms have been described: neural reserve and neural compensation. On the one hand, neural reserve refers to the efficiency and resilience of pre-existing cognitive networks that may be capable of maintaining cognitive function despite brain pathology (Barulli & Stern, 2013; Stern, 2012). Several investigations suggest that this mechanism is related to bilingualism's contribution to CR because healthy older bilinguals show increased GM volume in the anterior cingulate cortex (Abutalebi, Guidi, et al., 2015) and higher WM integrity in the corpus callosum and superior longitudinal fasciculi (Anderson, Grundy, et al., 2018b; Luk et al., 2011), compared to monolinguals. On the other hand, neural compensation occurs when brain networks that are not normally used for a certain cognitive function acquire relevance and compensate for brain alterations in other regions (Barulli & Stern, 2013). Evidence supporting this mechanism in bilinguals comes from studies investigating brain connectivity using neuroimaging techniques (Perani & Abutalebi, 2015a). A previous fluorodeoxyglucose-positron emission tomography (FDG-PET) study in patients with dementia found increased and positive metabolic connectivity in bilinguals compared to monolinguals between the default mode network (DMN) and the executive control network (ECN) and brain areas related to language control, such as the cingulate cortex and the inferior frontal gyrus (Perani et al., 2017). In fMRI investigations carried out with samples of healthy older adults, bilinguals showed

more frontal-parietal and frontal-occipital functional connectivity (FC) (Luk et al., 2011) and stronger intrinsic connectivity than monolinguals in the frontoparietal control network and DMN, as well as stronger correlations between intrinsic connectivity of this control network and task-related increases in activity in prefrontal and parietal regions (Grady et al., 2015).

Neuroplastic effects in circuits linked to the executive and attentional demands of language processing have been proposed as the neural mechanism through which bilingualism compensates for brain damage in dementia (Perani et al., 2017; Perani & Abutalebi, 2015a). However, the mechanism of neural compensation due to bilingualism has not been explored in preclinical stages of the disease, such as MCI. Moreover, the previous evidence regarding this mechanism is inconclusive because of the use of healthy samples (Grady et al., 2015; Luk et al., 2011) or neuroimaging techniques that do not directly investigate the temporal synchronization between brain regions (Perani et al., 2017). Therefore, the aim of our research was to study the role of bilingualism in CR by comparing FC at rest in bilingual and monolingual subjects suffering from MCI. Based on the previous study on this topic (Perani et al., 2017), bilinguals were expected to show higher FC in regions included in the language network, ECN, and DMN, when compared with monolinguals. To comprehensively investigate brain patterns in MCI bilinguals and monolinguals during resting-state, we also explored possible bilingualism effects on the synchronization and amplitude of regional spontaneous activity.

METHODS

Participants

Eighty-one MCI individuals were recruited for this study (40 women; mean age = 73.83 ± 5.69). All of the participants were selected from dementia units of the Valencian Community public healthcare system. The diagnostic and inclusion criteria were the following: (1) subjective memory complaints (self-reported or confirmed by an informant), (2) objective memory impairment assessed with the logical memory subtest II of the Wechsler Memory Scale-III (WMS-III) (Wechsler, 1997), (3) essentially intact activities in daily living, (4) no evidence of dementia, and (5) a Clinical Dementia Rating score of 0.5. Participants were excluded if they had: (1) other nervous system diseases, such as a brain tumor, cerebrovascular disease, encephalitis, or epilepsy, or met the criteria for dementia; (2) a Geriatric Depression Scale (Martínez de la Iglesia et al., 2002; Yesavage et al., 1982) score > 6; (3) visible cerebral abnormalities, such as leukoaraiosis and infarction, reported by a radiologist with experience in magnetic resonance images; and (4) a current psychiatric disorder or use of psychoactive medication.

All the participants were born in Spain and resided permanently in the Spanish region of Valencia. During a clinical interview, they were asked about their use of languages. Participants who reported only speaking Spanish were considered monolinguals (n=50), whereas those who reported Catalan as their native language, Spanish as a second language, and active daily use of both were considered bilinguals

(n=31). The two groups shared similar everyday life settings and circumstances, such as neighborhood of residence and school/workplace environment. It should be noted that, for the sake of simplicity, we use the term monolingual to refer to the participants who only speak Spanish. Nevertheless, they could also be referred to as *passive bilinguals* because monolinguals who permanently reside in Valencia and do not speak Catalan are generally able to understand it.

All the participants were informed of the nature of the research, and they provided written informed consent prior to their participation in the study. All the study procedures were approved by the Clinical Research Ethics Committee of the Bellvitge University Hospital (Clinical Trial Registration number: PR020/15) and conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Neuropsychological assessment

All the participants underwent a structured clinical interview and a neuropsychological assessment, including the following tests: Mini-Mental State Examination (MMSE) (Folstein et al., 1975), Functional Activities Questionnaire (FAQ) (Pfeffer et al., 1982), Boston Naming Test (Serrano et al., 2001b), a Word List Acquisition and Recall test, two fluency tests (phonetic and semantic), a remote memory test, and the Clock-drawing Test (Cacho et al., 1996a). Descriptive statistics of the sociodemographic variables and results of a two-sample t test for each of the neuropsychological tests are reported in Table 1. There were no significant differences between the groups on any neuropsychological or sociodemographic variables.

Functional MRI acquisition

Images were acquired on a 3T MRI scanner (Siemens Magnetom Trio, Erlangen, Germany). Participants were placed in a supine position inside the scanner, and their heads were immobilized with cushions to reduce motion. During the acquisition, they were instructed to simply rest with their eyes closed, trying to let their minds go blank and not to fall asleep. A total of 270 volumes were collected over 9 min using a gradient-echo T2*-weighted echo-planar imaging sequence (TR=2000 ms; TE=30 ms; matrix, 64 x 64; FOV, 224 x 224 cm; flip angle, 90°; 33 slices, parallel to the hippocampus; slice thickness, 3.5 mm; slice gap, 0.5 mm).

Table 1. Sociodemographic and neuropsychological variables for bilinguals and monolinguals with MCI.

	Bilinguals (N = 31)		Monolinguals (N = 50)		Statistical differences	P
Sex	M/F = 19/12		M/F = 22/28		$\chi2 = 2.29$	0.13
	M	SD	M	SD	t	p
Age	73.61	5.85	73.74	5.47	0.99	0.92
Years of schooling	8.45	2.41	8.82	3.64	0.50	0.62
MMSE	27.32	2.09	27.14	2.56	-0.33	0.74
FAQ	4.16	3.29	3.78	3.34	-0.50	0.62
Boston Naming Test	9.74	1.24	9.32	1.50	-1.31	0.19
Phonetic fluency	8.74	2.32	8.22	2.19	-1.02	0.31
Semantic fluency	10.84	2.22	10.54	2.37	-0.57	0.57
Delayed recall	1.19	0.98	1.02	0.71	-0.92	0.36
Immediate recall	3.22	0.87	2.99	0.88	-1.14	0.26
Remote memory	9.58	1.03	9.16	1.49	-1.38	0.17
Clock-drawing test	7.16	1.44	7.06	1.87	-0.25	0.80

MMSE, Mini-Mental State Examination; FAQ, Functional Activities Questionnaire; N, sample size; M/F, males/females; $\chi2$, chi-squared test; M, Mean; SD, Standard Deviation; t, t value for two-sample t test.

Image preprocessing and statistical analyses

We used Data Processing and Analysis for Brain Imaging (DPABI V4.2_190919, http://rfmri.org/dpabi) to carry out resting-state fMRI data processing. Preprocessing included: (1) removal of the first ten volumes of each raw fMRI dataset; (2) slice timing correction; (3) realignment using a six-parameter (rigid body) linear transformation; (4) spatial normalization to the Montreal Neurological Institute (MNI) space (voxel size 3 × 3 × 3 mm); (5) removal of spurious variance through linear regression: 24 parameters from the head motion correction, linear, and quadratic trends, and the first five principal components associated with WM and cerebrospinal fluid (Behzadi et al., 2007); (6) spatial smoothing with a 4-mm FWHM Gaussian Kernel; and (7) band-pass temporal filtering (0.01–0.1 Hz).

Participants with more than 1 mm/degree of movement in any of the six directions or fewer than 120 volumes with framewise displacement (FD) < 0.5 mm (Jenkinson et al., 2002) (ensuring at least 4 minutes of rest with low FD) were excluded from the analyses.

Resting-state FC analysis

A seed-based correlation analysis was performed using *a priori* regions of interest (ROIs). ROIs were defined from the CONN network cortical ROI atlas (HCP-ICA) included in the CONN toolbox (https://web.conn-toolbox.org/). Following the previous study on this topic (Perani et al., 2017), we focused our analysis on the following networks: DMN, ECN, and the language network. To avoid the introduction of different amounts of noise derived from the signal average of regions with different sizes, we used the representative local maxima provided on the atlas to create spherical masks (5mm radius) as our seeds. Specifically, the medial prefrontal cortex (MNI: 1, 55, -3), left (MNI: -39, -77, 33) and right parietal gyri (MNI: 47, -67, 29), and posterior cingulate cortex (MNI: 1, -61, 38) were considered seeds for the DMN. Left (MNI: -43, 33, 28) and right (MNI: 41, 38, 30) prefrontal cortices and left (MNI: -46, -58, 49) and right (MNI: 52, -52, 45) posterior parietal cortices were considered ROIs for the ECN. Finally, for the language network, left (MNI: -51, 26, 2) and right (MNI: 54, 28, 1) inferior frontal gyri and the left (MNI: -57, -47, 15) and right (MNI: 59, -42, 13) posterior parts of the superior temporal gyri (pSTG) were used. After the estimation of individual correlation maps, group-level voxelwise analyses restricted to an inclusive mask comprising the brain networks under study (i.e. the DMN, ECN and language network) were performed.

Regional Homogeneity (ReHo) analysis

We used the ReHo method to explore group differences in regional synchronization of fMRI time courses between neighboring voxels. For this analysis, Steps 6 and 7 of preprocessing were modified. After spatial normalization, band-pass temporal filtering (0.01–0.1 Hz) was applied, and Kendall's coefficient of concordance was calculated between each voxel's BOLD time series and those of its 19 neighbors. The ReHo value of each voxel was divided by the global mean ReHo of the whole-brain mask, and the resulting ReHo maps were smoothed with a 4-mm full width at half maximum (FWHM) Gaussian kernel (Chao-Gan & Yu-Feng, 2010). Then, group-level whole-brain voxelwise analyses were performed.

Fractional amplitude of low-frequency fluctuations (fALFF) analysis

Differences in the amplitude of regional spontaneous activity between groups were explored using the fALFF method. For this analysis, Step 7 of preprocessing was modified. After performing spatial smoothing, the time series of each voxel was transformed into the frequency domain and band-pass filtered (0.01 – 0.08 Hz). Then, the square root was calculated at each frequency of the power spectrum, the averaged square root (i.e., ALFF) was obtained at each voxel, and a ratio of total amplitude within the low frequency range to the total amplitude of the detectable frequency range was calculated (i.e., fALFF). Lastly, group-level whole-brain voxelwise analyses were performed.

Statistical analyses

Group differences in FC, ReHo, and fALFF values were investigated using a two-sample t-test, with FD values as a covariate, as implemented in SPM12 (Statistical Parametric Mapping 12; Wellcome Trust Centre for Neuroimaging, University College, London, UK; http://www.fil.ion.ucl.ac.uk/spm/). The statistical significance was determined using cluster-based inference at a threshold of $p < 0.05$, family-wise error (FWE) corrected, with a primary voxel-level threshold of $p < 0.001$ uncorrected.

Correlation with cognitive status

We explored the relationship between the differences in resting-state measures found in our sample and their cognitive status. To do so, for each brain region showing significant differences in group analyses of any of the measures, a 5 mm^3 sphere mask centered in its local maxima was defined (see Table 2 for MNI coordinates). Then, the specific values of the voxels within the mask were averaged in each subject separately, and these averaged values were used to perform a correlation analysis with the MMSE scores, using $p < 0.05$ as a statistically significant threshold, for the sample as a whole and for each group separately.

Table 2. List of regions with higher FC, and subcortical nuclei with higher fALFF in bilinguals compared to monolinguals, based on the AAL atlas(Tzourio-Mazoyer et al., 2002) and a recently proposed thalamus segmentation(Najdenovska et al., 2018).

Seed/Type of analysis	**Regions**	**K**	**MNI coordinates (x, y, z)**	**t**
Left pSTG (LN)	Left precuneus (BA 7)	59	-3, -57, 45	4.62
	Right precuneus (BA 7)		3, -63, 51	4.25
fALFF	Left thalamus MDN	41	-9, -15, 0	4.52
	Left thalamus CMPN		-12, -24, 0	4.36
	Right thalamus AN	37	15, -9, 3	3.95
	Right thalamus VLVN		18, -15, 3	3.75

pSTG, posterior superior temporal gyrus; LN, language network; fALFF, fractional amplitude of low-frequency fluctuations; K, cluster size in voxels; BA, Brodmann's area; MNI, Montreal Neurological Institute; t, t value for two-sample t test; MDN, mediodorsal nucleus; CMPN, central-medial pulvinar nucleus; AN, anterior nucleus; VLVN, ventral-lateroventral nucleus.

RESULTS

Seed-based connectivity analysis

We found significant differences between bilinguals and monolinguals in the FC of the left pSTG seed of the language network. Specifically, the connectivity of this region with the precuneus was higher in bilinguals than in monolinguals (Fig 1; Table 2). The opposite contrast did not show any significant results (monolinguals > bilinguals). No other significant differences were found in any other seed.

ReHo analysis

We found no significant differences in regional synchronization in bilinguals compared to monolinguals.

fALFF analysis

We found a higher amplitude of regional spontaneous activity in bilinguals compared to monolinguals in the thalamus bilaterally (Fig 2; Table 2). Specifically, the differences appeared in the left mediodorsal and central-medial pulvinar and right anterior and lateroventral nuclei of the thalamus (Najdenovska et al., 2018). The opposite contrast did not show any significant results.

Correlation with cognitive status

We found a significant correlation between our sample's performance on MMSE and FC between the left pSTG seed of the language network and the precuneus ($R = 0.272$, $p = 0.014$). We found no significant correlations for the groups separately or for the differences in fALFF.

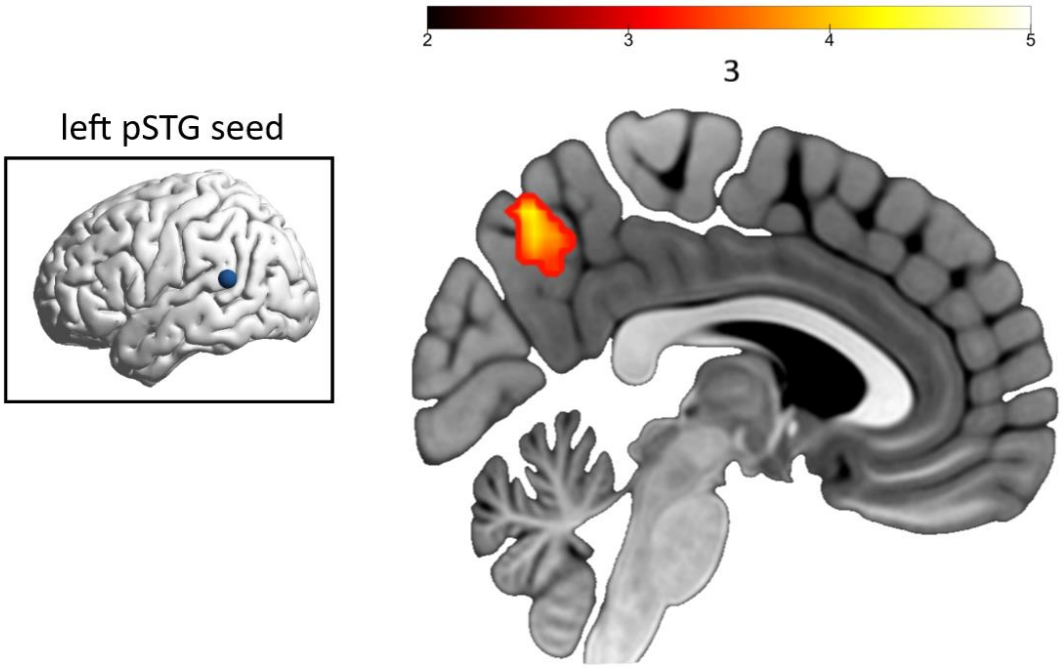

Figure 1. Differences in FC of the left pSTG between bilinguals and monolinguals. In blue, the location of the left pSTG seed. The color bar represents the t-value of areas with significantly higher FC with this seed in bilinguals compared to monolinguals.

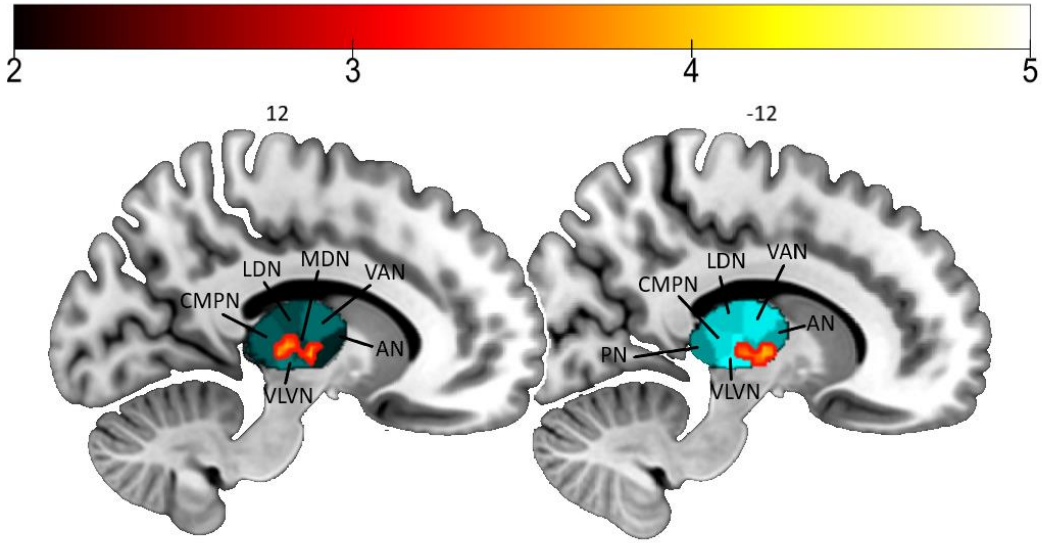

Figure 2. Differences between bilinguals and monolinguals in fALFF. The color bar represents the t-value of areas with significantly higher fALFF in bilinguals compared to monolinguals. Thalamus nuclei are shortened as follows: PN, pulvinar; CMPN, central-medial pulvinar; LDN, laterodorsal; VAN, ventral anterior; VLVN, lateroventral; AN, anterior; MDN, mediodorsal.

DISCUSSION

In this study, we investigated the FC, ReHo, and fALFF differences between bilingual and monolingual MCI subjects who had no significant differences in age, years of schooling, proportion of men and women, or performance on neuropsychological testing. Bilinguals showed higher FC than monolinguals between the left pSTG of the language network and the precuneus, and higher fALFF in several nuclei of the thalamus. Moreover, FC values between the pSTG and precuneus correlated with the MMSE scores in the whole sample. These results suggest that the experience of bilingualism promotes CR through neural compensation.

Our results are consistent with previous evidence suggesting that bilingualism may contribute to neural compensation in dementia (Luk et al., 2011; Perani et al., 2017). A previous study showed enhanced WM integrity and more functional connections involving frontal, parietal, and occipital lobes in healthy older bilinguals compared to monolinguals (Luk et al., 2011). These results were subsequently interpreted as a possible compensatory mechanism that would provide reserve and compensate for GM deterioration (Guzmán-Vélez & Tranel, 2015). In a later study analyzing metabolic connectivity in a sample of patients with dementia, bilinguals showed increased connectivity compared to monolinguals between the precuneus/posterior cingulum and the anterior cingulum, orbitofrontal cortex, thalamus, and caudate nucleus, all described as crucial brain regions for language control in bilinguals (Perani et al., 2017). Since bilinguals also showed more hypometabolism than monolinguals, the increased connectivity was also interpreted as a compensatory mechanism by which the bilingual brain would be able to cope better with neurodegeneration (Perani et al., 2017).

Along the same lines, our study showed that, in a sample of subjects with MCI, bilinguals exhibited higher levels of FC than monolinguals between the left pSTG, an area within the language network, and the precuneus, a region typically affected in dementia. Therefore, we interpret our findings as neural compensation and not neural reserve, validating previous evidence using indirect measures of interregional connectivity (Perani et al., 2017). Moreover, we also found a positive correlation between MMSE performance and FC between the precuneus and pSTG for the whole sample, further supporting the relationship between cognitive status and FC between these brain regions. Perani et al. also found increased anterior-posterior metabolic connectivity in the ECN in bilinguals compared to monolinguals (Perani et al., 2017). However, we found no significant FC differences between bilinguals and monolinguals in the ECN seeds. This may be due to the different characteristics of our samples: in their investigation, participants were dementia patients, whereas our work involved MCI subjects. In the first stages of Alzheimer's disease, which is the most common form of dementia in elder populations, brain pathology, such as β-amyloid accumulation (Palmqvist et al., 2017), tau deposition (Hall et al., 2017), and hypometabolism (Sperling et al., 2011), is mainly limited to DMN areas, and especially to the precuneus/posterior cingulum. Therefore, the fact that the differences in our sample are restricted to the

FC of this region may be due to the early stage of the disease in our sample. Along the same lines as previous investigations (Grady et al., 2015; Luk et al., 2011; Perani et al., 2017), our results suggest that bilingualism may be acting as a CR factor through neural compensation mechanisms.

We also found higher fALFF in bilinguals compared to monolinguals in multiple nuclei of the thalamus. Previous studies show that fALFF values tend to decrease in prodromal AD and MCI (Cha et al., 2015; Zeng et al., 2019). Regarding the thalamus and its role in dementia, a review based on post-mortem studies, animal models, and non-invasive imaging investigations suggests that the loss of episodic memory in early stages of dementia is not mainly related to hippocampus dysfunction, but rather to broader neurodegeneration of the Papez circuit, an extended memory system that involves the limbic thalamus (Aggleton et al., 2016). Thus, previous studies found reduced thalamic volumes in amnestic MCI subjects (Pedro et al., 2012; Sorg et al., 2007; Yi et al., 2016) and correlations between thalamic volume and cognitive status in MCI (Pedro et al., 2012; Yi et al., 2016). Moreover, bilateral atrophy in the dorsomedial thalamus, reductions in WM integrity in the anterodorsal nucleus, and smaller internal medullary lamina were found in dementia patients compared to controls (Zarei et al., 2010). In our study, the differences found in fALFF in the thalamus were restricted to the left mediodorsal and central-medial pulvinar and right anterior and lateroventral nuclei, based on the human thalamic segmentation proposed in a recent investigation (Najdenovska et al., 2018). Although the relationship between memory impairment and thalamic lesions has been described for years (Carlesimo et al., 2011; Danet et al., 2017; Fedio & Van Buren, 1975; Harding et al., 2000), there is a lack of agreement about the specific nuclei responsible for this relationship: early investigations found verbal memory deficits due to left pulvinar nucleus stimulation (Fedio & Van Buren, 1975), neuronal loss in the anterior nuclei due to Korsakoff's syndrome was associated with amnesia (Harding et al., 2000), and mediodorsal nucleus lesions due to left thalamic stroke were related to impaired recollection (Danet et al., 2017). Based on these studies, we tested if the differences in fALFF in the thalamus found in our sample were correlated with the scores of any of the memory tests used, but we found no significant results. A possible explanation for this lack of relationship between memory status and the amplitude of regional spontaneous activity in the thalamus could be the low variability in the memory scores of the participants in our sample. Moreover, previous studies state that functional alterations in fMRI can be detected prior to the manifestation of cognitive decline and clinical deterioration (Sheline & Raichle, 2013; Sperling et al., 2011). Thus, another possibility is that the differences found in our sample in fALFF in the thalamus might not have manifested behaviorally yet. Also importantly, other investigations suggest that the thalamus is related to language processing and bilingualism, based on evidence showing that corticothalamo-cortical connections have a pivotal impact on language processing through feedback mechanisms (Crosson, 2013) and that the thalamus is expanded in young simultaneous bilinguals compared to monolinguals (Burgaleta et al., 2016). Thus, our results suggest that bilingualism may act as a CR factor by means of

a higher amplitude of regional spontaneous activity in the thalamus, specifically in nuclei that show atrophy in dementia (Zarei et al., 2010) and are related to memory impairment (Danet et al., 2017; Fedio & Van Buren, 1975; Harding et al., 2000).

As a general conclusion, our results show that, in a sample of MCI subjects with the same disease severity, proportion of men and women, years of schooling, and sociocultural characteristics, bilinguals manifest higher FC than monolinguals between the pSTG of the language network and the precuneus, and higher fALFF in the thalamus. These results expand our knowledge about the effects of the active use of two languages on brain function, and they support the role of bilingualism as a CR factor that protects against dementia through neural compensation.

Author contribution statement

V. C. and L. M-M. conceptualized the study and were responsible for implementation of data analyses. V. C., L. M-M., M-Á. P-G., A. M-P., N. A., J A-V. and E. V-R. were involved in interpreting findings, drafting and revising the manuscript.

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Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Study 4

Dynamic effects of immersive bilingualism on cortical and subcortical grey matter volumes

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Abstract

Bilingualism has been shown to induce neuroplasticity in the brain, but conflicting evidence regarding its specific effects in gray matter continues to emerge, probably due to methodological differences between studies, as well as approaches that may miss the variability and dynamicity of bilingual experience. In our study, we devised a continuous score of bilingual experiences and we investigated their non-linear effects on regional GM volume in a sample of young healthy participants from an immersive and naturalistic bilingual environment. We focused our analyses on cortical and subcortical regions that had been previously proposed as part of the bilingual speech pipeline and language control network. Our results showed a non-linear relationship between bilingualism score and grey matter volume of the inferior frontal gyrus. We also found linear increases in volumes of putamen and cerebellum as a function of bilingualism score. These results go in line with predictions for immersive and naturalistic bilingual environments with increased intensity and diversity of language use and provide further evidence supporting the dynamicity of bilingualism's effects on brain structure.

Introduction

Bilingualism - the experience of being exposed to two languages and manage them in everyday life - has been shown to induce neuroplasticity in the brain (Grundy et al., 2017). During language production, bilinguals need to select one language and suppress the other, while adequately articulating the target language, which results in increased demands for linguistic control and, consequently, in changes in brain structure and function to accommodate these heightened demands (Tao et al., 2021). Different models have attempted to describe the location and characteristics of these changes, and the particular features of the bilingual experience that contribute to them. For instance, the Adaptive Control Hypothesis (ACH) proposed that any effects of bilingualism on brain structure are dependent on the interactional context in which the individual uses their languages and the specific control processes that different contexts entail: *single language contexts* in which languages are used separately in different environments; *dual-language contexts* in which both languages are used but separately with different speakers; and *dense code-switching contexts* where speakers use both languages interleaving them in their discourse (Green & Abutalebi, 2013). Based on previous evidence, they propose a brain network for language control and speech, composed by inferior frontal, parietal, anterior cingulate, motor and premotor cortices, thalamus, caudate nucleus, putamen, cerebellum and insula (Abutalebi & Green, 2016). These regions are hypothesized to be differentially affected by bilingual experience depending on the interactional context: while dense code-switching contexts would engage more the cerebellum and left inferior frontal cortex, dual or multiple language interactional contexts would engage bilateral inferior frontal, anterior cingulate and parietal cortices, caudate nucleus, putamen and the thalamus.

Other authors have proposed that a brain adaptation pattern arises with increased *length* of immersion in bilingual environments, characterized by an initial tissue volume increase in frontostriatal regions, followed by reductions in volume and lower functional recruitment of frontal executive regions, as well as greater recruitment and further expansions of posterior and subcortical areas, a phenomenon they call the "bilingual anterior-to-posterior and subcortical shift" (BAPSS; Grundy et al., 2017). However, mixed evidence regarding the specific brain changes produced by bilingual *experience* continues to emerge. Namely, when investigating grey matter (GM) differences between bilinguals and monolinguals, the former generally show higher volume, density and cortical thickness, as well as shape expansions, in cortical, subcortical and cerebellar areas, but some studies have also found results in the opposite direction – lower volumes in bilinguals – or no differences at all between the groups (see Tao et al., 2021 for a systematic review).

Apparent inconsistencies between studies when investigating bilingualism and GM structure may stem from multiple sources. Methodological issues – e.g., the use of different measures – and sample differences have been suggested as the main origins of variation (García-Pentón et al., 2015). In fact, many investigations carried out to date used samples of bilinguals with very distinct characteristics. While some studies only considered simultaneous bilinguals – that is, bilinguals who first learned both languages at the same time (Burgaleta et al., 2016), others only included bilinguals who were not simultaneously exposed to both languages but acquired the second language (L2) early in life (Olulade et al., 2016), or late sequential bilinguals whose age of acquisition (AoA) of L2 was greater than seven (Deluca, Rothman, Bialystok, et al., 2019; Pliatsikas et al., 2017). Moreover, the age cutoffs for different groups of bilinguals – simultaneous, early or late – are arbitrary and sometimes differ between studies (Klein et al., 2014; Mechelli et al., 2004b; Ressel et al., 2012), which adds to the confusion. Levels of immersion in L2 also remarkably vary between studies, with some investigations comparing monolinguals to proficient bilinguals that frequently use L2 (Deluca, Rothman, Bialystok, et al., 2019), while others investigate non-immersed bilinguals (Korenar et al., 2021). The Unifying the Bilingual Experience Trajectories (UBET) framework (DeLuca et al., 2020), which brings together previous models on the trajectory of neurocognitive adaptations due to bilingualism, emphasizes that different characteristics of bilingual experiences – intensity and diversity, language switching, relative proficiency, and duration – lead to adaptations in efficiency and control demands that have different consequences on cognition and brain structure. In particular, they hypothesize that increased duration and a balanced proficiency between the languages will increase *efficiency*, associated with increases in GM volume of subcortical and posterior regions, and return to baseline volumes in cortical areas that had expanded in previous initial stages of the bilingual experience. They also propose that increases in diversity, intensity and language switching will increase *control demands*, resulting in GM volume

increases in areas involved in control processes such as the inferior frontal gyrus (IFG), anterior cingulate cortex (ACC) or inferior parietal lobule, as an adaptation to these demands. Moreover, they draw attention to the consequences of the socio-linguistic environment on the interaction between bilingualism characteristics and their consequences. For instance, in countries where only one language is official and widely used in society, the use of a second one will probably be restricted to specific community contexts, which might result in compartmentalized usage of languages, different language proficiency levels, or low levels of immersion (Claussenius-Kalman et al., 2020; Vaughn et al., 2019). This type of bilingual use would be expected to require increased executive control demands whenever the least practiced language is used, with more recruitment of frontal and cortical structures, in contrast to environments with a balanced use of languages and opportunities of intense immersion, which are expected to shorten the latency by which efficiency effects materialize (DeLuca et al., 2020).

Crucially, many studies that investigated effects of bilingualism on GM structure treated bilingualism as a categorical variable, an approach that has been recently challenged (Anderson, Mak, et al., 2018; Deluca, Rothman, Bialystok, et al., 2019; Luk & Bialystok, 2013; Pliatsikas et al., 2019). When dividing participants in two groups based on their experience with languages and treating each group as a homogeneous category, relevant bilingual variability within the groups is likely missed (Grundy et al., 2017), since few people have 'pure' and indistinguishable monolingual and bilingual experiences (Luk & Bialystok, 2013). Consequently, it has been argued that bilingualism would be better described as a continuum arising from bilingual experience-based factors, since these show *when* bilingualism starts to influence the system and *how* it interacts with it (Deluca, Rothman, Bialystok, et al., 2019). Following up on the criticism on the categorical approach, recent studies have started to investigate the effects of *quantified* bilingualism on GM structure, reporting effects such as significant correlations between length of L2 immersion and globus pallidus expansions (Pliatsikas et al., 2017), and reshaping of left thalamus and right caudate nucleus volumes and decreases in left middle temporal gyrus as a function of amount of exposure to L2 (Burgaleta et al., 2016). To investigate similar effects, recent studies have looked at how structural changes can be predicted by bilingualism composite "scores" provided by tools such as the Language and Social Background Questionnaire (LSBQ, Anderson et al., 2018), the Language Experience and Proficiency Questionnaire (LEAP-Q, Kaushanskaya et al., 2020), and the Language History Questionnaire (LHQ3, Li et al., 2020), all of which measure bilingualism experience-based factors such as language proficiency, AoA, or frequency of use in different contexts. For example, Deluca and colleagues (Deluca, Rothman, Bialystok, et al., 2019) used as predictors of brain change scores derived from the LSBQ, including L2 use in social/community settings, and in home settings, as well as L2 AoA and length of immersion. Results showed that L2 AoA positively correlated with GM expansions in the left nucleus accumbens and bilateral thalamus, length of L2 immersion predicted

reshaping in right caudate nucleus, expansions in right putamen and contractions in bilateral thalamus and nucleus accumbens, and social use also predicted significant expansions in left caudate nucleus, left nucleus accumbens and right thalamus. Other investigations have also found significant relationships between specific aspects of the bilingual experience and GM structure, such as negative correlations between both AoA and current exposure to L2 and GM volume in right IFG (Wei et al., 2015), reductions in left thalamus and right caudate nucleus, but expansions in left middle temporal gyrus, as a function of amount of time listening and speaking the dominant language (Burgaleta et al., 2016), and positive correlations between expansions in right globus pallidus and length of immersion in a country where L2 is dominant (Pliatsikas et al., 2017). Interestingly, another study found accent scores to be significantly correlated with GM volume in left putamen only in sequential bilinguals - the more native-like they sounded, the more left putaminal volume they showed (Berken et al., 2016). Similar patterns have been reported in studies looking at the relationship between WM integrity and AoA of L2 (Nichols & Joanisse, 2016; Rossi et al., 2017), length of L2 training/immersion (Mamiya et al., 2016), and L2 proficiency (Nichols & Joanisse, 2016; Singh et al., 2018). Taken together, this evidence suggests that the relationship between bilingual experience and brain changes may be better grasped by approaches that quantify the bilingual experience rather than more traditional categorical descriptions of bilingualism.

However, it still remains the case that even investigations that used correlational approaches might fail to describe the full patterns underlying bilingualism-induced neuroplasticity because of the use of linear approaches. These approaches assume continuous growth or reduction of brain structures as a function of bilingual experience, which is an unlikely pattern due to the mixed findings of multiple bilingualism studies (Tao et al., 2021); indeed, theories on experience-based neuroplasticity have assumed non-linear volumetric changes in the brain, with volumetric increases during skill acquisition followed by decreases that suggest efficient brain reorganisation (Lövdén et al., 2013). Therefore, non-linear approaches may be better suited to describe the changing tendencies of brain adaptations along the bilingual experience. The Dynamic Restructuring Model (DRM), a recent proposal that attempts to coherently merge all the apparently inconsistent evidence, describes bilingualism's effects on brain structure as dynamic and non-linear, that is, following patterns of expansion and renormalization (Pliatsikas, 2020). Specifically, the DRM proposes three main stages of bilingual experience, characterized by different brain adaptations: initial exposure, consolidation, and peak efficiency. At the initial exposure stage, the model proposes that cortical GM volumes increase especially in anterior regions related to executive control, and parietal and temporal areas related to specific aspects of language learning. Subcortical and cerebellar GM volumes are also proposed to expand in this stage, due to the increases in demands for language control and selection between motor programmes. These expansions revert and renormalize cortically in the consolidation stage, potentially due to the optimization of lexical learning and control through the

elimination of redundant local connections and conservation of only the most efficient. Still, cerebellar and subcortical regions continue increasing in volume, since bilinguals still need to exert language control and selection. The last stage, which is described by the author as the most difficult to characterize due to the scarcity of evidence, would be distinguished by further cerebellar increases, renormalization of the caudate nucleus and stabilization of the putamen and globus pallidus.

Notably, a recent study investigating young healthy bilinguals provides evidence in support of these non-linear patterns of GM changes (Korenar et al., 2021). Korenar and colleagues used generalized additive mixed models (GAMMs) to investigate non-linear effects of bilingual experience, as measured by a composite score that is calculated by the LSBQ (Anderson, Mak, et al., 2018), on regional subcortical volumes. They found linear volume increases in putamen and thalamus as a function of bilingualism, but non-linear patterns of expansion-renormalization in bilateral caudate nuclei and expansion-plateauing in the nucleus accumbens. These results were interpreted in terms of the DRM predictions (Pliatsikas, 2020): the continuous increase in volume for putamen and thalamus goes in line with the constant need for bilinguals to select motor programmes of the target language and exert cognitive control, whereas the observed pattern in caudate nucleus reflects its central role in lexical control and selection, crucial in initial stages of bilingual experience, but likely optimised as experience increases. Moreover, the pattern observed in nucleus accumbens is interpreted to reflect the initial reward in pursuing social interactions that might reach a plateau when bilinguals reach language efficiency. Nevertheless, this study focused only on subcortical structures, and investigated a very specific sample of bilinguals: highly proficient non-immersed speakers of an L2 and with limited opportunity for active naturalistic bilingual language use. Thus, it remains to be determined whether non-linear bilingualism's effects on brain structure extend to cortical regions and to populations with more sustained long-term immersive bilingual experiences.

In the present study, our main objective was to investigate non-linear effects of bilingual experiences on the GM structure in a healthy sample of bilinguals from the region of València. Both Spanish and Catalan are official languages widely used in society in that region, so bilinguals have the opportunity to use both of them in an active and naturalistic context. Our sample presented a wide variety of bilingual experiences, ranging from simultaneous immersed to late non-immersed bilinguals, in order to fully capture the variability of bilingual experiences and their dynamic effects. We developed a bilingualism score from a questionnaire that was appropriate to the particular linguistic environment of our participants, and this score was used as a predictor of grey matter volume in specific regions. Following up on recent work (Korenar et al., 2021; Pliatsikas et al., 2020), we used GAMMs to account for non-linear volumetric effects of bilingualism, by focusing on the regions of the speech pipeline and language control network proposed in the ACH (Green & Abutalebi, 2013). This method enabled us to model

complex patterns of GM volume changes as a function of bilingual experiences, which constitutes one of the main strengths and novel aspects of our investigation, as opposed to previous studies that used categorical and linear approaches. This also allows us to account for non-linear GM changes due to age, previously described to follow an inverted U shape of initial volume increases during childhood, followed by abrupt reductions in adolescence and more stable pruning during adulthood (Giedd et al., 1999). For example, such patterns have been documented in the parietal lobe, also extending to medial and superior frontal cortices, the cingulum, postcentral cortex and occipital lobe (Tamnes et al., 2010). These patterns have been reported to differ between bilinguals and monolinguals during childhood and adolescence, with bilinguals showing less age-related reductions of frontal and parietal regions (Pliatsikas et al., 2020). Following up on previous investigations (Burgaleta et al., 2016; Deluca, Rothman, Bialystok, et al., 2019; Korenar et al., 2021; Pliatsikas et al., 2017), we expected to find linear increases in GM volume of putamen, thalamus and cerebellum as a function of bilingualism score, as well as increases followed by reductions in the caudate nucleus. Due to the characteristics of the immersive bilingual environment of our sample, where a balanced used of the two languages is common, and in line with previous models' predictions (DeLuca et al., 2020; Grundy et al., 2017; Pliatsikas, 2020), we expected to expand on previous evidence (Korenar et al., 2021) by finding volume increases in cortical areas – IFG, ACC, and parietal cortex – as a function of bilingualism score, accommodating for the continuous control demands exerted by a context of high diversity and intensity of use, but also a shortened latency for the return to baseline volumes due to increasing efficiency.

Materials and methods

Participants

Data from 334 healthy participants was included in this study (147 females; 187 males; mean age = 23, SD = 6, range = 18-53). All participants were right-handed, had normal or corrected-to-normal vision, and reported no previous history of neurological, psychiatric or language disorders. All participants were born in Spain and living in the region of València at the time of testing. This is a territory where both Catalan and Spanish are taught during formal education and co-officially used in public administration. Since both languages are understood by most of the population (Generalitat Valenciana. Direcció General de Política Lingüística i Gestió del Multilingüïsme, 2015), a person can choose to use one or the other depending on the context, motivated by factors such as personal preferences, habits or perceived command on the languages of the interlocutor and oneself. As a consequence, participants in our sample spoke fluently only Spanish or Spanish and Catalan, and lived a complex variety of bilingual experiences, close to being "monolingual" and at different degrees of "bilingual". This means that some of them had simultaneously acquired Spanish and Catalan (46%), while others acquired the second language later in life (54%). Moreover, some of them had a balanced use of both languages to different

degrees (46%), which entailed different degrees of immersion in Catalan (years of immersion range = 0-52), while others were clearly exposed to one language over the other in their daily lives (64%).

Data from 60 of our participants had already been used in a previous study (Burgaleta et al., 2016) that serves as basis for our investigation. Therefore, this data was only used for the extraction of the bilingualism score based on our bilingualism questionnaire (See Data analysis – Bilingualism score) and subsequently excluded from further analyses, resulting in a final sample of 274 subjects (115 females; 159 males; mean age = 23, SD = 6, range = 18-53; 45.7% of simultaneous bilinguals, 42.6% immersed, 67.4% non-immersed; years of immersion range = 0-49).

Written informed consent before scanning was obtained from each subject and they received monetary compensation for their time and effort. The study was approved by the Ethics Committee of the Universitat Jaume I.

Bilingualism questionnaire

To assess the characteristics of the bilingual experiences of our participants, they were administered an in-house questionnaire. This questionnaire contained two sections. In the first one, demographic information was gathered, and participants were asked about their proficiency (from 1=perfect, to 4=very low), general frequency of use in percentages and AoA of Catalan and Spanish. In the second part, information regarding frequency of use (proportion of Spanish/Catalan use) in specific contexts (home, school and others) and periods of time (childhood, adolescence, adulthood) was gathered (see Supplementary Information for original questionnaire and a translation into English). This resulted in a comprehensive collection of information regarding lifelong bilingual experiences of the participants in our sample.

MRI data acquisition

Images were acquired on a 1.5-T Siemens Avanto scanner (Erlangen, Germany). Participants were placed inside the scanner in the supine position, and their heads were immobilized with cushions. Whole-brain 3-D images were collected for 6 minutes using a T1-weighted MPRAGE sequence, with the following parameters: TE = 3.8 ms; TR = 2200 ms; flip angle = 15°; matrix = 256 × 256 × 160 mm; voxel size = 1 mm3.

2.4. Data analysis

2.4.1. Image preprocessing

All analyses were performed using the standard preprocessing pipeline of CAT12 (Computational Anatomy Toolbox; C. Gaser, Jena University Hospital, Jena, Germany; http://dbm.neuro.uni-jena.de/cat/). After an initial bias correction of intensity non-uniformities, individual volumes of GM, WM, and cerebrospinal fluid were estimated applying the standard segmentation procedure of the toolbox, and images were registered to the template provided. Then, to study region-specific volumetric differences, region of interest (ROI) analysis implemented in CAT12 was performed. In this analysis, also called region-based morphometry (RBM), an anatomical atlas is transformed into native subject space, and the sum of the local GM inside the ROIs of the atlas is estimated. We restricted our analysis to the language control and speech production network proposed in the ACH (Green & Abutalebi, 2013), including IFG, ACC, parietal, motor and premotor cortices, thalamus, caudate, putamen, cerebellum and insula (see Table 1 for mean volumes of ROIs by hemisphere). Volumes of all ROIs were extracted using the LONI Probabilistic Brain Atlas (LPBA40; Shattuck et al., 2008) provided by the toolbox, except for left and right cerebellum, thalamus and ACC, extracted using the Computational Brain Anatomy (CoBrA) atlas (https://github.com/cobralab/atlases) and the automated anatomical labelling atlas 3 (AAL3; Rolls et al., 2020), because these subdivisions were not defined in the LPBA40. Finally, total intracranial volume (TIV) was estimated.

Table 1. Mean and standard deviation of GM volumes (cm^3) of our ROIs.

	Mean GM volume (Standard Deviation)	
	Left hemisphere	Right hemisphere
Inferior frontal gyrus	24.78 (2.96)	25.41 (3.05)
Parietal (supramarginal gyrus)	8.94 (1.10)	8.52 (1.09)
Anterior cingulate cortex	51.74 (11.78)	44.32 (10.34)
Precentral gyrus	12.53 (1.46)	12.40 (1.36)
Middle frontal gyrus	24.78 (2.96)	25.41 (3.05)
Thalamus	4.58 (0.47)	4.94 (0.50)
Caudate	3.68 (0.44)	3.55 (0.42)
Putamen	4.61 (0.53)	4.60 (0.52)
Cerebellum	50.04 (4.90)	51.13 (4.97)
Insula	6.55 (0.70)	6.50 (0.74)

2.4.2. Bilingualism score

In order to obtain a single score that reflected the degree of bilingualism of our participants, an exploratory factor analysis (EFA) was carried out from the data obtained in our bilingualism questionnaire, following the procedure used in a previous study (Anderson, Mak, et al., 2018).

All analyses were performed using Rstudio (R version 3.6.3). First, a matrix of correlations was estimated between the 41 bilingualism items in our questionnaire, using *mixedCor* function from the *psych* package. 18 items fulfilled the criterion of correlating higher than r=0.3 or lower than r=-0.3 with more than 50% of the rest of the items of the questionnaire. This implied discarding items related to Spanish proficiency (understanding, reading, writing, listening and fluency), probably due to the low variability in these scores found in our sample (e.g., for Spanish comprehension, mean=1.03, SD=0.18). A first EFA was carried out using the correlation matrix of those 18 items, and the inspection of their loadings led to the exclusion of 4 more, since they could not be clearly associated to a single factor (they were found to load strongly or very similarly in more than one). After this, 14 items were left to be analyzed (see Table 2). The Kaiser-Meyer-Olin (KMO) test (Kaiser & Rice, 2016) verified the sampling adequacy for our analysis (KMO = 0.92) and all the individual KMO values for the items were higher than 0.85. Bartlett's test for sphericity indicated that correlations between our items were sufficiently

large for factor analysis ($\chi 2(91) = 6759.24$, $p<0.001$), and we got an alpha of 0.97, indicating a high internal consistency of the items in our questionnaire.

Next, a parallel analysis was performed using the matrix of correlations of the remaining 14 items, in order to determine the number of factors to be retained in the EFA. The output and scree plot suggested 3 factors. An EFA was carried out using an ordinary-least-squares minimum residual approach and an oblique rotation (*promax*), obtaining three factors and its factor loadings (Table 2). The three factors in combination explained 85% of the variance. Inspection of the distribution of the loadings revealed that Factor 1 is related to use of Catalan and Spanish at school and Catalan proficiency, Factor 2 reflects general use of both languages in contexts outside home and school, and Factor 3 represents use at home.

After obtention of factor structure, scores for each of the factors were calculated using *factor.scores* function in R and using the Harman method, which finds weights based on "idealized" variables (Grice, 2001). Lastly, a composite bilingualism score was computed by summing the factor scores weighted by each factor's variance (Anderson, Mak, et al., 2018). The final score ranged from -1.25 to 0.67 (SD = 0.47, skewness = -0.987, kurtosis = 0.127; see Supplementary Information for a graphical representation of the distribution). We verified the meaning of our score by exploring its relationship with the items of our questionnaire and found that the higher bilingual scores were present in the participants who reported a more balanced use of Catalan and Spanish, as well as balanced proficiency (high proficiency in both languages), while lower scores were found in the participants who reported unbalanced use and lower Catalan proficiency. Thus, our general bilingualism score reflects lifelong balanced use of both languages and proficiency. It is also important to note that one of the factors that forms our composite score contains proficiency in Catalan, since a balanced use of both languages at school (a significant amount of school hours in Catalan, at least 30%) is relevantly related to perception of proficiency on that language, as opposed to proficiency in Spanish, which shows little variation in scores due to its dominant role in society, expressed in specific contexts such as speaking to new people, in department stores or when using social networks (Generalitat Valenciana. Direcció General de Política Lingüística i Gestió del Multilingüïsme, 2015). Finally, our score might be reminiscent of language entropy (Gullifer & Titone, 2020) in that it measures the amount of balance between languages, but it also contains information regarding balance in proficiency and lifelong use.

Table 2. Standardized loadings of each item and factor, as a result of our EFA, with the strongest loading for each item indicated in bold.
%=percentage, Cat=Catalan, Sp=Spanish

	Use at home	**Proficiency and use at school**	**General use in other contexts**
% of time hearing Cat	0.16	-0.21	**0.94**
% of time hearing Sp	-0.08	0.16	**-0.91**
Cat/Sp use at home - child	**0.83**	0.19	0.02
Cat/Sp use at school - child	0.06	**0.59**	0.24
Cat/Sp use at home - adolescent	**0.82**	0.11	0.12
Cat/Sp use at school - adolescent	-0.14	**0.65**	0.41
Cat/Sp use at home - adult	**0.82**	0.09	0.15
Cat/Sp use at workplace - adult	-0.10	0.27	**0.61**
Cat/Sp use another context - adult	0.03	0.23	**0.64**
Writing in Cat	-0.01	**1.01**	-0.07
Pronunciation in Cat	0.18	**0.87**	-0.05
Fluency in Cat	0.25	**0.84**	-0.06
Reading in Cat	0.02	**1.03**	-0.10
Understanding of Cat	0.08	**0.95**	-0.07

2.4.3. Statistical analysis

Data were analysed using R (version 3.6.3.; https://www.r-project.org/), applying GAMMs by using *gam()* function of the mgcv package (Wood, 2011). GAMMs are generalized linear mixed models with linear predictors that involve a sum of smooth functions of covariates or splines (Wood, 2017) – i.e., the linear component of the model is replaced with an additive component (Hastie & Tibshirani, 1995), allowing to model non-linear data. These splines are only applied if there is enough evidence for a curve in the data, since wiggliness (number of curves) penalizes the estimated model fit. GAMMs compute the estimated degrees of freedom (edf), which indicate whether the predictor is in a non-linear (edf>1) or a linear relationship (edf=1) with the dependent variable. We ran a series of GAMMs in order to investigate the effects of individual bilingual experiences as measured by our bilingualism composite score on GM of each one of our ROIs.

In a first-level analyses, we used GAMMs in which we fitted a regression spline for the main effect of bilingualism score on GM volume of each ROI, with participant as a random effect, and also considering the main effect of TIV in order to control for the different head sizes of our participants. We examined the interaction effect of bilingualism score and age on GM volumes, due to the large age range in our sample and accounting for non-linear brain changes related to age and bilingualism that have been previously reported (Pliatsikas et al., 2020). We also included the interaction of bilingualism score and hemisphere in our analyses, to account for previous evidence of lateralized bilingualism effects (Deluca, Rothman, Bialystok, et al., 2019). To do so, following up on previous studies (Korenar et al., 2021; Pliatsikas et al., 2020), we included hemisphere in our models as an ordered factor with two levels (left-right) and we ran two GAMMs, each one with one hemisphere level as reference. The interaction effect between bilingualism score and hemisphere would only be considered reliable if significant in both models.

In a second-level analyses, we analyzed the main effect of bilingual score on GM volumes collapsed across hemisphere, due to the lack of significant interactions with this variable at the first level, and including age, hemisphere and TIV as covariates. We also included participant as a random effect.

For all our results, we considered $p<0.05$ as a threshold of significance, after correcting for family-wise error rate (FWE) using the Bonferroni correction.

2.4.4. Assessment of model fits

In order to assess the model fits of all the second-level models, we used the gam.check() function mgcv (Wood, 2011). All the final models converged with 6 to 9 iterations, and the number of functions which

gave rise to the regression splines were in all cases higher than the estimated degrees of freedom. For all variables of interest, p-values above the 0.05 significance threshold there were obtained, and the k-index was in all cases close to or above 1, which suggests that there were no significant or missed patterns in the residuals of the models (Wood, 2017). See Tables in Supplementary Information for details.

Results

In the first-level analyses, we found that neither the interaction between bilingualism score and hemisphere nor between bilingualism and age were significant predictors in any of the ROI volumes (see Supplementary Information). Consequently, we carried out our second-level analyses collapsing the data across hemisphere for all ROIs and including hemisphere and age as covariates of no interest.

In the second-level analyses, bilingualism score emerged as a significant predictor of GM in three structures: putamen ($p=0.034$, FWE corrected), cerebellum ($p=0.018$, FWE corrected) and IFG ($p=0.021$, FWE corrected). Specifically, putaminal and cerebellar volumes showed linear increases as a function of increasing bilingual experiences. For GM volume in the IFG, bilingualism emerged as a non-linear predictor that showed an initial decrease, followed by an increase in the middle part of the bilingualism spectrum, and a final decrease at the end of the continuum, resulting in an "S" shaped distribution (see Figure 1 for details). Hemisphere emerged as a significant predictor of GM volumes of all regions except for insula, putamen and precentral gyrus, and TIV and age emerged as significant predictors for all ROIs ($p<0.05$, FWE corrected; see Figure 1 for details).

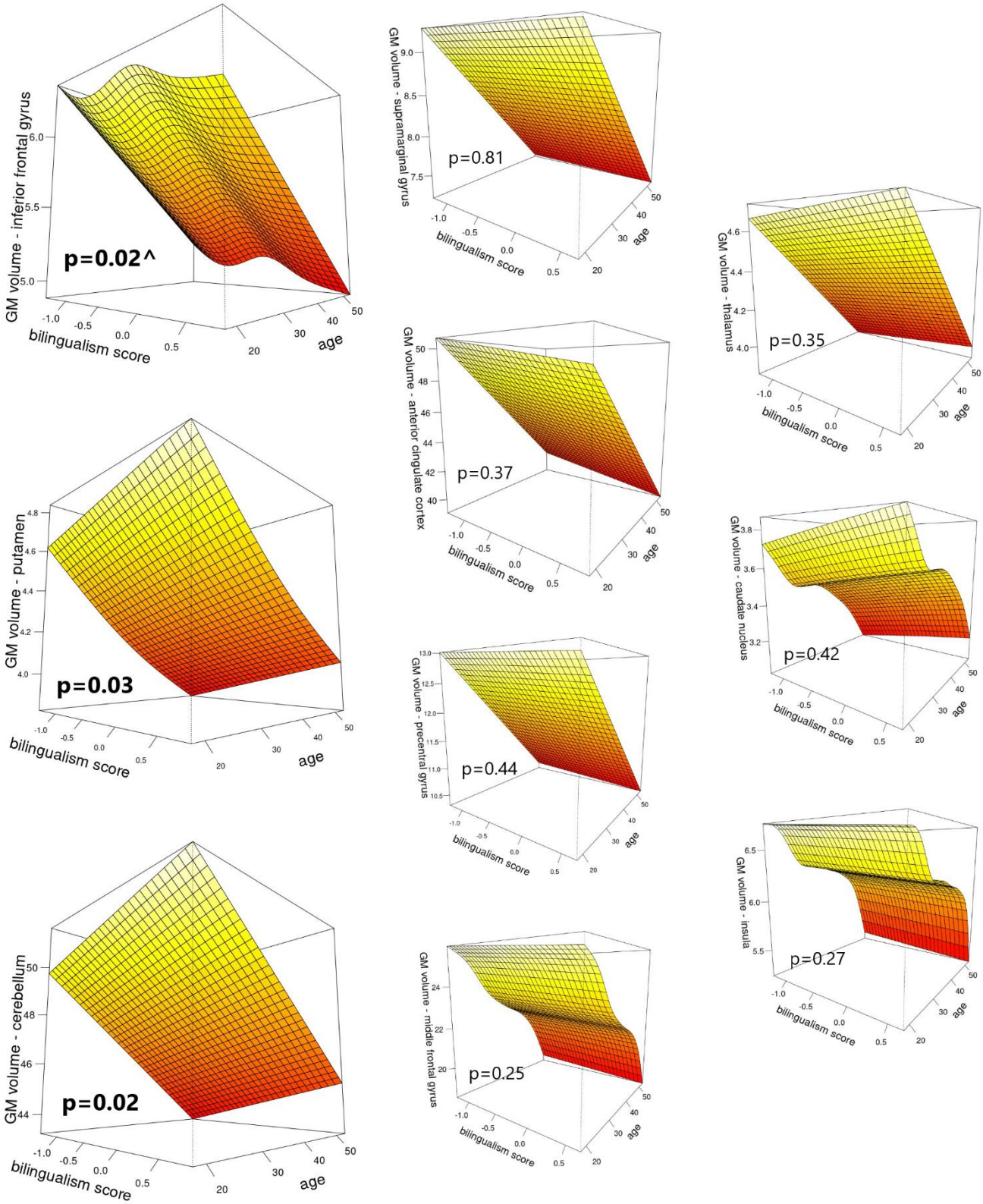

Figure 1. Visual representation of bilingualism score and age as predictors of GM volumes (cm^3) in: A) inferior frontal gyrus, B) putamen, C) cerebellum, D) supramarginal gyrus, E) anterior cingulate cortex, F) precentral gyrus, G) middle frontal gyrus, H) thalamus, I) caudate nucleus and J) insula. P-values correspond to the main effect of bilingualism score. ^ indicates edf>1, denoting a non-linear effect.

Discussion

In the present study, we investigated the effect of quantified bilingual experiences on regional GM volumes. To do so, we focused on a healthy sample of bilinguals living in a society where both Spanish and Catalan are actively used, in contrast to environments where languages are used in more compartmentalized manner (Claussenius-Kalman et al., 2020; Vaughn et al., 2019). Due to the language use characteristics of this environment, our sample included a wide variety of bilingual experiences, from simultaneous highly immersed to late bilinguals with little exposure to L2. In order to fully capture this variety, we considered bilingualism as a continuum, avoiding the use of two separate categories for our participants – i.e., 'bilinguals' and 'monolinguals'. We developed a bilingualism score from data of language use and proficiency, following up from previously published methods (Anderson, Mak, et al., 2018). Finally, we used non-linear models in order to account for dynamic effects of bilingualism on GM volumes, that is, expansion and renormalization patterns (Korenar et al., 2021), in a series of regions that have been implicated in bilingual language control (Abutalebi & Green, 2016). We found a non-linear relationship between our bilingualism score and GM volume in the IFG; specifically, in the lower and higher parts of the continuum of bilingual experiences, there was a decrease of volume as a function of bilingualism, while we found increases in the middle part of the continuum. We also found that GM putaminal and cerebellar volumes increased linearly as a function of bilingualism. None of these effects interacted with hemisphere, and no other significant effects were observed. The next paragraphs will elaborate on the significant findings and discuss them in the context of similar effects that have been reported in the literature.

The IFG is one of the core cortical areas implicated in language control (Abutalebi & Green, 2016), and its GM volume has been shown to increase in L2 learners with brief experience - 3 weeks to 4 months - compared to monolinguals (Hosoda et al., 2013; Legault et al., 2019; Stein et al., 2012). Based on these findings, the IFG was one of the cortical regions predicted to increase its volume in initial stages of bilingualism and later renormalize as duration of bilingual experience increases (Grundy et al., 2017; Pliatsikas, 2020). This suggestion partly matches the pattern of our current findings: The volume reductions we found in IFG at the lower end of the bilingualism continuum could be explained by the characteristics of our sample: immersed bilinguals with such limited bilingual experiences could be considered "passive bilinguals" (Calabria et al., 2020; Costumero et al., 2020), i.e., they have been exposed to a second language and are able to understand it, but currently have limited opportunities to use it and/or switch between languages. Thus, IFG might have increased its volume at an earlier point of their bilingual experience and renormalization might be already in place as the opportunities to use both languages start to increase. This would also go in line with recent evidence showing that forced switching implies increased brain activity in right IFG as measured by magnetoencephalography (MEG),

an effect that is absent during natural switching (Zhu et al., 2022). Given the bilingual characteristics of the region where we conducted our study, where a big majority of the population is able to understand both languages, switching is probably more natural than enforced by the context – if the interlocutor understands both languages, changes from one to the other can be performed freely, not because they are required for successful communication. Therefore, reductions in IFG volume might be related to an increase in experience with naturalistic switching and reduced involvement of the IFG. It should be noted that we did not ask our participants if they performed forced or natural switching, so this limits our interpretation. Finally, the UBET predicts that increased intensity and diversity of language use will reduce the latency by which efficiency adaptations and automation happen as a function of duration of use (DeLuca et al., 2020). Our study was carried out in an environment where two languages are broadly used and opportunities for interacting using both are plentiful, which might increase and diversify the exposure to L2 in the earliest stages of acquisition of the language and accelerate the process of optimisation and pruning of GM cortical volumes.

Our results also showed an unexpected increase of IFG volumes in the middle of the bilingual experience spectrum, right after the initial decrease, which itself was followed by a decrease at the highest levels of bilingual experience. This effect might be caused by a change in the nature of the cognitive demands that bilingualism poses after the first stages of bilingual experience, and before reaching full efficiency (Pliatsikas, 2020), such as the exposure to novel bilingual naturalistic contexts, which would suppose renewed high control demands and might be accompanied by increases in IFG volume, which also seem to normalise again with increasing experience. This pattern escapes the predictions of previous models, which makes it hard to interpret in more detail. To the best of our knowledge, such an effect had not been reported before, but this might be due to the fact that previous studies with similar socio-linguistic characteristics did not use continuous non-linear approaches on cortical GM volumes. Taken at face value, this finding suggests that the dynamicity of the effects of bilingualism in immersive environments may hold even for cortical regions, not just subcortical or the cerebellum as it was previously thought (Deluca, Rothman, & Pliatsikas, 2019; Pliatsikas, 2020) and calls for more evidence from similar samples that are highly immersed for long periods, which will help elaborate on the relevant theories.

Our results further corroborate suggestions that bilingualism increases the volume of the putamen (Abutalebi et al., 2013; Burgaleta et al., 2016; Pliatsikas et al., 2017), and that these effects may be a function of measures of bilingual experiences, such as length of immersion in the L2 (Deluca, Rothman, Bialystok, et al., 2019), or the general degree of bilingualism (Korenar et al., 2021). This region receives inputs from parietal associative areas and is connected to motor regions (Cacciola et al., 2017), which goes in line with evidence showing its involvement in phonological processing (Tettamanti et al., 2005), language control (Hervais-Adelman et al., 2017), motor programming (Garbin et al., 2010), and

articulation of L2 (Berken et al., 2016, 2017; Klein et al., 1994, 1995, 2006; Simmonds et al., 2011). Therefore, it is hypothesized that is more often recruited by bilinguals than monolinguals, leading to volume increases, since the first learn and continuously use a wider range of speech sounds than the second (Burgaleta et al., 2016), and need to control motor programmes between the two languages (Pliatsikas, 2020). Crucially, this effect might be independent of immersion, since it has been reported in immersed and non-immersed bilinguals (Deluca, Rothman, Bialystok, et al., 2019; Korenar et al., 2021), and may be related to simultaneous acquisition and native-like accent proficiency (Berken et al., 2016).

Similar to the putamen, our results also corroborate previous evidence showing GM volume increases in the cerebellum of immersed bilinguals (Burgaleta et al., 2016; Filippi et al., 2011; Pliatsikas et al., 2014). The cerebellum is critical to language control due to its connections to the inferior frontal cortex and thalamus (Abutalebi & Green, 2016). It has also been suggested to participate in error-based learning of complex structural rules, as a part of the procedural memory system (Ullman, 2004). Notably, GM density in the cerebellum has been linked to efficiency in suppressing the first language when using in the second (Filippi et al., 2011) and cerebellar volume is directly related to the speed of processing of grammatical rules in L2 (Pliatsikas et al., 2014). All this evidence suggests that immersive bilingual environments entail high demands of language control and grammatical processing, which involves a special recruitment of the cerebellum and an increase in its volume in all stages of the immersed bilingual experience (Deluca, Rothman, & Pliatsikas, 2019; Pliatsikas, 2020).

Some major cortical regions that lacked significant changes in our results were ACC and inferior parietal cortex. The inferior parietal lobule is thought to be crucial for the integration of semantics and phonology of recently learned vocabulary (Richardson et al., 2010), a process that might have already taken place even in our less experienced bilingual participants, since they could be considered ‘passive bilinguals’ (Calabria et al., 2020; Costumero et al., 2020). Alternatively, the ACC is associated to conflict monitoring, which is hypothesized to be especially required in dual-language interactional contexts (Green & Abutalebi, 2013). However, in territories where Catalan and Spanish are widely used, bilinguals tend to mix both languages during the same interaction (Garbin et al., 2011; Rodriguez-Fornells et al., 2006), resulting in a bilingual experience closer to dense code-switching, where opportunistic planning is hypothesized to be more relevant for the interaction than conflict monitoring (Green & Abutalebi, 2013). Moreover, voluntary switching, as opposed to imposed by external cues, has been shown to require less ACC and prefrontal MEG activation (Blanco-Elorrieta & Pylkkänen, 2017). Since most of the population in the region where we carried out our study understands both languages, we interpret that switching is probably more natural than forced, and this could explain the absence of significant effects in the ACC as a function of bilingual experience. The fact that we found

significant effects only in IFG and cerebellum cortically also goes in line with ACH predictions for dense code-switching interactional contexts, where special recruitment of these regions is expected (Abutalebi & Green, 2016). Still, we did not measure the characteristics of our participants' conversational context, so these interpretations remain speculative. Future research should try to measure bilingualism experiences not only focusing on usage diversity, intensity, duration, and proficiency, but also on the characteristics of interactional contexts where participants make use of their languages, e.g., nature of switching practices. As for the subcortical structures described in the ACH, we did not find the expected significant changes as a function of bilingualism for the caudate nucleus and thalamus. Volumes of caudate nucleus are expected to increase in bilinguals who start acquiring vocabulary of an L2, and renormalize with increased experiences (Pliatsikas, 2020). However, previous evidence suggests that these changes are restricted to bilinguals with limited immersion, due to less proficiency and practice of L2, and would not be necessary for bilinguals in an active immersive environment, an interpretation that goes in line with the immersive context where our bilinguals find themselves and the lack of significant results we observed in this region (Pliatsikas et al., 2017). Regarding the thalamus, it is believed to intervene in the selection of relevant lexical and semantic representations in bilinguals (Abutalebi & Green, 2016), but previous studies have emphasized the specialized contribution of its nuclei to different language functions, such as naming or active speech listening, and advocated for investigating these nuclei separately (Burgaleta et al., 2016; Llano, 2013). Thus, the lack of regional subdivisions in our analyses might have masked GM volume changes in different thalamic nuclei as a function of bilingual experience.

To summarize, in this study we investigated the dynamic effects of bilingualism on GM volumes of healthy participants with a wide variety of bilingual experiences, living in a naturalistic and immersive bilingual environment. We reported a non-linear relationship between IFG and bilingualism score, a pattern that largely goes in line with predictions for effects in environments with high bilingual immersion, increased diversity and intensity of language use. We also reported linear putaminal and cerebellar GM volume increases as a function of bilingualism, which might reflect a growing need to control for motor programmes and grammatical processing. Our results further support the dynamic nature of bilingualism's effects on brain structure and show that this dynamicity is also present in immersive environments.

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Supplementary Information

1. Bilingualism questionnaire (Spanish original)

Código:

CUESTIONARIO DE USO DE LAS LENGUAS

Edad…….

Lugar de nacimiento…….

Lugar de residencia actual…….

Si no es donde naciste, desde cuando vives en el lugar actual…….

Lugar de nacimiento del padre…….

Lugar de nacimiento de la madre…….

A qué edad comenzaste a escuchar de forma continuada el catalán…….

A qué edad comenzaste a utilizar (hablar) el catalán…….

Cómo (dónde) aprendiste el catalán…….

A qué edad comenzaste a escuchar de forma continuada el castellano…….

A qué edad comenzaste a utilizar (hablar) el castellano…….

a) Indica la lengua (catalán, castellano, ambas u otras) que usualmente utilizas para hablar con:
   - Padre: Madre: Hermano/as: Novio/a:
b) Si de pequeño hablabas con tus padres o hermanos en alguna otra lengua de la que utilizas actualmente, indica a qié edad se va a producir el cambio:
   - Padre: Madre: Hermano/as: Novio/a:
c) ¿Qué otras lenguas puedes utilizar (hablar, leer, escribir)? …….
   - ¿A qué edad iniciaste el aprendizaje formal de estas lenguas? …….

Señala la opción que mejor te representa en cada una de las siguientes preguntas:

Qué nivel de comprensión tienes de estas lenguas:

Francés:	perfectamente	bien	suficiente	muy poco	nada
Inglés:	perfectamente	bien	suficiente	muy poco	nada
Catalán:	perfectamente	bien	suficiente	muy poco	nada
Castellano:	perfectamente	bien	suficiente	muy poco	nada

Qué nivel de lectura tienes de estas lenguas:

Francés:	perfectamente	bien	suficiente	muy poco	nada

Inglés:	perfectamente	bien	suficiente	muy poco	nada
Catalán:	perfectamente	bien	suficiente	muy poco	nada
Castellano:	perfectamente	bien	suficiente	muy poco	nada

Cómo hablas en estas lenguas (fluidez):

Francés:	perfectamente	bien	suficiente	muy poco	nada
Inglés:	perfectamente	bien	suficiente	muy poco	nada
Catalán:	perfectamente	bien	suficiente	muy poco	nada
Castellano:	perfectamente	bien	suficiente	muy poco	nada

Cómo hablas en estas lenguas (corrección de pronunciación):

Francés:	perfectamente	bien	suficiente	muy poco	nada
Inglés:	perfectamente	bien	suficiente	muy poco	nada
Catalán:	perfectamente	bien	suficiente	muy poco	nada
Castellano:	perfectamente	bien	suficiente	muy poco	nada

Cómo escribes en estas lenguas:

Francés:	perfectamente	bien	suficiente	muy poco	nada
Inglés:	perfectamente	bien	suficiente	muy poco	nada
Catalán:	perfectamente	bien	suficiente	muy poco	nada
Castellano:	perfectamente	bien	suficiente	muy poco	nada

¿En qué lengua te sientes más cómodo/a?

Catalán Castellano En ambas igual

¿Si desafortunadamente hubieras sufrido un accidente cerebral que te supusiera la pérdida de una lengua, cuál de las dos preferirías conservar (sin tener en cuenta criterios prácticos)?

Catalán Castellano

Si tienes (o tuvieras) un perro o un gato, en qué lengua le hablas (o hablarías)

Catalán Castellano En ambas igual

¿Percibes diferencias dialectales?

En castellano:	No	Sólo entre algunos dialectos	Sí, claramente
En catalán:	No	Sólo entre algunos dialectos	Sí, claramente

Evalúa el porcentaje de tiempo al día que dedicas a escuchar cada lengua (incluyendo clases, programas

de TV, radio, etc)

Catalán Castellano Otras

Evalúa el porcentaje de tiempo al día que dedicas a hablar en cada lengua

Catalán Castellano Otras

En respuesta a las preguntas que vienen a continuación, señala con un círculo la frecuencia con la que utilizaste el castellano y el catalán a las diferentes edades y en las diferentes situaciones reseñadas. Para ello, utiliza la siguiente escala:

1-Solo castellano

2-Castellano frecuentemente, catalán raramente

3-Principalmente castellano, utilizando el catalán al menos una cuarta parte del tiempo

4-Uso equitativo de castellano y catalán

5-Principalmente catalán, utilizando el castellano al menos una cuarta parte del tiempo

6-Catalán frecuentemente, castellano raramente

7-Solo catalán

1. ¿Siendo un niño pequeño, antes de iniciar la etapa escolar?

- 1 2 3 4 5 6 7

2. ¿Siendo un niño, en la etapa de educación primaria?

EN LA ESCUELA

- 1 2 3 4 5 6 7

EN CASA

- 1 2 3 4 5 6 7

EN OTROS LUGARES

- 1 2 3 4 5 6 7

3. ¿En la pubertad, en la etapa de educación secundaria y en el bachillerato?

EN LA ESCUELA

- 1 2 3 4 5 6 7

EN CASA

- 1 2 3 4 5 6 7

EN OTROS LUGARES

- 1 2 3 4 5 6 7

4. ¿En la edad adulta?

EN LA UNIVERSIDAD O EN EL TRABAJO

- 1 2 3 4 5 6 7

EN CASA

- 1 2 3 4 5 6 7

EN OTROS LUGARES

- 1 2 3 4 5 6 7

2. Bilingualism questionnaire (English translation)

Code:

USE OF LANGUAGES QUESTIONNAIRE

Age…….

Birthplace…….

Current place of residence…….

If that is not where you were born, since when do you live in that location…….

Father's birthplace…….

Mother's birthplace…….

At what age did you start hearing Catalan continuously…….

At what age did you start using (speaking) Catalan…….

How (where) did you learn Catalan…….

At what age did you start hearing Spanish continuously …….

At what age did you start using (speaking) Spanish…….

d) Indicate the language (Catalan, Spanish, both or others) that you normally use to speak with your:
   - Father: Mother: Brother/Sister/s: Couple:

e) If you spoke a different language from the one you use now with your parents or brother/sister/s, indicate at what age did you change:
   - Father: Mother: Brother/Sister/s: Couple:

f) What other languages can you use (speak, read, write)? …….
   - At what age did you start formal learning of those languages? …….

Mark the option that represents you best in each one of the following questions:

What level of comprehension do you have of the following languages?

French:	perfect	good	sufficient	very little	nothing
English:	perfect	good	sufficient	very little	nothing
Catalan:	perfect	good	sufficient	very little	nothing
Spanish:	perfect	good	sufficient	very little	nothing

What is your reading level in the following languages?

French:	perfect	good	sufficient	very little	nothing
English:	perfect	good	sufficient	very little	nothing

Catalan:	perfect	good	sufficient	very little	nothing
Spanish:	perfect	good	sufficient	very little	nothing

How do you speak the following languages (fluency)?

French:	perfect	good	sufficient	very little	nothing
English:	perfect	good	sufficient	very little	nothing
Catalan:	perfect	good	sufficient	very little	nothing
Spanish:	perfect	good	sufficient	very little	nothing

How do you speak the following languages (pronunciation correctness)?

French:	perfect	good	sufficient	very little	nothing
English:	perfect	good	sufficient	very little	nothing
Catalan:	perfect	good	sufficient	very little	nothing
Spanish:	perfect	good	sufficient	very little	nothing

How well do you write in the following languages?

French:	perfect	good	sufficient	very little	nothing
English:	perfect	good	sufficient	very little	nothing
Catalan:	perfect	good	sufficient	very little	nothing
Spanish:	perfect	good	sufficient	very little	nothing

What language do you feel more comfortable with?

Catalan Spanish Both equally

If you unfortunately suffered from brain damage and you had to lose one language, which one would you prefer to keep (without considering practical criteria)?

Catalan Spanish

If you have (or had) a dog or a cat, what language do you (or would you) use with them?

Catalan Spanish Both equally

Do you perceive dialectal differences?

In Spanish:	No	Only between some dialects	Yes, clearly
In Catalan:	No	Only between some dialects	Yes, clearly

Evaluate the percentage of time in a day that you dedicate to listening to each language (including courses, TV programs, radio…)

Catalan Spanish Others

Evaluate the percentage of time in a day that you dedicate to speaking in each language

Catalan Spanish Others

In the following questions, mark with a circle the frequency of use of Spanish and Catalan at different ages and situations. To do so, use the following scale:

1-Only Spanish

2-Spanish frequently, Catalan rarely

3-Mainly Spanish, using Catalan at least ¼ of the time

4-Equal use of Spanish and Catalan

5-Mainly Catalan, using Spanish at least ¼ of the time

6-Catalan frequently, Spanish rarely

7-Only Catalan

1. When being a toddler, before starting the schooling period.

- 1 2 3 4 5 6 7

2. When being a child, at elementary school.

AT SCHOOL

- 1 2 3 4 5 6 7

AT HOME

- 1 2 3 4 5 6 7

IN OTHER ENVIRONMENTS

- 1 2 3 4 5 6 7

3. At puberty, during secondary school and high school.

AT SCHOOL

- 1 2 3 4 5 6 7

AT HOME

- 1 2 3 4 5 6 7

IN OTHER ENVIRONMENTS

- 1 2 3 4 5 6 7

4.During adulthood.

AT THE UNIVERSITY OR WORKPLACE

- 1 2 3 4 5 6 7

AT HOME

- 1 2 3 4 5 6 7

IN OTHER ENVIRONMENTS

- 1 2 3 4 5 6 7

3. Results from the first-level models (p-values).

Inferior frontal gyrus

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.064	0.074
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.479	0.479
Bilingualism score x hemisphere	0.417	0.417

Parietal (supramarginal gyrus)

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.404	0.578
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.427	0.427
Bilingualism score x hemisphere	0.582	0.582

Anterior cingulate cortex

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.864	0.8807
TIV	<0.001	<0.001
Age	0.022	<0.028
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.819	0.819
Bilingualism score x hemisphere	0.553	0.553

Motor cortex

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.568	0.857
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	0.136	0.136

Bilingualism score x age	0.536	0.341
Bilingualism score x hemisphere	0.451	0.387

Premotor cortex

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.243	0.199
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.067	0.067
Bilingualism score x hemisphere	0.379	0.379

Thalamus

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.945	0.394
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.885	0.885
Bilingualism score x hemisphere	0.352	0.352

Caudate

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.913	0.838
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.552	0.552
Bilingualism score x hemisphere	0.848	0.848

Putamen

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.849	0.7961
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	0.64	0.64

Bilingualism score x age	0.308	0.308
Bilingualism score x hemisphere	0.890	0.890

Cerebellum

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.488	0.493
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	<0.001	<0.001
Bilingualism score x age	0.947	0.947
Bilingualism score x hemisphere	0.965	0.965

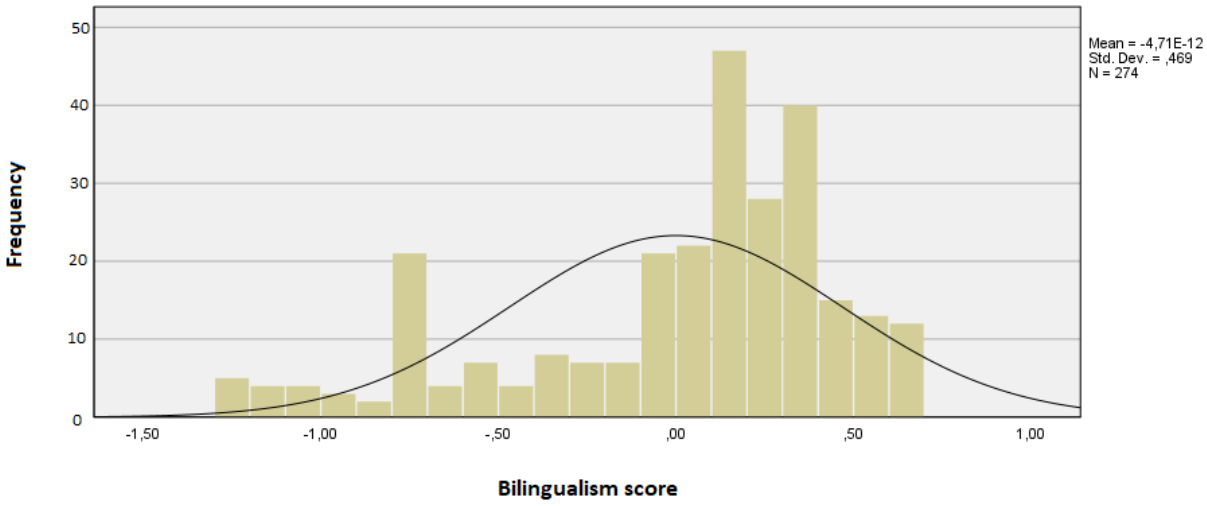
Insula

Reference level of hemisphere	**Left hemisphere**	**Right hemisphere**
Bilingualism score	0.877	0.743
TIV	<0.001	<0.001
Age	<0.001	<0.001
Hemisphere	0.209	0.209
Bilingualism score x age	0.988	0.988
Bilingualism score x hemisphere	0.736	0.736

4. Assessment of model fits

ROI	**Smooth term**	**K (number of basis functions)**	**Estimated degrees of freedom**	**k-index**	**p-value of significant patterns in residuals**
IFG	**Bilingualism score**	8	3.84	0.91	0.22
Putamen	**Age**	2	1.52	0.99	0.47

5. Range and distribution of bilingual scores



Study 5

Structural but not functional connectivity differences within default mode network indicate conversion to dementia.

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Under review

Abstract

Introduction: Malfunctioning of the default mode network (DMN) has been consistently related to mild cognitive impairment (MCI) and Alzheimer's disease (AD). However, evidence on differences in this network between MCI converters (MCI-c) and non-converters (MCI-nc), which could mark progression to AD, is still inconsistent. Therefore, the aim of our study was to multimodally investigate the DMN in dementia. Methods: To do so, we measured gray matter (GM) volume, white matter (WM) integrity, and functional connectivity (FC) at rest in healthy elderly controls, MCI-c, MCI-nc and AD patients, matched on sociodemographic variables. Results: We found significant differences between AD patients and controls in the structure of most of the regions of the DMN. MCI-c only differed from MCI-nc in GM volume of the medial temporal lobe (MTL) bilaterally and WM integrity of the parahippocampal cingulum connecting the left MTL and precuneus. We found significant correlations between integrity in those regions and global neuropsychological status, as well as an excellent discrimination ability between converters and non-converters for their combination. However, we found no significant differences in FC. Conclusion: These results further support the relationship between abnormalities in the DMN and AD, and suggest that structural measures could be more accurate than resting-state estimates as markers of conversion from MCI to AD.

INTRODUCTION

Alzheimer's Disease (AD) is increasingly conceptualized as a disconnection syndrome, involving not only gray matter (GM) atrophy and accumulation of pathological proteins in specific brain regions, but also disrupted functioning of brain networks (Brier et al., 2014; Mito et al., 2018a). Alterations in functional connectivity (FC) have been found in the default mode (DMN), salience, and limbic networks, in patients with AD and mild cognitive impairment (MCI) (Badhwar et al., 2017b). However, recent studies tend to focus on the study of the DMN, since AD has been shown to especially affect the functioning of this network (Mohan et al., 2016). Namely, there seems to be a spatial overlap between DMN abnormalities and AD histopathology (Dennis & Thompson, 2014). Evidence shows structural white matter (WM) deterioration in AD patients in fibers connecting DMN nodes (Mito et al., 2018a; Tucholka et al., 2018), and an accelerated aging pattern of DMN disconnection in AD patients compared to age-matched controls, with decreases in resting-state FC between its nodes (Binnewijzend et al., 2012; L. Wang et al., 2006). Also importantly, resting-state FC abnormalities in the DMN have been shown to increase with disease severity (Wu et al., 2011).

When investigating brain network alterations in mild cognitive impairment (MCI), studies report DMN abnormalities similar to those found in dementia patients, but to a lesser extent – that is, with integrity values that fall between those of dementia patients and healthy elderly controls (Binnewijzend et al., 2012). MCI patients have also been found to show lower FC between hippocampi and posterior cingulate cortex (PCC) compared to healthy elderly controls (Sorg et al., 2007), decreased whole-brain connectivity in PCC and precuneus (Drzezga et al., 2011), reduced connectivity between DMN nodes and between regions of the cortico-striatal-thalamic loop (Tam et al., 2015) and decreases in FC within the DMN and between the hippocampus and the DMN (Gilligan et al., 2019). Regarding WM structure, a meta-analysis reported reliable abnormalities in WM integrity in MCI patients in the fornix, uncinate fasciculus and parahippocampal cingulum (Yu et al., 2017), further confirmed by later studies (Gilligan et al., 2019; Mito et al., 2018a).

All this evidence suggests that DMN alterations are typically implicated in both AD and MCI. However, it is estimated that 85-90% of MCI cases per year remain stable and do not progress to AD (R. Roberts & Knopman, 2013). It is therefore relevant to identify the neural substrates that differentiate MCI converters (MCI-c) from non-converters (MCI-nc), which could mark deterioration leading to AD. Previous studies have linked medial temporal atrophy to progression from MCI to AD (Brueggen et al., 2015; DeCarli et al., 2007). As for structural connectivity, previous studies focusing on WM integrity have found significant differences between MCI-c and MCI-nc in the corpus callosum (van Bruggen et al., 2012), and uncinated fasciculus (Hiyoshi-

Taniguchi et al., 2015), and reported thalamus (Brueggen et al., 2015), left hippocampus and cingulate (Marcos Dolado et al., 2019), and bilateral corticospinal, right hippocampal cingulum, right inferior fronto-occipital, left inferior longitudinal, right superior longitudinal and left uncinate fasciculi integrity values as predictors of progression (Stone et al., 2021). Regarding FC, a study found a significant predictive effect of a goodness-of-fit index of the DMN (i.e., the degree to which DMN maps of MCI patients matched those of controls), on progression from MCI to AD (Petrella et al., 2011). Based on this evidence, a general overview arises on GM structure markers of disease progression, which seem relatively consistent. However, to the best of our knowledge, evidence on WM integrity and resting-state FC still seems to be comparatively scarce and inconsistent.

Considering this, the main objective of our study was to determine the contribution of brain alterations in the DMN to conversion from MCI to AD. With this aim, we carried out a multimodal study, focusing on a sample of MCI-c, MCI-nc, AD patients and healthy elderly control participants. We compared measures of three neuroimaging modalities between the groups: GM volume, WM integrity, and resting-state FC. Our hypothesis was that MCI-c would show significantly lower WM integrity than MCI-nc in fibers corresponding to the left hippocampus and cingulum, lower GM volume in the left MTL, and lower FC within the DMN. We also expected to find the same pattern when comparing AD patients to healthy controls. Finally, we also explored how AD-related alterations within the neuroimaging modalities related to global neuropsychological status.

METHODS

Participants

One hundred and three elderly individuals were recruited for this study, all of them selected from dementia units of the Valencian Community public healthcare system. Our sample consisted of 20 healthy elderly subjects (11 women; mean age=73.15, SD=4.45), 20 patients with a diagnose of probable AD (13 women; mean age=74.95, SD=2.95), 31 patients with MCI that converted to dementia (22 women; mean age=74.26; SD=6.10) and 32 that did not convert (16 women; mean age=73.22, SD=4.98). MCI patients were classified as converters or non-converters after a follow-up with periodic neuropsychological assessments and clinical interviews every 6 months, although MR data was acquired only in the first clinical visit. Those who received a probable AD diagnosis during the follow-up - within a period of two years after the functional magnetic resonance imaging (MRI) session - were considered converters (MCI-c), whereas patients who remained stable after this period were classified as non-converters (MCI-nc). Probable AD and MCI diagnoses were performed by experienced neurologists, based on clinical criteria (see Supplementary material for details).

All participants were informed of the nature of the research, and provided written informed consent before their participation in the study. All study procedures were approved by the Ethics Committee of the Universitat Jaume I of Castelló and conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Neuropsychological assessment

Participants underwent a structured clinical interview and a neuropsychological assessment, which included: a short form of the Boston Naming test (BNT) (Serrano et al., 2001b), a Word List Acquisition and Recall test (immediate and delayed recall), two Fluency tests (semantic and phonetic), a remote memory test, and the clock-drawing test (Cacho et al., 1996a). With the purpose of simplification, a composite global neuropsychological score was calculated by obtaining the mean of the standardized values of the measures. See Table 1 for details on sociodemographic variables and neuropsychological performance of the four groups.

Table 1. Sociodemographic and neuropsychological variables of four groups.

	Healthy elderly (N = 20)		**MCI non-converters** (N = 32)		**MCI Converters** (N = 31)		**AD patients** (N = 20)		Statistical differences	P
Gender	M/W = 9/11		M/W = 16/16		M/W = 9/22		M/W = 7/13		$\chi2 = 3.31$	0.35
	M	SD	M	SD	M	SD	M	SD	F	
Age	73.15	4.45	73.22	4.98	74.26	6.10	74.95	2.95	0.71	0.55
Years of schooling	8.15	2.73	8.41	3.14	9.10	3.74	8.63	4.10	0.26	0.86
BNT	11.95	0.22	9.78	1.00	8.87	1.69	7.40	3.02	25.31	0.00
Phonetic Fluency	13.20	2.02	9.44	1.39	8.23	2.63	4.95	2.96	45.50	0.00
Semantic Fluency	17.05	2.87	11.31	1.87	10.06	3.37	7.70	3.16	40.12	0.00
Immediate recall	6.50	0.57	2.85	0.57	3.04	1.03	1.85	1.22	109.62	0.00
Delayed recall	6.65	0.75	1.22	0.42	1.03	1.07	0.20	0.52	328.40	0.00
Remote memory	11.70	1.34	9.47	0.92	9.39	1.54	5.65	2.56	48.97	0.00
Clock drawing test	9	0	7.38	1.19	6.55	1.69	4.05	2.82	30.34	0.00
Global NPS score	1.33	0.23	0.00	0.19	-0.21	0.46	-1.04	0.67	113.79	0.00

BNT, Boston Naming Test; NPS, neuropsychological; N, sample size; M/W, men/women; $\chi2$, chi-squared test; M, Mean; SD, Standard Deviation; F, F value for ANOVA.

Image preprocessing and analyses

Information on MRI acquisition can be found in Supplementary material.

Region-based morphometry analysis. Structural GM analyses were performed with CAT12 (Computational Anatomy Toolbox; C. Gaser, Jena University Hospital, Jena, Germany; http://dbm.neuro.uni-jena.de/cat/) using their standard preprocessing pipeline. A first quality check was conducted to detect images affected by important inhomogeneity or movement artefacts. After an initial bias correction of intensity non-uniformities, individual volumes of GM, WM, and cerebrospinal fluid were estimated applying segmentation, and images were registered to the shooting template provided in the CAT12 toolbox. Then, the region of interest (ROI) analysis implemented in CAT12 was performed. In this analysis, also called Region-based morphometry (RBM), a mask in standard space is transformed into native subject space, and the sum of the local GM inside the mask is estimated. We restricted our analysis to the nodes of an atlas of the dorsal DMN (Shirer et al., 2012) (i.e., precuneus, left and right MTL, left and right medial frontal cortex, left and right angular gyrus, middle cingulum, and thalamus).

DTI analysis. All DTI data were processed using the FMRIB Software Library (FSL) (Smith et al., 2004). Diffusion weighted images were corrected for eddy current distortions using *eddycorrect*, brain extraction and deletion of non-brain tissue were performed using *bet* (Brain Extraction Tool) (Smith, 2002), and *dtifit* was applied to extract fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AxD) and radial diffusivity (RD). Tract-based spatial statistics of FA, MD, AxD, and RD was carried out using TBSS (Smith et al., 2006). Non-linear registration of all FA individual images to a common space was performed using the tool FNIRT (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FNIRT), and a mean FA skeleton was created and thinned in order to represent the center of all tracts common to the subjects. Finally, each subject's aligned FA data were projected onto this skeleton. The same process was used for MD, AxD, and RD. We calculated the mean FA, MD, AxD and RD of those fibers connecting the dorsal DMN, based on a probabilistic DTI atlas (Figley et al., 2015) of fMRI-guided tractography between previously defined DMN nodes (Shirer et al., 2012). We focused on the streamlines with a probability of 25% or higher of belonging to the DMN, following the procedure of a previous study (Prillwitz et al., 2018). Seven fibers of interest remained, connecting the following nodes: (a) precuneus – left MTL; (b) precuneus – right MTL; (c) medial frontal cortex – precuneus; (d) medial frontal cortex – middle

cingulum; (e) medial frontal cortex – thalamus; (f) precuneus – middle cingulum; (g) thalamus – left MTL.

ROI to ROI resting-state FC analysis. We used Data Processing and Analysis for Brain Imaging (DPABI V4.2_190919, http://rfmri.org/dpabi) to carry out resting-state data processing. Preprocessing included: (1) removal of the first ten volumes of each raw dataset; (2) slice timing correction; (3) realignment using a six-parameter (rigid body) linear transformation; (4) spatial normalization to the Montreal Neurological Institute (MNI) space (voxel size 3 × 3 × 3 mm); (5) removal of spurious variance through linear regression: 24 parameters from the head motion correction, linear, and quadratic trends, and the first five principal components associated with WM and cerebrospinal fluid (Behzadi et al., 2007); (6) spatial smoothing with a 4-mm FWHM Gaussian Kernel; and (7) band-pass temporal filtering (0.01–0.1 Hz). None of the participants had more than 2 mm/degree of movement in any of the six directions or fewer than 120 volumes with framewise displacement (FD) < 0.5 mm (Jenkinson et al., 2002), ensuring at least 4 minutes of rest with low FD. Moreover, there were no significant differences between the groups in FD. ROI time courses were extracted from regions of the DMN (Shirer et al., 2012), then FC between ROIs was estimated using Pearson's correlation and r to z transformation was applied using the Fisher's method. We focused our analyses on the connectivity between the regions linked by the fibers of interest of our DTI analyses (i.e., precuneus, left and right MTL, medial frontal cortex, middle cingulum, and thalamus). To avoid the introduction of different amounts of noise derived from the signal average of regions with different sizes, we used the centroids of the ROIs provided on the atlas to create spherical masks (5mm radius) as our seeds. Specifically, we used the following: precuneus (MNI: 1, -53, 28), left (MNI: -24, -29, -13) and right (MNI: 27, -23, -17) MTL, medial frontal cortex (MNI: -3, 49, 14), middle cingulum (MNI: 3, -15, 36) and thalamus (MNI: -1, -8, 4).

Statistical analyses

Group differences in GM volume, WM diffusion and ROI to ROI resting-state FC were investigated with between group comparisons using one-way ANOVA $p<0.05$ FWE-corrected (Bonferroni method). Planned post hoc comparisons between AD-healthy controls and MCI-c-MCI-nc were performed for each significant ANOVA. These analyses were performed in SPSS 23 (IBM Corp.). When using GM volumes, total intracranial volume (TIV) was included as a covariate.

We carried out post-hoc correlations as implemented in SPSS 23 to explore the relationship between modality estimates in regions that showed to be relevant for conversion from MCI to AD and global neuropsychological score. These analyses were restricted to participants that presented a disease (MCI-c, MCI-nc and AD).

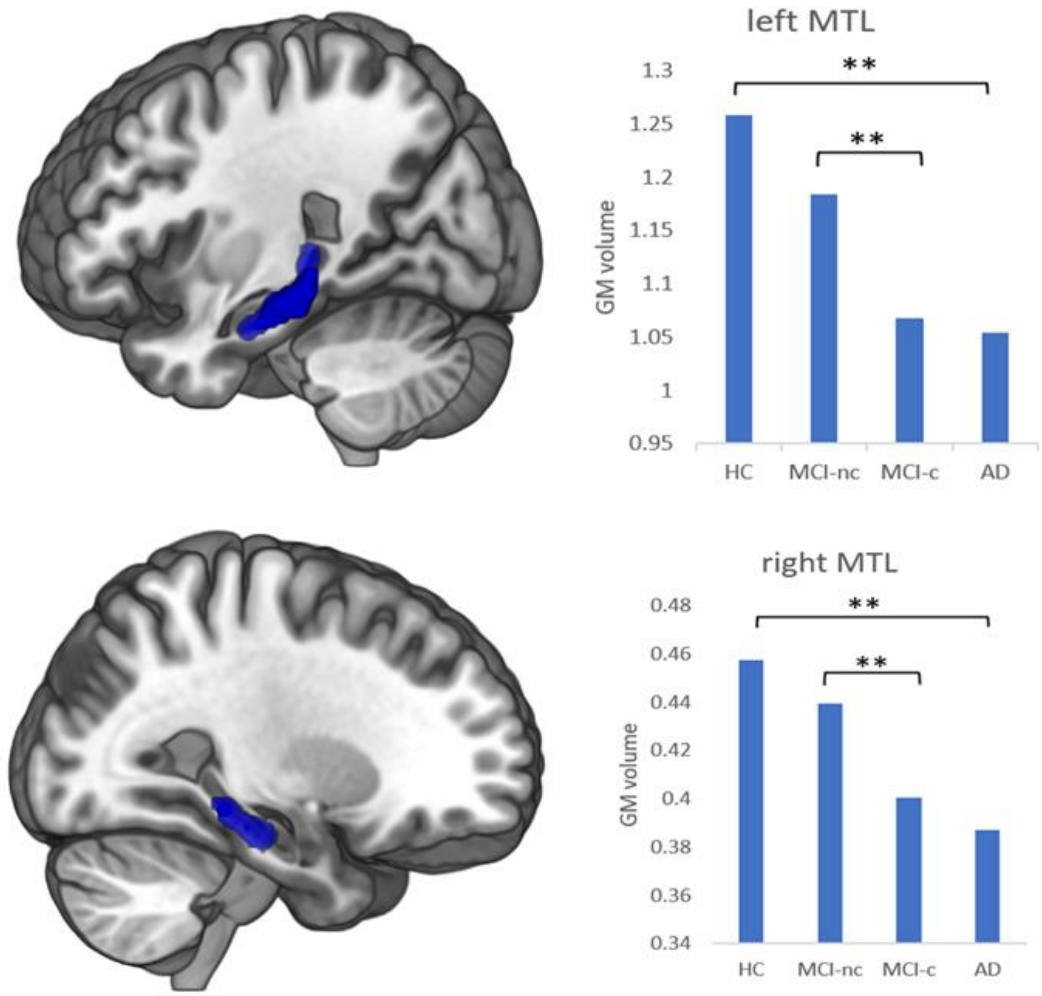
Finally, receiver operator curves (ROC) and their area under these curve (AUC) were calculated for the modality estimates in regions that turned out to be relevant for conversion, for their combination, and for the composite global neuropsychological score, in order to assess their ability to discriminate between the categories of MCI-c and MCI-nc. In this analysis, GM volumes were corrected for TIV using the power proportion method (D. Liu et al., 2014) as implemented in R (https://github.com/tkoscik/tkmisc), and a mean of the two hemispheres was used, while the sign of RD values was reversed in order to transform them to the same discriminative direction as GM volume values. Following up on a previous study (K. Liu et al., 2017), logistic regression analysis was used to combine the measures relevant for conversion in a single ROC.

RESULTS

Region-based morphometry analysis

We found significant differences between the groups in GM values of the left and right MTL ($F=12.49$, $p<0.05$, FWE-corrected; Figure 1). In post hoc two-sample analyses, we found that these results were driven by AD patients showing lower GM volume than elderly controls and MCI-c showing lower GM volumes than MCI-nc in both regions. Finally, we also found significant positive correlations between the global neuropsychological score and GM volumes in left ($R=0.231$, $p=0.040$) and right MTL ($R=0.221$, $p=0.049$) in patients (MCI-c, MCI-nc and AD).

Figure 1. GM volume differences between healthy elderly controls, MCI-c, MCI-nc and AD patients (ANOVA and post-hoc Two Sample t-Test). Asterisks indicate significant differences.



DTI analysis

We found significant differences between the groups in all DTI measures and fibers of the DMN, except for the tracts connecting the medial frontal cortex and middle cingulum, and thalamus and left MTL ($p<0.05$, FWE-corrected; Figure 2). In post hoc two sample analyses, we found that these differences were the aftereffect of AD patients showing lower WM integrity than healthy elderly controls. We also found that MCI-c showed higher RD than MCI-nc in the WM fiber linking the precuneus and left MTL (Figure 2). Finally, RD of the parahippocampal cingulum significantly negatively correlated with the global neuropsychological score in patients (MCI-c, MCI-nc and AD) ($R=-0.325$, $p=0.003$).

ROI to ROI resting-state FC analysis

We found no significant differences between the groups in FC between DMN regions using the predefined threshold of $p < 0.05$ FWE corrected. However, considering uncorrected results, differences in FC between the precuneus and left MTL ($F=3.67$, $p=0.015$ uncorrected) were observed. This effect was driven by lower FC in AD patients than healthy controls.

ROC analysis

The ROC for discrimination between MCI-c and MCI-nc are shown in Figure 3. The global neuropsychological score showed an AUC of 0.733 (SE=0.056, p=0.000, lower boundary=0.702, upper boundary=0.921). RD values of the parahippocampal cingulum with inverted sign showed an AUC of 0.720 (SE=0.065, p=0.003, lower boundary=0.592, upper boundary=0.847). Bilateral GM values of the MTL showed an AUC of 0.715 (SE=0.067, p=0.004, lower boundary=0.584, upper boundary=0.845). The combination of bilateral GM values of the MTL and RD values of parahippocampal cingulum showed an AUC of 0.806 (SE=0.057, p=0.000, lower boundary=0.695, upper boundary=0.918).

Figure 2. WM integrity differences between healthy elderly controls, MCI-c, MCI-nc and AD patients (ANOVA and post-hoc Two Sample t-Test). Asterisks indicate significant differences.

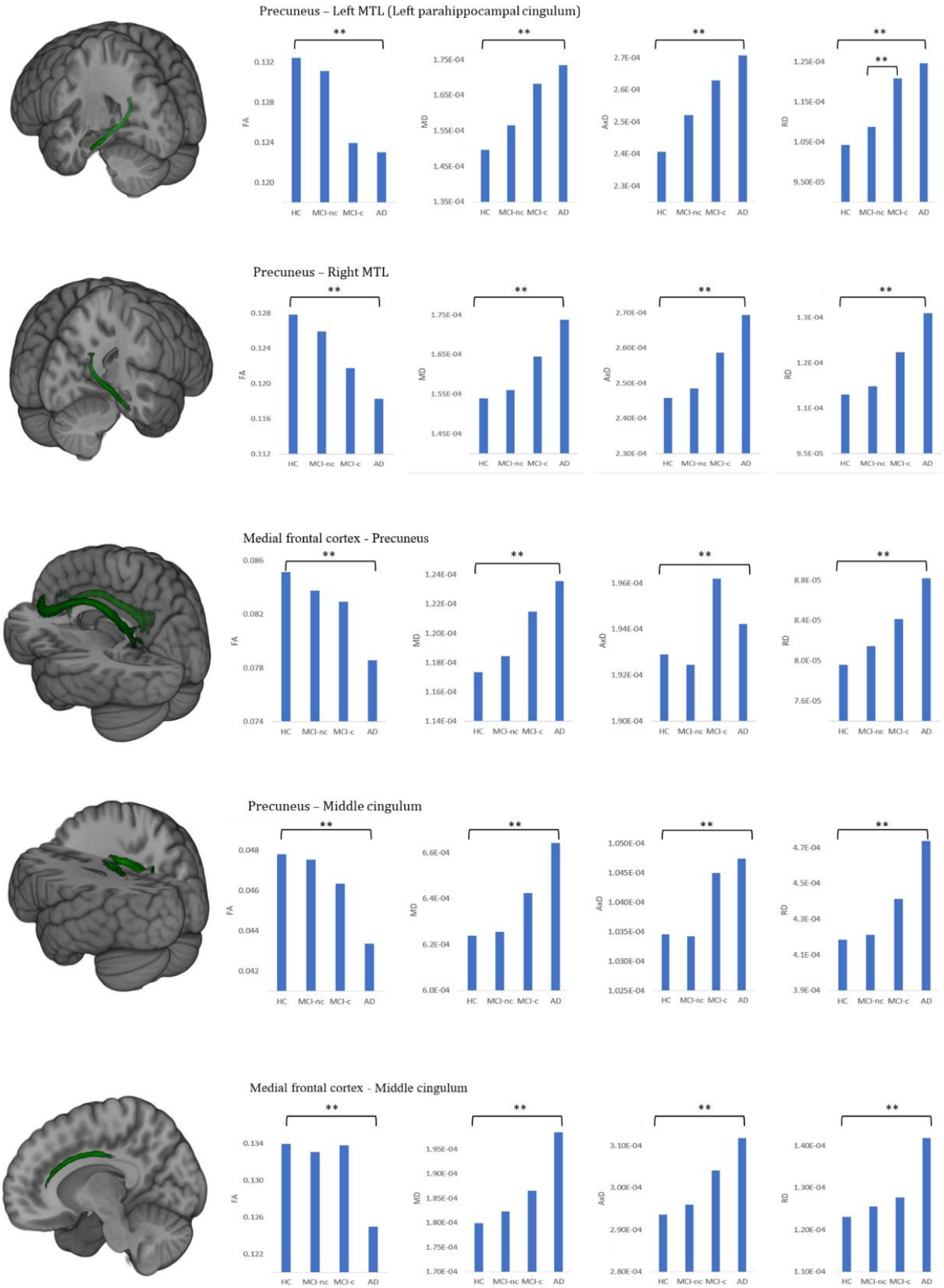
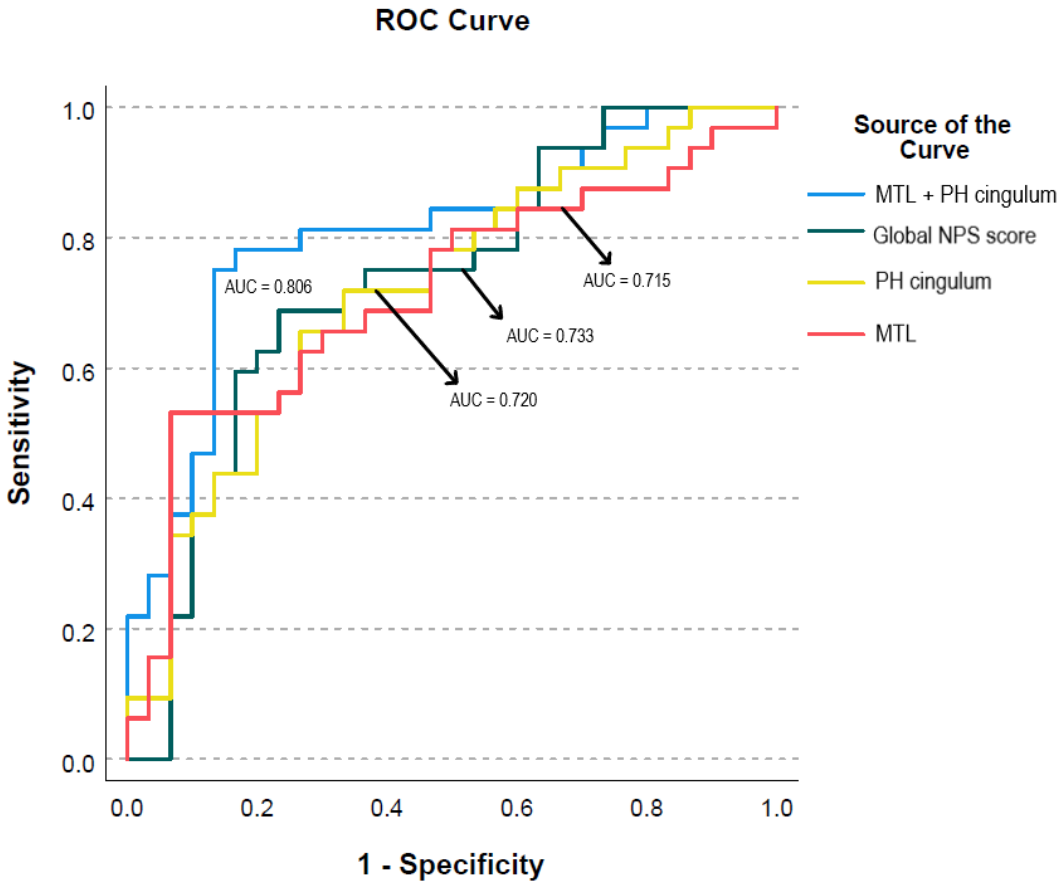


Figure 3. ROCs for the global neuropsychological score (in green), WM integrity of the parahippocampal cingulum (negative RD values, in yellow), GM volumes of bilateral MTL (in red), and the combination of GM and WM measures (in blue). ROC= receiver operator curve, AUC = area under the curve, MTL= middle temporal lobe, PH=parahippocampal, NPS=neuropsychological.



DISCUSSION

The DMN is characterized by being active in the brain at rest and becoming deactivated when a task is performed, indicating a state of alertness but not active involvement in a task (Mohan et al., 2016). Some of its components, such as the parietal, posterior cingulate, and precuneus, are associated with recollection (Vincent et al., 2006), memory retrieval and consolidation (Randy L. Buckner et al., 2008). Neuropathology in the DMN seems to be consistently found in AD, but the reason why this happens remains unknown (Dennis & Thompson, 2014; Desgranges et al., 2011). The "metabolism hypothesis", suggests that the DMN's continuous high baseline activity increases the cascade that leads to dementia pathology (Randy L. Buckner et al., 2005), while others suggest that the DMN, among other multimodal networks, is associated with multiple cognitive functions and supports the integration of information, being especially vulnerable to early and fast spread of pathology for that very reason (Mišić et al., 2015).

In this prospective multimodal MRI study, we investigated structural and functional alterations of the DMN as possible markers of transition from MCI to dementia. Specifically, we analyzed GM volume, WM integrity and FC of a single sample of elderly participants with similar age, years of schooling and proportion of men and women, that differed in their diagnoses: healthy elderly controls, MCI patients that converted to AD during a follow-up of two years, MCI that remained stable, and AD patients. Our analyses revealed that AD patients showed significantly lower WM integrity than elderly controls in most of the DMN fibers, lower GM volumes in bilateral MTL and lower FC between the precuneus and left MTL. MCI-c showed lower WM integrity than MCI-nc in the fiber connecting the precuneus and left MTL, and lower GM volume in bilateral MTL, but no differences in FC within the DMN.

Our results are in agreement with previous findings of atrophy in the MTL in AD patients, described since long (R. L. Buckner, 2005). Regarding progression from MCI to AD, our results coincide with previous evidence showing that MCI-c differ from MCI-nc in GM atrophy in medial temporal regions (Brueggen et al., 2015; DeCarli et al., 2007). Specifically, our results show that MCI patients that converted to AD in a period of two years or less had lower GM volumes in the MTL than those who did not convert. Moreover, GM volume of the MTL emerged as a marker with acceptable ability of discrimination between MCI-c and MCI-nc, as measured by their area under the ROC (Hosmer et al., 2013). We also found significant correlations between GM volume of bilateral MTL and global neuropsychological score in patients, which supports the relationship between atrophy of the MTL and general cognitive impairment.

We also provide evidence supporting findings of WM density loss in the DMN (Mito et al., 2018a) and reductions in the numbers of fibers connecting this network (Tucholka et al., 2018) in dementia, by showing that AD patients present lower WM integrity than healthy elderly participants between most of the nodes of the DMN as measured by all DTI measures. Crucially, we found that MCI-c presented lower integrity (higher RD) in the fiber of DMN that connects the precuneus and left MTL, the parahippocampal cingulum, which has been previously associated to episodic memory in MCI (C. Metzler-Baddeley et al., 2012). Previous evidence pointed towards the relevance of this fiber in conversion to AD: WM integrity in left hippocampus and left cingulate had been previously described as predictor of disease progression (Marcos Dolado et al., 2019) and FA reductions in MCI-c compared to healthy controls in the parahippocampal cingulum had also been found, but significant differences between MCI-c and MCI-nc in this tract were absent (Ito et al., 2015a). Therefore, we provide new evidence in favor of the relevance of the parahippocampal cingulum for conversion to AD. Notably, RD was the only DTI measure that emerged as sensitive to MCI progression in the parahippocampal cingulum, in agreement with previous evidence describing RD as the DTI metric that best discriminates between different stages of the disease (Becerra-Laparra et al., 2020). Furthermore, RD in the parahippocampal cingulum showed an acceptable discrimination ability between MCI-c and MCI-nc (Hosmer et al., 2013) and correlated significantly with global neuropsychological score in patients, further corroborating its contribution to disease status and general cognitive function. Also noteworthy, the discrimination ability of RD in the parahippocampal cingulum and GM in bilateral MTL combined turned out to be excellent (Hosmer et al., 2013), better than the acceptable discrimination capacities obtained for them separately and for the global neuropsychological score, which supports the relevance of these structural measures as markers of conversion from MCI to AD.

Finally, our data goes in line with previous evidence showing that AD patients present lower resting-state FC between the precuneus and left MTL (Binnewijzend et al., 2012; Greicius et al., 2004; L. Wang et al., 2006), since the tendencies towards FC differences found in our sample between these regions, although uncorrected, were driven by differences between healthy controls and AD. Importantly, we found no significant differences between MCI-c and MCI-nc in FC in the DMN. A recent meta-analysis on resting-state FC studies investigating DMN abnormalities in MCI found substantial inconsistency and low replicability in results, which led them to conclude that DMN resting-state connectivity does not qualify as a useful biomarker of disease progression or AD risk (Eyler et al., 2019), which could explain the lack of differences in FC between MCI-c and MCI-nc in our results.

An additional explanation for the lack of significant differences in resting-state FC when comparing our MCI groups might be the sample size of our investigation. Although it is larger than the ones used in most previous studies, according to a previous meta-analysis (Eyler et al., 2019), others affirm that sample sizes should exceed by far 100 in order to optimize replicability (Turner et al., 2018). Finally, another limitation of our study is the lack of tau or amyloid measurements, which are recommended by the National Institute on Aging and Alzheimer's Association as biomarkers for AD and MCI diagnosis in research, in an attempt to focus the definition of the disease on the pathological processes rather than the symptoms (Jack et al., 2018). Future studies should take on this approach if their objective is to characterize the sequence of pathological incidents that lead to AD.

In sum, our study shows that the structure of the DMN is widely altered in AD patients compared to controls. However, when measured at baseline, MCI patients that converted to AD during a 2 years follow-up and those who remained stable only differed in GM integrity of bilateral MTL and its left WM connections with the precuneus, with no differences in resting-state FC between the nodes of the DMN. Therefore, our results support the notion that GM abnormalities of medial temporal areas are key for disease progression, and adds new evidence regarding the relevance of WM integrity between the precuneus and left MTL for conversion from MCI to AD. Our evidence also supports the involvement of DMN abnormalities in dementia, but suggests that structural measures of GM and WM atrophy in the MTL could work better than connectivity of the DMN as indicators of conversion from MCI to AD.

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Supplementary material

Clinical criteria for probable AD and MCI diagnoses

The participants of the AD group met revised criteria for probable AD (McKhann et al., 2011) and showed a Clinical Dementia Rating (CDR) (Morris, 1997) score of 1 (mild AD). The inclusion criteria for the MCI group included 1) memory complaints, self-reported or confirmed by an informant; 2) objective memory impairment assessed with the logical memory subtest II from the Wechsler memory scale-III (WMS III) (Wechsler, 1997); 3) essentially intact activities in daily living; 4) no evidence of dementia; and 5) a score of 0.5 on the CDR. All MCI patients were type amnestic. The healthy control group included participants that had no memory complaints, showed no impairment in their performance on the neuropsychological assessment, and a CDR score of 0. Criteria that implied exclusion from the study were the following: (1) suffering from other nervous system diseases such as a brain tumor, cerebrovascular disease, encephalitis, or epilepsy, or meeting the criteria for other dementia different from AD; (2) a score higher than 6 on the Geriatric Depression Scale (Martínez de la Iglesia et al., 2002; Yesavage et al., 1982) (3) visible abnormalities in magnetic resonance images, such as leukoaraiosis and infarction, reported by an experienced radiologist; and (4) suffering from a current psychiatric disorder or using psychoactive medication.

MRI acquisition

Images were acquired on a 3T MRI scanner (Siemens Magnetom Trio, Erlangen, Germany), using a 12-channel head coil. Participants were placed in a supine position inside the scanner, and their heads were immobilized with cushions to reduce motion. Whole-brain 3-D images were collected using sagittal T1-weighted images (MPRAGE sequence, 176 slices, 256x256 matrix, TR=2300 ms, TE=2.98 ms, flip angle=9º, spatial resolution 1x1x1 mm). Axial diffusion tensor images (DTI) were acquired with an echo-planar imaging sequence (EPI) with 20 gradient directions, with the following scan parameters: TR = 10300 ms, TE = 104 ms, b0/b = 0/1000s/mm2, FOV = 256 mm, matrix = 128x128, flip angle = 90º, number of slices = 70, slice thickness = 2 mm, gap = 0mm. Finally, during the resting-state functional imaging acquisition, participants were instructed to just rest with their eyes closed, trying to let their minds go blank and not to fall asleep. A total of 270 volumes were collected over 9 min using a gradient-echo T2*-weighted echo-planar imaging sequence (TR=2000 ms; TE=30 ms; matrix, 64 x 64; FOV, 224 x 224 cm; flip angle, 90°; 33 slices, parallel to the hippocampus; slice thickness, 3.5 mm; slice gap, 0.5 mm).

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Study 6

Activity in Memory Brain Networks During Encoding Differentiates Mild Cognitive Impairment Converters from Non-Converters

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Abstract.

Alzheimer's disease (AD) has been associated with memory impairment due to alterations in the medial temporal lobe (MTL) and the precuneus. Therefore, the goal of this study was to investigate the effects of AD on the brain networks associated with the hippocampus and precuneus during an encoding memory task. 68 mild cognitive impairment patients (MCI), 21 AD patients, and 20 healthy controls (HC) were included. Participants were instructed to memorize landscapes while undergoing fMRI scanning, followed by a recognition test. MCI were followed up clinically for 18 months to track conversion status. Independent component analysis (ICA) was performed to investigate AD effects on precuneus and MTL networks during memory encoding. Behavioral analyses indicate that HC had a better performance than MCI converters (MCIc) and AD. ICA showed that MCIc had significantly higher activation in the MTL-associated network than MCI non converters (MCIn) and AD, including bilateral hippocampus, parahippocampus, and fusiform gyrus. Furthermore, the precuneus-associated network fitted the default mode network, showing a negative correlation with behavioral performance. These findings indicate that the hyperactivation of the hippocampal network displayed by MCIc has potential discrimination capacity to distinguish them of MCIn, and could be interpreted as a compensatory mechanism.

Keywords: Alzheimer's disease, hippocampus, independent component analysis, memory, precuneus

INTRODUCTION

Alzheimer's disease (AD) is a complex disease characterized by cognitive dysfunction, with memory impairment as the representative symptom. The relationship between this disease and life expectancy can be seen in the number of deaths attributed to AD, which increased by 71% between 2000 and 2013 [1]. Furthermore, the number of people who suffer from AD is expected to double in 20 years, reaching a total of 115.4 million affected people in 2050 [2, 3].

AD exhibits a general neuronal impairment that affects memory and other cognitive domains. Previous studies attributed the memory impairment to damage of the perforant pathway of the medial temporal lobe (MTL), leading to a disconnection between the entorhinal cortex and hippocampal structures [4–7]. However, recent models suggest that clinical symptoms of AD may reflect a dysfunction of distributed brain networks, rather than a region-specific neuronal impairment [6, 8]. Thus, as Grady et al. [9] indicated, it is expected that "memory and other

cognitive abilities are the result of the integrated activity in networks of regions, rather than activity in any one region in isolation". In recent years, neuroimaging studies have contributed to understanding the neurobiological alterations associated with memory impairment in AD. Several studies have consistently reported decreased functional magnetic resonance imaging (fMRI) activation in MTL regions in patients with AD, compared to older control subjects, during episodic encoding tasks with a wide variety of stimuli, such as name-face associations, geometric shapes, verbal stimuli, or scenes [10–17]. However, these studies focused on investigating segregated brain activations, ignoring how AD affects the underlying brain networks involved in memory encoding. More recently, AD research has paid special attention to the default mode network (DMN) because one of the main nodes of this network, the pre- cuneus/posterior cingulate, has been shown to be relevant in memory processes [18, 19]. The DMN includes the posterior cingulate, precuneus, lateral parietal lobe, and medial prefrontal cortex, and it is usually deactivated during memory encoding and other cognitively demanding tasks focused on processing external stimuli [20–22]. The DMN shows abnormal responses in the posterior cingulate and precuneus during a memory task in AD patients and high-risk (of AD) subjects. Namely, these sub- jects tend to manifest a paradoxical increase in fMRI activity above baseline [21–23], whereas healthy young people exhibit beneficial deactivations in these regions during memory tasks [24, 25].

Nevertheless, despite the remarkable advances in our understanding of the neurological alterations found in AD in the past two decades, at the time when a clinical diagnosis can be made, significant neuronal losses and irreversible neuropathological changes have already taken place [26]. Thus, it is crucial to work on developing early biomarkers that enable us to detect alterations in brain functions before the neuronal damage becomes irreversible, with the aim of successfully intervening in the course of the disease. For this reason, research on AD has shifted its focus to prodromal stages of AD, when mild cognitive impairment (MCI) patients are susceptible to developing AD [27–30]. The results obtained in studies on the brain activation associated with memory encoding in MCI patients vary consider- ably. Whereas some studies report a decreased fMRI activation in the MTL in MCI patients compared to older control subjects [31–34], other studies did not find any differences between these groups during memory encoding, although they reported a differential brain activation during memory recovery [35]. Furthermore, several studies reported an MTL hyper-activation in MCI patients compared to older control subjects during stimuli encoding [36–39]. In this regard, the increased activity in the MTL might be interpreted as a compensatory mechanism associated with the prodromal phase of AD. In this way, the previous findings may be reconciled if the activation throughout the course of AD has an "inverse U curve" shape, because MCI patients could be distributed across a continuum in terms of their degree of MTL activation [18].

In the present study, we assumed the involvement of the hippocampus and precuneus in the development of AD within the context which considers this pathology as a dysfunction in distributed brain networks. For this reason, we used independent component analysis (ICA) to investigate the effects of AD on the brain networks associated with these structures. Unlike previous studies using conventional general linear model (GLM) analysis, ICA may serve to reveal the hidden factors that underlie fMRI signals, allowing the study of the specific time course associated with a component (i.e., a functional net- work) separately from the signals associated to other components (i.e., other networks and/or artifacts). Thus, the analysis of task-related modulations in the time courses of the functional networks generated by ICA can provide new insights into the brain's functional organization that are not observed in conventional GLM analysis [40]. In this regard, we expected to identify the structures that work in a coordinated network with the precuneus and hippocampal areas during a memory encoding task. For the picture- encoding task selected for this study, strong bilateral activations have previously been reported in the tem- poral lobe [41, 42], especially in the parahippocampal formation, hippocampus, and fusiform and lingual gyrus. Furthermore, we investigated how AD impairs the activity of these networks. Based on the reviewed literature, we hypothesized that MCI patients would show a hyperactivation in these networks compared to other groups. We also expected that AD patients would display hypoactivation in the hippocampal net- work compared to healthy controls (HC) or MCI. Conversely, we expected that AD patients would not show proper deactivation of the DMN, compared to HC or MCI.

MATERIAL AND METHODS

Participants

The study included 109 participants organized in three groups: 68 MCI patients (mean age: 73.34±5.17, gender (M/F): 28/40) and 21 AD patients (mean age: 75±3.83, gender (M/F): 8/13), both recruited from dementia units of the Valencian Community Healthcare System, and 20 healthy older volunteers (mean age: 72.85±5.51, gender (M/F): 10/10). AD and MCI diagnoses were made by experienced neurologists and based on clinical and neuropsychological evidence. The AD group was composed of patients who met revised criteria for probable AD [43] and showed a Clinical Dementia Rating (CDR) score of 1 (mild AD). For the MCI group, the inclusion criteria included 1) memory complaints (self-reported or confirmed by an informant); 2) objective memory impairment assessed with the logical memory subtest II from the Wechsler memory scale-III (WMS III); 3) essentially intact activities in daily living; 4) no evidence of dementia; and 5) a CDR score of 0.5. Cognitively normal individuals were included in the HC group if they had no memory complaints, normal performance on the neuropsychological assessment (see below), and a CDR

score of 0. Participants were excluded if they had any of the following clinical characteristics: 1) other nervous system diseases such as a brain tumor, cerebrovascular disease, encephalitis, or epilepsy, or that they met the criteria for other dementias different from AD; 2) a Geriatric Depression Scale score ≥ 6 [44, 45]; 3) visible abnormalities reported by an experienced radiologist in magnetic resonance images, such as leukoaraiosis and infarction; 4) current psychiatric disorder or use of psychoactive medication. All participants underwent a structured clinical interview and a neuropsychological assessment, which included the Mini-Mental State Examination (MMSE) [46, 47], Functional Activities Questionnaire (FAQ) [48], short form of the Boston naming test [49], Digit subtest (forward and backward) from the WMS-III [50], Similarities subtests from the Wechsler adult intelligence scale-III (WAIS-III) [51], and logical memory subtests (I and II) from the WMS-III [50]. The MCI patients were followed up clinically with periodic neuropsychological assessments and clinical interviews every 6 months for one and a half years, although the MR data was acquired only once in the first clinical visit. The MCI patients who received an AD diagnosis during the follow-up period (within a period of 18 months after the fMRI recording) were classified as MCI converters (MCIc; N= 21), whereas patients who remained MCI after this period were classified as MCI non-converters (MICn; N= 28). Nineteen MCI participants did not complete the follow-up period and, consequently, were excluded from the analyses involving the longitudinal recording. Statistics for neuropsychological assessment and demographic data are presented in Table 1.

Participants were informed of the nature of the research, and they provided their written informed consent prior to their participation in the study. This research study was approved by the Institutional Review Board of the Jaume I of Castelló University. All the study procedures conformed to the Code of Ethics of the World Medical Association.

Table 1. Demographic, clinical and neuropsychological data of all participants.

Neuropsychological and demographic data	**HC**	**MCIn**	**MCIc**	**AD**	
N	20	28	21	21	**Differences**
Age	72.85±5.50	71.89±5.38	75.81±4.68	75.00±5.01	$F_{3,86}$= 3.21; p<0.05
Sex (men/women)	10/10	13/15	7/14	8/13	χ^2 = 1.53; p=0.68
MMSE	29.50±0.83	28.46±1.67	26.00±2.49	22.90±3.48	$F_{3,84}$= 32.93;p<0.001
FAQ	0.50±0.61	4.21±1.47	6.76±5.88	13.76±7.08	$F_{3,85}$= 30.22; p<0.001
Boston	11.95±0.22	9.64±1.16	8.57±1.78	7.38±3.67	$F_{3,85}$= 16.57; p<0.001
WMS-III: Digit subtest					
Forward	7.25±0.44	5.11±0.83	5.14±1.42	4.81±1.78	$F_{3,85}$= 19.15; p<0.001
Backward	6.20±0.95	3.68±0.55	3.24±0.89	2.57±1.40	$F_{3,85}$= 54.49; p<0.001
WAIS-III: Similarities subtests	17.75±2.31	13.28±2.12	9.43±3.40	6.95±4.19	$F_{3,85}$= 51.89; p<0.001
WMS-III: Logical memory subtests					
Short-term	13.35±1.60	10.79±2.41	8.85±3.59	3.55±2.91	$F_{3,84}$= 47.04; p<0.001
Long-term	11.20±2.46	8.36±1.97	6.85±3.04	2.05±2.31	$F_{3,84}$= 50.86; p<0.001
Memory task					
Hits percentage (%)	77.43±9.75	71.82±16.01	63.82±11.11	65.07±11.23	$F_{3,84}$= 3.37, p<0.05

HC = Healthy controls, MCIn = Mild Cognitive Impaired no-converter, MCIc = Mild Cognitive Impaired converter, AD=Alzheimer Disease

Experimental design and stimuli

The task was based on previous studies investigating memory function [37]. Participants performed a memory encoding task (see Fig. 1) while undergoing fMRI scanning. They had to memorize a total of 48 different landscape pictures displayed with VisuaStim goggles (Resonance Technology, Northridge,

CA, USA). The activation task consisted of two conditions that were alternated in 16 blocks: 1) Baseline (8 blocks): Subjects viewed a white fixation cross centered on a black screen for 18 s; 2) Encoding (8 blocks): Subjects viewed 6 different landscapes, each presented for 2.5 s and with an interstimulus interval of 500 ms; this block had a duration of 18 s. The total duration of the task was 288 s. The task was programmed and presented with the Presentation software (Neurobehavioral Systems, Albany, CA, USA).

Immediately after the fMRI scan, the participants performed a recognition test. It consisted of distinguishing the landscapes they had seen during the scanner presentation from other landscapes that were not presented during the fMRI scan. Target and non-target images were randomly selected from the same pool of landscapes prior to the study. Correct responses (hits) were recorded for further analysis.

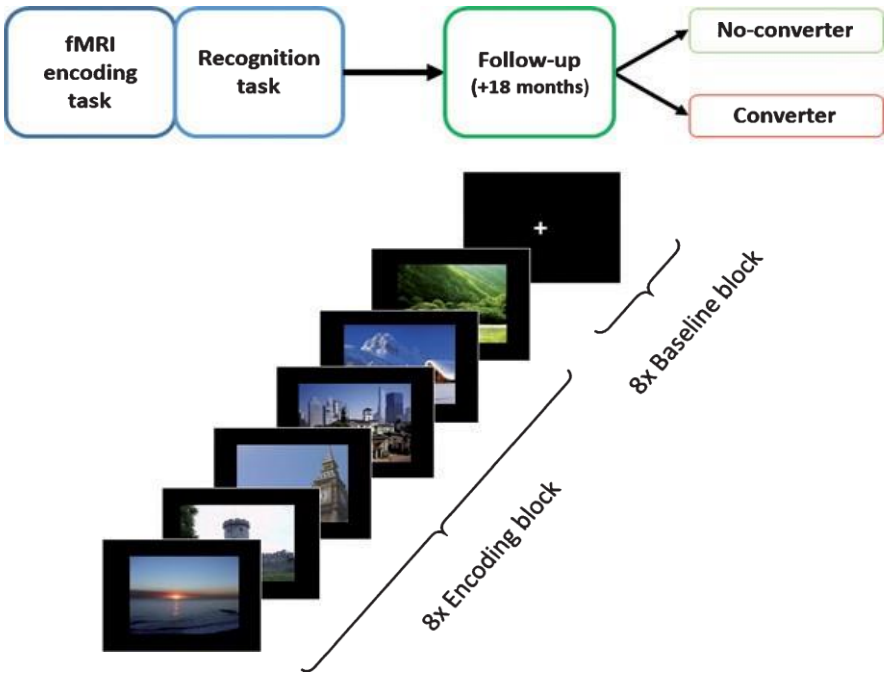

Fig. 1. Experimental procedure (top) and fMRI encoding task (bot- tom). The encoding task was composed from a total of 16 blocks (8 encoding blocks + 8 baseline blocks). While encoding blocks, participants had to memorize 6 different landscapes, each displayed for 2.5 s with an interstimulus interval of 500 ms.

Data acquisition

Images were acquired on a 3T scanner (Siemens Trio). Participants were placed in a supine position in the MRI scanner, and their heads were immobilized with cushions to reduce motion artifacts. For the fMRI, a total of 120 volumes were recorded over 4'8 min using a gradient-echo T2*-weighted echo- planar imaging sequence (TR = 2400 ms; TE = 30 ms; matrix, 64 x 64; voxel size, 3.8 x 3.8 mm; flip angle, 90◦; slice thickness, 3.5 mm; slice gap, 0.5 mm). We acquired 33 interleaved axial slices parallel to the anterior–posterior commissure plane covering the entire brain. Before the functional fMRI sequences, a high-resolution structural T1-weighted MPRAGE sequence was acquired (TR = 2300 ms, TE = 2.98 ms; flip angle 9◦, matrix = 256 x 256; voxel size = 1 mm).

Data processing

Functional MRI data were preprocessed using SPM12 (Wellcome Trust Center for Neuroimaging, London, UK). It included noise filtering (automatic detection and repairing bad slices) with the ArtRepair toolbox for SPM (http://cibsr.stanford.edu/tools/humanbrain-project/artrepair-software.html), realignment to correct for motion-related artifacts, spatial normalization into the standard Montreal Neurological Institute (MNI) space, and smoothing using a Gaussian kernel with

a full-width at half-maximum (FWHM) of 8 mm. Inspection of realignment parameters showed that none of the participants moved their heads more than 2 mm/degrees in any of the six directions during functional data recording.

Group independent component analysis

Group independent components analysis was performed using the GIFT toolbox (v3.0b, http://icatb.sourceforge.net) and the Infomax algorithm [52] to obtain functional networks that underlie fMRI data [53]. The group spatial ICA is performed on all the subjects at once and provides an independent component spatial map and a single associated ICA time course for every component, subject, and session. Significant between-group differences are determined by a second-level analysis of the ICA results. The objective of our study was to investigate between-group differences in the activity of the precuneus and hippocampus networks during memory encoding. Thus, we performed a GLM analysis in the components' time courses, estimated by ICA, to determine how the activity of the different brain networks was modulated by the experimental conditions. To study between-group comparisons, we used the beta-weights obtained from the GLM in second-level analyses. Fifty iterations of the ICA analysis were performed with the ICASSO software to ensure the stability of the estimated components. ICA dimensionality was set at 24 independent components based on minimum description length criteria [54]. Prior to the ICA, the intensity of images was normalized, and data dimensionality was reduced through a principal-components analysis (PCA). After ICA decomposition, individual independent component maps and time courses were computed using the GICA-3 back-reconstruction approach.

Component selection

In order to determine the functional networks comprising the hippocampus and precuneus, spatial correlations were run between the averaged spatial maps of each component and region of interest (ROI) of these structures. Probabilistic masks of the bilateral hippocampus and precuneus were extracted from subcortical and cortical Harvard-Oxford atlases, respectively. The components that showed the highest correlation with each ROI were selected for the subsequent analyses. After the identification of the components of interest, subject-specific spatial maps for these components were used to determine the regions belonging to the hippocampal and pre- cuneus networks through whole brain voxel-wise one-sample t-test analyses. Following previous studies [55, 56], the statistical threshold for these analyses was set at $p < 1 \times 10^{-13}$ FDR-corrected).

Statistical analyses

In order to study how the hippocampal and pre- cuneus networks were modulated during the task, a GLM, as implemented in the GIFT toolbox, was performed for each subject and component. The subject-specific time courses for each component of interest were set as dependent variable. The GLM design matrix included as independent variables a regressor defining the occurrence memory blocks and the six parameters that modeled residual motion. The task regressor was convolved with the canonical hemodynamic response function. Once the analyses had been performed, the beta-weights associated with the memory task were used to perform the second- level analyses.

Second-level analyses were conducted to study between-group differences in the activity of the hippocampal and precuneus networks. In these analyses, the averaged beta-weights for each group derived from the longitudinal classification (HC, MCIn, MCIc, and AD) were compared using a one-way ANCOVA as implemented in SPSS 23 (IBM Corp.), with age as covariate.

Voxel based morphometry

To complement the functional measures obtained using ICA analysis, we studied regional atrophy in our sample. Specifically, we performed a voxel-based morphometry (VBM) using CAT12 (Computational Anatomy Toolbox; C. Gaser, Jena University Hospital, Jena, Germany; http://dbm.neuro.uni- jena.de/cat/). We used standard VBM preprocessing which included bias correction of intensity non- uniformities, spatial normalization to MNI template using the Diffeomorphic Anatomic Registration Through Exponentiated Lie algebra (DARTEL) algorithm [57], tissue segmentation into grey matter (GM), white matter, and cerebrospinal fluid, modulation using the Jacobian determinants and smooth with an 8 mm full-width-at-half-maximum Gaussian kernel.

We investigated focal GM volume differences in a priori ROIs, specifically using the same hippocampus and precuneus from the Harvard-Oxford that we used to select the components of interest in the previous ICA analysis, but, in this case, with a minimum 50% probability threshold (see Fig. 4a). The GM volumes were obtained for each ROI and each subject from the modulated and smoothed images. Next, a one-way ANCOVA was performed using IBM SPSS Statistics 23 (IBM Corp) in order to compare GM volumes in target regions between the four experimental groups (HC, MCIn, MCIc, and AD), controlling by age and total intracranial volume (TIV).

Recognition test analysis

SPSS 23 (IBM Corp) was used to process the behavioral data. The percentage of correct responses during the post-scanner recognition test was used as the accuracy index. A one-way ANCOVA

[Group (HC, MCIn, MCIc, and AD); covariate: age] was con- ducted in order to assess potential differences among groups in the accuracy ratio. This analysis was followed by Bonferroni post-hoc tests. Additionally, we studied the relationship between the participant's performance and the activity of the components of interest. Specifically, we investigated whether the activity of the hippocampal and precuneus networks were positively and negatively correlated with the percentage of hits, respectively. To test these hypotheses, we performed partial correlations between beta-weights for each network of interest and the accuracy index, including age as covariate. Similar partial correlation analyses were performed to study the relationship between hippocampus and precuneus volumes obtained in VMB analysis and participants performance using age and TIV as covariates.

RESULTS

Neuropsychological results

As expected, neuropsychological results revealed statistically significant between-group differences. The MMSE scores distinguished: a) HC compared to the MCIc ($p < 0.001$) and AD groups ($p < 0.001$); b) MCIn compared to MCIc ($p < 0.01$) and AD ($p < 0.001$); and c) MCI compared to AD ($p < 0.001$). All the other neuropsychological tests revealed statistically significant differences ($p < 0.001$) between HC and each patient group (MCIn, MCIc, and AD). In addition, on these neuropsychological tests, a progressive performance impairment was observed across groups (HC>MCIn>MCIc>AD). Addition- ally, the MCIn group showed statistically significant better performance than the AD group ($p < 0.01$) on the FAQ, Boston, Backward digits, Similarities, and Logic Memory (short and long-term) tests. Finally, AD had statistically significant worse performance on the Logic Memory tests, compared to MCIc ($p < 0.01$).

Independent component analysis results

Spatial correlations revealed that C6 ($r = 0.147$) was the component that correlated most with the hip- pocampus, and C19 ($r = 0.40$) was the component that correlated most with the precuneus. As Fig. 2a and Table 2a show, the hippocampal network included the bilateral hippocampus, parahippocampus, lingual gyrus, fusiform gyrus, cerebellum (lobules IV and V), and cerebellar vermis (lobules III, IV and V). The one-way ANCOVA revealed significant differences between groups in the activity of this network during the task ($F3,85 = 3.38$, $p = 0.022$). As Fig. 2b shows, Bonferroni post-hoc analysis revealed that MCIc patients have a significantly higher activation ($p < 0.05$) in this network than MCIn and AD patients. As Fig. 3a and Table 2b show, the precuneus net- work matched the key regions of the DMN, and included the right precuneus, bilateral angular gyrus,

midcingulate gyrus, middle temporal gyrus, mid- dle frontal lobe, superior frontal lobe, cerebellum (lobule VI), vermis (lobules VI and VII), superior temporal pole, and left orbitofrontal medial. The one- way ANCOVA revealed no significant differences between groups in the activity of this network during the task (F3,85 = 0.64, p = 0.577; see Fig. 3b).

Finally, given that our groups presented differences in age, we replicated the analyses investigating between-group differences in network activity, but using a subsample of participants matched on this variable. The matching was carried out with the MatchIt function implemented in R Project for Statistical Computing and using the "nearest" method. This procedure provided four matched groups of 20 participants each (HC = 72.85 ± 5.509, MCIn = 72.25 ± 5.004, MCIc = 75.45 ± 4.501, AD = 74.80 ±3.820; F(3,76) = 2.075, p = 0.111). The analyses using this subsample revealed similar results that the analyses with the whole sample: MCIc patients showed a greater activity compared to MCIn patients (t38 = 2.146, p < 0.05) and also compared to AD patients (t38 = 2.224, p < 0.05) in the hippocampus network. Furthermore, no significant differences were found in the precuneus network.

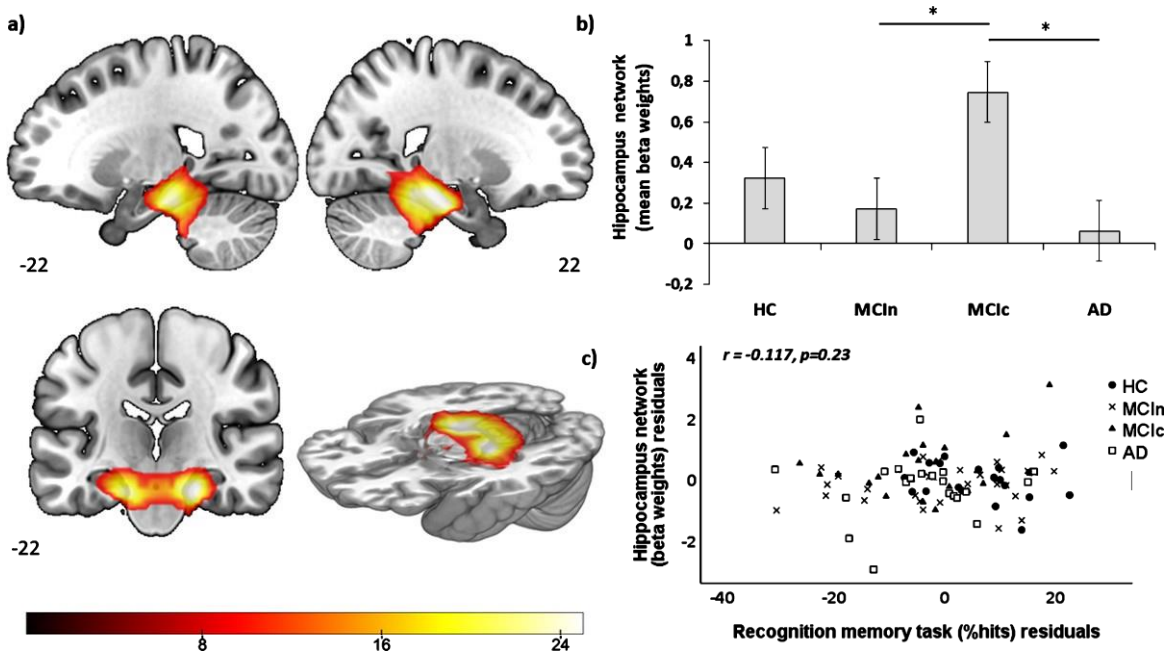

Fig. 2. a) Hippocampal network spatial map. Results FDR-corrected at p <1 10–13; k = 30. Colored bars express t-scores. b) Between-group differences in hippocampal network activity during fMRI task (memory encoding > baseline). MCIc showed a significant hyperactivation compared to MCIn and AD patients. c) Non-significant correlation between hippocampal network activity and hits on the recognition memory task covariate by age.

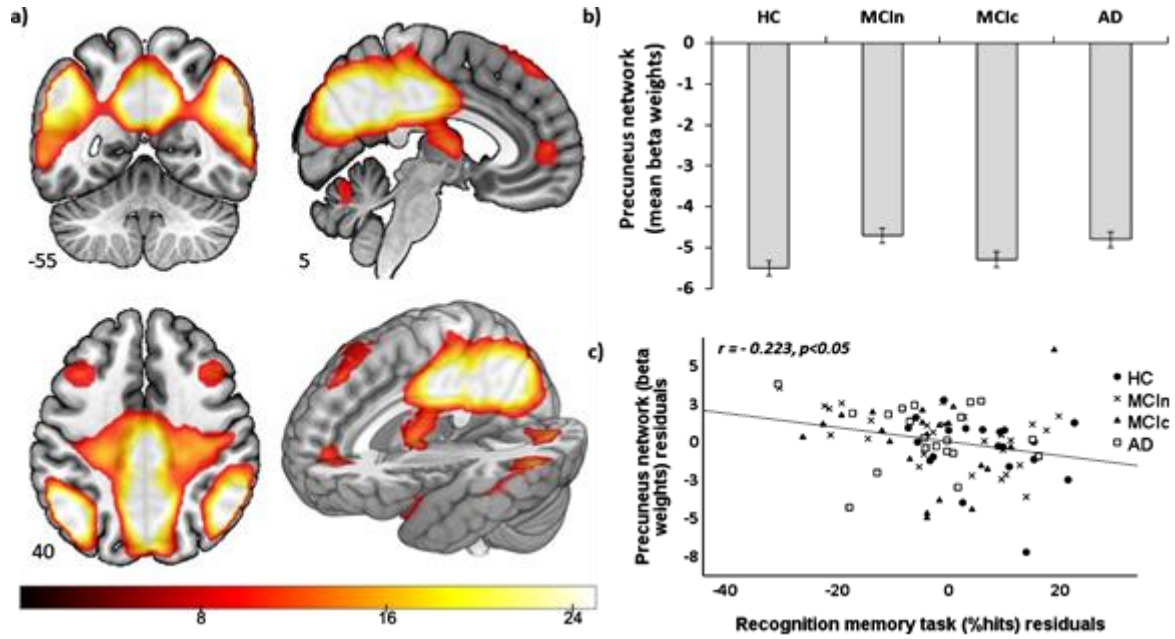

Fig. 3. a) Precuneus network spatial map. Results FDR-corrected at p <1 10–13; k = 30. Colored bars express t-scores. b) Non-significant between-group differences in precuneus network activity during fMRI task (memory encoding > baseline). c) Negative correlation between precuneus network activity and hits on the recognition memory task covariate by age.

Table 2. Brian regions belonging to hippocampus and precuneus networks ($p < 1\times10^{-13}$ FDR-corrected, k = 30).

Regions	R/L	BA	MNI coordinates			Voxels number	T	K size
			x	*y*	*z*			
a) Hippocampus Network (Component 6)								
Cerebellum, lobules IV, V	L	30	-15	-31	-19	276	29.10	*2638*
Parahippocampus	L	30	-15	-31	-16	120	29.08	
Parahippocampus	R	30	18	-25	-19	151	28.08	
Fusiform gyrus	L	30	-15	-34	-16	90	27.58	
Lingual gyrus	L	27/30	-12	-34	-10	117	26.00	
Cerebellum, lobules IV, V	R	30	18	-34	-16	217	25.55	
Fusiform gyrus	R	30	21	-37	-16	90	24.66	
Lingual gyrus	R	30	18	-37	-13	161	23.39	
Vermis, lobule III	-	-	6	-37	-13	62	22.67	
Hippocampus	R	30	24	-22	-16	72	21.90	
Hippocampus	L	30	-15	-28	-10	94	20.66	
Vermis, lobule IV, V	-	-	-3	-49	-22	106	18.78	
b) Precuneus Network (Component 19)								
Precuneus	R	23	6	-55	35	595	39.47	*9929*
Angular gyrus	R	39	51	-55	38	381	37.67	
Angular gyrus	L	39	-51	-64	32	349	36.61	
Midcingulate gyrus	R	23	0	-22	35	398	33.98	
Midcingulate gyrus	L	23	-3	40	38	384	32.14	
Middle temporal gyrus	R	21	60	-52	11	586	22.08	
Middle temporal gyrus	L	21	-63	-37	-7	569	18.80	
Middle frontal lobe	R	9	42	20	44	147	13.88	*315*
Superior frontal lobe	R	8	21	26	59	67	12.47	
Middle frontal lobe	L	9	-42	17	47	225	18.18	*258*
Superior frontal lobe	L	8	-15	26	62	30	10.41	
Cerebellum Crus1	L		-33	-67	-34	60	14.66	*161*
Cerebellum, lobule VI	R	18	15	-67	-25	25	10.88	
Vermis, lobule VI			0	-73	-16	26	9.97	
Cerebellum, lobule VI	L	18	-9	-64	-19	27	9.69	
Vermis, lobule VII			3	-70	-25	11	9.31	
Orbitofrontal medial lobe	L	10	-9	47	-4	46	12.29	*153*
Superior medial frontal lobe	R	10	3	50	4	18	11.69	
Superior temporal pole	L	38	-36	14	-22	81	15.70	*116*
Superior temporal pole	R	38	39	17	-25	32	12.01	*38*

R/L = Right/Left, BA = Brodmann Areas, MNI = Montreal Neurological Institute, Voxel number = Number of voxels of each structure into the cluster, K size = cluster size measured by voxels.

Table 3. Regional atrophy, GM-volume between groups differences ($p < 0.001$, FWEc corrected).

Between-group differences	R/L	K size	MNI coordinates			Z value	T value
			x	y	z		
a) HC > MCIn							
Fusiform	L	1041	-32	-18	-26	4.02	4.24
Hippocampus	L		-33	-23	-17	3.53	3.69
Parahippocampus	L		-24	6	-24	3.48	3.62
Amygdala	L		-29	0	-27	3.17	3.28
b) HC > MCIc							
Inferior temporal lobe	L	1267	-62	-33	-20	4.81	5.19
Middle temporal lobe	L		-59	2	-29	3.99	4.21
Fusiform	L	2002	-30	3	-39	4.33	4.61
Amygdala	L		-26	3	-23	3.92	4.13
c) HC > AD							
Superior temporal pole	R	5046	27	8	-23	5.17	5.64
Hippocampus	R		24	-11	-14	4.34	4.61
Fusiform	R		27	-35	-15	4.26	4.56
Inferior orbitofrontal	R		26	27	-15	4.21	4.46
Parahippocampus	R		35	-26	-17	3.88	4.07
Inferior temporal	R		35	9	-42	3.80	3.98
Middle temporal pole	R		30	14	-33	3.71	3.88
Superior temporal pole	L	5304	-26	5	-23	5.00	5.43
Fusiform	L		-27	-33	-18	4.92	5.33
Inferior temporal	L		-32	5	-39	4.42	4.72
Hippocampus	L		-18	-35	0	4.56	4.89
Parahippocampus	L		-30	-30	-12	4.55	4.87

R/L = Right/Left, MNI = Montreal Neurological Institute, K size = cluster size measured by voxels

Voxel based morphometry results

One-way ANCOVAs revealed statistically significant differences across groups in GM hippocampus volume ($F_{3,82} = 6.938$, $p < 0.001$), but not in GM precuneus volume ($F_{3,82} = 2.016$, $p = 0.118$). Specifically, the main differences in GM hippocampus volume were found between HC and MCIn patients ($p < 0.05$), HC and MCIc patients ($p < 0.05$), and HC and AD patients ($p < 0.001$). Overall, these results showed hippocampus atrophy in patients compared to HC (see Fig. 4b).

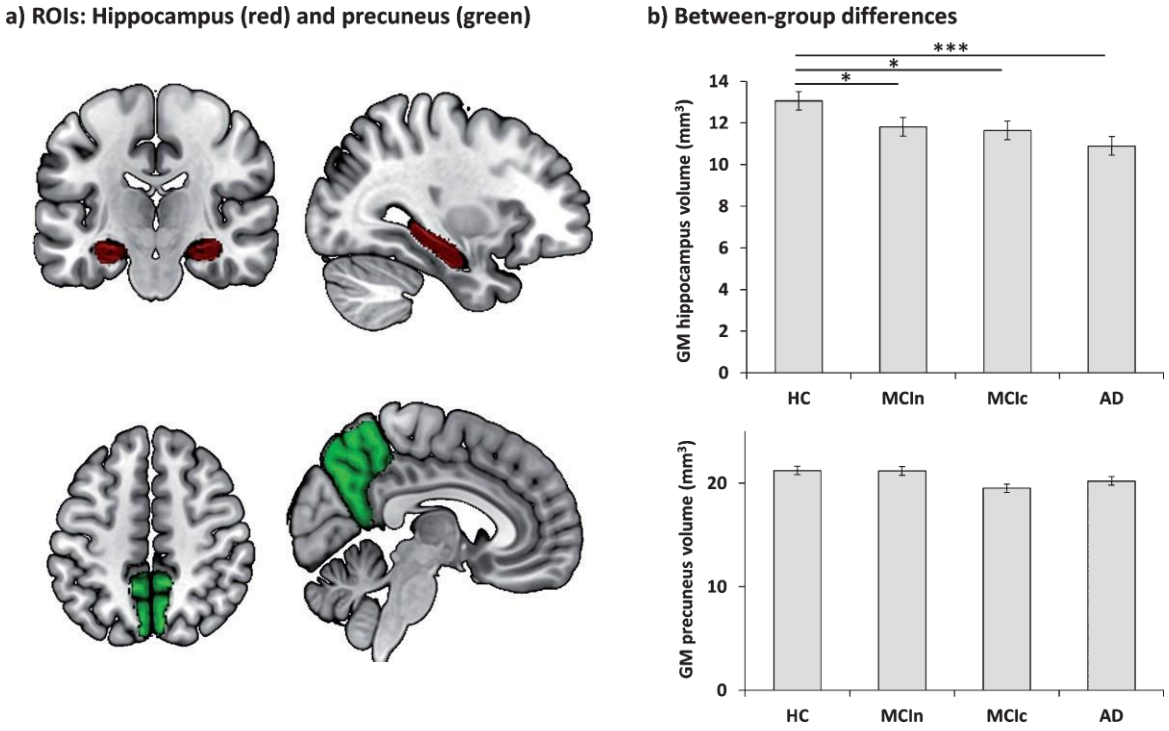

Fig. 4. a) Hippocampus (red) and precuneus (green) ROIs taken from the Harvard-Oxford atlas used for VBM analysis. b) Between-group differences in GM hippocampus volume ($F_{3,82} = 6.938$, $p < 0.001$) and GM precuneus volume ($F_{3,82} = 2.016$, $p = 0.118$).

Recognition test results

Task performance of each group was measured by the mean of the percentage of hits: HC = 77.43 ± 9.75%; MCIn = 71.82 ± 16.01%; MCIc = 63.82 ± 11.11% and AD = 65.07 ± 11.23%. The one-way ANCOVA revealed a statistically significant difference between groups ($F_{3,83} = 3.37$, $p < 0.05$).

Additionally, as Fig. 2c shows, the activity of the precuneus network showed a negative correlation with participants' performance ($r = –0.223$, $p < 0.05$). No significant correlations were observed between the hippocampal network and accuracy ($r = 0.117$, $p = 0.23$). Furthermore, a significant positive correlation was found between GM hippocampus volume and participants' performance ($r = 0.385$, $p < 0.001$).

DISCUSSION

The present fMRI research focused on studying the underlying networks associated with the key regions related to memory impairments in AD: hippocampus and precuneus [21–23, 58–60]. To accomplish this, we performed an ICA analysis and obtained the functional connectivity networks associated with these structures, along with their specific temporal courses during a memory encoding task (landscape encoding versus fixation). Additionally, we performed a VBM analysis to obtain complementary brain atrophy measures. On the one hand, our results showed that the hippocampal network revealed differential activation between groups; specifically, MCIc patients showed hyperactivation in this network compared to MCIn and AD patients. On the other hand, the pre- cuneus network matched the key regions of the DMN, and its activity was negatively correlated with task performance. Additionally, we found GM volume differences between HC and all the patient groups (MCIn, MCIc, and AD) in the hippocampus. Over- all, our findings support the hypothesis that DMN deactivation is relevant in memory encoding, and they suggest that hippocampal network activity may serve to detect MCI subjects at risk of conversion to AD.

Classically, the MTL has been the key region in the search for predictive biomarkers during the prodromal phase of AD. It has been studied from a volumetric perspective, where we can find lost hippocampal volume to be an early biomarker of AD in subjects with MCI [61–64]. Moreover, functional research has associated AD patients with an MTL hypoactivation compared to healthy older controls [10–17]. Regarding MCI, patients' results have been heterogeneous, and several studies have associated MCI patients with increased MTL activation during a memory task when compared to healthy-older controls. To explain this, Sperling et al. [65, 66] proposed the inverse-U hypothesis, which argues that MTL activation could be course-dependent, such that MTL activation increases as degeneration of this region begins, acting as a compensatory mechanism. The increasing activity continues until reaching a breaking point when MTL degeneration is too severe, making a compensatory mechanism impossible. A recent meta-analysis [67] collected different studies that compared brain activity during episodic memory tasks in healthy older controls, MCI patients, and AD patients, using fMRI. This study concluded that HC had significantly higher activation in the hippocampus than AD patients, whereas MCI patients showed a significant hyperactivation in the cerebellum and lingual gyrus compared to HC. The regions reported in this meta-analysis corresponded to the hippocampal network obtained in the present study, which included the cerebellum (lobules IV and V), lingual gyrus, parahippocampus, vermis (lobules III, IV and V), hippocampus, and fusiform gyrus. The ICA procedure followed in our study enables the estimation of the specific temporal signal associated with this network. Thus, our study adds to the previous literature by demonstrating that: 1) these regions make up a functional network and 2) that

this network shows a hyperactivation in MCIc patients during memory encoding. Our findings do not reveal significant differences between HC and MCIn or between HC and AD patients; nevertheless, the differential hyperactivation shown by the MCIc group is able to discriminate between "low-risk" (MCIn) and "high-risk" (MCIc) MCI patients. Following previous research, hyperactivation of the MTL during the task is predictive of greater brain atrophy [37, 39, 67, 68]. Additionally, event-related fMRI studies [38, 39, 69] found that this hyperactivation occurred specifically on successful trials; therefore, Sperling suggested that "the increased activity may serve as a compensatory mechanism in the setting of early AD pathology" [18].

As expected, the precuneus network matched the key regions of the DMN, including the precuneus, bilateral angular gyrus, and medial regions of the prefrontal cortex. The pattern of activity of this net- work is inconsistent with our hypothesis that MCI and AD patients would show less hypoactivation than HC in this network. Nevertheless, the activation pat- tern showed a significant negative correlation with the participants' performance; that is, the worse the performance, the greater the activation. As we have seen in the recognition test results, MCIc and AD patients obtained significantly less accuracy than HC, but these groups do not show a differential activation in the DMN. This finding supports the idea that AD patients show a decline of the beneficial deactivation of DMN regions during task performance, which has been previously associated with better performance on memory tasks [21, 70–72].

As a complementary analysis, we investigated volumetric measures of hippocampus and precuneus in our sample. Previous research indicates that hippocampus atrophy may serve as an early biomarker of AD in subjects with MCI [61–64]. In this study we found significant higher GM hippocampal volume in HC than MCIn, MCIc, and AD. Furthermore, the volume of hippocampus correlated with the percentage of hits of the recognition task. However, we did not find significant differences between our MCIn and MCIc groups. Taken together, the results of this study may have clinical implications. In our sample, hippocampus volume was able to discriminate between patients and controls, while hippocampus network activity was able to discriminate between MCIn and MCIc. These results suggest that the hyperactivity of the hippocampus network may be a potential marker in detecting individuals at risk of conversion to AD, at least in the following 18 months after recording. By contrast, this measure would not be sensitive enough to discriminate between HC and early AD. Given the inverse-U shaped pattern, HC and early AD may have similar activation indices. In this regard, the study of hippocampus volume would be a better measure, given that it follows a linear descending pattern. Finally, our study is limited in the use of a fixation cross as a baseline condition. A scrambled image rather than a black screen with a white fixation cross would have been more appropriate to disentangle pure vision effects from landscape encoding.

In conclusion, our findings show differential hip- pocampal network activity between MCI patients at “high-risk” of developing AD and “low-risk” MCI patients. Specifically, we found that “high-risk” MCI patients present a hyperactivation in this network which is in line with the assumption of a compensatory mechanism within this stage of AD. Furthermore, although no significant differences in activation were found between groups, the pre- cuneus network was inversely related to participants’ performance, which means that participants with greater precuneus-network deactivation obtained better results on the memory task.

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GENERAL DISCUSSION

The general aim of this thesis was to investigate the neural basis of bilingualism and dementia and its implications for cognitive reserve. To do so, we looked at differences in brain volume, integrity of WM and FC at rest between bilinguals and monolinguals (in our case, *passive bilinguals*) in a prodromal dementia stage, MCI. We also investigated the neural underpinnings of different dementia stages, and the differences between prodromal patients that eventually developed AD and those who did not convert, by measuring brain volume, WM integrity, resting-state FC, ReHo and fALFF and precuneus and MTL network activation during an encoding task. Finally, we also studied the dynamic effects of degree of bilingualism in GM volumes of a healthy sample of participants.

Our cross-sectional findings support previous evidence by showing that, in a sample of bilinguals and monolinguals/*passive bilinguals* with MCI that suffered from the same amount of cognitive impairment, bilinguals showed general reduced brain volume compared to monolinguals. When investigating regionally, reduced GM volume in right supramarginal and left lingual gyrus, reported as affected by AD and MCI in previous meta-analyses (Yang et al., 2012). This is in consonance with the idea that bilinguals require more neuropathology in order to present the same clinical manifestation of the disease than monolinguals, which had been previously shown in AD patients (Perani et al., 2017; Schweizer et al., 2012), but, to the best of our knowledge, never in a prodromal dementia stage. Bilinguals also presented lower WM integrity in the fornix, a fibre crucial to episodic memory that is typically impaired in different dementia stages (Aggleton et al., 2000; Bubb, Kinnavane, & Aggleton, 2017; Ibrahim, Young, & Larner, 2009; Mao et al., 2018; Xu & Ponce, 2018). However, bilinguals also showed higher integrity than monolinguals in the PH cingulum, a WM tract that is also part of the hippocampal–diencephalic-cingulate network and is more related to memory based on contextual cues and familiarity (Bubb et al., 2017), and in the UF, a WM tract related to verbal and auditory memory (Von Der Heide, Skipper, Klobusicky, & Olson, 2013). Importantly, WM integrity in the PH cingulum correlated with immediate recall performance only in bilinguals, although this result did not reach significance when correcting for multiple comparisons. A similar pattern had been previously observed in a study comparing healthy controls and patients with MCI, in which patients showed higher atrophy in the fornix and PH cingulum, but free recall correlated with integrity in the fornix only in controls, while in patients, the strongest relationship was found with WM integrity in the PH cingulum (Claudia Metzler-Baddeley et al., 2012). This was interpreted as a compensatory mechanism by which the PH cingulum may support memory performance through familiarity-based memory processes, once the fornix is impaired.

Following up on this interpretation, our results support the hypothesis that bilinguals present more pathology in AD-related regions, in this case expressed in less WM integrity in the fornix, and that there may be a compensation in order to maintain the same cognitive level than monolinguals and support memory processes, which might be expressed in terms of WM integrity in the PH cingulum and UF. Finally, we also found higher resting-state FC in bilinguals compared to monolinguals between the pSTG of the language network and precuneus of the DMN, which correlated with general cognitive status for the whole sample, and higher fALFF in the thalamus. This can be interpreted as a further mechanism by which bilingualism compensates for impaired memory processes and protects against dementia. These results validate previous findings obtained using metabolic measures to infer connectivity between brain regions (Perani et al., 2017), and suggest that functional compensatory effects of bilingualism against dementia rely on connections between regions of the language network and the DMN.

Crucially, our longitudinal results show that, during a 6 to 9 month follow-up, bilinguals with MCI presented a slower global cognitive decline than monolinguals/passive bilinguals, suggesting that the protective effect of bilingualism against dementia is sustained up until early stages of AD. This also fits in with previous reports of a delay in the onset of the disease in bilinguals compared to monolinguals (Alladi et al., 2013; Bialystok et al., 2007; Calabria et al., 2020; Craik et al., 2010; Perani et al., 2017; Woumans et al., 2015) – if both groups present a similar level of cognitive impairment in prodromal stages, but the decline of monolinguals is faster, they will fulfil behavioural criteria for the disease before bilinguals. Additionally, bilinguals showed a significantly slower longitudinal brain volume loss compared to monolinguals, which also goes in line with the longitudinal maintenance of the neuroprotective effect of bilingualism against dementia. In spite of that trend, they still presented lower volume than monolinguals after the follow-up, which coincides with previous cross-sectional evidence in AD samples (Schweizer et al., 2012).

Linking our results with the concept of cognitive reserve (Steffener & Stern, 2012), we provide evidence supporting the protective effect of bilingualism against dementia and its two underlying mechanisms: neural reserve and neural compensation. Neural reserve, expressed as resilience against pathology of brain networks used for the cognitive function of interest, manifests itself in our MCI sample as higher atrophy in bilinguals in AD-related regions and fibers (i.e., fornix, supramarginal and lingual gyri), but the same level of cognitive impairment than monolinguals, and less longitudinal atrophy and cognitive loss. Neural compensation, in turn, is evidenced in the MCI bilinguals of our sample by higher WM integrity in alternative circuits related to memory (PH cingulum and UF), higher FC between regions of the DMN and the language network (precuneus

and pSTG) and higher fALFF in the thalamus, showing that bilinguals implicate alternative regions and networks in order to compensate for pathology.

The fact that bilingual experience has these consequences for brain atrophy and function could be related to the processes that it entails: from increased control demands (Abutalebi & Green, 2016) to other specific language processes, such as articulation, switching, and semantic and morphosyntax processing. These processes are dependent on specific brain regions that coincide with circuits relevant for memory and AD, such as subcortical, temporal and parietal regions and their connections (Sala et al., 2021). Thus, the increased demands that bilingualism poses on the brain induce plastic changes on these networks, complementarily providing bilinguals with cognitive reserve against dementia, and allowing them to maintain the same level of cognitive function than monolinguals when suffering from more AD pathology (Sala et al., 2021). Our results go in line with this explanation, since the mechanisms of cognitive reserve of our MCI bilinguals were found to be located in regions related to both bilingual experience and memory processes. For instance, the left pSTG is part of the language network (Shirer, Ryali, Rykhlevskaia, Menon, & Greicius, 2012) and shows increased connectivity with a region of the DMN, the precuneus, in the MCI bilinguals of our sample compared to monolinguals. The thalamus, part of the extended memory system (Aggleton et al., 2016) and expanded in young bilinguals compared to monolinguals (Burgaleta, Sanjuán, Ventura-Campos, Sebastian-Galles, & Ávila, 2016) also showed more fALFF in our MCI bilinguals than in monolinguals. Finally, the fibers that show more WM integrity in the bilinguals of our sample have both been proposed to be related to memory - familiarity-based for the PH cingulum and verbal and auditory for the UF (C. Metzler-Baddeley et al., 2012; Von Der Heide et al., 2013) – and to connect regions relevant for bilingualism (Abutalebi, Canini, Della Rosa, Green, & Weekes, 2015; Mechelli et al., 2004). All these findings support the compensatory effects of bilingualism against AD brain pathology by means of involvement of alternative brain circuits.

It is noteworthy that the monolinguals in our sample could be considered *passive bilinguals*, that is, they understand both languages and have been exposed to them since long, even if they only actively use one of them (Calabria et al., 2020). Thus, the main difference between the experience with languages of an *active* and a *passive* bilingual (monolingual) of our sample is the *use* they make of both languages. This is very relevant for the interpretation of our results, and grants them special interest and uniqueness: the observed protective effect of bilingualism could be more related to the lifelong active use of two languages than to proficiency, age of acquisition or other bilingualism factors. However, we did not measure any of those factors in our sample, and although we can make general inferences about them from the social and demographic characteristics of our sample, this

interpretation is still speculative. This might also explain the differences between our results and a previous study carried out with another sample of bilinguals and monolinguals with MCI (Duncan et al., 2018), in which monolinguals should probably not be considered *passive bilinguals*, and supports the relevance of awareness of type of bilingualism when carrying out research and comparing bilingualism studies.

In order to investigate the neural characteristics of dementia, we also explored the differences between AD patients, MCI patients who eventually converted to AD, MCI patients that remained stable, and healthy elderly controls, in GM volume, WM integrity, resting-state FC and network activity during an encoding task. We focused our analyses on brain regions that had previously proved to be especially affected in AD – the DMN and its connections (Mohan et al., 2016; Mormino et al., 2011). As expected, there were significant differences between AD patients and healthy controls in cognitive impairment, GM volume of bilateral MTL and WM integrity of all fibers connecting the regions of the DMN. Values of these measures for MCI patients fell between healthy controls and AD patients, which goes in line with our hypotheses and the expected progressive cognitive and brain deterioration that happens along the AD continuum (Binnewijzend et al., 2012; Chandra et al., 2018; Mito et al., 2018; Tucholka et al., 2018; Yang et al., 2012). Regarding differences between patients that progressed to AD and those who remained stable during the follow-up, we found that MCI converters showed lower GM volume in left MTL, WM integrity in the PH cingulum and hyperactivation of the MTL network when performing an encoding task than non-converters. These results coincide with previous evidence emphasizing the relevance of the MTL in conversion (Brueggen et al., 2015; DeCarli et al., 2007; Ito et al., 2015; Marcos Dolado et al., 2019). Interestingly, the WM tract that marks conversion to AD according to our data, is the same fiber where we found higher WM integrity in bilinguals with MCI compared to monolinguals, which further supports the relevance that bilingualism could have as a protective factor against the development of dementia. Finally, we found no significant differences in FC neither between AD patients and healthy controls nor between MCI converters and non-converters, supporting the idea that connectivity between regions of the DMN might not be an adequate marker of conversion to AD (Eyler et al., 2019).

Finally, in order to better understand the dynamic effects of bilingualism on the healthy brain, we explored GM volume changes as a function of bilingualism score, using an approach that accounted for non-linear changes. We found a significant linear positive relationship between bilingualism and GM volume of putamen and cerebellum, which corroborates previous findings (Abutalebi et al.,

2013; Burgaleta et al., 2016; Filippi et al., 2011; Pliatsikas, DeLuca, Moschopoulou, & Saddy, 2017; Pliatsikas, Johnstone, & Marinis, 2014) and is congruent with the increase in control for grammatical and motor programmes that bilingualism entails (Filippi et al., 2011; Garbin et al., 2010; Hervais-Adelman, Moser-Mercer, Murray, & Golestani, 2017; Pliatsikas et al., 2014; Simmonds, Wise, Dhanjal, & Leech, 2011). We also found a significant relationship between bilingual experience and GM volume in the IFG, following a non-linear pattern of decreases in the lower and higher parts of the bilingual scores, but an increase in the middle of the continuum. These results coincide with previous proposals for cortical volume changes due to bilingualism, characterized by initial increases and later renormalization as bilingual experience increases (Grundy et al., 2017; Pliatsikas, 2020), because the participants situated at the lower end of the bilingualism scores could be considered *passive bilinguals* (Calabria et al., 2020) and their IFG volumes might have already increased before the initial renormalization found in our results. This also coincides with the reduced delay in the appearance of efficiency and cortical renormalization proposed for bilingual environments of high diversity and intensity of language use (DeLuca et al., 2020). In general, these results provide further evidence supporting the non-linearity of brain structural changes as a function of bilingual experience. The absence of any significant effect of bilingualism on the supramarginal gyrus in this study is also of interest, since this region showed increased volume in monolinguals compared to bilinguals in our investigation with a sample of MCI patients. This suggests that the effects of bilingualism on the volume of this region might only appear in interaction with AD status.

To summarize, the main conclusions of the studies included in this thesis are the following:

1. Cross-sectionally, bilinguals with MCI, at the same level of cognitive impairment as monolinguals/passive bilinguals, showed higher brain atrophy in right supramarginal gyrus and left lingual gyrus. Longitudinally, in a brief 6-month follow-up, bilinguals with MCI showed less decline than monolinguals in brain atrophy and cognitive functions.
2. Bilinguals with MCI also showed lower WM integrity in the fornix than monolinguals, but higher WM integrity in the PH cingulum and UF, at the same level of cognitive impairment.
3. Bilinguals with MCI presented higher FC between the pSTG, a region of the language network, and the precuneus of the DMN.
4. There were significant differences between healthy controls, MCI and AD patients in cognitive impairment, GM volume of bilateral MTL and WM integrity of the DMN, with a general pattern of increased abnormalities as a function of disease progression. MCI converters differed from

non-converters in that they showed lower GM volume of the MTL bilaterally and lower WM integrity of the PH cingulum, connecting the left MTL and precuneus.

5. MCI converters showed hyperactivation in the brain network associated to the MTL during an encoding task when compared to non-converters and AD patients.
6. In a healthy sample, there were linear increases in putaminal and cerebellar volumes as a function of bilingualism, as well as a non-linear relationship (S-shaped) between IFG volume and bilingualism score.

As a general conclusion, the ensemble of our studies suggests that the neural underpinnings of the protection of bilingualism against dementia is the usage of alternative circuits - the structural and functional connections of the MTL, DMN and language network – as a neural compensation mechanism, in the presence of higher structural atrophy than monolinguals in fibers and regions related to AD pathology, such as the fornix and supramarginal and lingual gyrus. This protection seems to start in prodromal disease stages and be sustained longitudinally until AD dementia starts. Moreover, the alternative MTL connection used as a compensation in bilinguals, the PH cingulum, coincides with the one especially relevant for conversion from MCI to AD. Finally, a brain region that shows different affectation in MCI depending on bilingualism status, the right supramarginal gyrus, does not show volumetric changes in our study with healthy participants, suggesting specific interactions between bilingualism and dementia that would only appear when the mechanisms of the disease are already in motion.

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