Original Article

Aging Effects on Whole-Brain Functional Connectivity in Adults Free of Cognitive and Psychiatric Disorders

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Abstract

Aging is associated with decreased resting-state functional connectivity (RSFC) within the default mode network (DMN), but most functional imaging studies have restricted the analysis to specific brain regions or networks, a strategy not appropriate to describe system-wide changes. Moreover, few investigations have employed operational psychiatric interviewing procedures to select participants; this is an important limitation since mental disorders are prevalent and underdiagnosed and can be associated with RSFC abnormalities. In this study, resting-state fMRI was acquired from 59 adults free of cognitive and psychiatric disorders according to standardized criteria and based on extensive neuropsychological and clinical assessments. We tested for associations between age and whole-brain RSFC using Partial Least Squares, a multivariate technique. We found that normal aging is not only characterized by decreased RSFC within the DMN but also by ubiquitous increases in internetwork positive correlations and focal internetwork losses of anticorrelations (involving mainly connections between the DMN and the attentional networks). Our results reinforce the notion that the aging brain undergoes a dedifferentiation processes with loss of functional diversity. These findings advance the characterization of healthy aging effects on RSFC and highlight the importance of adopting a broad, system-wide perspective to analyze brain connectivity.

Key words: brain, elderly, functional magnetic resonance imaging, normal aging, resting state

Introduction

Resting-state functional connectivity (RSFC) has been increasingly used in the investigations of the effects of aging on the brain (Mevel et al. 2011; Grady 2012). Functional MRI (fMRI) acquired while the subject is not performing any specific task (resting state) is a feasible and ubiquitous method that has yielded critical results such as the frequently described age-related decrease in RSFC within the default mode network (DMN) (Damoiseaux et al. 2008; Hafkemeijer et al. 2012; Ferreira and Busatto 2013).

Aging is known to show complex changes that differentially affect multiple brain regions and systems (Eyler et al. 2011;
Goh 2011; Grady 2012). However, most fMRI studies investigating the aging effects on RSFC have focused on specific networks (Ferreira and Busatto 2013). Results yielded by analyses of limited anatomical scope such as those investigating changes only within the DMN (Koch et al. 2010; Wang et al. 2010) or focusing on specific brain networks or regions such as the language system (Antonenko et al. 2012), subcortical regions (Ystad et al. 2010) or the motor system (Wu, Zang, Wang, Long, Hallett et al. 2007; Wu, Zang, Wang, Long, Li et al. 2007) should be interpreted in light of this feature. In contrast, broader explorations can provide novel insights by describing patterns of change that otherwise are missed (Deco et al. 2011).

For instance, whole-brain functional connectivity studies have suggested that aging influences connectivity patterns not only in the DMN and but also in attention networks, sensory-motor systems and subcortical regions (Tomasi and Volkow 2012; Chan et al. 2014; Geerligs et al. 2015). Moreover, these studies have raised awareness to the importance of exploratory whole-brain investigations: They documented that age-related changes in connectivity are heterogeneous across different brain networks and showed that both increases and decreases in connectivity can span multiple brain systems.

Previous work has provided some evidence that aging has an impact not only on within-network connectivity but also on the integration and segregation between different brain networks (Ferreira and Busatto 2013). When estimating integration and segregation using functional connectivity, it is important to understand that 2 brain regions can present positive or negative correlations between their neural activities (the latter are also described as “anticorrelations”). Positive correlations are much more commonly reported than anticorrelations but both are important for functional specialization, cognitive development and between-network integration (Uddin et al. 2009; Hawellek et al. 2011; Bonnelle et al. 2012; Barber et al. 2013; Chai et al. 2014). These aspects are very relevant in this field because there is evidence that aging leads to a dedifferentiation process, characterized by a decrease in functional specificity of multiple brain systems (Park and Reuter-Lorenz 2009; Goh 2011), and this might lead to a decreased density of anticorrelations (Geerligs et al. 2015). Notwithstanding, the decision to include or exclude anticorrelations during the analysis process is still not homogenous across different studies; for instance among whole-brain investigations, a number of authors have excluded negative correlations (Tomasi and Volkow 2012; Chan et al. 2014). Because others have shown that there are age-related effects both on negative and positive correlations (Meier et al. 2012; Geerligs et al. 2015), including the whole range of functional connectivity values is a relevant aspect in the investigation of the aging brain.

In this study, we used a multivariate approach to characterize age-related changes in whole-brain RSFC in a sample of adults from different age groups—young adults, middle aged, and elderly. Our predictions were 2-fold: We expected to identify decreases in within-network coherence, especially, within the DMN but also age-related markers of loss of differentiation between different networks as indicated by increases in positive correlations and decreases in anticorrelations.

One critical limitation of previous studies of aging effects on RSFC is that few used a combination of neuropsychological assessment and structured psychiatric interviews to identify undiagnosed psychiatric conditions or mild cognitive impairment. This is clearly relevant because cognitive and psychiatric disorders are highly prevalent in the general population (Ferri et al. 2005; Steel et al. 2014) and are associated with abnormalities in RSFC (Greicius 2008; Seeley et al. 2009). Accordingly, we used a careful and active investigation to select a sample free of cognitive deficits and lifetime psychiatric disorders.

Also important is the removal of the potentially confounding effects of the ε4 allele of the apolipoprotein E gene (APOE) due to its influence on RSFC (Reinvang et al. 2013). However, this has been rarely performed in RSFC involving adults from different age groups. In this study, we identified ε4 carriers in order to perform the analysis before and after removing the effect of APOE ε4 on RSFC data.

**Methods**

The Ethics Committee of the Medical School of University of São Paulo approved this project (protocol 026/11) and subjects provided informed consent to participate.

**Inclusion and Exclusion Criteria**

We selected participants with age between 18 and 25, 40 and 55, and 65 and 75 years. They had to have at least an incomplete undergraduate course (+11 years of education), their native language had to be Portuguese, and they had to be right-handed (at least +70 on the Edinburgh Inventory) (Oldfield 1971). All participants had to be able to perform all activities of daily living independently and to be active members of the community. Participants were excluded if they presented any lifetime diagnosis of psychiatric disorder (with the exception of isolated specific phobia), cognitive deficits, MRI contraindications, brain structural abnormalities, history of head trauma with loss of consciousness, current pregnancy, or current severe medical conditions.

**Recruitment, Selection, and Assessment of Participants**

Participants were recruited through printed and electronic online announcements. Screening of potential participants was performed using online forms, questions via email, telephone, or personal contact. During this nonstructured preliminary assessment, we investigated sociodemographic characteristics, handedness, MRI contraindications, memory complaints, medical conditions, and history of psychiatric diagnosis and treatment. We only scheduled the clinical assessments for participants who reported no previous history of psychiatric disorder, no lifetime use of drugs to treat psychiatric conditions, and did not present significant memory complaints.

Participants were assessed with the Structured Clinical Interview for DSM Disorders (SCID) (First et al. 2002). The neuropsychological assessment included tests to evaluate attention, memory, language, frontal-executive functions, speed of information processing, and intelligence quotient. The tests were as follows: vocabulary and matrix from the Wechsler Adult Intelligence Scale, digit span (forward and backward), verbal fluency (letters FAS and categories: animals and male names), logical memory (Wechsler Memory Scale third Edition), Wisconsin Card Sorting Test (WCST), Color Word Interference Test, the Short Cognitive Performance Test (SKT) (Lefebvre and Erzigkeit 1997; Flaks et al. 2006), and the mini-mental state examination (MMSE) (Folstein et al. 1975).

We excluded participants presenting current or past psychiatric diagnosis and/or cognitive deficit (cognitive performance of <1.5 standard deviation from the expected average or below the percentile 10 in any cognitive domain or MMSE score of <26). The assessment was interrupted at the moment we identified any exclusion criteria.
After screening 3832 volunteers, we performed 138 face-to-face assessments of potential participants; 54 were excluded and 84 were deemed eligible to the MRI session. Data from the 59 participants who completed the MRI session and did not fulfill any exclusion criterion were included in the present analysis (19 young adults, 20 middle aged, and 20 elderly). The sample selection process is illustrated in Figure 1.

Cognitive Scores

The raw score of each neuropsychological test was transformed to z-score (z = (raw – mean)/standard deviation). In some of the cognitive tests, higher scores indicate worse performance (as in tests in which the raw score is equal to the time to complete the task). In those cases, given that the raw test score was negatively associated with cognitive performance, the signal of the z-score was inverted (i.e., multiplied by −1) so that we could sum the final z-scores to obtain composite scores.

We calculated sets of composite cognitive scores using 2 approaches.

Firstly, we performed an unrotated principal components analysis on Matlab (version R2014a; The MathWorks, Inc.) of all cognitive tests listed earlier. We selected the first 4 components, which represented the majority of variance of the data (62%). The first component (33% of the variance) represented the overall cognitive performance; based on this component, we used the loadings of each cognitive test as weights to calculate a weighted general cognitive score from the normalized scores from the tests. The second component (12% of the variance) was heavily influenced by performance on the 3 items of the WCST (number of perseverative mistakes, number of categories and failure to maintain sets); thus, we summed the z-scores of the 3 WCST-items into a single WCST score. The verbal memory tests from the Wechsler Memory Scale were strongly associated with the third component (9% of the variance), and therefore we built a verbal memory score by summing the z-scores of these 2 tests. Finally, the fourth component (8% of the variance) was mostly influenced by the digit span tests (forward and backward); thus, we calculated a digit span score by summing the normalized scores from both tests. The detailed results from principal component analysis are presented in Supplementary Figure 1.

In the second approach, we calculated 4 composite scores based on 4 theoretical cognitive domains (attention, executive function, processing speed and memory) by summing the z-scores of the individual tests (Grimm and Grimm 1993; Van der Elst 2006; Andrews-Hanna et al. 2007). The tests that were included to calculate the composite scores of each theoretical domain were as follows:

1. processing speed: picture naming—SKT, number naming—SKT, number ordering—SKT, number reordering—SKT, symbol counting—SKT, Trials 1 and 2 of the Color Word Interference Test
2. memory: immediate and delayed recall of the logical memory test—Wechsler Memory Scale 3rd Edition; immediate and delayed recall—SKT
3. attention: forward digit span
4. executive function: backward digit span, letter reverse naming—SKT, Trials 3 and 4 of the Color Word Interference Test, WCST: number of categories, perseverative errors and failure to maintain sets; verbal fluency: letters FAS and categories.

Apolipoprotein E Genotyping

We collected blood sample (10 mL) to perform DNA extraction for APOE genotyping. DNA was extracted from peripheral blood by...
Imaging

The MRI session was scheduled in <1 month after the clinical and neuropsychological assessments. Resting-state fMRI was acquired from 59 participants using a Philips Achieva 3T scanner at the Institute of Radiology of the Faculty of Medicine, University of São Paulo. We used a T2*-weighted echo planar imaging sequence with the following parameters: TE 30 ms, TR 2000 ms, flip angle 80°, field of view 240 × 240, matrix 80 × 80, slice thickness 4 mm (voxel size 3 × 3 × 4 mm), number of slices 31–32, gap 0.5 mm, Sense 2.5, Softtone 3.7—aquatic noise reduction (Rondinoni et al. 2013). Participants were told to keep their eyes open looking at a fixation cross. During 6 min and 48 s, we acquired 204 volumes; the first 4 volumes were discarded so that we had 200 volumes per subject. During the image acquisition, we used 4 electrocardiogram electrodes affixed to the subjects’ chest and a respiration band placed at the abdomen to collect signals from the heart beats and respiratory movements (sampled at 500 Hz). The resting-state fMRI was performed immediately after the reference scan and, on the day of MRI, no cognitive tasks or tests were administered before the MRI session.

An anatomical 3D T1-weighted scan of the whole brain was acquired immediately after the resting-state fMRI acquisition. The T1 parameters were as follows: TR 7 ms, TE 3.2 ms, flip angle 8°, Sense 1.5, field of view 240 × 240, matrix 240 × 240, 180 slices of 1 mm each with no gap, yielding a voxel size of 1 × 1 × 1 mm.

Preprocessing


We removed confounds due to cardiac pulsation, respiratory cycle, fluctuations in heart rate, and respiration volume using the respiratory and electrocardiogram data collected during fMRI acquisition as input to AZTEC; then, using AFNI, we performed slice timing correction and motion correction—all subjects presented <2 mm (translation) and <3 degrees (rotation).

The fMRI normalization procedure began using the anatomical T1-weighted images, which underwent intensity uniformization using AFNI to aid spatial registration, and manual skull stripping by experienced technicians using MRcro (http://www.mccauslandcenter.sc.edu/mricro/). Using AFNI, the skull-stripped images were then: coregistered to the subject’s 100th fMRI volume (mid-run volume) and linearly and nonlinearly normalized to match AFNI’s T1 MNI template. Finally, the concatenated linear and nonlinear transformations were applied in 1 step to the entire fMRI volumes of each subject to complete the normalization.

We then regressed out motion parameters estimates (estimated at the motion correction step) and signal from 4 voxels in the deep white matter and 4 cerebrospinal fluid voxels (at the anterior and posterior horns of the lateral ventricles) using Matlab. After that, we performed despiking, temporal filtering (at 0.01–0.08 Hz), and spatial smoothing (6 mm at FWHM) with AFNI. Finally, motion censoring was performed as described by Power et al. (2012) and based on the AFNI’s implementation (afni_restproc.py). Motion censoring is used to remove frames presenting both high-motion indices and excessive changes in BOLD signal, suggesting a strong influence of noise due to motion. Briefly, frame-wise head displacement (based on the motion parameters estimates) and root mean square intensity change of BOLD signal across the whole brain were calculated. Cut-off limits for frame-wise displacement and whole-brain BOLD change were set at 0.5 and 0.5%, respectively, as suggested by Power et al. (2012). Frames whose indices exceeded both these thresholds were marked; frames 1 back and 2 forward from these marked frames were also marked for censoring. The marked frames were removed from the final preprocessed images (Power et al. 2012).

Processing

We used a parcellation atlas built by an independent group of researchers with the aim of maximizing the functional homogeneity within each brain subunit during resting state (Shen et al. 2013) available at http://www.nitrc.org/frs/?group_id=51. We used this publicly available atlas with a parcellation scheme that partitions the brain into 278 spatially contiguous regions. The average time series from each of the regions was extracted using AFNI. For each subject, we calculated the correlation coefficient between the time series of every possible pair of regions and then performed Fisher r-to-z transformation with Matlab. Thus, for each subject, we estimated the values of 38 503 resting functional connections.

Confounding and Moderating Variables

Motion effect is considered an important confounding variable in studies of functional connectivity and aging (Power et al. 2012). In addition to the preprocessing steps described earlier (regressing out motion parameters estimates from the BOLD signal and motion censoring of compromised frames), we also removed linear effects of mean frame-wise displacement from connectivity data in all analyses by regressing it out from functional connectivity data.

Aging is known to be associated with changes in BOLD signal variability (Grady 2012; Garrett et al. 2013), and such age-related increased variability may affect patterns of age-related connectivity changes (Ricciardi et al. 2013). In order to take account of such confounding influence, we calculated for each subject the mean BOLD signal variability (i.e., the standard deviation of the BOLD signal) across the 278 brain regions. In all analyses, we used this mean variability score to remove the linear effects of mean BOLD variability from functional connectivity data.

Finally, the relationship between age (or cognitive performance) and functional connectivity may be also mediated or moderated by APOE genotype and overall atrophy resulting in reduced brain size (Sheline et al. 2010; Ferreira and Busatto 2013; Reinvang et al. 2013; Geerligs et al. 2015; Marstaller et al. 2015). Therefore, we repeated the analyses after controlling for each of these 2 variables (by regressing out linear effects of

salting-out (Lahtinen et al. 1994). Two single-nucleotide polymorphisms (SNPs) (rs7412 and rs429358) were evaluated in order to determine the APOE genotype using real-time polymerase chain reaction (PCR) SNP genotyping systems (TaqMan® Assays—Life Technologies), as follows: TaqMan PCR Mastermix 1×/µL, TaqMan SNP genotyping assay 1×/µL, genomic DNA 10 ng/µL, and ultrapure water to complete a 7 µL volume were mixed in each well of an optical plate. Allelic discrimination was evaluated in a 7500 Real-Time PCR system (Life Technologies) comparing fluorescence levels before and after amplification (45 cycles of 15 s at 95°C and 1 min at 60°C) (Forlenza et al. 2010). We classified participants as ε4-carriers and noncarriers (binary variable), as commonly performed in the literature (Reinvang et al. 2013).
each variable from the connectivity data). To estimate overall brain tissue volumes, the skull-stripped anatomical T1 images were segmented into gray and white matter using FSL’s FAST (Oxford’s Functional MRI Software of the Brain Library, Automated Segmentation Tool) (Zhang et al. 2001). The sum of gray and white matter volumes afforded the index of overall brain tissue volume that was used to control for atrophy effects.

Statistical Analysis
Age was treated as a continuous variable in the analyses of its relationship with cognitive performance and functional connectivity, unless otherwise stated.

To characterize the relationship between functional connectivity and the clinical variables (age and cognitive performance), we used a multivariate technique, partial least squares (PLS) implemented in Matlab (http://www.rotman-baycrest.on.ca) (McIntosh et al. 1996; Krishnan et al. 2011). In PLS, data are centered and normalized and then a matrix of correlation between the clinical and the neuroimaging data is computed. For instance, to characterize the associations between age and brain connectivity, a matrix of correlations between age and all brain connections was calculated in this step. The next step is the singular value decomposition (SVD), which is a generalization related to eigen decomposition (Abdi and Williams 2013). With SVD, the aims are to extract signals from noisy data sets (Wall et al. 2003) and to transform the original data (which often contains highly intercorrelated variables) into a set of orthogonal factors that best reconstitutes the decomposed data (McIntosh et al. 2004; Krishnan et al. 2011; Abdi and Williams 2013). The matrix of correlations is decomposed by SVD into 3 matrices: a matrix of singular values, a matrix of the saliences for the behavioral variables (behavioral saliences) and one of the saliences for the brain data (brain saliences). Latent variables are calculated by projecting the original matrices containing the brain and the clinical data onto their respective saliences (Calculated by SVD). Mathematically, this procedure is the dot product of the matrix containing the data (either brain related or the clinical variables) by the matrix containing the respective saliences (Krishnan et al. 2011). In particular, brain scores are the dot product of the neuroimaging data (in this case, functional connectivity) and its respective saliences. The brain scores reveal how strongly each individual expresses the pattern of brain connectivity extracted from the data by the SVD procedure (McIntosh et al. 2004; Krishnan et al. 2011; Abdi and Williams 2013).

Statistical significance was tested with a permutation procedure (10,000 permutations), which resulted in a distribution of singular values (significance threshold was set at \( P<0.05 \)). The assessment of statistical significance in PLS is performed through the permutation test and is applied at the level of the patterns extracted by SVD, not at the level of each single brain connection. Therefore, no correction for multiple comparisons is required (McIntosh and Lobaugh 2004).

To test the reliability of the results, we used a bootstrap procedure, that is, sampling with replacement (10,000 bootstrap samples). PLS analysis was repeated for each bootstrap sample. This procedure results in a distribution of correlations between the clinical variables and the brain scores. This distribution was used to assess the reliability of the brain-clinical variables correlation (McIntosh et al. 2004). If the 95% confidence interval did not contain 0, the correlation was considered reliable. Moreover, the bootstrap procedure results in a distribution of brain saliences; bootstrap ratios are computed by dividing the mean of the bootstrapped distribution by its standard deviation, and we considered reliable those with bootstrap ratio of \( \geq 3 \). This step was performed to determine which connections showed effects that are stable across multiple subsamples, thus protecting against outliers. It does not involve assessment of statistical significance so no corrections for multiple comparisons are necessary (such assessment is performed once through the permutation test) (McIntosh and Lobaugh 2004).

The primary focus of this study was to characterize the relationship between age and functional connectivity, and the main PLS analysis was performed to test for such association. We also performed additional analyses to test for associations between connectivity and cognitive performance as estimated by the weighted general cognitive score, the 3 groups of scores identified on principal component analysis (WCST-score, verbal memory score, and digit span score), and the 4 theoretical cognitive scores (attention, processing speed, executive function, and memory) – see section Cognitive scores.

Classification of Age-related Findings
In order to better characterize the patterns of age-related changes in functional connectivity, we classified the connections presenting significant association with age. For each significant connection, we checked whether functional connectivity was positively or negatively correlated with age and calculated the mean connectivity among the young subjects and the mean among the elderly. With this information, age-related increases in connectivity were classified as follows: 1) increased magnitude of positive correlation, 2) shift from negative to positive correlation or 3) decreased magnitude of negative correlation, whereas decreases were classified as 4) decreased magnitude of positive correlation, 5) shift from positive to negative correlation, or 6) increases in magnitude of negative correlation.

Anatomic and Network Labels
Each brain region received an anatomical label based on the widely used Automated Anatomical Labeling atlas (available at http://www.gin.cnrs.fr/spip.php/article2178lang=en%20n%20200146604) (Tzourio-Mazoyer et al. 2002). The anatomical label given to each brain region was the name of the region from the Automated Anatomical Labeling atlas containing the largest number of voxels.

In addition to the anatomical labels, we wished to take into account previously described brain networks. Thus, we categorized each of the 278 regions as pertaining to 1 of the 7 networks identified by Yeo et al. (2011). For each brain region, the network label was based on the network with the largest number of voxels in accordance with the parcellation atlas from this study (available at http://surfer.nmr.mgh.harvard.edu/swiki/CorticalParcellation_Yeo2011).

Results
Sample Characteristics
Figure 1 shows the details about the sample selection process. It should be noted that 28% of the 138 volunteers with no history of cognitive or psychiatric disorder presented, at the face-to-face
assessments, cognitive deficits or psychiatric conditions. The active investigation of psychiatric disorder using the SCID (First et al. 2002) was especially important for the selection of the elderly sample: 24% of the elderly presented a psychiatric condition that led to the exclusion from the study (Fig. 1).

The histogram on Figure 2 shows the age distribution of the sample, and Table 1 describes the characteristics of each age group. The groups are similar regarding sample size, gender distribution and number of APOE e4 carriers. The sample presented high educational level due to our inclusion criteria (established a priori). The middle-aged and elderly subjects had more years of high educational level due to our inclusion criteria (established a priori). The middle-aged and elderly subjects had more years of high educational level due to our inclusion criteria (established a priori). The middle-aged and elderly subjects had more years of high educational level due to our inclusion criteria (established a priori). The middle-aged and elderly subjects had more years of high educational level due to our inclusion criteria (established a priori).

Relationship between Age and Functional Connectivity
We identified a pattern of brain functional connections with significant age-related changes in RSFC ($p = 0.0165$; 95% confidence interval of the correlation between age and the connectivity pattern: $0.50–0.75$; [bootstrap ratio] $> 3$). Of the 1665 brain connections included in this pattern, 1648 (99%) presented a positive correlation between age and RSFC whereas only 17 (1%) were characterized by a negative association with age.

We classified the age-related increases and decreases and found that most connections presented age-related increases in the magnitude of positive correlation (95%). The second most common type of connections were those presenting shifts from negative to positive correlation as age increases (4%). There were few connections characterized by decreases in the magnitude of positive correlation (1%). Figure 3 shows the number of connections and an example of each type of connection presenting age-related significant changes.

To visualize the location of each significant connection, we displayed this result on a square matrix (Fig. 4). When classifying and ordering the regions in accordance with brain hemispheres and anatomical location, we could not identify a clear pattern (Fig. 4, left lower triangle). On the other hand, after using an atlas of brain networks (Yeo et al. 2011) to classify and order the regions, we could observe that connections presenting shifts from negative to positive correlations clustered at the intersections between: 1) the DMN and the dorsal attention network and 2) the DMN and the ventral attention network. (Fig. 4, upper right triangle).

In order to better characterize the relationship between brain networks and each type of age-associated change in RSFC, we used the software Circos—www.circos.ca (Krzyszkowski et al. 2009)—to display a circular ideogram representing brain networks and ribbons as brain connections. The pattern of brain connections presenting age-related changes in RSFC was predominantly characterized by: 1) diffuse increases in the magnitude of positive correlations and 2) focal shifts from negative to positive correlation—these focal changes involved mainly the DMN, especially, its relationship with the attention networks (Fig. 5).

Intra- versus Internetwork Connections
Internetwork connections were, by far, the most common among those showing age-related changes (92.7% of all connections presenting age-related changes). Because of the whole-brain atlas we used to parcellate the brain (Shen et al. 2013) and the combinatorial nature of massive region-based pairwise connectivity analyses, the number of possible internetwork connections was much higher than the number of intranetwork pairs (33,254 internetwork versus 5249 intranetwork possible pairs of regions). Nevertheless, the number of internetwork connections presenting significant age-related change represented 4.6% of the number of internetwork investigated, whereas significant intranetwork connections corresponded to 2.3% of all intranetwork pairs (Table 2).

Connections presenting decreases in the magnitude of positive correlation were special because they comprised a significant number of intranetwork connections (53%), and this was not observed in the other types of age-related changes: All other contained a small proportion (7% or 3%) or no intranetwork connections (Fig. 6). The internetwork connections presenting decreases in the magnitude of positive correlation were located mainly within the DMN (78%).

Controlling for APOE and Brain Atrophy
We found a similar pattern of age-related changes after removing the effects of the APOE e4 allele from RSFC data by residualization ($p = 0.0027$; 95% confidence interval of the correlation between age and the connectivity pattern: 0.51–0.78; [bootstrap ratio] $> 3$) — see Supplementary Figures 2–5.
After controlling for the effects of age and connectivity approached but did not reach statistical significance \( (P = 0.0788; 95\% \text{ confidence interval of the correlation between age and the connectivity pattern: } 0.47 – 0.76) \). There were fewer connections showing a reliable association \( (|\text{bootstrap ratio}| \geq 3) \), but the overall pattern of age-related changes was similar to the one found before controlling for brain atrophy: diffuse increases in the magnitude of positive correlations (mainly internetwork) and focal loss of anticorrelations (mostly between the DMN and the attentional networks) – see Supplementary Figures 6–9.

Finally, we tested for associations between brain tissue volume and functional connectivity using PLS and we did not identify significant results \( (P = 0.3697) \).

**Relationship between Cognitive Performance and Functional Connectivity**

We did not find any significant pattern of RSFC associated with overall cognitive performance \( (P = 0.44) \). No significant association was found between connectivity and the set of 3 other composite cognitive scores derived from principal component analysis \( \text{WCST score, verbal memory score, and digit span score; } P = 0.35) \). We also tested for associations between connectivity and performance on the set of 4 theoretical cognitive domains \( \text{(attention, memory, processing speed, and executive function)} \), but no significant pattern emerged either \( (P = 0.32) \). Results remained not significant after removing linear effects of age from both RSFC and behavioral data by residualization \( (\text{for RSFC x general cognitive performance: } P = 0.12; \text{RSFC x 3 scores from principal component analysis: } P = 0.36; \text{RSFC x the set of 4 theoretical cognitive domains: } P = 0.07) \). Removing the effect of APOE ε4 allele from RSFC data also did not affect the results \( (\text{for RSFC x general cognitive performance: } P = 0.42; \text{RSFC x 3 scores from principal component analysis: } P = 0.34; \text{RSFC x the set of 4 cognitive domains: } P = 0.32) \). After controlling for the effects of brain atrophy, the results remained not significant \( \text{RSFC x general cognitive performance: } P = 0.42; \text{RSFC x 3 scores from principal component analysis: } P = 0.36; \text{RSFC x the set of 4 cognitive domains: } P = 0.33) \.

**Post hoc Analyses**

After controlling for the effects of age on cognitive performance, we divided participants in high- and low-performing participants...
and, using PLS, we could not characterize a difference in functional connectivity between these 2 groups ($P = 0.3444$).

We also compared ε4 carriers to noncarriers and could not identify between-group differences in connectivity ($P = 0.3642$).

**Discussion**

In a sample of adults free of cognitive or psychiatric disorders, we characterized the pattern of age-related changes in whole-brain RSFC. The main features of this pattern were as follows: 1) diffuse internetwork increases in positive correlations, 2) focal loss of anticorrelations, and 3) decreases in intranetwork connectivity.

### Diffuse Increases in Positive Correlation

Age-related increases in the magnitude of positive correlations were profuse and affected mainly internetwork connections (Fig. 5). This finding has been reported before in a number of brain regions and networks such as frontoparietal attention networks, somatomotor, and somatosensory systems (Ferreira and Busatto 2013). However, ours is the first study to report such increases in such a ubiquitous fashion. One previous fMRI study that identified extensive increases in connectivity associated with aging used a classifier approach (support vector machine) to discriminate young adults from elderly individuals using RSFC data (Meier et al. 2012). The authors...
found that >31% of the “weight” used by the classifier was derived from connections showing increases in the magnitude of positive correlations. The majority (63%) of the weight from these connections originated from pairs of regions from different networks, reinforcing the notion that age-related increases in the magnitude of positive correlations are especially present between different networks.

A recent whole-brain RSFC study also identified increases in positive correlations in an elderly sample when compared with a sample of young adults (Geerligs et al. 2015). The authors of this study employed graph theory metrics and found that the participation coefficients were increased in the elderly—another evidence that the elderly present greater internetwork connectivity (participation coefficient is an index of the number of between module connections in relation to the total number of connections of a node). In that study, there was a predominance of the visual system among the connections presenting between-network increases in the magnitude of positive correlations. In the present investigation, we also identified a strong contribution from the visual network (Fig. 5).

Meinzer et al. (2013) investigated the effects of transcranial direct current stimulation and identified widespread increases in positive correlations when an elderly group which received sham treatment was compared with a young (control) group. Interestingly, active electrical stimulation over the left inferior frontal gyrus not only improved the cognitive performance of the elderly group but also partially reversed the age-related changes in RSFC (Meinzer et al. 2013).

We found widespread age-related increases in the magnitude of positive correlations between different networks. Others, using graph theory metrics, have shown that these can lead to less differentiated brain modules and even to a merging between networks that are known to be functionally independent in young adults (Geerligs et al. 2015). Two other studies that applied graph-theoretic analyses to investigate age-related changes in RSFC also found that aging is associated with loss of segregation between different networks, with the older participants showing an
increased number of connections between different functional brain systems or modules (Chan et al. 2014; Song et al. 2014). These findings are consistent with the dedifferentiation hypothesis of aging (Park and Reuter-Lorenz 2009) and reinforce the notion that fMRI data acquired during resting state can be useful to characterize this phenomenon.

The overall increase in brain synchronization can also be related to a decrease in complexity measures of brain function that have been described in normal elderly both using empirical and simulation approaches (Grady and Garrett 2014). Interestingly, when complexity of EEG and magnetoencephalography data is estimated using coarse timescales, aging is also related to decreases in the complexity of neural activity as measured by these techniques (McIntosh et al. 2014) (the BOLD fMRI signal we used to estimate RSFC represents a very coarse timescale in relation to brain activity). Our findings of overall increase in between-network synchronicity and the focal losses of between-network anticorrelation (see below), in combination with the results from previous studies, clearly reinforce the notion that the aging brain loses functional diversity and complexity.

Focal Loss of Anticorrelations

Current evidence suggests that anticorrelations are a relevant feature of brain function, and changes in anticorrelations are found in a number of mental disorders such as schizophrenia, attention-deficit hyperactivity disorder and Alzheimer’s disease (Whitfield-Gabrieli and Ford 2012).

We found that the DMN was importantly involved in the connections presenting loss of anticorrelation (shifts from negative to positive correlation and decreases in the magnitude of negative correlations). The loss of anticorrelation was especially present in connections between the DMN and the attentional networks (Fig. 5). It is important to note that almost all connections showing shifts from negative to positive correlations were internetwork (Fig. 6).

These findings are in accordance with the previous literature, and age-related losses of anticorrelations have been described by independent researchers (Wu et al. 2011; Meier et al. 2012). In addition, the importance of internetwork connections among those presenting decreases in anticorrelation was highlighted by one study in which all connections presenting this type of change were between different networks (Meier et al. 2012). The authors from that study also found a strong presence of the DMN and the sensorimotor regions among the pairs of brain regions presenting loss of anticorrelations.

These patterns are especially relevant when we contrast our findings with results from neurodevelopmental studies. Anticorrelations are known to increase in magnitude during development between childhood and early adulthood (Barber et al. 2013;
of brain disconnectivity (Wu et al. 2011). These points of view suggest that they represent a special type of integration so that segregation among networks (Fox et al. 2005), whereas others normal aging.

lescence and early adulthood is particularly vulnerable during the anticorrelated state between these networks acquired during adolescence and early adulthood is particularly vulnerable during normal aging.

Some authors have stated that anticorrelations are markers of segregation among networks (Fox et al. 2005), whereas others suggest that they represent a special type of integration so that decreases in anticorrelation should be interpreted as a marker of brain disconnectivity (Wu et al. 2011). These points of view can sound puzzling or contradictory at first, but they are in fact complementary: There is strong evidence that structural integration is not only important for functional integration (positive correlations) but also for functional segregation (anticorrelations), and both features are necessary for optimal information processing (Stam 2010; Hawellek et al. 2011; Bonnelle et al. 2012).

In our study, no connection presented significant age-related increase in the magnitude of anticorrelation. This seems to be in line with previous studies because we are aware of only one previous work showing an increase in the magnitude of negative correlations in the elderly (Geerligs et al. 2015). In that study, the authors found both age-associated increases and decreases in the magnitude of anticorrelations. The increases were located between the cingulo-opercular network and the somatomotor network and within the cingulo-opercular network. Interestingly, they also reported that performance on a verbal learning task was correlated with the magnitude of negative correlations between the cingulo-opercular network and the DMN (Geerligs et al. 2015).

Loss of Within-Network Coherence

Connections showing age-related decreases in positive correlations were much less common than those with increases. However, the set of connections characterized by decreases in the magnitude of positive correlations presented a feature that was not found in any other type of connection: A significant part (53%) of decreases in positive correlations was found to be intranetwork connections (Fig. 6). The significant participation of intranetwork connections among those presenting decreases in positive correlations is in accordance with the prevailing notion derived from the current literature: The most common finding in RSFC studies has been age-related decreases in RSFC within the DMN (Ferreira and Busatto 2013). In our results, within DMN connections represented 78% of all intranetwork decreases in the magnitude of positive correlations (Fig. 6).

It should be noted that we used an atlas of brain regions that typically present a high degree of internal coherence and age-related decreases in the magnitude of positive correlations have been mostly located within networks, especially in the DMN (Ferreira and Busatto 2013). Thus, the fact that in our study the decreases in positive correlations represented just a small part of all connections presenting significant age-related changes may have been influenced by the data analysis strategy chosen for our study. Therefore, our findings should not be interpreted strictly as evidence that decreases in positive correlations are of little relevance to the neurophysiology of aging; rather, we show here that they are part of a bigger picture that comprises other types of age-related changes.

Methodological Considerations

We used a whole-brain parcellation atlas and estimated the RSFC between 278 contiguous functional subunits (Shen et al. 2013). We applied the same parcellation scheme to all subjects, regardless of age. Aging is associated with changes in brain structure, and these can modulate the effects related to partial volumes and spatial blurring. Thus, these effects may have been heterogeneous in our sample of adults from different age ranges. Besides, although all 278 volumes of interest were located in gray matter regions (cortical and subcortical), it is possible that variable amounts of signal from white matter were also included in some of these regions. This is a common limitation of studies that apply a fixed set of 1 or more volumes of interest to extract BOLD signal from the whole sample. In spite of these limitations, this strategy allowed us to carry out a desired hypothesis-free analysis, increasing reproducibility (the atlas is publicly available) and favoring the identification of age-related changes between different functional subunits over the whole brain. These combined features are very rarely found in imaging studies in this field, and the data analysis strategy of this study allowed us to characterize a pattern of age-related change that has not been reported before.

The BOLD signal depends on neurovascular coupling and cerebrovascular reactivity, both of which are known to undergo age-related changes (D’Esposito et al. 2003; Lu et al. 2011). These changes can have an impact on functional connectivity and may cause both increases and decreases in connectivity values (Liu 2013). Ideally, one should use a reliable index of neurovascular coupling and vascular reactivity to normalize the BOLD signal. Candidate strategies for such normalization include BOLD response to hypercapnia (e.g., during breath-holding tasks) and calculation of ratios between the BOLD signal amplitude during a task and the signal amplitude acquired during rest (Kannurpatti et al. 2011; Liu 2013). In our study, we could not take advantage of those options since fMRI runs were performed only during the resting state. Therefore, we cannot entirely rule out the possibility that our results could have been influenced by the effect of age-related changes in neurovascular coupling or vascular reactivity, rather than solely reflecting age-related changes in neural function. This limitation is shared by previous whole-brain functional connectivity studies in the field of normal aging (Meier et al. 2012; Tomasi and Volkow 2012; Chan et al. 2014; Geerligs et al. 2015; Song et al. 2014).

Age has an effect on test–retest reliability of RSFC, and this effect is not homogenous across different brain networks (Song et al. 2012). As in most previous RSFC studies, we had only one fMRI run; therefore, we could not measure test–retest reliability in our sample.

The assessment of statistical significance in PLS was performed at the level of the patterns extracted by SVD—not at the level of each single brain connection—so that no correction for
multiple comparisons is needed (McIntosh and Lobaugh 2004). Interpretation of results from PLS analyses is thus different from interpretations of massive univariate analyses. In PLS, the significant finding is the whole pattern that has been characterized. In other words, the whole set of brain connections comprising the reported result have been tested for significance, and single brain elements have not been considered as multiple independent variables.

It is known that older subjects present greater head motion during fMRI than young adults, and this can affect the estimation of functional connectivity (Power et al. 2012; Van Dijk et al. 2012). We found no significant correlation between maximum absolute displacement and age, both for translation ($r = −0.11; P = 0.40$) and rotation parameters ($r = -0.16; P = 0.23$). However, age was positively correlated with mean frame-wise displacement ($r = 0.36; P = 0.005$; frame-wise displacement was calculated as described by Power et al. (2012)). As commonly performed in studies in this field, we regressed out the motion parameters from the BOLD signal to minimize the impact of this confounding factor. This procedure is commonly used to address this issue, but it does not completely remove motion effects (Van Dijk et al. 2012). Thus, we also performed the motion-censoring step as described by Power et al. (2012). Finally, we controlled for the effects of mean frame-wise displacement on functional connectivity estimates in all analyses. It should be noted that in general, most of the variation in RSFC across subjects is not related to movement (Van Dijk et al. 2012), and there is no standard procedure that has been shown to unequivocally eliminate this confounding factor (Buckner et al. 2013).

In this study, our aim was to select a sample of adults free of psychiatric disorders to minimize the confounding effects that these conditions have on RSFC. Scarcity of resources for mental health and stigma about mental disorders are widespread, and this restrains very significantly access to adequate diagnosis and treatment (Saxena et al. 2007). Associated with such constraints, the high prevalence of psychiatric disorders leads to a significant number of people who are unaware of their condition—lifetime prevalence has been estimated at 29% of the general population by a recent meta-analysis (Steel et al. 2014). Nevertheless, including individuals who report having no mental disorders without further psychiatric investigation is common practice among fMRI studies. On one hand, including psychiatric interviews in the sample selection process can be very time-consuming (~90 additional minutes per assessment in this study); on the other hand, it is known that psychiatric conditions are associated with changes in brain function (Greicius 2008; Seeley et al. 2009) so that failure to identify them may complicate the interpretation of neuroimaging findings. To the best of our knowledge, there is no previous RSFC study of the normal aging brain that have used a broad neuropsychological evaluation in combination with the gold standard tool for psychiatric diagnosis, the SCID (First et al. 2002), to select a sample free of cognitive and psychiatric disorders. Our comprehensive evaluation was important because we excluded 14% of the participants due to a psychiatric condition that had not been reported by participants during the triage procedures; among the elderly, this proportion was even higher: 24% (Fig. 1). Thus, the results presented here represent the first characterization of age-related changes in RSFC in a sample of adults with no lifetime psychiatric disorder.

Given that the relationship between age and functional connectivity may be affected by brain size, we repeated the analyses after controlling for the effects of overall brain atrophy on functional connectivity. We obtained results that approached but did not reach the threshold for statistical significance ($P = 0.08$), thus indicating that controlling for brain tissue volume leads to a decrease in the strength of the association between age and connectivity. This is consistent with the hypothesis that brain atrophy might influence functional connectivity changes (Damoiseaux et al. 2012). Although we found a decrease in the number of connections after controlling for the effects of brain atrophy, the overall distribution of changes across networks was very similar to the one found before controlling for brain tissue volume (i.e., diffuse increases in the magnitude of positive correlations, focal loss of anticorrelation involving the DMN and decreases in the magnitude of positive correlation—see Supplementary Figs 6–9). Moreover, it is relevant to highlight that we did not identify a significant association between overall brain volume and functional connectivity ($P = 0.37$). These results indicate that although brain atrophy may partially mediate the relationship between age and connectivity, its effects are limited. Furthermore, our results suggest that the pattern of distribution of age-related changes in connectivity across brain networks is independent of atrophy. A number of previous fMRI investigations that focused on specific brain regions have found that age-related changes in functional connectivity remain significant after controlling for brain atrophy (Damoiseaux et al. 2008; Onoda et al. 2012; Langner et al. 2015). A whole-brain study reported that a few but not all graph-theoretic metrics were correlated with brain volume, and the authors found that age-related changes in connectivity could not be solely explained by brain atrophy (Geerligs et al. 2015). Most previous whole-brain studies in this field have not controlled for atrophy effects on connectivity (Meier et al. 2012; Tomasi and Volkow 2012; Chan et al. 2014; Song et al. 2014), and it would be important to further characterize the mediating effects of brain atrophy on the relationship between aging and connectivity in future investigations.

Finally, we also addressed the potential confound effect of the APOE ε4 allele due to its known influence on RSFC (Reinvang et al. 2013). We included ε4 carriers and, after removal of ε4 effects, the pattern of age-related changes remained very similar to the one we identified before (Figs 3–6 and Supplementary Figs 2–5). This indicates that the pattern of aging effects on RSFC was not secondary to APOE genotype.

**Future Perspectives**

We have characterized a pattern of age-related changes in RSFC that would not be found had we focused on a few brain regions or networks as is common in both seed-based studies and independent component analyses. It is now clear that distinct patterns of RSFC emerge from choosing different perspectives to extract information and analyze data. The importance of adopting a broader perspective of brain functional connectivity has been highlighted by others (Power et al. 2010). However, there are still many obstacles to an accurate and definitive identification of distinct brain regions so that there is no “gold standard” for brain parcellation yet (Van Essen 2013). Thus, future studies in the field of aging-related brain changes could benefit from adopting a multiple perspective approach to yield complementary results.

Describing the results from functional connectivity fMRI studies as simple increases and decreases in functional connectivity can lead to misleading interpretations. This is because functional connectivity results from correlations, which can be positive or negative. Therefore, “increases” in RSFC may imply an increment in the magnitude of positive correlation or a loss of anticorrelation. Thus, without a clear description, a positive association between RSFC and cognitive performance can have 2 different
meanings: Better performance might be related to greater magnitude of positive correlations or to decreased anticorrelations. However, in some articles, this distinction is not clearly described, perhaps because anticorrelations are much less common than positive correlations. The importance of this distinction has been clear both in our study and in previous investigations (Meier et al. 2012; Geerligs et al. 2015). More specific and clear descriptions such as increased or reduced positive correlation or increases or decreases in anticorrelation should be used when appropriate. When anticorrelations are not found or are excluded from analysis, this should also be clearly stated.

The neurobiological significance of different changes in RSFC is still a matter of debate. Dopaminergic deficits, white matter disruptions, gray matter atrophy, and amyloid deposition are frequently cited as culprits (Ferreira and Busatto 2013). Age is related to all these processes so that it is plausible that they all play roles in shaping brain functional architecture. It would be very relevant to identify how each of these phenomena determines age-related changes in brain functional connectivity.

Another main avenue for research is the impact of amyloid burden on RSFC. Amyloid deposition is known to have a significant effect on connectivity (Sheline and Raichle 2013) and 20–40% of the elderly population present asymptomatic amyloidosis (Jack et al. 2014). As amyloid imaging techniques become more available, the importance of distinguishing the samples of normal elderly in amyloid negative and positive is emerging as an important feature of new studies (Koch et al. 2014).

Conclusion

In this RSFC study of adults with no cognitive deficits and no lifetime psychiatric disorders, the functional connectivity hallmark of normal brain aging was a diffuse increase in internetwork positive correlations with a focal loss of anticorrelations, involving mainly the connections between the DMN and the attentional networks. The careful sample selection process of this study is unprecedented in the field of RSFC of normal aging, and our whole-brain, hypothesis-free strategy allowed us to better characterize a pattern of functional changes that extends beyond the commonly described age-related decreases in RSFC. Based on our results, age is not only related to decreases in RSFC within the DMN but also to diffuse increases in positive correlations between different networks and focal losses of anticorrelations, especially between the DMN and the attentional networks. These results reinforce the notion that the aging brain undergoes a dedifferentiation process and that late neurodevelopmental stages are particularly vulnerable to aging.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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References


