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Title: THE CEREBELLUM IN IDIOPATHIC CERVICAL DYSTONIA: A SPECIFIC PATTERN OF STRUCTURAL ABNORMALITIES?

Article Type: Full Length Article

Keywords: idiopathic cervical dystonia, cerebellum, MRI, voxel-based morphometry, cerebellar peduncles

Corresponding Author: Dr. Sirio Cocozza, M.D.

Corresponding Author's Institution: University

First Author: Giuseppe Pontillo, MD

Order of Authors: Giuseppe Pontillo, MD; Anna Castagna; Elena A Vola; Antonella Macerollo; Silvio Peluso; Camilla Russo; Francesca Baglio; Fiore Manganelli; Arturo Brunetti; Sirio Cocozza; Marcello Esposito

Abstract: Introduction
In recent years, cerebellar abnormalities have gained increasing attention as possible physiopathological substratum of idiopathic cervical dystonia (ICD), but a consistent pattern of cerebellar structural modifications has not yet been established. We systematically investigated the presence of volumetric alterations of cerebellar gray (GM) and white matter (WM) in ICD patients, as well as their clinical relevance.
Methods
In this two-centers prospective cross-sectional study, from May 2013 to December 2017, 27 patients with ICD and 27 age- and sex-comparable healthy controls underwent brain MRI including 3D T1-weighted sequences for volumetric analyses. Between-group differences in terms of gray matter and cerebellar peduncles volumes were investigated using both region of interest (ROI)-based and voxel-based approaches using the SUIT tool (SPM12), and significant volumetric changes were correlated with clinical impairment (as measured with the Tsui score) and presence of tremor.
Results
ICD patients showed significant volumetric reduction of cerebellar GM in the anterior lobe and lobule VI, resulting from both ROI-based ($p \leq 0.009$) and voxel-based ($p \leq 0.04$) analyses, while small clusters of reduced WM volume were found in the right cerebellum and left midbrain ($p=0.04$), along with reduced volume of the bilateral superior ($p=0.04$) and middle ($p=0.03$) cerebellar peduncles. Furthermore, higher middle cerebellar peduncles volume was associated with the presence of tremor ($p=0.04$).
Conclusion
Our data show evidence of a specific pattern of cerebellar structural abnormalities in ICD patients, with volume loss mainly involving cortical GM regions related to the somatotopic representation of the affected body parts and, to a lesser extent, cerebellar peduncles.

Author Declaration

Parkinsonism & Related Disorders is committed to proper scientific conduct and the protection of animal and human research subjects. Submission of this manuscript implies compliance with the following ethical requirements. Please affirm that you are representing all of the authors in stating compliance with these policies by checking the box at the end of this section.

1. Studies with human subjects must have been conducted in accordance with the Declaration of Helsinki. All persons must have provided informed consent prior to being included in the study.

2. Studies with animal subjects must have been conducted in accordance with the Guide for the Care and Use of Laboratory Subjects as adopted by the US National Institutes of Health and/or according to the requirements of all applicable local, national and international standards.

3. Protocols with animal or human subjects must have been approved by the relevant local committee(s) charged with ensuring subject protection. Studies that entail pain or distress will be assessed in terms of the balance between the distress inflicted and the likelihood of benefit.

4. The authors declare that the manuscript is original, that it is not being considered for publication elsewhere, and that it will not be submitted elsewhere while still under consideration for Parkinsonism & Related Disorders or after it has been accepted by Parkinsonism & Related Disorders.

5. All authors have seen and approved the manuscript in the form submitted to the journal. The authors declare that they have conformed to the highest standards of ethical conduct in the submission of accurate data and that they acknowledge the work of others when applicable.

6. All sources of financial support for the work have been declared in the Acknowledgements section of the manuscript. Any additional conflicts of interest must also be declared. Please include declarations of any consultancy or research funding received from relevant companies from three years prior to performance of the research until the time of manuscript submission. If the research is supported by internal funds, that should be stated as well.

To indicate compliance with the preceding declaration and that you have obtained agreement from all of the authors of this paper to declare their compliance as well, please place an x here: _X_

In cases of uncertainty please contact an editor for advice.

UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"
DIPARTIMENTO DI SCIENZE BIOMEDICHE AVANZATE

Naples, August 17th, 2020

Dear Editor,

We are respectfully submitting a revised version of the manuscript PARKRELDIS-D-20-00653 entitled **"THE CEREBELLUM IN IDIOPATHIC CERVICAL DYSTONIA: A SPECIFIC PATTERN OF STRUCTURAL ABNORMALITIES?".**

We are grateful for the detailed feedback we have received on our Manuscript. Addressing all the concerns raised gave us the opportunity of substantially improving the completeness of the Manuscript, and substantiating the validity of the Results.

Enclosed please find a point-by-point answer to the comments by the Reviewers, along with a revised version of the Manuscript, with modifications highlighted in track mode.

All authors have read the manuscript and take full responsibility for the data, the analyses and interpretation, and the conduct of the research.

The paper has not been previously published, and it is not under simultaneous consideration by another journal. I also herein confirm that no ghost writing by anyone not named on the author list is included in the manuscript and that the conflict of interest form has been completed and submitted by all authors.

We sincerely hope that the revised version of out work will now meet the standards for publication in ***Parkinsonism and related disorders.***

UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"
DIPARTIMENTO DI SCIENZE BIOMEDICHE AVANZATE

Kind Regards,

Sirio Cocozza, MD

Department of Advanced Biomedical Sciences

University "Federico II", Via Pansini, 5, 80131 - Naples - Italy

e-mail: sirio.cocozza@unina.it Twitter: @NeuroN_Lab

TEL: +390817462560; FAX: +390817463561

UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"
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Reviewer #1:

In this study GM and WM changes in ICD patients and controls were explored. ICD patients showed significant volumetric reduction of cerebellar GM in ROI and whole-brain analyses. The paper is well-written, concise, technical sound and I enjoyed reading. It is worth noting that the authors used a cerebellum toolbox to perform the analyses. I only have a few minor comments that might help to improve the paper.

Methods:

#1 Do you think TFCE is appropriate for the small cerebellar VBM clusters and in combination with SUIT? What did you observe without TFCE (just using FWE correction)? If you also observed GM atrophy without TFCE I think this should be added to the results. If not, please comment on the discrepancy.

We thank the referee for the valid observation, which gives us the opportunity to further clarify our methods. In our opinion, in the specific setting of our study, the use of a non-parametric approach based on permutations in conjunction with TFCE (Smith and Nichols, 2009; doi: 10.1016/j.neuroimage.2008.03.061) has several advantages over more classical parametric methods based on Gaussian random field theory (Worsley, et al. 1996; doi: 10.1002/(SICI)1097-0193(1996)4:1<58::AID-HBM4>3.0.CO;2-O). Firstly, TFCE generally provides better sensitivity and stability compared to other methods. Furthermore, it does not require the definition of an initial, arbitrary, cluster-forming threshold. Finally, non-parametric approaches require a smaller amount of smoothing (or even no smoothing), thus leading to greater spatial accuracy according to the

matched filer theorem (Smith and Nichols, 2009; doi: 10.1016/j.neuroimage.2008.03.061). Of note, this spatial specificity potentially allows to precisely locate the effects of interest within small, contiguous, but functionally distinct, anatomical structures like the cerebellar lobules.

Nevertheless, for the sake of completeness we also repeated the VBM analysis using the "classical" parametric approach implemented in SPM12, after smoothing the images with a 3 mm FWHM isotropic Gaussian kernel, with a cluster-forming threshold of $p \leq 0.001$ uncorrected and FWE correction at cluster level (with significance level set at $p \leq 0.05$), showing a similar pattern of GM atrophy in ICD patients compared to HC, although with smaller clusters (probably due to the lesser sensitivity of the method). An additional file including a Table and a Figure is attached to this revision, showing these results.

Given the substantial stability of our Results, coupled to the aforementioned reasons to a priori select a permutations-based approach in conjunction with TFCE, and in order not to overcrowd the Manuscript, we refrained from including this additional analysis in the revised version of the Manuscript.

We hope this clarifies.

#2 You performed t-test for age. Was age normally distributed?

We thank the referee for giving us the opportunity to elucidate this point. As is now stated in the Statistical Analysis section of the revised version of the Manuscript (Page 8), assumptions for parametric statistics (including normal distribution of continuous variables) were preliminarily checked before the statistical analyses.

Results:

#3 "between neurological data". I recommend to specify what was correlated with each other exactly. The term "neurological data" is too general.

Following the Reviewer's suggestion, the exact clinical variables tested for correlation with MRI metrics are now expressed in detail in the Results section of the revised Manuscript (Page 12).

Discussion:

#4 Using data from a 1.5 and 3.0 T scanner for VBM is challenging and the authors mentioned this limitation. In my VBM experiences it is not always sufficient just to enter field strength as covariate into the model, as there are many other factors which can cause bias in that case. Did you in principle reveal the same results when analyzing the two cohorts separately (consider to add this to the supplement)?

We agree with the Referee that using data from scanners with different field strengths may represent an important source of bias when analyzing structural MRI data. However, automated methods for brain volumes quantification are known to be robust across different field strengths (Heinen, et al. 2016; doi: 10.1371/journal.pone.0165719), while including scanning parameters in the statistical model as nuisance regressors represent a widely accepted method to remove scanner-related effects (Chen, et al. 2014; doi:10.1016/j.jneumeth.2014.04.023). Furthermore, an equal number of ICD patients and age- and sex-comparable HC were enrolled at each site. Finally, as is now more clearly stated in the Statistical Analysis and corresponding Results sections of the revised manuscript, the interaction term "scanner per group" was also included in the statistical models

comparing ICD patients with HC, demonstrating no significant effect of scanner field strength on between-group volume differences. Nevertheless, following the Reviewer's suggestion, we performed additional subgroup analyses investigating cerebellar volumetric modifications in ICD patients vs HC for each site separately. The subgroup analyses, now included in the revised version of the Supplementary Materials, demonstrated similar directions and comparable magnitude of the effect sizes of the differences between patients and controls across sites, while no supra-threshold alterations emerged at either the ROI-based or the voxel-based analyses, most probably due to the small sample sizes and corresponding reduced statistical power and increased likelihood of a Type II error.

#5 Please discuss the (potential) relationship between cerebellar changes and BoNT treatment.

We thank the referee for the interesting suggestion. Accordingly, this issue is now debated in the Discussion section of the revised version of the Manuscript (Page 15).

UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"
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Reviewer #2: *This manuscript is a two-center cross-sectional study of 27 ICD patients compared with 27 sex and age-matched healthy controls that aimed to analyze the cerebellar GM and WM. The authors used the SUIT tool for GM and WM analyses in a ROI-based approach but also performed a voxel-based analysis without delimitating ROIs. The images were obtained in two scanners of different fields and the results of the ROI analyses were reported by lobules, being the right + left + vermis an 'unique' structure. Despite that, the findings are interesting, the text is clear and well-written.*

Major points:

1) Have the authors studied the right and left lobules, as well as the vermis, separately using the SUIT tool? If yes, what have you found? It would be interesting to see these analyses in the supplementary material. Observing the figure 1, it seems to me that the right lobules are significantly more affected than the left, so it is not clear why the authors counted the two sides and the vermis all together. I would like to see whether each lobule (left apart from right and the respective vermian portion) would survive to the statistics and multiple comparison corrections. Furthermore, all the previous studies (Draganski et al; Prell et al; Piccinin et al; Pantano et al) describe their findings in a lateralized manner, so it would facilitate to compare with them. In addition, it would be relevant to see whether the damage in the WM corresponds to the same side as the GM.

We thank the referee for the intriguing suggestion. The reasons for considering each cerebellar lobule and peduncle as the sum of the right and left portions are manifold. First, even if the

cerebellum morpho-functional architecture is certainly characterized by a certain degree of asymmetry, cerebellar lobules (considered as the sum of right, left and vermian portions) are commonly considered as unique morpho-functional subunits (Stoodley, et al. 2010; doi: 10.1016/j.cortex.2009.11.008). Furthermore, symptom lateralization is not always evident and hardly quantifiable in ICD (Pantano, et al. 2011, doi: 10.3174/ajnr.A2242), making it difficult to establish a meaningful correspondence between the lateralization of clinical symptoms and the asymmetry of brain imaging findings. Finally, reducing the number of statistical tests mitigates the multiple comparisons problem.

Nevertheless, following the reviewer's suggestion, in the revised version of the manuscript we added an ancillary analysis exploring cerebellar volumes separately for each side (Page 9), finding a nearly symmetrical atrophy pattern, predominantly involving the GM of the anterior cerebellum and lobule VI and cerebellar peduncles (mainly the MCP), with slight right-side predominance, in accordance with the results of the voxel-based analyses (Pages 11 and 12, Supplementary Table 2). However, due to the large number of tests, these findings did not retain statistical significance after multiple comparisons correction.

2) The patients' recruitment and the MRI scan were made in two different centers and, as the authors mentioned, it brings some limitations and cautions to the study. Although the authors have endeavored to minimize the interference of different fields, it is still unclear to me whether the results are "field-dependent", that is, due exclusively to the images obtained in 3T. Thus, I recommend that the authors perform a sub-analysis of patients vs controls acquired in the 1.5T and other analysis of patients vs controls acquired in the 3T. This will help us to understand whether all

subjects contribute to the results or this is a study of 12 patients.

Please see response to Reviewer #1, Query #4.

3) Still regarding the different fields, it would be interesting to provide noise measurements to see that the images of the two machines are at least comparable. You can find the types of noise measurement using this link: https://mriqc.readthedocs.io/en/stable/

We thank the referee for the valid suggestion. Accordingly, an additional image quality assessment has been included in the revised version of the Supplementary Material. As expected, brain MRI scans obtained at 3T were characterized by significantly less image noise and better overall image quality compared to those acquired with a 1.5T scanner. However, despite this predictable discrepancy, all the proper adjustments (please see Query #2) were made in order to remove scanner-related effects and actually detect disease-related brain modifications.

We hope this clarifies

4) The authors could also provide the demographic data of patients and controls grouped by center and not only by the total of patients and total of controls in table 1 (supplementary material). This will facilitate for the reader to see the differences of all variables.

According to the Reviewer's suggestion, Supplementary Table 1 has been modified in order to include demographic and clinical data of ICD patients and HC grouped by center.

Minor points:

1) In the abstract the authors referred to the methodology as "ROI-based" and "VBM-based"

which confounds the reader since the "ROI-based" that you used is also "VBM-based". Furthermore, when you state "ROI-based" without detailing it, the reader can first think in "manual segmentation". Since the SUIT tool was thought to be a more sophisticated and less human-dependent method, I suggest the authors to include the information that the "ROI-based" was performed using the SUIT tool in the abstract.

We thank the Reviewer for drawing attention to this possible source of confusion. Accordingly, the revised version of the Abstract has been modified to include more detailed information regarding image analysis approaches.

2) The authors stated: "Following variables: Tsui score as a measure of the severity of symptoms, disease duration (DD) and duration of BoNT treatment". When I first read the methods, it was not clear if the authors used only the total Tsui score, or the total and the tremor sub-item or the total and all the sub-itens. Please, clarify which sub-itens you have analyzed in the clinical correlation.

We thank the referee for drawing attention to this point, and we apologize for the missing information. As is now clearly stated in the Materials and Methods section of the revised Manuscript (Pages 6 and 9), only the total Tsui score was considered as an overall measure of symptom severity.

3) Table 1 caption: make it clear that is the sum of hemispheric and vermian portions

According to the Reviewer's suggestion, in the revised version of the Manuscript Table 1 caption has been expanded to include details about cerebellar volumes computation.

4) Why did the authors used FWER correction for VBM analyses and FDR for SUIT instead of using the same correction for everything?

We thank the referee for drawing attention to this apparent discrepancy, and giving us the opportunity to further clarify our Methods.

For the ROI-based analysis (including a number of statistical tests ranging between 10 and 20), we adopted FDR-correction with the Benjamini-Hochberg procedure since it is less conservative compared to other FWER-correction methods (e.g. the Bonferroni method), leading to increased power (Benjamini & Hochberg, 1995; doi: 10.2307/2346101). On the other hand, in the setting of the voxel-wise analysis with cluster-based inference using the TFCE approach, no clear benefit of one method over the other was identifiable a priori (Smith and Nichols, 2009; doi: 10.1016/j.neuroimage.2008.03.061), so that we decided to adopt the more conventional FWER correction method. We hope this clarifies.

5) In the "results" section (between-group comparison), the first paragraph regarding lobular GM and peduncular WM analysis contains numbers of p-values that are uncorrected while in the next paragraph (VBM analyses) the p-values in the text are FWE-corrected. Please, make it clear (explaining whether you are showing a corrected or uncorrected value) in the text or change all of them to corrected p-values.

We thank the referee for pointing out this issue and we apologize for the discrepancy.

In the revised version of the Manuscript, FDR-adjusted *p* values regarding ROI-based analyses have been added in the Results section (Page 11).

6) In addition, the tables are a little bit confusing for the same reason. Table 1 exhibits uncorrected p-values while table 2 shows FWE-corrected. I suggest adding an extra column in table 1 with the FDR-corrected p-values.

According to the reviewer's suggestion, in the revised version of Table 1, FDR-adjusted *p*s were reported along with uncorrected values.

7) In the VBM analysis, the WM results are too small in volume. Once you consider this result a valid one, so I suggest the authors to include in the methods the information that you chose an 'extend threshold (k)=0'.

We thank the Referee for pointing out this issue, and we apologize for the lack of clarity in the previous version of the Manuscript.

As is now stated in the Statistical Analysis section of the revised Manuscript (Page 9), a minimum extent threshold k=50 was adopted in order to avoid possible false positive results.

8) "In order to explore the afferent and efferent connections of the cerebellum, we also investigated possible cerebellar WM structural modifications, demonstrating a slight volumetric reduction of the middle and superior cerebellar peduncles, containing the main afferent and efferent branches of the cortico-ponto-cerebello-thalamo-cortical loop, respectively". I suppose that when authors state "in order to explore" it means an "exploratory analysis" however, I would

appreciate if the authors add in this paragraph the information that this is an uncorrected result (punc < .05).

Following the reviewer's observation, the corresponding part of the Discussion section of the revised Manuscript has been slightly rephrased in order to clarify that these results did not survive multiple comparisons correction.

Reviewer #1:

In this study GM and WM changes in ICD patients and controls were explored. ICD patients showed significant volumetric reduction of cerebellar GM in ROI and whole-brain analyses. The paper is well-written, concise, technical sound and I enjoyed reading. It is worth noting that the authors used a cerebellum toolbox to perform the analyses. I only have a few minor comments that might help to improve the paper.

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According to the reviewer's suggestion, in the revised version of Table 1, FDR-adjusted *p*s were reported along with uncorrected values.

7) In the VBM analysis, the WM results are too small in volume. Once you consider this result a valid one, so I suggest the authors to include in the methods the information that you chose an 'extend threshold (k)=0'.

We thank the Referee for pointing out this issue, and we apologize for the lack of clarity in the previous version of the Manuscript.

As is now stated in the Statistical Analysis section of the revised Manuscript (Page 9), a minimum extent threshold k=50 was adopted in order to avoid possible false positive results.

8) "In order to explore the afferent and efferent connections of the cerebellum, we also investigated possible cerebellar WM structural modifications, demonstrating a slight volumetric reduction of the middle and superior cerebellar peduncles, containing the main afferent and efferent branches of the cortico-ponto-cerebello-thalamo-cortical loop, respectively". I suppose that when authors state "in order to explore" it means an "exploratory analysis" however, I would appreciate if the authors add in this paragraph the information that this is an uncorrected result (punc < .05).

Following the reviewer's observation, the corresponding part of the Discussion section of the revised Manuscript has been slightly rephrased in order to clarify that these results did not survive multiple comparisons correction.

THE CEREBELLUM IN IDIOPATHIC CERVICAL DYSTONIA: A SPECIFIC PATTERN OF STRUCTURAL ABNORMALITIES?

HIGHLIGHTS

- ICD patients show volume loss of cerebellar GM in the anterior lobe and lobule VI
- Cerebellar atrophy is consistent across ROI-based and voxel-based analyses
- Slight volumetric reduction of cerebellar peduncles is also present
- Volume of the middle cerebellar peduncle correlates with the presence of tremor

*Revised manuscript (with revisions highlighted)

THE CEREBELLUM IN IDIOPATHIC CERVICAL DYSTONIA: A SPECIFIC PATTERN OF STRUCTURAL ABNORMALITIES?

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ABBREVIATIONS

ICD = idiopathic cervical dystonia

HC = healthy controls

BoNT = botulinum neurotoxin

GM = gray matter

WM = white matter

DD = disease duration

VBM = voxel-based morphometry

SCP = superior cerebellar peduncle

MCP = middle cerebellar peduncle

ICP = inferior cerebellar peduncle

ABSTRACT

Introduction

In recent years, cerebellar abnormalities have gained increasing attention as possible physiopathological substratum of idiopathic cervical dystonia (ICD), but a consistent pattern of cerebellar structural modifications has not yet been established. We systematically investigated the presence of volumetric alterations of cerebellar gray (GM) and white matter (WM) in ICD patients, as well as their clinical relevance.

Methods

In this two-centers prospective cross-sectional study, from May 2013 to December 2017, 27 patients with ICD and 27 age- and sex-comparable healthy controls underwent brain MRI including 3D T1-weighted sequences for volumetric analyses. Between-group differences in terms of gray matter and cerebellar peduncles volumes were investigated using both region of interest (ROI)-based and voxel-based approaches using the SUIT tool (SPM12), and significant volumetric changes were correlated with clinical impairment (as measured with the Tsui score) and presence of tremor.

Results

ICD patients showed significant volumetric reduction of cerebellar GM in the anterior lobe and lobule VI, resulting from both ROI-based ($p \leq 0.009$) and voxel-based ($p \leq 0.04$) analyses, while small clusters of reduced WM volume were found in the right cerebellum and left midbrain ($p=0.04$), along with reduced volume of the bilateral superior ($p=0.04$) and middle ($p=0.03$) cerebellar peduncles. Furthermore, higher middle cerebellar peduncles volume was associated with the presence of tremor ($p=0.04$).

Conclusion

Our data show evidence of a specific pattern of cerebellar structural abnormalities in ICD patients, with volume loss mainly involving cortical GM regions related to the somatotopic representation of the affected body parts and, to a lesser extent, cerebellar peduncles.

INTRODUCTION

Idiopathic Cervical Dystonia (ICD) is a chronic neurologic disorder characterized by involuntary sustained contractions of cervical musculature resulting in abnormal movements or postural changes of the head, neck and shoulders[1]. Considered as a disorder of motor programs controlling semiautomatic movements or postures[2], it represents the most common of the adult-onset focal dystonias[3]. Despite the number of investigations on ICD pathogenesis has grown considerably over the past decades, its exact physiopathological substratum remains largely unknown[2, 4]. Originally classified as a basal ganglia disease, it is now regarded as a network disorder due to abnormalities not only in the basal ganglia, but also in other interconnected structures including the cerebral cortex and the cerebellum[2, 4-6]. Preliminary evidences from animal models and human clinic-pathological observations have been confirmed by a growing number of experimental neurophysiological and neuroimaging investigations which demonstrated the association between dystonia and alterations of cerebellar activity, connectivity and structure[2, 4-6]. Regarding structural abnormalities, Voxel-Based Morphometry (VBM) studies reported variable volumetric changes in the cerebellum of dystonic patients[7-10]. However, to date, a consistent pattern of cerebellar morphometric alterations in ICD patients has not yet been established. Furthermore, no data exist on the distinct involvement of specific cerebellar lobules and of cerebellar peduncles, which could explain the role of different morphofunctional subunits in ICD pathophysiology, thus helping unravel the nature of cerebellar pathology in these patients.

From this background, aim of our study was to investigate the presence of volumetric alterations of cerebellar gray (GM) and white matter (WM) in ICD patients using both region of interest

(ROI)-based and voxel-based approaches, as well as their possible contribution to clinical impairment in this condition.

MATERIALS AND METHODS

Subjects

In this prospective cross-sectional study, from May 2013 to December 2017, right-handed patients with ICD[1] along with age- and sex-comparable right-handed healthy controls (HC) were enrolled from the University "Federico II" (Naples, Italy) and the IRCCS "Fondazione Don Gnocchi" (Milan, Italy). Exclusion criteria included: age<18 years and the presence of any other relevant neurologic/psychiatric disease or systemic condition that could affect the CNS. All patients were receiving botulinum neurotoxin (BoNT) injections following standard treatment regimens[1] and underwent the MRI examination during the wearing-off phase, before receiving the treatment. Within one week from MRI, patients were clinically assessed, and the following variables were obtained: total Tsui score[11] as an overall measure of the severity of symptoms, disease duration (DD) and duration of BoNT treatment.

The protocol was approved by each respective ethics committee, and written informed consent was obtained from all participants before the beginning of the study in accordance with the Declaration of Helsinki.

MRI data acquisition and analysis

All images were acquired using two different MRI scanners (3T Magnetom Trio and 1.5T Magnetom Avanto, Siemens Healthineers). The acquisition protocol included a 3D T1-weighted Magnetization Prepared RApid Gradient-Echo (MPRAGE) with a 1mm-isotropic resolution used for volumetric analyses and a 2D T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) or a 2D dual-echo turbo spin echo sequence for incidental lesions detection. Details of the acquired sequences are reported in the Supplementary Material. Before image processing, an experienced radiologist with more than 20 years of practice in the field of neuroimaging (AB) preliminarily checked both sequences to exclude the presence of posterior fossa lesions or malformations.

For all subjects included in the analysis, global and lobular cerebellar GM volumes were calculated on 3D T1-weighted images using the Spatially Unbiased Infratentorial Toolbox (SUIT) version 3.4[12], implemented in the Statistical Parametric Mapping (SPM12) software (http://www.fil.ion.ucl.ac.uk/spm), as described in previous works[13].

Briefly, for each subject, the cerebellum was automatically identified and isolated to obtain a cerebellar segmentation mask, which was then visually inspected and manually adjusted when necessary. Next, the isolated cerebellum was normalised to the SUIT atlas template and resliced in the atlas space. Finally, by applying an inverse transformation matrix derived from the previous coregistration step, the SUIT atlas was aligned to the native subject space, and lobular volumes were computed as the sum of their hemispheric and vermian portions (Figure 1A). Following the traditional division of the cerebellum in anterior (lobules I-V), posterior (lobules VI-IX) and flocculonodular (lobule X) lobes[14], anterior and posterior cerebellar volumes were also calculated as the sum of lobules I–V and VI–IX, respectively.

A similar approach was adopted in order to obtain an atlas-based segmentation of cerebellar peduncles using a diffusion MRI-based probabilistic atlas of the cerebellar WM obtained from tractography data of 90 subjects participating in the Human Connectome Project mapped onto a common reference space (SUIT atlas space)[15]. This atlas provides probability maps of each cerebellar peduncle: each voxel value ranges from 0 to 1 and represents the proportion of subjects in which that same voxel was part of the bundle. Thus, we thresholded the probability of each cerebellar peduncle at 0.5 in order to obtain binary ROIs corresponding to the superior (SCP), middle (MCP) and inferior (ICP) cerebellar peduncles. Finally, the same inverse transformation matrix derived from the previously described processing steps was used to warp cerebellar peduncles ROIs in each subject's native space and compute individual bilateral

peduncular volumes (Figure 1B). As a quality check, an expert (AB) visually inspected the outcome of the registrations to exclude CSF contamination.

Furthermore, to investigate possible local volume differences at a voxel level, voxel-base morphometry (VBM) analyses[16] were also carried out. In particular, normalized GM maps were modulated by scaling by the inverse of the amount of the volume changes due to spatial registration, in order to preserve the local GM amount, and then spatially smoothed using a 1 mm Full Width at Half Maximum isotropic Gaussian kernel[17]. The same procedure was also applied to normalized WM maps.

Finally, for each subject, the Total Intracranial Volume (TIV) was also estimated using the standard procedure implemented in the Computational Anatomy Toolbox (CAT12, http://www.neuro.uni-jena.de/cat) and used as confound in subsequent statistical analyses in order to correct for the effect of individual head size. To exhaustively investigate the possible effect of scanner field strength on cerebellar volumes, additional image quality assessment and subgroup volumetric analyses were performed on scans from the two sites (reported in the Supplementary Material).

Statistical analysis

Unless otherwise specified, all analyses were carried out using Statistical Package for Social Science (IBM SPSS Statistics 25), with a significance level set at $p \leq 0.05$, corrected for the false discovery rate (FDR) using the Benjamini-Hochberg procedure. Assumptions for parametric tests were preliminarily checked, with normality of continuous variables assessed via the Shapiro-Wilk's test.

Differences between ICD and HC groups in terms of age, sex and scanner's field strength were probed by Student's t and Pearson's χ^2 tests, respectively.

Group differences regarding cerebellar ROI volumes were tested by ANCOVA analyses, including age, sex, scanner and TIV as confounding covariates. The interaction term scanner (1.5T vs 3T) per group (ICD vs HC) was included in the model to test the possible influence of scanner field strength on between-group volume differences. As an ancillary analysis, between-group differences were also assessed separately for each lobule's right, left and vermian portions, as well as for each peduncle's right and left side, in order to investigate possible lateralized effects.

For the VBM analysis, the normalized, modulated and smoothed GM and WM maps were statistically analyzed to assess local volume differences between the two groups using a nonparametric approach based on permutations applied to the general linear model[18] via SPM's Threshold Free Cluster Enhancement (TFCE) toolbox (http://www.neuro.uni-jena.de/tfce), including age, sex, TIV and scanner as confounding variables. Five thousand permutations were generated, and cluster-like structures were enhanced using the TFCE approach[17], with a significance level set at $p \leq 0.05$, corrected for multiple comparisons across space using the family-wise error rate (FWER), and an extent-threshold k=50 voxels to avoid false positive results.

When regional differences in terms of local GM or WM volume emerged between the two groups, the corresponding first eigenvariate was extracted from the cluster and corrected for the effect of age, sex, TIV and scanner in HC: for each metric, the linear relationship with these variables was modelled in the HC group and used to compute standardized residuals in all subjects. The relationship between the so obtained Z-scores and clinical variables was assessed via linear (total Tsui score, DD, BoNT treatment duration) and binary logistic (tremor) regression analyses, validated using the bootstrap method with 5000 replications. Likewise,

adjusted Z-scores of other cerebellar volumes that emerged as significantly different at the between-group ROI analyses were entered in similar regression analyses. Significance level for regression models was not adjusted for multiple testing given the exploratory nature of the analyses.

RESULTS

Subjects

27 patients with ICD (mean age 50.4±11.3 years, F/M=14/13) and 27 HC of comparable age and sex (mean age 51.7±11.5 years, F/M=14/13) were enrolled in the study from the University "Federico II" (12 ICD: mean age 50.1±14.0 years, F/M=5/7; 12 HC: mean age 50.3±14.3 years, F/M=5/7) and the "Don Gnocchi" Foundation (15 ICD: mean age 50.6±9.0 years, F/M=9/6; 15 HC: mean age 52.8±9.0 years, F/M=9/6). Mean DD for ICD patients was 7.1 years (standard deviation: 6.3), with a median Tsui score of 8 (interquartile range: 5 - 10). Tremor was present in 12 (out of 27) patients.

Demographic and clinical characteristics of all subjects included in the analysis are reported in Supplementary Table 1.

Between-group comparisons

When investigating possible differences in terms of cerebellar GM volumes, ICD patients showed a significant volumetric reduction of the anterior cerebellum compared to HC (15.4±1.5 vs 16.1±1.5, ICD vs HC; p=0.006, FDR-adjusted p=0.05). At a lobular level, ICD patients demonstrated significant atrophy of cerebellar lobules I-IV (7.0±0.6 vs 7.3±0.7, ICD vs HC; p=0.004, FDR-adjusted p=0.05), V (8.4±0.9 vs 8.8±0.8, ICD vs HC; p=0.01, FDR-adjusted p=0.05) and VI (19.6±2.1 vs 20.5±1.8, ICD vs HC; p=0.009, FDR-adjusted p=0.05) (Table 1).

Regarding cerebellar WM tracts, ICD patients showed reduced volume of the bilateral SCP (0.7±0.1 vs 0.7±0.1, ICD vs HC; p=0.04) and MCP (9.5±0.9 vs 9.9±1.1, ICD vs HC; p=0.03), which did not retain statistical significance after correcting for multiple comparisons (Table 1). There was no significant effect of the scanner per group interaction term. The ancillary analysis

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demonstrated nearly symmetrical cerebellar GM and WM atrophy patterns, with slight right-side predominance (Supplementary Table 2).

At the VBM analyses, clusters of reduced GM volume in both right (FWER-corrected p=0.01) and left (FWER-corrected p=0.04) cerebellar lobules IV, V and VI emerged in ICD patients compared to HC (Table 2, Figure 2A), along with small clusters of reduced WM volume in the right cerebellum (FWER-corrected p=0.04) and the left midbrain (FWER-corrected p=0.04) (Table 2, Figure 2B). No significant between-group differences emerged for the ICD>HC contrast.

Relationship between MRI features and clinical data

When exploring the clinical correlates of the observed MRI alterations, no significant relationship was found between MRI metrics and either the total Tsui score, DD or BoNT treatment duration, with~~neurological data and MRI metrics, except for~~ an association between the presence of tremor and the bilateral MCP volume (Nagelkerke R^2 = 0.190, p = 0.04; B = 0.736 [bias-corrected and accelerated bootstrap 95% confidence interval = - 0.137 to 2.491, p = 0.04]).

DISCUSSION

In this study, we investigated the presence of possible structural modifications in the cerebellum of ICD patients, demonstrating a specific spatial pattern of decreased cerebellar GM and (to a lesser extent) WM volumes, resulting from both ROI-based and voxel-based analyses.

In recent years, modifications of cerebellar structure and function have gained increasing attention as a possible physiopathological substratum of primary dystonia, which has been the object of a paradigm shift: from being considered a basal ganglia disease to a complex network disorder involving cerebellar, cortical and subcortical motor (and non-motor) areas, along with their reciprocal connections[2, 4, 5].

Nevertheless, discordant evidence exists regarding volumetric changes in the cerebellum of dystonic patients, with several structural MRI studies variably reporting increases[7, 19, 20], decreases[8, 9], or no modifications[10, 21, 22] of cerebellar GM volumes, with inconstant spatial patterns. This apparent inconsistency may be attributable to different factors, including small size and heterogeneity of the patient cohorts (often including different types of focal or segmental dystonia[8, 9, 19, 20, 22]), and methodological differences (with most studies using whole-brain rather than cerebellum-oriented approaches[7, 10, 19-22]), all severely hindering meaningful comparisons between studies.

In our work, we focused on a homogenous sample of ICD patients, using cerebellum-tailored analyses with complementary ROI-based and voxel-based approaches, both demonstrating consistent volume loss of cerebellar GM at the level of the anterior lobe and lobule VI in patients compared to HC. Of note, these results partially overlap with those of a recent study adopting a similar cerebellum-oriented approach[9], which has proven to be more sensitive and accurate compared to whole-brain analyses for the characterization of infratentorial structural

abnormalities[12]. Interestingly, a slight right-side predominance emerged at both the ROI-based and voxel-based analyses, in accordance with the already reported asymmetry of brain imaging findings in this condition[7, 21].

According to the topographic organization of the cerebellum, lobules of the anterior lobe and lobule VI contain the representation of sensorimotor functions, participating in the coordination of fine movements of the extremities as well as in the control of posture and gait[14]. These regions, densely connected with spinal cord, brainstem and cerebral cortical areas involved in sensorimotor processing, show a precise somatotopic arrangement[14]. Interestingly, for both sensory projections carrying cutaneo-kinesthetic information via the trigemino-cerebellar tracts and afferent and efferent branches of the motor cortico-ponto-cerebello-thalamic-cortical loop, the representation of the head/neck and face/mouth lies principally in lobule VI, with some extension into lobules V and IV[14, 23]. In this light, the observed volume loss in the cerebellum may express the selective vulnerability of specific cerebellar cortical areas containing the representation of the affected body parts (head/neck for ICD patients), demonstrating a link between cerebellar involvement and the topography of dystonic symptoms[2, 4]. However, it remains unclear if cerebellar cortical atrophy represents a primary abnormality or a secondary effect resulting from damage in other salient supratentorial areas and/or in projection tracts interconnecting them, which has been also demonstrated in ICD patients[24].

In order to explore the afferent and efferent connections of the cerebellum, we also investigated possible cerebellar WM structural modifications, demonstrating a slight volumetric reduction (not surviving multiple comparisons correction) of the middle and superior cerebellar peduncles, containing the main afferent and efferent branches of the cortico-ponto-cerebello-thalamo-cortical loop, respectively[24]. These results are in line with previous studies reporting

microstructural damage of cerebellar peduncles in dystonic patients[25], and support the hypothesis of a sensorimotor network disorder, underpinned by structural and functional modifications involving different nodes at the level of cerebral cortex, basal ganglia and cerebellum, as well as their reciprocal connections[2, 4, 5].

When looking at the relationship between the observed structural modifications and clinical variables, a positive correlation emerged between the volume of the bilateral middle cerebellar peduncles and the presence of tremor. These results are in contrast with findings in other tremorous conditions (mainly essential tremor), in which an association between tremor and cerebellar peduncles' macro- and micro-structural damage has been described[26], suggesting that cerebellar involvement contributes to the genesis of tremor in cervical dystonia through distinct physiopathological mechanisms[27]. Regarding the relationship with BoNT treatment, which has been anecdotally linked to changes in brain structure[28], no significant association between treatment duration and cerebellar atrophy emerged, possibly due to the low variance of BoNT treatment duration in our sample.

Some limitations of the present study need to be acknowledged. Firstly, MRI exams were acquired at different field strengths, introducing a possible source of bias. However, demographic and clinical characteristics of the subjects examined with the two scanners were highly homogeneous, and all statistical analyses included field strength as a confounding variable, thus greatly limiting possible scanner-related bias and even increasing the generalizability of our results. Furthermore, a more extensive clinical examination including finer evaluations of motor, as well as sensory, cognitive, neuropsychiatric and autonomic domains may have allowed for greater insight into the contribution of cerebellar modifications to the development of motor and non-motor symptoms, which are known to occur in dystonic

patients[29]. The implementation of other advanced MRI techniques focusing on the analysis of brain structural and functional connectivity could have helped interpret the observed cerebellar modifications in the framework of a more complex network disorder[2, 4, 5]. Finally, a longitudinal evaluation might have provided the means to unravel the causal relationship between cerebellar, cortical, basal ganglia and interconnecting WM tracts abnormalities, as well as to investigate the potential reversibility of brain structural modifications in response to BoNT treatment.

In conclusion, our data show evidence of a specific pattern of cerebellar structural abnormalities in ICD patients, with volume loss mainly involving cortical GM of the anterior cerebellum and lobule VI, consistent across both ROI-based and voxel-based approaches and seemingly related to the somatotopic representation of the affected body parts. These results, notwithstanding the abovementioned limitations, may shed novel light on the nature of cerebellar modifications in ICD and their role in the physiopathology of this condition.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Conflicts of interest (activities not related to the present article)

S.C. reports fees for speaking from Sanofi and Amicus

The remaining authors have no conflicts of interest to declare.

TABLES

Table 1. Cerebellar volumes for all subjects included in the analysis. Volumes of cerebellar lobules (considered as the sum of right, left and vermian portions) and cerebellar peduncles (considered as the sum of right and left components) are presented, along with the effect sizes (Cohen's *d*) and *p*-values of the between-group differences.

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Volumes	ICD (N=27)	HC (N=27)	Cohen's *d*	Uncorrected *p* (FDR-adjusted *p*)
Cerebellum	121.5±11.0	124.8±9.4	0.62	0.05* (0.12)
Anterior Lobe	15.4±1.5	16.1±1.5	0.87	**0.006 (0.05)**
Posterior Lobe	106.1±9.5	108.7±8.1	0.56	0.07 (0.13)
Lobules I-IV	7.0±0.6	7.3±0.7	0.91	**0.004 (0.05)**
Lobule V	8.4±0.9	8.8±0.8	0.78	**0.01 (0.05)**
Lobule VI	19.6±2.1	20.5±1.8	0.83	**0.009 (0.05)**
Crus I	26.9±2.8	27.5±2.4	0.50	0.11 (0.18)
Crus II	20.0±1.7	20.2±1.7	0.23	0.44 (0.49)
Lobule VIIB	10.3±1.0	10.6±0.9	0.42	0.17 (0.21)
Lobule VIIIA	11.2±1.0	11.5±0.8	0.50	0.11 (0.18)
Lobule VIIIB	9.0±0.8	9.2±0.6	0.45	0.14 (0.19)
Lobule IX	7.4±0.8	7.6±0.7	0.23	0.45 (0.49)
Lobule X	1.8±0.2	1.8±0.1	0.04	0.89 (0.90)
SCP	0.7±0.1	0.7±0.1	0.63	0.04 (0.11)*
MCP	9.5±0.9	9.9±1.1	0.67	0.03 (0.10)*
ICP	3.0±0.3	3.1±0.3	0.59	0.06 (0.13)

Cerebellar Volumes (in ml) are expressed as mean ± SD.

Significant differences are reported in bold.
**Not significant after FDR-correction.*

ICD, Idiopathic Cervical Dystonia; HC, Healthy Controls; SCP, Superior Cerebellar Peduncle; MCP, Middle Cerebellar Peduncle; ICP: Inferior Cerebellar Peduncle.

Table 2. Results of the voxel-based analyses. Clusters of decreased GM and WM volume in ICD patients compared to HC are presented, along with significance level (FWE-corrected) and the corresponding local maxima's effect sizes, T values and anatomical labels. No significant differences emerged when testing the ICD > HC contrast. Coordinates refer to mm from the anterior commissure in MNI space, with anatomical labeling according to[30].

	Cluster Volume (ml)	*p*-value (FWE-corr)	Cohen's *d*	T	MNI Coordinates (mm) X	Y	Z	Anatomical Label
GM	9.70	0.01	1.33	4.61	25	-72	-20	Right Cerebellar Lobule VI
		0.01	1.38	4.77	14	-52	-13	Right Cerebellar Lobules IV-V
	2.01	0.04	1.00	3.47	-8	-60	-11	Left Cerebellar Lobules IV-V
		0.04	1.02	3.54	-15	-58	-25	Left Cerebellar Lobule VI
WM	0.09	0.04	1.34	4.26	24	-62	-31	Right Cerebellum
	0.39	0.04	1.09	3.76	-12	-18	-13	Left Midbrain

GM, Gray Matter; WM, White Matter; ICD, Idiopathic Cervical Dystonia; HC, Healthy Controls.

FIGURES

Figure 1. Results of the segmentation of cerebellar lobules and peduncles. (A) In a 53-year-old female patient, the SUIT cerebellar atlas is aligned in the native subject space and superimposed on (*from left to right*) axial, coronal and sagittal reconstructions obtained from the 3D T1-weighted sequence. **(B)** In a 54-year-old male patient, atlas-derived cerebellar peduncles ROIs are aligned in the native subject space and superimposed on coronal (*left column*) and axial (*right column*) reconstructions obtained from the 3D T1-weighted sequence.

Figure 2. Results of the voxel-based analyses. Thresholded statistical maps (*in red-yellow*) for the ICD < HC contrast regarding GM **(A)** and WM **(B)** volumes are superimposed on the SUIT T1-weighted template in axial planes.

ICD, Idiopathic Cervical Dystonia; HC, Healthy Controls; GM, Gray Matter; WM, White Matter.

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THE CEREBELLUM IN IDIOPATHIC CERVICAL DYSTONIA: A SPECIFIC PATTERN OF STRUCTURAL ABNORMALITIES?

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ABBREVIATIONS

ICD = idiopathic cervical dystonia

HC = healthy controls

BoNT = botulinum neurotoxin

GM = gray matter

WM = white matter

DD = disease duration

VBM = voxel-based morphometry

SCP = superior cerebellar peduncle

MCP = middle cerebellar peduncle

ICP = inferior cerebellar peduncle

ABSTRACT

Introduction

In recent years, cerebellar abnormalities have gained increasing attention as possible physiopathological substratum of idiopathic cervical dystonia (ICD), but a consistent pattern of cerebellar structural modifications has not yet been established. We systematically investigated the presence of volumetric alterations of cerebellar gray (GM) and white matter (WM) in ICD patients, as well as their clinical relevance.

Methods

In this two-centers prospective cross-sectional study, from May 2013 to December 2017, 27 patients with ICD and 27 age- and sex-comparable healthy controls underwent brain MRI including 3D T1-weighted sequences for volumetric analyses. Between-group differences in terms of gray matter and cerebellar peduncles volumes were investigated using both region of interest (ROI)-based and voxel-based approaches using the SUIT tool (SPM12), and significant volumetric changes were correlated with clinical impairment (as measured with the Tsui score) and presence of tremor.

Results

ICD patients showed significant volumetric reduction of cerebellar GM in the anterior lobe and lobule VI, resulting from both ROI-based ($p \leq 0.009$) and voxel-based ($p \leq 0.04$) analyses, while small clusters of reduced WM volume were found in the right cerebellum and left midbrain ($p=0.04$), along with reduced volume of the bilateral superior ($p=0.04$) and middle ($p=0.03$) cerebellar peduncles. Furthermore, higher middle cerebellar peduncles volume was associated with the presence of tremor ($p=0.04$).

Conclusion

Our data show evidence of a specific pattern of cerebellar structural abnormalities in ICD patients, with volume loss mainly involving cortical GM regions related to the somatotopic representation of the affected body parts and, to a lesser extent, cerebellar peduncles.

INTRODUCTION

Idiopathic Cervical Dystonia (ICD) is a chronic neurologic disorder characterized by involuntary sustained contractions of cervical musculature resulting in abnormal movements or postural changes of the head, neck and shoulders[1]. Considered as a disorder of motor programs controlling semiautomatic movements or postures[2], it represents the most common of the adult-onset focal dystonias[3]. Despite the number of investigations on ICD pathogenesis has grown considerably over the past decades, its exact physiopathological substratum remains largely unknown[2, 4]. Originally classified as a basal ganglia disease, it is now regarded as a network disorder due to abnormalities not only in the basal ganglia, but also in other interconnected structures including the cerebral cortex and the cerebellum[2, 4-6]. Preliminary evidences from animal models and human clinic-pathological observations have been confirmed by a growing number of experimental neurophysiological and neuroimaging investigations which demonstrated the association between dystonia and alterations of cerebellar activity, connectivity and structure[2, 4-6]. Regarding structural abnormalities, Voxel-Based Morphometry (VBM) studies reported variable volumetric changes in the cerebellum of dystonic patients[7-10]. However, to date, a consistent pattern of cerebellar morphometric alterations in ICD patients has not yet been established. Furthermore, no data exist on the distinct involvement of specific cerebellar lobules and of cerebellar peduncles, which could explain the role of different morphofunctional subunits in ICD pathophysiology, thus helping unravel the nature of cerebellar pathology in these patients.

From this background, aim of our study was to investigate the presence of volumetric alterations of cerebellar gray (GM) and white matter (WM) in ICD patients using both region of interest

(ROI)-based and voxel-based approaches, as well as their possible contribution to clinical impairment in this condition.

MATERIALS AND METHODS

Subjects

In this prospective cross-sectional study, from May 2013 to December 2017, right-handed patients with ICD[1] along with age- and sex-comparable right-handed healthy controls (HC) were enrolled from the University "Federico II" (Naples, Italy) and the IRCCS "Fondazione Don Gnocchi" (Milan, Italy). Exclusion criteria included: age<18 years and the presence of any other relevant neurologic/psychiatric disease or systemic condition that could affect the CNS. All patients were receiving botulinum neurotoxin (BoNT) injections following standard treatment regimens[1] and underwent the MRI examination during the wearing-off phase, before receiving the treatment. Within one week from MRI, patients were clinically assessed, and the following variables were obtained: total Tsui score[11] as an overall measure of the severity of symptoms, disease duration (DD) and duration of BoNT treatment.

The protocol was approved by each respective ethics committee, and written informed consent was obtained from all participants before the beginning of the study in accordance with the Declaration of Helsinki.

MRI data acquisition and analysis

All images were acquired using two different MRI scanners (3T Magnetom Trio and 1.5T Magnetom Avanto, Siemens Healthineers). The acquisition protocol included a 3D T1-weighted Magnetization Prepared RApid Gradient-Echo (MPRAGE) with a 1mm-isotropic resolution used for volumetric analyses and a 2D T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) or a 2D dual-echo turbo spin echo sequence for incidental lesions detection. Details of the acquired sequences are reported in the Supplementary Material. Before image processing, an experienced radiologist with more than 20 years of practice in the field of neuroimaging (AB) preliminarily checked both sequences to exclude the presence of posterior fossa lesions or malformations.

For all subjects included in the analysis, global and lobular cerebellar GM volumes were calculated on 3D T1-weighted images using the Spatially Unbiased Infratentorial Toolbox (SUIT) version 3.4[12], implemented in the Statistical Parametric Mapping (SPM12) software (http://www.fil.ion.ucl.ac.uk/spm), as described in previous works[13].

Briefly, for each subject, the cerebellum was automatically identified and isolated to obtain a cerebellar segmentation mask, which was then visually inspected and manually adjusted when necessary. Next, the isolated cerebellum was normalised to the SUIT atlas template and resliced in the atlas space. Finally, by applying an inverse transformation matrix derived from the previous coregistration step, the SUIT atlas was aligned to the native subject space, and lobular volumes were computed as the sum of their hemispheric and vermian portions (Figure 1A). Following the traditional division of the cerebellum in anterior (lobules I-V), posterior (lobules VI-IX) and flocculonodular (lobule X) lobes[14], anterior and posterior cerebellar volumes were also calculated as the sum of lobules I–V and VI–IX, respectively.

A similar approach was adopted in order to obtain an atlas-based segmentation of cerebellar peduncles using a diffusion MRI-based probabilistic atlas of the cerebellar WM obtained from tractography data of 90 subjects participating in the Human Connectome Project mapped onto a common reference space (SUIT atlas space)[15]. This atlas provides probability maps of each cerebellar peduncle: each voxel value ranges from 0 to 1 and represents the proportion of subjects in which that same voxel was part of the bundle. Thus, we thresholded the probability of each cerebellar peduncle at 0.5 in order to obtain binary ROIs corresponding to the superior (SCP), middle (MCP) and inferior (ICP) cerebellar peduncles. Finally, the same inverse transformation matrix derived from the previously described processing steps was used to warp cerebellar peduncles ROIs in each subject’s native space and compute individual bilateral

peduncular volumes (Figure 1B). As a quality check, an expert (AB) visually inspected the outcome of the registrations to exclude CSF contamination.

Furthermore, to investigate possible local volume differences at a voxel level, voxel-base morphometry (VBM) analyses[16] were also carried out. In particular, normalized GM maps were modulated by scaling by the inverse of the amount of the volume changes due to spatial registration, in order to preserve the local GM amount, and then spatially smoothed using a 1 mm Full Width at Half Maximum isotropic Gaussian kernel[17]. The same procedure was also applied to normalized WM maps.

Finally, for each subject, the Total Intracranial Volume (TIV) was also estimated using the standard procedure implemented in the Computational Anatomy Toolbox (CAT12, http://www.neuro.uni-jena.de/cat) and used as confound in subsequent statistical analyses in order to correct for the effect of individual head size. To exhaustively investigate the possible effect of scanner field strength on cerebellar volumes, additional image quality assessment and subgroup volumetric analyses were performed on scans from the two sites (reported in the Supplementary Material).

Statistical analysis

Unless otherwise specified, all analyses were carried out using Statistical Package for Social Science (IBM SPSS Statistics 25), with a significance level set at $p \leq 0.05$, corrected for the false discovery rate (FDR) using the Benjamini-Hochberg procedure. Assumptions for parametric tests were preliminarily checked, with normality of continuous variables assessed via the Shapiro-Wilk's test.

Differences between ICD and HC groups in terms of age, sex and scanner's field strength were probed by Student's t and Pearson's χ^2 tests, respectively.

Group differences regarding cerebellar ROI volumes were tested by ANCOVA analyses, including age, sex, scanner and TIV as confounding covariates. The interaction term scanner (1.5T vs 3T) per group (ICD vs HC) was included in the model to test the possible influence of scanner field strength on between-group volume differences. As an ancillary analysis, between-group differences were also assessed separately for each lobule's right, left and vermian portions, as well as for each peduncle's right and left side, in order to investigate possible lateralized effects.

For the VBM analysis, the normalized, modulated and smoothed GM and WM maps were statistically analyzed to assess local volume differences between the two groups using a nonparametric approach based on permutations applied to the general linear model[18] via SPM's Threshold Free Cluster Enhancement (TFCE) toolbox (http://www.neuro.uni-jena.de/tfce), including age, sex, TIV and scanner as confounding variables. Five thousand permutations were generated, and cluster-like structures were enhanced using the TFCE approach[17], with a significance level set at $p \leq 0.05$, corrected for multiple comparisons across space using the family-wise error rate (FWER), and an extent-threshold k=50 voxels to avoid false positive results.

When regional differences in terms of local GM or WM volume emerged between the two groups, the corresponding first eigenvariate was extracted from the cluster and corrected for the effect of age, sex, TIV and scanner in HC: for each metric, the linear relationship with these variables was modelled in the HC group and used to compute standardized residuals in all subjects. The relationship between the so obtained Z-scores and clinical variables was assessed via linear (total Tsui score, DD, BoNT treatment duration) and binary logistic (tremor) regression analyses, validated using the bootstrap method with 5000 replications. Likewise,

adjusted Z-scores of other cerebellar volumes that emerged as significantly different at the between-group ROI analyses were entered in similar regression analyses. Significance level for regression models was not adjusted for multiple testing given the exploratory nature of the analyses.

RESULTS

Subjects

27 patients with ICD (mean age 50.4±11.3 years, F/M=14/13) and 27 HC of comparable age and sex (mean age 51.7±11.5 years, F/M=14/13) were enrolled in the study from the University "Federico II" (12 ICD: mean age 50.1±14.0 years, F/M=5/7; 12 HC: mean age 50.3±14.3 years, F/M=5/7) and the "Don Gnocchi" Foundation (15 ICD: mean age 50.6±9.0 years, F/M=9/6; 15 HC: mean age 52.8±9.0 years, F/M=9/6). Mean DD for ICD patients was 7.1 years (standard deviation: 6.3), with a median Tsui score of 8 (interquartile range: 5 - 10). Tremor was present in 12 (out of 27) patients.

Demographic and clinical characteristics of all subjects included in the analysis are reported in Supplementary Table 1.

Between-group comparisons

When investigating possible differences in terms of cerebellar GM volumes, ICD patients showed a significant volumetric reduction of the anterior cerebellum compared to HC (15.4±1.5 vs 16.1±1.5, ICD vs HC; p=0.006, FDR-adjusted p=0.05). At a lobular level, ICD patients demonstrated significant atrophy of cerebellar lobules I-IV (7.0±0.6 vs 7.3±0.7, ICD vs HC; p=0.004, FDR-adjusted p=0.05), V (8.4±0.9 vs 8.8±0.8, ICD vs HC; p=0.01, FDR-adjusted p=0.05) and VI (19.6±2.1 vs 20.5±1.8, ICD vs HC; p=0.009, FDR-adjusted p=0.05) (Table 1).

Regarding cerebellar WM tracts, ICD patients showed reduced volume of the bilateral SCP (0.7±0.1 vs 0.7±0.1, ICD vs HC; p=0.04) and MCP (9.5±0.9 vs 9.9±1.1, ICD vs HC; p=0.03), which did not retain statistical significance after correcting for multiple comparisons (Table 1).

There was no significant effect of the scanner per group interaction term. The ancillary analysis

demonstrated nearly symmetrical cerebellar GM and WM atrophy patterns, with slight right-side predominance (Supplementary Table 2).

At the VBM analyses, clusters of reduced GM volume in both right (FWER-corrected p=0.01) and left (FWER-corrected p=0.04) cerebellar lobules IV, V and VI emerged in ICD patients compared to HC (Table 2, Figure 2A), along with small clusters of reduced WM volume in the right cerebellum (FWER-corrected p=0.04) and the left midbrain (FWER-corrected p=0.04) (Table 2, Figure 2B). No significant between-group differences emerged for the ICD>HC contrast.

Relationship between MRI features and clinical data

When exploring the clinical correlates of the observed MRI alterations, no significant relationship was found between MRI metrics and either the total Tsui score, DD or BoNT treatment duration, with an association between the presence of tremor and the bilateral MCP volume (Nagelkerke R^2 = 0.190, p = 0.04; B = 0.736 [bias-corrected and accelerated bootstrap 95% confidence interval = - 0.137 to 2.491, p = 0.04]).

DISCUSSION

In this study, we investigated the presence of possible structural modifications in the cerebellum of ICD patients, demonstrating a specific spatial pattern of decreased cerebellar GM and (to a lesser extent) WM volumes, resulting from both ROI-based and voxel-based analyses.

In recent years, modifications of cerebellar structure and function have gained increasing attention as a possible physiopathological substratum of primary dystonia, which has been the object of a paradigm shift: from being considered a basal ganglia disease to a complex network disorder involving cerebellar, cortical and subcortical motor (and non-motor) areas, along with their reciprocal connections[2, 4, 5].

Nevertheless, discordant evidence exists regarding volumetric changes in the cerebellum of dystonic patients, with several structural MRI studies variably reporting increases[7, 19, 20], decreases[8, 9], or no modifications[10, 21, 22] of cerebellar GM volumes, with inconstant spatial patterns. This apparent inconsistency may be attributable to different factors, including small size and heterogeneity of the patient cohorts (often including different types of focal or segmental dystonia[8, 9, 19, 20, 22]), and methodological differences (with most studies using whole-brain rather than cerebellum-oriented approaches[7, 10, 19-22]), all severely hindering meaningful comparisons between studies.

In our work, we focused on a homogenous sample of ICD patients, using cerebellum-tailored analyses with complementary ROI-based and voxel-based approaches, both demonstrating consistent volume loss of cerebellar GM at the level of the anterior lobe and lobule VI in patients compared to HC. Of note, these results partially overlap with those of a recent study adopting a similar cerebellum-oriented approach[9], which has proven to be more sensitive and accurate compared to whole-brain analyses for the characterization of infratentorial structural

abnormalities[12]. Interestingly, a slight right-side predominance emerged at both the ROI-based and voxel-based analyses, in accordance with the already reported asymmetry of brain imaging findings in this condition[7, 21].

According to the topographic organization of the cerebellum, lobules of the anterior lobe and lobule VI contain the representation of sensorimotor functions, participating in the coordination of fine movements of the extremities as well as in the control of posture and gait[14]. These regions, densely connected with spinal cord, brainstem and cerebral cortical areas involved in sensorimotor processing, show a precise somatotopic arrangement[14]. Interestingly, for both sensory projections carrying cutaneo-kinesthetic information via the trigemino-cerebellar tracts and afferent and efferent branches of the motor cortico-ponto-cerebello-thalamic-cortical loop, the representation of the head/neck and face/mouth lies principally in lobule VI, with some extension into lobules V and IV[14, 23]. In this light, the observed volume loss in the cerebellum may express the selective vulnerability of specific cerebellar cortical areas containing the representation of the affected body parts (head/neck for ICD patients), demonstrating a link between cerebellar involvement and the topography of dystonic symptoms[2, 4]. However, it remains unclear if cerebellar cortical atrophy represents a primary abnormality or a secondary effect resulting from damage in other salient supratentorial areas and/or in projection tracts interconnecting them, which has been also demonstrated in ICD patients[24].

In order to explore the afferent and efferent connections of the cerebellum, we also investigated possible cerebellar WM structural modifications, demonstrating a slight volumetric reduction (not surviving multiple comparisons correction) of the middle and superior cerebellar peduncles, containing the main afferent and efferent branches of the cortico-ponto-cerebello-thalamo-cortical loop, respectively[24]. These results are in line with previous studies reporting

microstructural damage of cerebellar peduncles in dystonic patients[25], and support the hypothesis of a sensorimotor network disorder, underpinned by structural and functional modifications involving different nodes at the level of cerebral cortex, basal ganglia and cerebellum, as well as their reciprocal connections[2, 4, 5].

When looking at the relationship between the observed structural modifications and clinical variables, a positive correlation emerged between the volume of the bilateral middle cerebellar peduncles and the presence of tremor. These results are in contrast with findings in other tremorous conditions (mainly essential tremor), in which an association between tremor and cerebellar peduncles' macro- and micro-structural damage has been described[26], suggesting that cerebellar involvement contributes to the genesis of tremor in cervical dystonia through distinct physiopathological mechanisms[27]. Regarding the relationship with BoNT treatment, which has been anecdotally linked to changes in brain structure[28], no significant association between treatment duration and cerebellar atrophy emerged, possibly due to the low variance of BoNT treatment duration in our sample.

Some limitations of the present study need to be acknowledged. Firstly, MRI exams were acquired at different field strengths, introducing a possible source of bias. However, demographic and clinical characteristics of the subjects examined with the two scanners were highly homogeneous, and all statistical analyses included field strength as a confounding variable, thus greatly limiting possible scanner-related bias and even increasing the generalizability of our results. Furthermore, a more extensive clinical examination including finer evaluations of motor, as well as sensory, cognitive, neuropsychiatric and autonomic domains may have allowed for greater insight into the contribution of cerebellar modifications to the development of motor and non-motor symptoms, which are known to occur in dystonic

patients[29]. The implementation of other advanced MRI techniques focusing on the analysis of brain structural and functional connectivity could have helped interpret the observed cerebellar modifications in the framework of a more complex network disorder[2, 4, 5]. Finally, a longitudinal evaluation might have provided the means to unravel the causal relationship between cerebellar, cortical, basal ganglia and interconnecting WM tracts abnormalities, as well as to investigate the potential reversibility of brain structural modifications in response to BoNT treatment

In conclusion, our data show evidence of a specific pattern of cerebellar structural abnormalities in ICD patients, with volume loss mainly involving cortical GM of the anterior cerebellum and lobule VI, consistent across both ROI-based and voxel-based approaches and seemingly related to the somatotopic representation of the affected body parts. These results, notwithstanding the abovementioned limitations, may shed novel light on the nature of cerebellar modifications in ICD and their role in the physiopathology of this condition.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Conflicts of interest (activities not related to the present article)

S.C. reports fees for speaking from Sanofi and Amicus

The remaining authors have no conflicts of interest to declare.

TABLES

Table 1. Cerebellar volumes for all subjects included in the analysis. Volumes of cerebellar lobules (considered as the sum of right, left and vermian portions) and cerebellar peduncles (considered as the sum of right and left components) are presented, along with the effect sizes (Cohen's *d*) and *p*-values of the between-group differences.

Volumes	**ICD (N=27)**	**HC (N=27)**	**Cohen's *d***	**Uncorrected *p* (FDR-adjusted *p*)**
Cerebellum	121.5±11.0	124.8±9.4	0.62	0.05* (0.12)
Anterior Lobe	15.4±1.5	16.1±1.5	0.87	**0.006 (0.05)**
Posterior Lobe	106.1±9.5	108.7±8.1	0.56	0.07 (0.13)
Lobules I-IV	7.0±0.6	7.3±0.7	0.91	**0.004 (0.05)**
Lobule V	8.4±0.9	8.8±0.8	0.78	**0.01 (0.05)**
Lobule VI	19.6±2.1	20.5±1.8	0.83	**0.009 (0.05)**
Crus I	26.9±2.8	27.5±2.4	0.50	0.11 (0.18)
Crus II	20.0±1.7	20.2±1.7	0.23	0.44 (0.49)
Lobule VIIB	10.3±1.0	10.6±0.9	0.42	0.17 (0.21)
Lobule VIIIA	11.2±1.0	11.5±0.8	0.50	0.11 (0.18)
Lobule VIIIB	9.0±0.8	9.2±0.6	0.45	0.14 (0.19)
Lobule IX	7.4±0.8	7.6±0.7	0.23	0.45 (0.49)
Lobule X	1.8±0.2	1.8±0.1	0.04	0.89 (0.90)
SCP	0.7±0.1	0.7±0.1	0.63	0.04 (0.11)*
MCP	9.5±0.9	9.9±1.1	0.67	0.03 (0.10)
ICP	3.0±0.3	3.1±0.3	0.59	0.06 (0.13)

Cerebellar Volumes (in ml) are expressed as mean ± SD.

Significant differences are reported in bold.
**Not significant after FDR-correction.*

ICD, Idiopathic Cervical Dystonia; HC, Healthy Controls; SCP, Superior Cerebellar Peduncle; MCP, Middle Cerebellar Peduncle; ICP: Inferior Cerebellar Peduncle.

Table 2. Results of the voxel-based analyses. Clusters of decreased GM and WM volume in ICD patients compared to HC are presented, along with significance level (FWE-corrected) and the corresponding local maxima's effect sizes, T values and anatomical labels. No significant differences emerged when testing the ICD > HC contrast. Coordinates refer to mm from the anterior commissure in MNI space, with anatomical labeling according to[30].

	Cluster Volume (ml)	*p*-value (FWE-corr)	Cohen's *d*	T	MNI Coordinates (mm) X	Y	Z	Anatomical Label
GM	9.70	0.01	1.33	4.61	25	-72	-20	Right Cerebellar Lobule VI
		0.01	1.38	4.77	14	-52	-13	Right Cerebellar Lobules IV-V
	2.01	0.04	1.00	3.47	-8	-60	-11	Left Cerebellar Lobules IV-V
		0.04	1.02	3.54	-15	-58	-25	Left Cerebellar Lobule VI
WM	0.09	0.04	1.34	4.26	24	-62	-31	Right Cerebellum
	0.39	0.04	1.09	3.76	-12	-18	-13	Left Midbrain

GM, Gray Matter; WM, White Matter; ICD, Idiopathic Cervical Dystonia; HC, Healthy Controls.

FIGURES

Figure 1. Results of the segmentation of cerebellar lobules and peduncles. (A) In a 53-year-old female patient, the SUIT cerebellar atlas is aligned in the native subject space and superimposed on (*from left to right*) axial, coronal and sagittal reconstructions obtained from the 3D T1-weighted sequence. **(B)** In a 54-year-old male patient, atlas-derived cerebellar peduncles ROIs are aligned in the native subject space and superimposed on coronal (*left column*) and axial (*right column*) reconstructions obtained from the 3D T1-weighted sequence.

Figure 2. Results of the voxel-based analyses. Thresholded statistical maps (*in red-yellow*) for the ICD < HC contrast regarding GM **(A)** and WM **(B)** volumes are superimposed on the SUIT T1-weighted template in axial planes.

ICD, Idiopathic Cervical Dystonia; HC, Healthy Controls; GM, Gray Matter; WM, White Matter.

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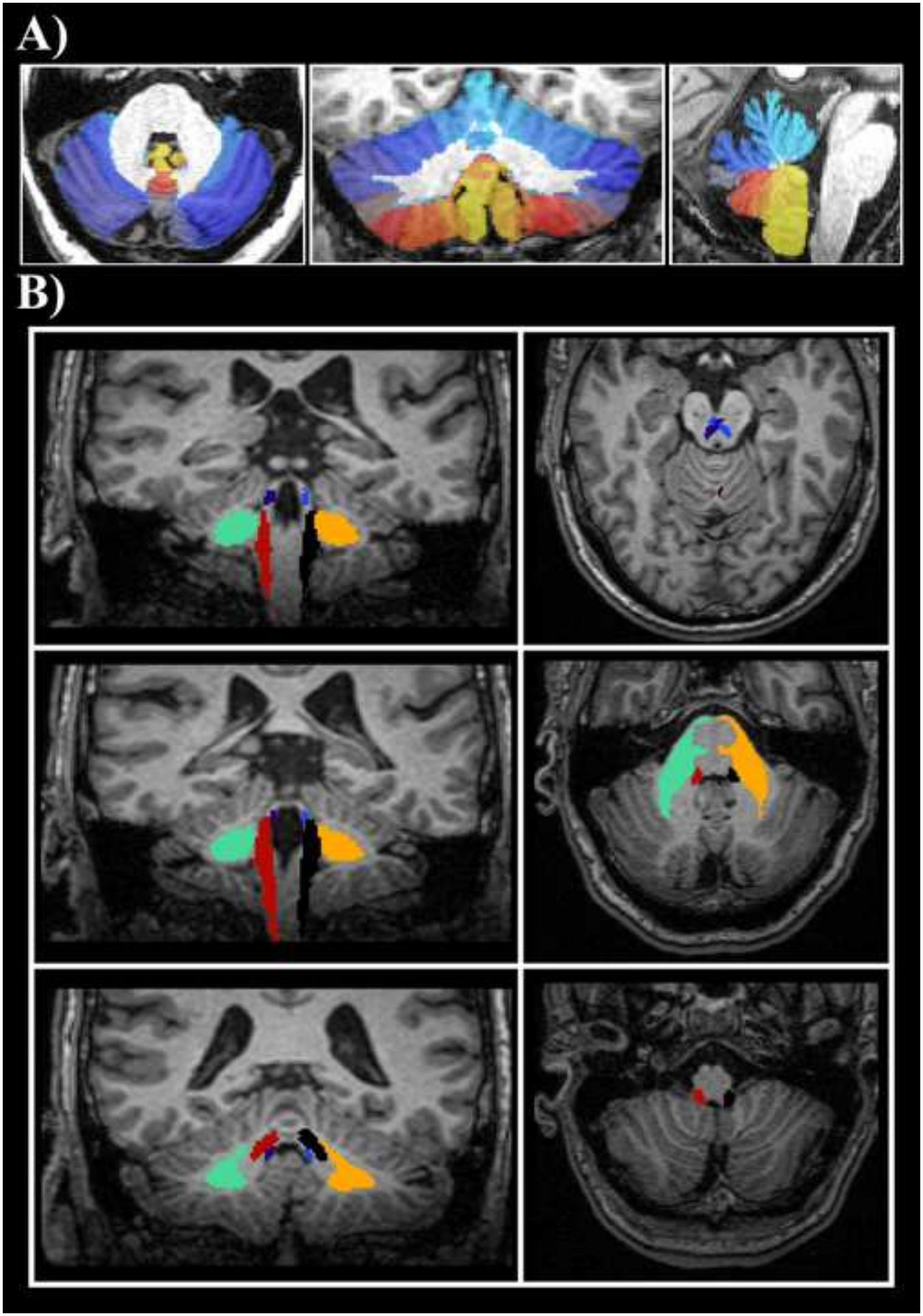

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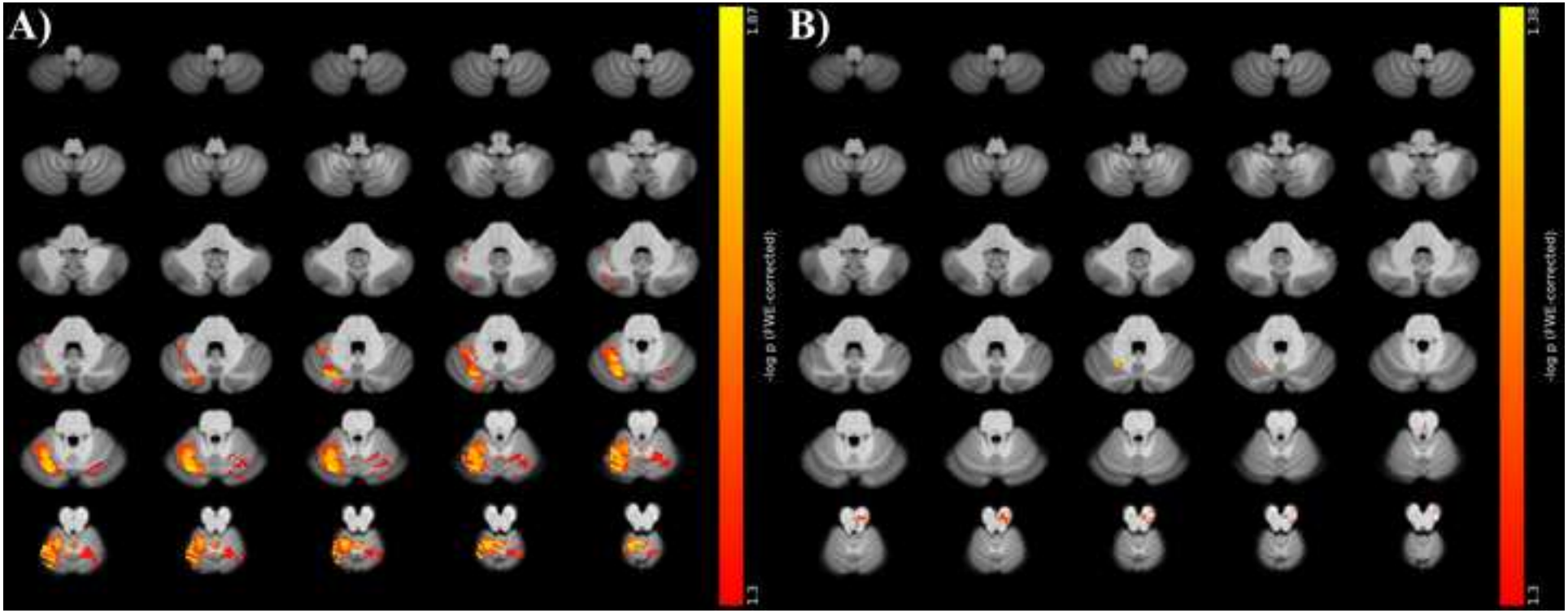

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