Cerebellar Clustering and Functional Connectivity During Pain Processing

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M. Diano[1,2] & F.D'Agata[3] & F. Cauda[1,2,4] & T. Costa[1,2] & E. Geda[5] & K.Sacco[1,4,5] &
S. Duca[2] & D. M.Torta[1,2] & G.C. Geminiani[1,2,4,6]

Abstract The cerebellum has been traditionally considered a sensory-motor structure, butmore recently has been relatedto other cognitive andaffective functions. Previous research and meta-analytic studies suggested that it could be involved in pain processing. Our aim was to distinguish the functional networkssubservedbythecerebellumduringpainprocessing. Weusedfunctionalmagnetic resonanceimaging(fMRI)on12 subjects undergoing mechanical pain stimulation and resting state acquisition. For the analysis of data, we used fuzzy c-mean to cluster cerebellar activity of each participant during nociception. The mean time courses of the clusters were used as regressors in a general linear model (GLM)analysis to explore brain functional connectivity (FC) of the cerebellar clusters. We compared our results with the resting state FC of the same cluster and explored with meta-analysis the behavior profile ofthe FC networks.We identified three significantclusters: clusterV,involvingthe culmen and quadrangular lobules (vermis IV-V, hemispheres IV-V-VI); cluster VI, involving the posterior quadrangular lobule and superior semilunar lobule (hemisphere VI, crus 1, crus 2), andcluster VII, involvingthe inferior semilunar lobule (VIIb, crus1,crus2).ClusterVwas more connectedduringpainwith sensory-motor areas,clusterVIwithcognitive areas, andclusterVII with emotional areas. Our results indicate that during the application of mechanical punctate stimuli, the cerebellum is not only involved in sensory functions but also with areas typically associated with cognitive and affective functions. Cerebellum seems to be involved in various aspects of nociception, reflecting the multidimensionality of pain perception.

Keywords Cerebellum .Pain .Fuzzyclustering .Functional connectivity

M. DianoandF.D'Agata contributedequallytothis work.

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* F.D'Agata federico.dagata@unito.it

[1]DepartmentofPsychology,UniversityofTurin,Turin,Italy [2]GCS-fMRI,Koelliker Hospital,Turin,Italy [3] DepartmentofNeuroscience,AOUS.GiovanniBattista,University ofTurin,ViaCherasco 15, 10126Turin,Italy

[4] NIT, Neuroscience Institute ofTurin,Turin, Italy

[5] ImagingandPlasticityLab,DepartmentofPsychology,Universityof Turin,Turin, Italy [6]NationalInstituteofNeuroscience-Italy,Turin,Italy

Introduction

Although in the past only sensory-motor functions were attributed to the cerebellum, there is now evidence of its involvement in many other complex functions, such as working memory, spatialprocessing,executivefunctions, emotionprocessing, language, memory, and associative learning (for a review, see[1]). The cerebellum has been considered to be involved in pain processing: for instance, in rats, pain stimulation evokes changes in neural activity inthe posterior cerebellar vermis[2],andin

humans, cerebellar lesion alteredpain perception[3].Asshowninalargefunctional magnetic resonance imaging–positron emission tomography (fMRI-PET) meta-analysis[4], the cerebellum is activated during painrelatedstimulation in conjunction with the areas typically associated with pain: the bilateral insula (with a right dominance),anterior cingulatecortex(ACC),bilateralprimary motor/sensory cortex (MI/SI), secondary somatosensory cortex (SII), posterior parietal cortex, andprefrontal cortex. Specifically, the nociceptive-related activation was observed in the deep cerebellar nuclei andanterior vermis IV-V, bilaterally in hemispheric lobules VI, VIIb, crus 1, and crus 2. Noxious stimulielicit other processes relatedto pain, butnotexclusive toit.Infact,painisa multidimensional construct[5–8]and includes many aspects that couldbe relatedto the cerebellum: motor reactions, orienting of attention, evaluation processes, anticipationandnegativeemotions,andconsciousness[9].All these functions may be involved in nocifensive withdrawal responses, sensory-motor integration, inhibition of action (e.g., freezing), anticipation of sensation, and emotion/ emotional memoryrecall[9].Althoughthere are manydifferent functions, some authors have postulated that the cerebellumdevelops onlyasingle computationalfunction:beingable to learn/predictthe outcome of an action usinginternalstored models(see[10]forageneraldescriptionoftheinternal modelidea).Somedata[9]suggestedthat computationalpowerof the cerebellum couldbe used for different tasks, dependingon the cerebellar areas involved and their cortical connections. This is well depicted by the very different areas activated during pain processing: the ipsilateral posterior cerebellum during the signal of impendingpain andthe bilateral anterior cerebellum during the actual pain. Taking into account the different spatiallocations of the anticipatory andpain activations, multiple parts of the cerebellum seem to be involved together with different large-scale networks that process painfuleventsindifferent ways[9].Inparticular,the cerebellumis directly andindirectly connected with some cortical and sub-cortical areas which are part of a centralpain modulation network:the periaqueductal gray (PAG) andperiventricular gray (PVG), the rostroventral medulla (RVM), the medial frontal and orbital cortices, the thalamus, the hypothalamus, andthe brainstem[11–17]. This pattern of multiple connections could be the reason of the heterogeneityof functions carried out by the cerebellum.

The aim of this study was to distinguishthe main cerebrocerebellar networks subserved by the cerebellum during mechanical punctuate stimuli processing.The nociceptive stimulus was deliveredby a pinprick stimulator that is composed byaflatcontactarea(edgecurvature)of0.25mmindiameter andthe punctuate stimulus can be administered withdifferent standardizedforces.We usedafuzzyclusteringtechnique on fMRI data to find cerebellar areas exhibiting different and independentbraindynamics.Wechoosethis analysisinstead of canonicalHemodynamicResponseFunction(HRF),implemented in a general linear model (GLM), because the latter onlyallowsidentifyingthe areaswith astandard responseto stimuli.Recently,different authorsdemonstratedthatthepainrelatedbloodoxygenation leveldependent(BOLD)activity is often complex and heterogeneous[18–20]and therefore a canonical GLM, being strictly linked to HRF, could be unsuitable to detect also the non-HRF response of the other pain-related networks.
After finding the cerebellar statistically independent clusters,obtained from fuzzyclustering technique,wedetermined, usingfunctional connectivity(FC)techniques,towhich cerebello-cortical networks they were connected, correlating clusters time courses with the whole brain voxel'stemporal profile. Furthermore, we compared the obtained pain-related FCmaps with theones derivedfrom restingstatedata from the same cerebellar clusters. This step couldhelpus to understand if the brain activity and connectivity are Bnormally^ present, or if this is specifically related tomechanical punctuate stimulation.
To clarify some functional meaning of the cortical areas that are more connected during nociception, we performed meta-analyses on them with the assistance of the BrainMap MRI/PET database(https://brainmap.org).
Lastly, we performed an Activation Likelihood Estimation (ALE), a meta-analytic statisticaltechnique, to determinate if the cerebellar independent clusters that we found are prevalently observed in literature in pain studies or can be also observed for other passive sensorialconditions, such as tactile stimulation.

Materials and Methods

Meta-analyseswere conductedwithSleuth2.2andGingerAle 2.3(http://www.brainmap.org/).
All other statistical analyses were conducted using BrainVoyager QX 2.3.1 (Brain Innovation, Maastricht, the Netherlands, http://www.brainvoyager.com).

Participants

Twelvehealthyvolunteers(5femalesand7males, meanage± SD=27±4years)tookpartinthisstudy.The volunteers were recruitedfromthestaffandstudentsoftheUniversityofTurin andallgave written informed consent. Allthevolunteers were right-handed according to the Edinburgh Assessment Scale [21],with no historiesof neurological or psychiatric disorders. Thisstudy conformedtothestandards requiredbytheDeclaration of Helsinki and was approved by the local ethics committee.

ExperimentalProcedure

During the imaging acquisition, the subjects were asked to remain still with their eyesclosed. Theparadigm(slow event-related design) was dividedinto four runs, with12 mechanical punctuate stimuliappliedduring each run. The stimulation was delivered on the back of the hands with a MRI-compatible 256mN pinprick.

The pinprick is a hand-helddevice that has been shown to selectively activatetypeIA-deltafiberswhichisincludedin the commonclinical evaluationof nociceptionintheGerman protocol.It activatesareasrelatedtotheelaborationofnoxious inputs[22]andtherefore constitutesa valid and more easily usable alternative to other kinds of nociceptive stimulation. This experimental paradigm was characterized by a slow event-related paradigm (inter-stimulus interval ~20 s), with pinprickstimulusdurationofabout1 sto correctly studythe complete BOLD response generatedby thepainful stimulation.

Theside andthestimulus location on thehand were chosen usingapseudorandomstrategy, maximumthreetimes consecutivelyon thesame hand,counterbalancedwithin subjects and avoiding stimulatingtwice the same point. The stimulihad a variable inter-stimulus interval of 18–22 s.To assess the intensity of the stimulation withoutintroducing any modification of the pain-induced BOLD response, the subjects were askedtoindicatethe meanofthepainintensity onlyatthe end of each run, using a scalefrom0to10,with0 corresponding to Bno pain intensity^ and 10 to Bthe highest pain intensity imaginable^ (group mean 3.3with standard deviation ±2.2).

For restingstate acquisition, the participant was instructed to laydown with their eyes closed andaskedto notfall asleep duringthe scan.The restingstate acquisition was setupprior to the mechanical punctuate stimulitask.

fMRI Acquisition

Data were acquired on a 1.5-Tesla INTERA scanner (Philips Medical Systems)with a SENSE high-field, high-resolution (MRIDC)head coil optimized for functional imaging.FunctionalT2*-weighted images were acquired using echoplanar (EPI) sequences, with a repetition time (TR) of 2000 ms, an echotime(TE)of50 ms, anda90°flip angle.The acquisition matrix was 64×64;the fieldof view (FoV) was 256 mm. For each run (four runs for mechanical punctuate paradigm, one run for resting state paradigm), a total of 240 (mechanical punctuate paradigm) or 200 volumes (resting state paradigm) were acquired. Each volume consistedof 19 axialslices, parallel to the anterior-posterior (AC-PC) commissure line and coveringthewholebrain;theslicethickness was5mmwitha 1-mm gap (4×4×5 mm^3 voxels). Three dummy scans were added at the beginning of functional scanning and the data discardedto reach a steady-state magnetization before acquisition of the experimental data.

Inthe same session,a setof3Dhigh-resolutionT1-weighted structural images was acquired for each participant. This data setwas acquired usingaFastFieldEcho(FFE)sequence, withaTRof8100 ms,ultra-shortTE25 ms, and a30°flip angle. The acquisition matrix was 256×256, and the FoV 256 mm.The set consistedof 107 contiguous sagittalimages covering the wholebrain. In-plane resolution was 0.9× 0.9 mm^2 and slice thickness 1.5 mm(0.9×0.9×1.5 mm^3 voxels).

fMRIPre-processing

BOLDimageswerepre-processedin orderto reducethenoise and remove the artefacts for all the functional runs: (1)slice scan time correction using a sinc interpolation algorithm; (2) thewhole volumes wereadjustedby meanintensity[23];(3) 3D motion correction using a trilinear/sinc interpolation algorithm (all volumes were spatiallyalignedto the first volume);
(4) whole volumes were spatially smoothed using a Gaussian kernel of 4 mm full width at half maximum (FWHM); (5) temporalfiltering(lineartrendremovals)andahighpassfilter to reduce cardiac and respiratory noise.
We performed a series of pre-processing steps to ease the coregistrationofthefunctionaldataintoaTalairach template, allowing inter-subject comparison. First, each subject'sfunctional scanswere coregistered on theirstructural scan. Second, the structuraldatawereBskullstripped^ (removal of the skull and scalp) and transformedinto the standardTalairach space [24]. Third, thefunctionaldata of each subject were coregistered using the transformation matrix derived from the previous steps. The Talairach transformation was performed using this procedure implemented in BrainVoyager software:(1)thebrainwasrotatedandalignedintotheanterior and posterior commissure plane; (2) the cerebrum borders were identified to allow the scale transformation of the 3D structuraldataintotheTalairach andTournoux standardbrain using an affine transformation.

ClusteringCerebellum Activity

In order to define clusters of activity in the cerebellum, we performed a technique that compared the time course signals of selected voxels, clustering themby their temporal similarity.
Fuzzyc-meanclusteringisadata-driven methodthatcompares the time courses of voxels, divides them into clusters, andlabels them with a value derivedfrom the distance from the clusters' centroids. This technique is named Bfuzzy^ because insteadof assigning everyelement to exactly one cluster, it produces for each voxel multiple cluster memberships with different probabilities[25].
Allthe centroids and memberships are constantlyupdated following the mathematical procedure described by Bezdek [26], which terminates when the interactions do not significantly change, establishedvia cluster algorithm distance measure. For the current fMRI dataset, the fuzziness coefficient was set to 0.4 as suggested in literature[27, 28].We limited the analysis tothe cerebellum with an exclusive mask, hand-drawn on the mean of allfunctional data.

We decidedto use a slow event-relatedparadigm because this design optimizes the clustering techniques, leaving the time necessaryto BOLD responseto expire between stimuli, so everyeventwas decoupledto the others. On the other side, the slow event-relatedparadigm entailedless signal respectto otherparadigms(i.e.,blockdesign)andpenalizedthestandard GLMapproachwith canonicalHRF.Wealso employedalittle spatialsmoothing(4 mm)inthepre-processingbecauseofthe smallness of cerebellar structures.

We usedSelf-organizinggroup-levelICA(SogICA,[29]) to summarize individual decomposed data sets on a group level and put together similar components between subjects [30]. Random effect group-level analyses (RFX) were computed on similar components extractedfrom fuzzyclustering ($p<0.01$), cluster-level corrected for multiple comparisons ($p<0.05$),usingaMonteCarlosimulation[31,32].We used classTAL (doi:10.1038/npre.2011.6142.2), a Matlab script that automatically classifies BrainVoyager statistical maps using afni(http://afni.nimh.nih.gov/afni)brain atlases. The script generates several useful output files (tables, region of interest (ROI)lateralization, figures).

The clusters, obtainedfrom the group analysis, were carefully examined to keep only the significant clusters unrelated to residual physiological or movement artefacts.

Functional Connectivity of the Clusters

Functionalconnectivity maps were computed asin[33]. BOLD time courses were extracted, both during nociception and resting state conditions, from each significant cerebellar clusterbyaveragingovervoxelswithin eachregionpreviously derivedfrom fuzzyclustering.

To reduce the noise derived from physiological processes (cardiac andrespiratoryrhythms), we included eightadditional covariates that modeled nuisance signals sampledfrom the white matter(WM) and cerebrospinalFluid(CSF), as well as from six motion parameters (three rotations andthree translations as savedbythe3Dmotioncorrection).Wederivedthe WM/CSF nuisance signals averagingthe time courses of the voxels in each subject's WM/CSF masks, andhand-drew the masks on the average of the subjects' functional volumes in order to be sure of sampling the right tissues. All predictors were z-normalized.

For eachcerebellarseedROIandeachsubject,anFCmap was computed on a voxel-wise basis for each condition: mechanical stimulation, resting state, and comparison between them.For each subject,theGLM[34]for multiple regression analysis resulted in ROI-based t-maps (SPMt). RFX group-level maps were computed on SPMt with a threshold of $p<0.01$ cluster-level corrected($p_{corr}<0.05$)for multiple comparisons[31, 32]and classified with classTAL Matlab script (doi:10.1038/npre.2011.6142.2).

Meta-analytic BehavioralProfile Analysis

BrainMapisadatabaseofpublishedfunctional neuroimaging studies (mainly PET and fMRI) that contains both metadata descriptions of experimental design andactivation locationsin theformofstereotactic coordinates[35].Atthepointof analysis, BrainMap contained2390 neuroimaging papers that analyze11,353 experiments using 99 unique paradigm classes, yielding to 91,039 locations or foci (February28, 2014).

We extracted the behavioral profiles[36]of theFC contrasts nociception>restingstate resultsto enrichthefunctional description of the cortico-cerebellar networks, in particular of the areas specifically synchronized by pain. We used the MANGO plugin Behavior Analysis 1.3: http://ric.uthscsa.edu/mango/plugin_behavioralanalysis.html. The plugin performed regional behavior analysis based on selected brain ROIs. The analysis was coordinate based, and results were presentedfor BrainMap'sfivemainbehavioral domains (Action, Cognition, Emotion, Interoception, and Perception) and 51 subdomains (e.g., Emotion-Fear). Only z-scores ≥3.0 are consideredsignificant($p\leqq0.05$withBonferronicorrection for multiple comparisons).

Meta-analysis

The ALE analysis is a quantitative meta-analytic method that can be used to estimate consistent activation across different imagingstudies[37]. ALE maps of coactivations are derived based on patterns of foci of interest, where multiple studies have reportedstatisticallysignificantpeakactivation.Tolimit the intersubject and interlaboratory variability, we used an algorithm that estimates the spatial uncertaintyof each focus, taking into accountthe possible differences amongthe neuroimaging studies[37]. The advantage of such an algorithm is thatitcomprisesa methodtocalculatetheabove-chanceclustering between experiments (i.e., random effects analysis) rather than between foci (fixed effects analysis).

We computed two ALE meta-analyses using BrainMap database and Sleuth 2.2 and GingerAle 2.3 software(http://www.brainmap.org).

WesentqueriestothedatabasewiththeSleuth2.2software [38]. The specific queries were as follows:

1 [Diagnosis = Normals]AND [Behavioral Domain=Perception Somesthesis]AND NOT [Behavioral Domain= Perception Somesthesis (Pain)];
2 [Diagnosis = Normals]AND [Behavioral Domain=Perception Somesthesis (Pain)].

The results of the queries were as follows:

Somesthesis102papers(fMRI,PET),1309 subjects,398 experiments, 2426 foci;

Pain 98 papers (fMRI, PET), 1379 subjects, 328 experiments, 3195 foci.

The complete list of the papers is in a supplementarydoc file.

Statistical maps with the regions of convergence were calculated usingGingerAle2.3software[37,39]intheTalairach space with the more conservative mask size and FWHM values subject-based.

For allanalyses, theselected pthresholdwasselected using theFalse Discovery Rate with positive dependence assumption, FDRpN<0.05 andminimum clusters extent Ke> 500 mm^3.Weused Chris Rorden's MRIcron software (http://www.nitrc.org/projects/mricron)to visualize and save images, overlayingtheGingerAle maps ontoaTalairachbrain template (created by[40]).

To comparethepain and somesthesis maps(pain>touch; touch>pain), all experiments contributing to either analysis were then pooled and randomly divided into two groups of thesamesize asthetwooriginal setsofexperiments.Thatis,if 102 experiments in BrainMap featured activation in ALE somesthesis and 98 featured activation in cluster ALE pain, the resulting pool of 200 experiments would be randomly divided into a group of 102 and a group of 98 experiments. ALE scores for these two randomly assembled groups were calculated and the difference between these ALE scores was recorded for each voxel in the brain. Repeating this process 10,000 times then yielded a null distribution for the differences in ALE scores. The observed difference in ALE scores wasthentestedagainstthis nulldistributionyieldinga pvalue for the difference at each voxel based on the proportion of equal or higher random differences. The resulting nonparametric p values were thresholded at p<0.05 and inclusively masked by the respective main effects.

We compared the pain>touch meta-analytic results with theindependentclusters thatwefoundinthe previous analyses.

Results

Cerebellum Clusters

We obtained eight clusters from the group analysis. These were carefully examined to keep only the significant clusters unrelated to residual physiological or movement artefacts. Two clusters were eliminated because their patterns were prevalently composed of CSF voxels; three clusters were eliminated because they containedWM voxels;three clusters were retained(see supplementaryFig. S1).

We observed three significant clusters(Figs.1,2,3)using fuzzyclustering RFX and we namedthem by the position of their center of mass in terms of cerebellar lobules:

- Cluster V (colored green, Figs. 1, 2, 3), located in the middle-upper part of the cerebellum; geometric center of mass coordinates x, y, z= −1mm, −50 mm, −11 mm; total volume10,592 mm^3;involvingthe culmen(vermis IV-V),anterior andposteriorquadrangularlobules(hemispheres IV-V-VI);
- ClusterVI(colored red,Figs. 1,2,3)involvingtheposterior quadrangular lobule (hemisphere VI) and superior semilunarlobule(crus1, crus2),with twodistinct activation clusters one to the left (geometric center of mass coordinates x, y, z= −29 mm, −57 mm, −30 mm; total volume 1,318 mm^3)and one to the rightof midline (geometric centerofmass coordinates x, y, z=30 mm, −58 mm. −27 mm;total volume 137 mm^3);
- Cluster VII(colored yellow,Fig. 3)involvingtheinferior semilunarlobule(VIIb, crus1, crus2);geometric center of mass coordinates x, y, z=21 mm, −72 mm, −36 mm;

^{3}

total volume 509 mm.

Functional Connectivity During Pain

The functional connectivity analysis of the cerebellar clusters during pain stimulation showed that each of them had a correlation with a different brain network. Cluster V (Fig. 1a, supplementary Fig. S2, supplementary Table S1)showed a positive correlation with the bilateral primary and secondary sensory-motor areas (MI, SI, SII), bilateral superior temporal gyrus, bilateral premotor areas (BA 6), bilateral visual areas (occipital cortex, cuneus, lingual gyrus), cingulate cortex (ACC and posterior cingulate), deep cerebellar nuclei, putamen, hippocampus, parahippocampal gyrus, midbrain, and red nucleus. Insula activation showed a bilateralposterior lo-calization,situated moreintherighthemisphere(60%right lateralized). We refer to this network as a Bsensory-motor network^ becausetheFC analysisshowsanaffinitywith cerebral areas typically associated with sensory-motor function (i.e., bilateralprimaryandsecondarysensory-motor areas MI, SI,SII).

TheclusterVFC also showed anegative correlation (Fig. 1a, supplementaryFig. S2,supplementaryTable S2)in the bilateral middle and superior frontalgyrus, bilateralinferior parietal lobule, bilateralsupramarginalgyrus, angular gyrus, andprecuneus.

RegardingclusterVI(Fig. 2a,supplementaryFig. S3,supplementaryTable S3),theFCshowedapositive correlationin the bilateral-middle frontalgyrus, superior and medial frontal gyrus, bilateral cingulate cortex (middle cingulate), bilateral inferior parietal lobule, bilateralpremotor areas (BA6), bilateral supramarginalgyrus, angular gyrus andprecuneus, bilateral anterior insula, andright thalamus. In this case,the anteriorinsula activationwassituated moreinthelefthemisphere (60% left lateralized).We refer to this network as the

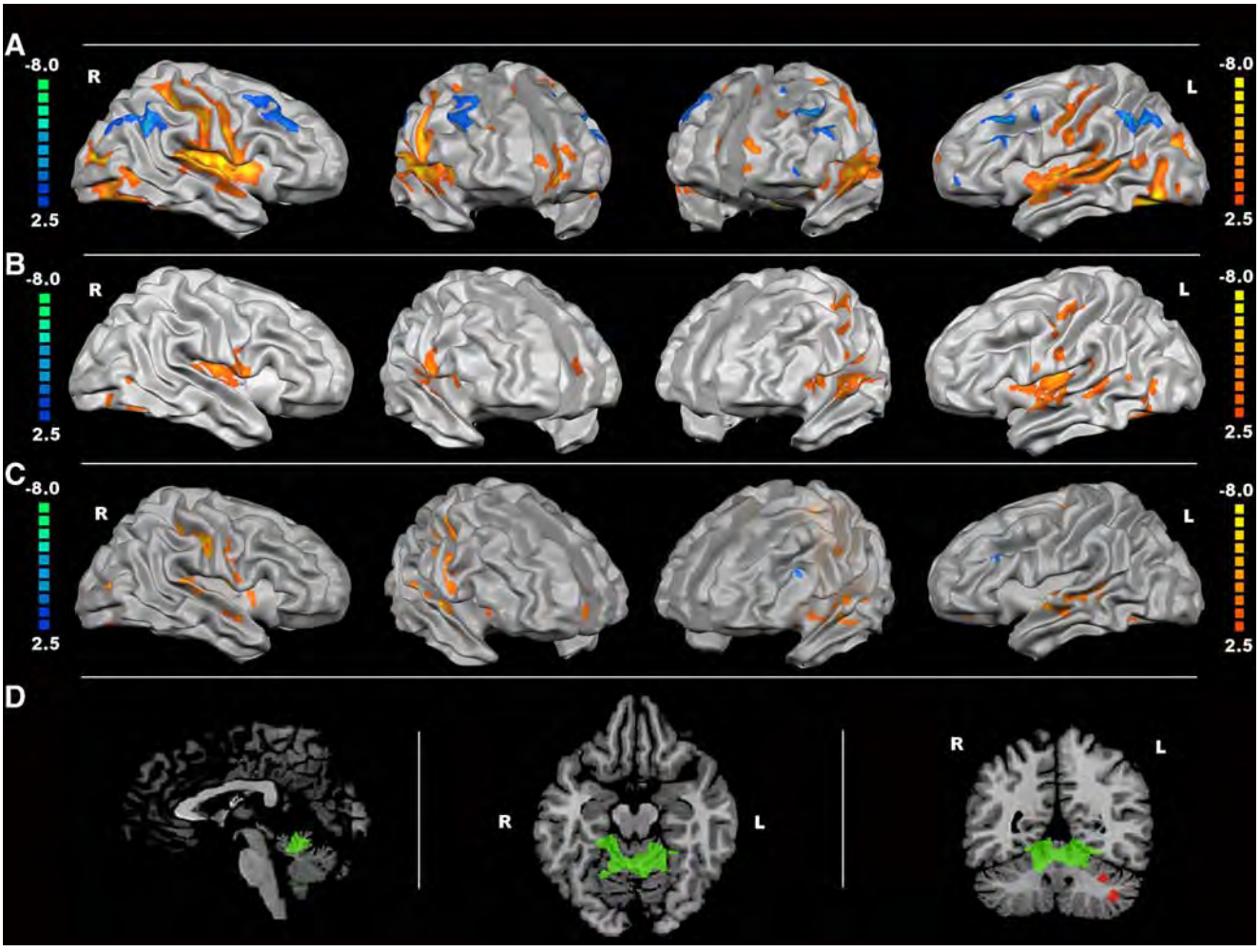

Fig.1 Pain-processingclustering: clusterV. aFunctionalconnectivityof cerebellar clusterV(green)during mechanical punctuate stimulation projected on a 3D template. Positive correlations included the bilateral MI, SI, SII, posterior insula, anterior cingulate cortex, midbrain, and hippocampus; negative correlation included the bilateral frontal gyrus andparietallobule. bFunctional connectivityofthecerebellarclusterV during resting state condition. c Comparison between pain and rest network,
conditions. Positive correlations included the right postcentral gyrus, bilateralsuperiortemporalgyrus,and posterior insulae. d Fuzzy clustering, map of cluster V projected on a 2D template. The cluster extended into the culmen(vermisIV-V) andanteriorand posterior quadrangularlobules(hemispheresIV-V-VI).Themapswere computed with BrainVoyager QX2.3, RFX $p<0.01$ cluster-level threshold corrected for multiple comparisons($p<0.05$)

Battentional network^ because theFC analysis shows an affinity with cerebral areas typically associated withattentional functions (i.e., dorsolateral prefrontal cortex, DLPFC, inferior parietal lobule).

Cluster VI showed negative correlation (Fig. 2a,supplementaryFig. S3,supplementaryTable S4)in the bilateralprimary and secondary sensory-motor areas (MI, SI), bilateral middle andsuperior temporalgyrus, bilateral visual areas (occipitalcortex,cuneus,lingualgyrus),bilateralposteriorinsula, cingulate cortex (ACCandposterior cingulate), putamen, and parahippocampalgyrus.

Many areas that were positively correlated with clusterV were negatively correlated with cluster VI, and many areas negativelycorrelatedwithclusterVwerepositivelycorrelated with cluster VI, so globally cluster V was correlated with motor and negatively correlated withthe attentional whileclusterVI wascorrelated with attentionalandnegatively correlated with the sensory-motor network (Figs. 1a vs. 2a, supplementaryFigs. S2 vs. S3,supplementaryTables S1 vs. S4, S3 vs. S2). In fact, the timecourses of clustersVand VI were inversely correlated($r= -0.31$, $p=0.031$).

For cluster VII, no FC survived the multiple comparison correction, so we used an uncorrectedthresholdof $p<0.01$to examine the connected network(see discussion below). ClusterVII(Fig. 3a,supplementaryFig.S4,supplementary Table S5)showedapositive correlationinthebilateralinferior, middle and superior frontalgyrus, bilateral amygdala, and parahippocampal gyrus.Werefer to this network asthe Bemotional network^ due to its FC with bilateral amygdala and parahippocampal gyri. Relating to negative correlation, the FC showed cingulate cortex (ACC, middle, andposterior) andsome othersmallsparse clusters,althoughthis patternwas

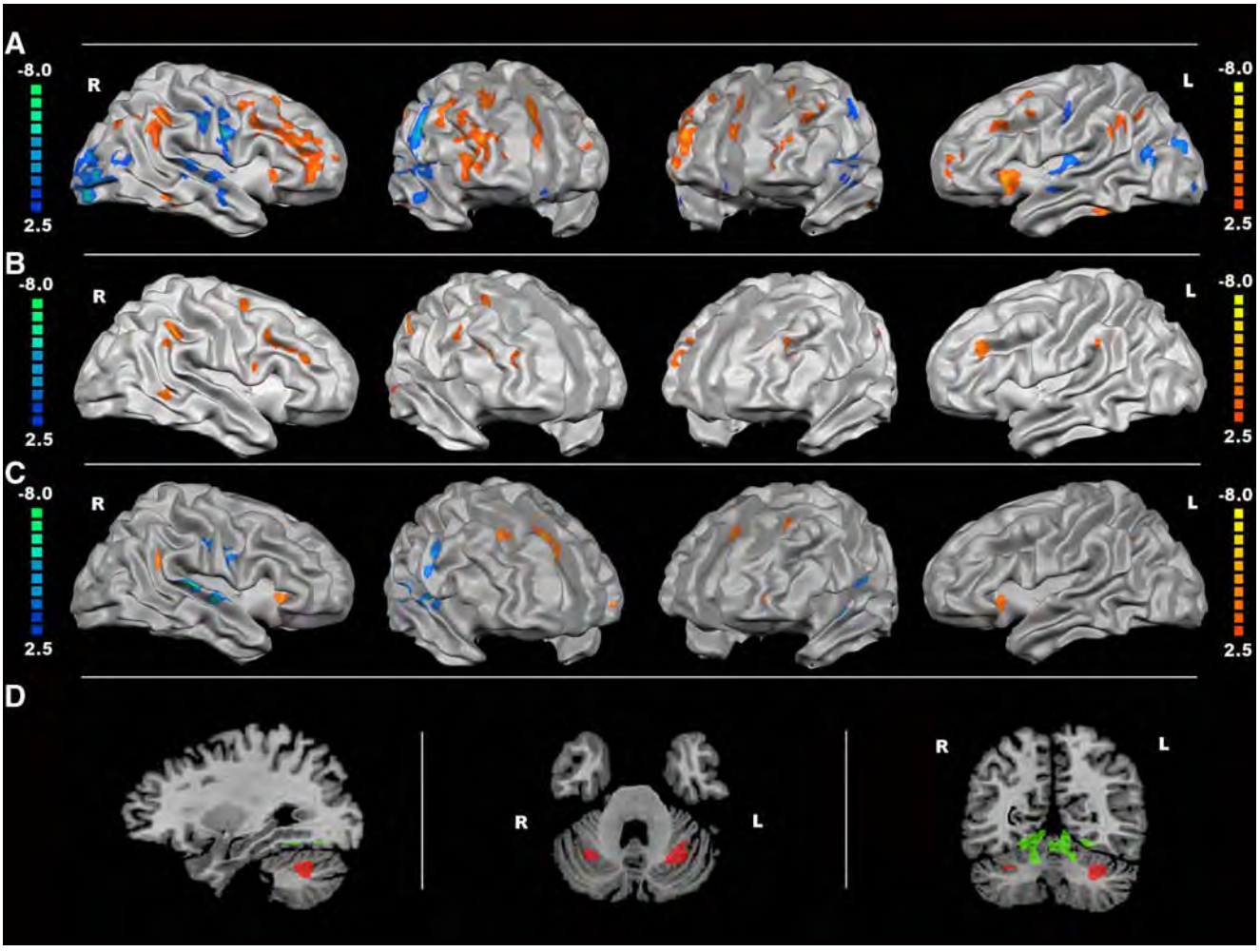

Fig.2 Pain-processingclustering: clusterVI.a Functional connectivity of cerebellar cluster VI(red)during mechanical punctuate stimulation projected on a 3D template. Positive correlations included the bilateral frontalgyrus andparietalcortex, middle cingulate, andbilateral anterior insula; negative correlationsincludedthebilateralMI,SI,posteriorinsula, anterior cingulate cortex, and parahippocampal gyrus. b Functional connectivityofthe cerebellarclusterVIduring restingstatecondition. c Comparison between pain and rest condition. Positive correlations included anterior cingulate cortex, anterior insula, and bilateral inferior parietallobule. dFuzzyclustering,mapofclusterVIprojected ona2D template. The cluster extended into the posterior quadrangular lobule (hemisphere VI) and superior semilunar lobule (crus 1, crus 2). The maps were computed using BrainVoyagerQX2.3, RFX $p<0.01$ cluster-levelthreshold correctedformultiplecomparisons($p<0.05$)

notclear-cut or verymeaningful(supplementaryFig. S4,supplementaryTable S6).

Functional Connectivity During RestingState

FC of the cerebellar clusters Vand VI during resting state showed positive correlations to different networks partially overlappingto that of the FC during pain andgenerally composedby smaller subsets (also less connected, $p_{pain}<p_{rest}$)of pain FC areas (compare Figs. 1a vs. 1b, 2a vs. 2b, supplementary Figs. S2 vs. S5, S3 vs. S6, supplementary Tables S1 vs. S7, S3 vs. S8). Negative correlations for cluster Vand VI during rest were absent or nearly absent (Figs. 1b, 2b,supplementaryFigs. S5, S6,supplementaryTables S9).

FC of clusterVIIwasnotsignificantoutsidethecerebellum (Fig 3b, supplementary Fig. S7, supplementary Table S10), whereasalarge networkcontainingincludingareasfromboth the motor andthe attentionalnetworks were negatively correlated withthis cluster (supplementaryFig. S7,supplementary Table S11).

DifferenceBetweenFunctionalConnectivityDuringPain andRestingState

We contrasted the FC during mechanical punctuate stimulation and resting state.TheBOLD activity of clusterVduring nociception task was increased in the postcentral gyrus (primary sensory cortex, mainly locatedin the righthemisphere), bilateral superior temporal gyrus, and posterior insulae (Fig. 1c, supplementaryFig. S8,supplementaryTable S12).
The BOLD activity of clusterVI during nociception task was increasedin the anterior cingulate cortex, anterior insula

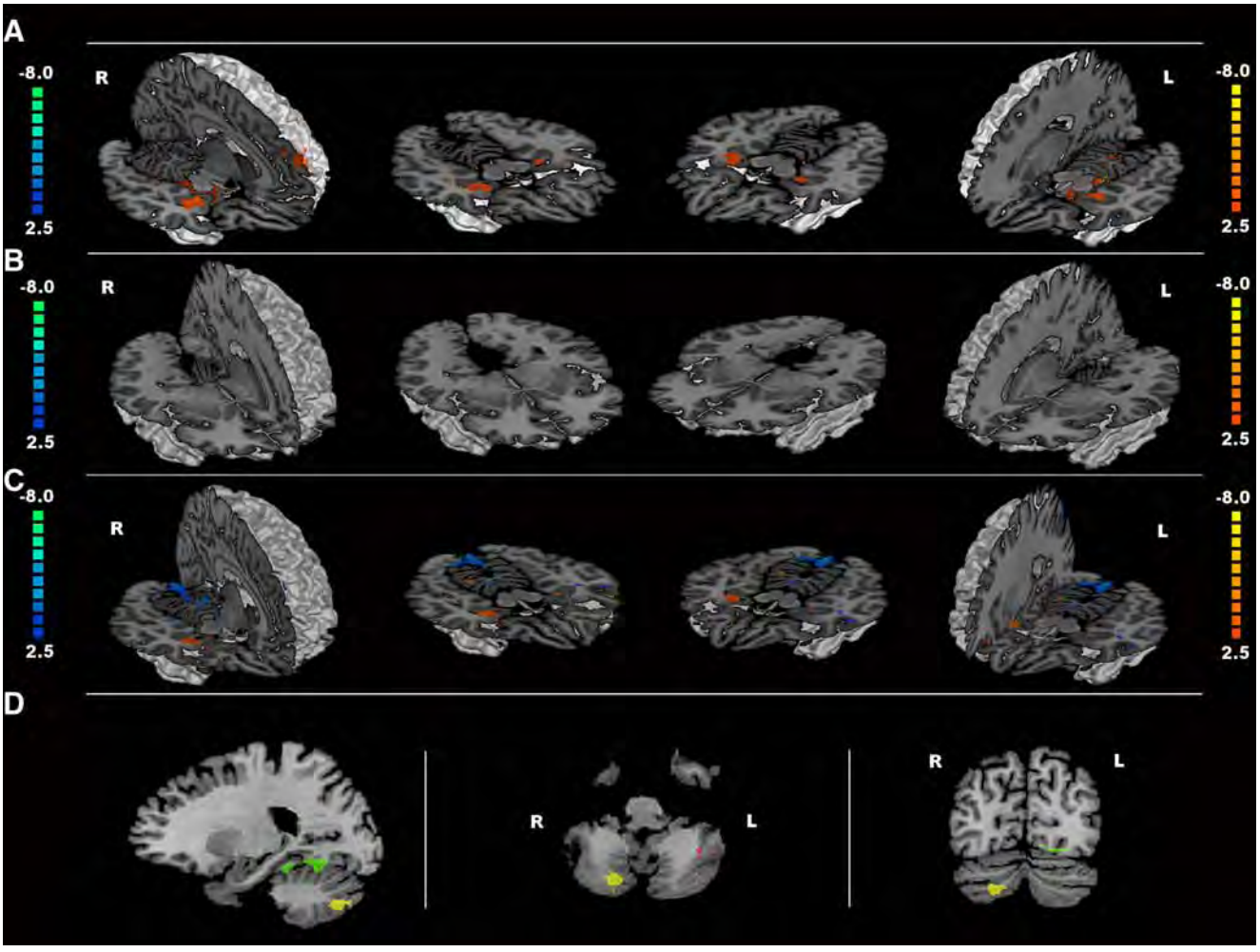

Fig.3 Pain-processing clustering: cluster VII. a Functional connectivity ofcerebellar cluster VII(yellow)duringmechanical punctuatestimulation projected on a 3D template. Positive correlations included the bilateral inferior,middle, andsuperior frontal gyrus,amygdala, and parahippocampal gyrus. b Functional connectivity of the cerebellar cluster VII during resting state condition. c Comparison between pain and rest condition. Positive correlations as in a. d Fuzzy clustering, map of cluster VII projected on a 2D template. The cluster extended intotheinferior semilunarlobule(VIIb,crus1,crus2).Themaps were computed using BrainVoyagerQX2.3, RFX p<0.01 uncorrected threshold

(mainlylocated on the left hemisphere), andbilateralinferior parietal lobule(Fig. 2c,supplementaryFig.S9,supplementary Table S13).

The BOLD activity of cluster VIIduring nociception task was increased in the amygdala (mainly located on the right hemisphere), right anterior insula, left putamen, andleftinferior frontal gyrus (Fig. 3c,supplementaryFig. S10,supplementaryTable S14).

Meta-analytic BehavioralProfile Analysis

Accordingto the BrainMap database, brain regions, we identifiedtobe connectedtoclusterV,havebeentypicallyfound to be active in tasks involving behavioral functions such as Action andPerception,whilethey are not usuallyinvolvedin Cognition andEmotion(Fig. 4a).

InthePerceptiondomain,it was noteworthythatAudition (z=6.16),VisionShape(z=3.08), andSomesthesisOther(z= 3.01) subdomains were particularly involvedwhile Someshesis Pain was not.

Brain regions connectedto cluster VI showed a behavioral profile involving Cognition functions (Fig 4b), in particular the subdomains of MemoryWorking Memory(z=7.39),Attention(z=5.64), and Reasoning(z=3.66). Tasks involving Somesthesis Painsubdomain(z=5.14) activated the areas, in contrast with tasksofthe generalPerception domain (Fig 4b).

Brain regions connectedtoclusterVIIshowedabehavioral profile involving Action and Emotion, in particular in the subdomain ofFear(z=3.56), while Perception andCognition tasks did not usually activate these regions (Fig 4c). Again, tasks involving Somesthesis Pain subdomain(z=3.49) activatedthe areasincontrastwiththegeneralfactor(Perception).

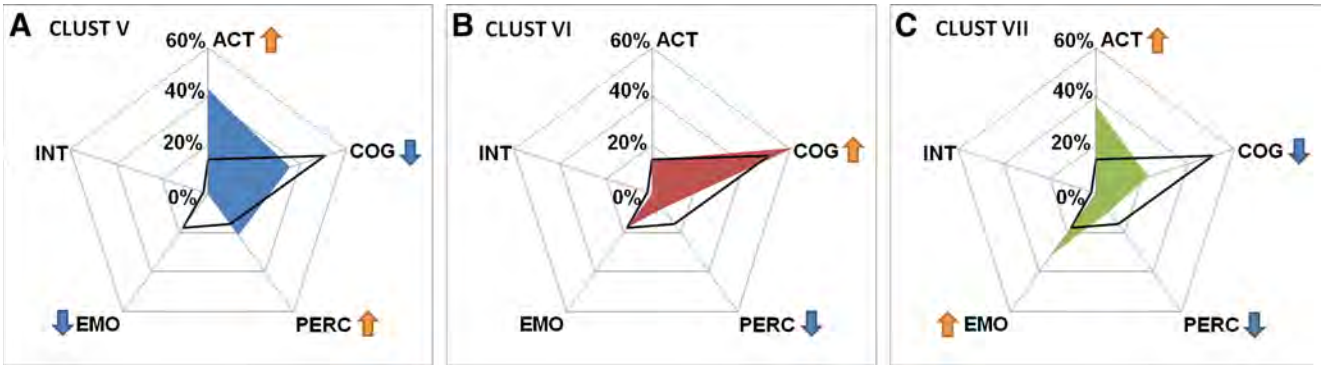

Fig.4 Behavioralprofilesof areaswithgreaterFCinpaincomparedto rest. a Pain>restFCbehavioralprofile ofclusterV(blue area). bPain> rest FCbehavioralprofileof clusterVI(red area). c Pain>rest FC behavioral profile of cluster VII(green area). All profiles were plotted on radar graphs with frequencies of the main behavioral domains of BrainMap database(blackline, ACT =Action, PERC = Perception, INT = Interoception, COG = Cognition, EMO = Emotion). Yellow arrows indicated increased specialization, blue arrows decreased specialization compared toBrainMapmeanfrequencies

Meta-analysis

Themeta-analysisshowedthatin touch(Fig. 5a,supplementary Table S15)andpain(Fig. 5b,supplementaryTable S16), our painandtouch conditions ledtooverlapping activationpatterns (Fig. 5a vs. 5b, supplementary Table S15 vs. S16): bilateral insula, bilateral postcentral gyrus, bilateral supramarginal gyrus, bilateralinferiorparietal lobule,bilateralthalamus, bilateral precentral gyrus, bilateral putamen, medial frontal gyrus, and paracentral lobule. Some of these areas, included in the pain neuromatrix, were more strongly activatedfor pain (e.g., insula, putamen, thalamus, see Fig. 5c)and some were more strongly activated fortouch(e.g., sensory-motor cortex,see Fig. 5a). Some areaswere observed onlyfor thepaincondition (Fig. 5c, supplementary Table S17), in particular bilateral amygdala,cingulate gyrus(BA 23), anterior (BA24, 32)and posteriorcingulatecortex (BA31).

In the touch condition, the cerebellum showed significant activationin two activationclusters, oneleft and oneright,in culmen/declive[IV-V-VI].Inthepain condition,therewere three activationclusters: onein the midlineof the cerebellum, in the vermis of the culmen [vIV-V], and two symmetrically, similar to the ones related to the touch condition, but more posterior,in thedeclive[VI,crus1]. Themedial one hadgeometric center of mass coordinates x, y, z=0 mm, −52 mm. −15mm,totalvolume1384mm^3;thelefthadgeometric center of mass coordinates x, y, z=−30 mm, −53 mm, −29 mm, total volume3136mm^3;andtherighthadgeometric centerof mass coordinates x, y, z=25 mm, −56 mm, −26 mm, total volume 1464mm^3.These clusters were significantly more activeinpain (Fig. 5c, supplementary Table S17), and in particular, the vermal activationwasevident onlyinpain (Fig. 5b,5c).

Discussion

The cerebellar activation during pain stimulation is often detected in neuroimaging studiesandit has been mostlyreported in the anterior vermis andposterior lobules[9], although the significance of these activations has been rarely commented upon andoften neglected.Initially,the cerebellar activity was considered as unspecific and linked to motor behavior or

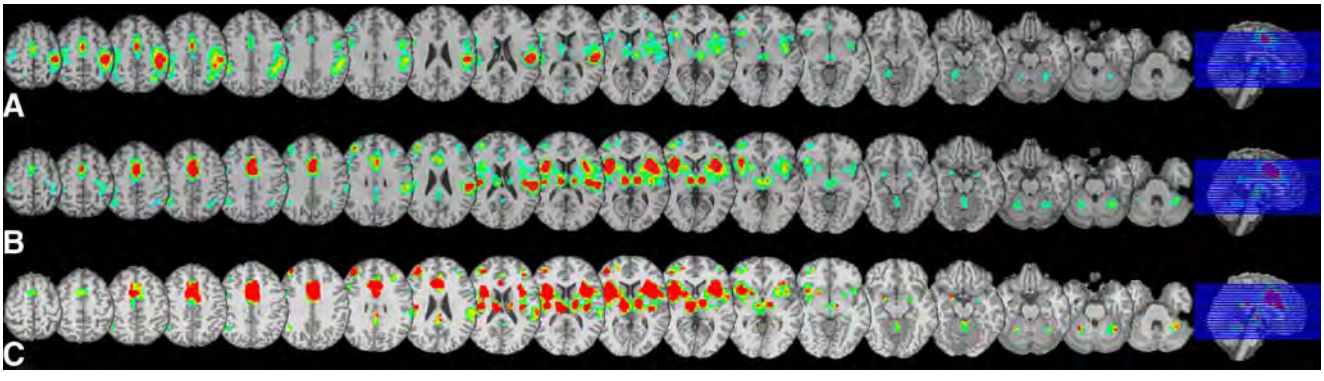

Fig.5 Meta-analysis: pain and somesthesis comparison. a ALE meta-criteria: [Diagnosis = Normals] AND [Behavioral Domain= Perception Somesthesis (Pain)]. b ALE meta-analysis with these criteria:[Diagnosis =Normals]AND[Behavioral Domain= Somesthesis(Pain)>PerceptionSomesthesis.Threshold wasFDRpN< 0.05 and minimum clusters extent Ke>500 mm^3. c ALE meta-analyses difference analysiswith these criteria:[Diagnosis =Normals]AND[Behavioral Domain= Perception Somesthesis(Pain)>Perception Domain =Perception Somesthesis] AND NOT [Behavioral Domain= Somesthesis(Pain)]

attentiveprocessing[41],butrecently thehypothesisofadirect involvementof the cerebelluminpainmodulationhasgained moreconsideration.In ourexperiment, wedescribedtheindependentcerebellarcontributionduringnociception.We useda fuzzy c-mean-based decomposition of the BOLD signal acquired in 12 subjects during mechanical pain stimulation to extractthree cerebellar functionalindependentclusters.Inthe followingparagraphs,wecommentuponthe resultsbased on thelocalizationofcerebellaractivity bothin ourexperimentand in the meta-analytic analyses, deepening our discussion with theaidoftheintrinsic cerebellarFCduringdifferent conditions.

ClustersVandVI

ClusterV wassituated mainlyinthesensory-motor cerebellum, localizedintheIV,Vlobulesandvermis(Fig. 1).TheFCcerebral networkrelatedto this clusterincludedthe bilateralprimaryand secondarysensory-motor areas,sowedecidedtocallthiscluster the Bsensory-motornetwork^;however,thisnetwork alsoincluded thebilateralposterior insula (moreactivated on theright side), cuneus,posteriorcingulatecortex, superior temporalgyrusand PAG(Fig. 1a).This networkisconsistentwiththemajor cerebral areas associated with pain processing as described in a recent reviewbyDuerdenand Albanese[4],butwealsoobservedareas notsocommonlyobservedinpain studies(the cuneusandPCC) that could be linked with the self-relevance/awareness of the intensity ofpainsensation[42], or witha specificaffinityfor A-delta fiber informationprocessing,elicitedbythe pinprick,in multi-sensory integrationregions[43].WefoundthePAGonlyin thesensory-motornetworkandit seemstobelinkedtothispart of thecerebellumandto theascendingpain/sensory anddescending limbic/emotional/motor cerebello-cortical loops[9,12,17].A pattern similarto thisnetwork, includingtheclusterV, wasalso found in thefMRI-basedneurologicsignature of pain recently reportedinthe NewEngland JournalofMedicine [44]: apattern extractedwith multivariate techniques whose meanactivity could well discriminate between noxious andharmlessheatstimuli.

ClusterVI was localizedinthecerebellumlobuleVI, crus1 and2.Thiscluster wasfunctionally connected witha network (Fig.2a)includingtheright thalamus,dorso-frontalparietal areas (with the DLPFC more localized in the right hemisphere), cingulatecortex,and anteriorinsula(leftlateralized).Inagreement withourresults,the DLFPC,inparticularintherighthemisphere, hasbeensuggestedashavingspecific painevaluationfeaturesas shownbysomestudies[9,20,45,46].Additionally,this network is similartothe networkcalledthe Bhow much^ system (lateral prefrontal cortex and anterior insula) by Baliki and colleagues [47], multisensorial (areas receiving inputs from different sensorial regions) brain regions encoding magnitude of stimuli. The DLPFC connectiontothe cerebellum is relevant,given that this regionisimplicatedin cognitivecontrolaswell asinpainmodulation[9].We foundparietal areastogether with frontalareas, both of which normally coactivate in attentional tasks, in particular with the spatial representation or ability to plan requiredfor such tasksand so we decidedtorefer to this cluster as Battentional^ [48].Thecerebellarclustersofthe sensory-motor and attentional networks were negatively correlated(r=−0.31): theincreaseofBOLD(derived fromthe event-relatedaverage analysis)inone correspondedtothedecreaseinthe other. Thisis alsoatypicalfindinginthecorrelation betweeninteroceptionand exteroception systems studied with functional connectivity, e.g., intheanteriorand posteriorinsula[33]orintheTaskPositive andTaskNegative networks[49].Baliki[47]suggeststhatthe subjective pain rating depends on the transfer of information fromthemulti-sensorysystemtotheanteriorinsulaand lateral prefrontalcortex,fromthe sensory-motortoattentionalnetwork.

Alternatively,thisnegativecorrelationcouldbelinkedtothe shiftingbetween attentionand automatic behaviororbetween anticipationand sensationofpain[20]. Mobbsandcolleagues [50]investigated spatialimminenceofthreatin an fMRI active avoidanceparadigminwhichvolunteerswere pursued through amazebyavirtualpredatorwiththeabilityto inflictpain.They found that as the virtual predator grew closer (the pain was unavoidable,asitwasin our study),brainactivity shifted from theventromedialprefrontal cortextotheperiaqueductalgray,a pattern thatisvery similartopriorobservations reportingthat as electric shock grew closer, brain responses shifted from rACC toPAG and the surrounding areas, including the thalamus, striatum,pallidum, hypothalamus,and cerebellum.

The relationship of these networks (sensory-motor and attentional) couldbe supportedbytheobservationofMoulton and colleagues[20]who found two distinct phases in pain processing: an Bearly phase,^ involving the anterior insula, frontal, and cingulate cortex associated with attentional, threat-detection, and evaluative processes; and a Blate phase, ^ involving the primary and secondary somato-sensorial cortex, related to perceptual intensity of noxious stimuli. The anterior and posterior insula were also split between evaluativeandsensory-motor networks,witharightlateralizationof the posterior insula (sensory-motor network) and a left lateralization of the anterior insula (evaluative network). This in line with the schema proposed by Craigwhere body feelings are transmittedfrom the posterior insula to the mid-and anterior insula to be integrated, with different hemispheric functional specializations of the insulae[51].Recently,

Mesmoudi et al.[52]proposed a cortical parcellation based on resting state data, dividing the whole brain activity into two distinctive patterns called Bthe dual intertwined rings architecture^. The cerebral functional architecture couldbe dividedinto two large families: a sensorimotor family includingvisual, somatic, and auditory areas and the other family more related to association cortices specialized in attention, language, and working memory. This categorization is in line with our cerebellar parcellation,indicating the two separate phases of the pain processing: one is more related with the sensory aspect (FCclusterV) andtheotheris morelinked tothe cognitive aspect(FCclusterVI).Asimilarfunctionalparcellation was seen in the clusterization of the cerebellum usingthe profiles of coactivation of many functional tasks[53].

So far, it remains unclear how much of what we have described is unspecific and how closely it is related to pain processing. In fact, the Bside effect^ of usingfuzzyclustering isthatwecannotbesureifthe extractedclustersbelongtoa specific process or we observe a Bbaseline^ clustered activity ofthese areas.Thisisthe reasonwhyweadded resting state acquisition into our experiment: this could lead us to profile the activityof these clusters under task andbaseline.

Thepain-relatedFCthat weobservedduringthetask could be part to the Bnormal^ activity of these cerebellar areas.To test the contribution of the baseline activity in our mechanical punctuate stimulation FC, we acquired resting state data whichallows one to investigate the intrinsic functional organizationofthebrain.Toachievethisaim, wecomparedtheFC derived from the mean time course of the cerebellar cluster during nociception and restingstate.AsshowninFigs. 1and 2, theFC of the clusters shared some areas during these two conditions, but the pain condition showed much more connectedareas(Figs. 1c,2c).RegardingtheclusterV,the connectivity was mainly shared in the posterior insula, somatosensory cortex, and precentral cortex; the cluster VI shared bilateralDLPC andinferior parietal lobule.

TheFC comparison indicatedtwo important aspects:

- The restingstate andthenociceptionFCwerepartlysimilar.Wefound a cerebellar restingFCsimilartothe one proposed in the literature (e.g.,[53–56]), showing the highcorrelation of theanterior part of thecerebellum with somatosensory-motor network and the posterior part of the cerebellum with parieto-frontal executive network [1, 57].
- The painful stimulus played a crucial role as a hemodynamic Bcoordinator.^ The nociception enhanced the cerebellar connection (Figs. 1c, 2c)with several networks involved in different aspects of pain processing and this was reflected in pain FC.

The FC networks, emerging from the clusters Vand VI during nociceptive stimuli compared to restingFC networks, showedabehavioralprofile coherentwiththeclassificationof thesensory-motor network(clusterV)andattentionalnetwork (clusterVI).Infact,clusterVsynchronizedwith moreAction andPerception areas(Fig. 4a)thatdidnot expressCognition or Emotion functions and cluster VI with more Cognition areasthatdid not expressPerceptionfunctions(Fig. 4b).Interestingly,theclusterVenhancedthe correlationwith areas activated by tasks belonging to Perception domains such as Vision, Audition, andSomesthesis, areas that couldbe related to a general monitoring of the environment (i.e., explorative behavior)andto movement(e.g., withdrawal or freezing), but nottoSomesthesisPainspecifically.Thiscouldbeinlinewith the interpretation that the pain neuromatrix, includingthe anterior motor cerebellum[44], couldbeakindofa moregeneral networkinvolvedin detecting and reacting to the occurrenceof salient/threateningsensoryevents[58].ClusterVI on the other hand showed an enhanced correlation with Attention, Working Memory, Memory, and Reasoning together withSomesthesisPain,which couldbeaspecific evaluative processing of the pain events supported by previously built internal models stored in the cerebellum.

Cluster VII

Cluster VII FC included a network composed only by few voxels, localized in the amygdala, parahippocampal, and limbic-frontal cortex (Fig. 3a). Because of its small size, it did not survive to the multiple comparison correction based upon cluster size. Indeed, the amygdala is a small area that couldbe too severely penalizedby this kind of statistical correction, butit is also critical in fear andpain memory processing. The amygdaloid complex is a well-known site of multimodalintegration receivinginputfrom visual, somatosensory, auditoryareasandprojectingtothehypothalamus,rednucleus of the stria teminalis, midbrain PAG, pons, medulla, and brainstem[59].These structures are involved in fear conditioning, emotional modulation, and integration, and support the idea that the amygdala plays an important role in neural circuits relatedtopainprocessing[59].Interestingly,this network overlapped with the small amygdalo-hippocampal-frontalnetworkfoundinarecentworktobelinkedto aversion stimuliprocessing[60],so we decidedto referto this clusteras Bemotional.^ With fMRI, the authors compared responses to noxious heat and unpleasant pictures, and evidenced a supramodal network composed of overlapping areas linked to aversion in the posterior cerebellum (specificallyin hemispheric lobule VI, crus1 andVIIb). Baumann and colleague [61]also showed that the vermis VIIand crus I, II were responsive to fearfulandangryfaces. In fact, these emotions are typically elicited by threatening stimuli and these cerebellar areasareprobablyrelatedtoprocessandrespondtothesekind of stimuli (i.e., resulting in autonomic and fight-or-flight responses)[61].This couldcorrespondtotheobservation made that stronger fear memories obtained by increasing the strength of conditioning are affected by the combined, but not independent, amygdala andcerebellarblockade[62]. Strong fear memories represent essential information to the survival, so a complex memory system including the amygdala and cerebellum might be a specialized evolutionary development of a more basic circuit. Recently, Schienle et al. [63]showedthattherightcrus2inthe cerebellumisfunctionally connected with the amygdala in response to emotional stimuli, underlying the idea that this area is recruited during emotionalprocessing.

It could be argued that the cluster VII should not be considered as a significant result and therefore discarded. However, we decided to include it in the present work, using an uncorrectedthreshold,becauseclusterVIIhadagood overlap with meta-analytic results of emotional activation of the cerebellum[1,64].Also, it hasbeen demonstratedthat lobule VIIb is functionally connectedwiththe vermis VII[53]. The cerebellar vermis is an area that participates during emotional memory and modulates the typicalphysiological response to adversestimuli asdemonstratedby manyexperimentsin humans and animals[62].Therefore,we could expect major activityof this area during nociception in human as due to the factthatthepainhasahighimpact onbiologicalpreservingof life creating fear memory conditioning and visceral primal aversive emotion. In animals (e.g., mice and rodents), the posterior vermis orlimbicvermis[62]playsa centralrolein fear conditioning, mediatedbypain;inhumans,the cerebellar areas involved could also include posterior cerebellar hemispheres together with the vermis as suggestedby our results. Adamaszek et al. provided preliminary evidence of that in a groupof cerebellar stroke patients with a specific impairment for emotionalprocessing and associated lesions in the posterior cerebellum[65]. This could be related to an integrated processing in emotion and cognition in humans[66]: an evolutionary frontalization of the emotion parallel to the frontal lobedevelopment, well representedbythis cerebellar network.

We didnot observe anysignificantFCduring restingstate for this cluster (Fig. 3b). The areas recruited by cluster VII networkduring nociceptionbelongedtoAction andEmotion, in particular to Fear. This is suggestive of fear conditioning whereanactionpatterncouldbelinkedtoanaversivestimulus.

Pain could be extremely effective in conditioning and cerebellumisakey areain animalstostorefearful memories [62].The subdomainSomesthesisPaintasks,includedinPerception domain, activated this network in contrast with the general Perception factor, supporting a specific effect of nociception in the promotion of associative learning processingin the cerebellum.

Meta-analysis

We compared pain and somesthesis perception to look if a simple tactile stimulation could elicit the same cerebellar areas.The results(Fig. 5)showedthatthree activationclusters (Fig. 5c)had more activation specifically in pain condition. The first was inside the vermis IV-V and it was completely insideinthelargerclustersVthatalsoincludedthe cerebellar lateral lobules IV-V (supplementary Fig. S11). The second and third activation cluster of the meta-analysis overlapped completelywithclusterVIfromourpaincondition.We could arguethatasimplepassivesensorialstimulationliketouchdid not elicit these cerebellar clusters.

We reliably detected with a nonstandard technique (fuzzy c-mean clustering andSogICA)the results of a meta-analytic approach with 200papers and2688 subjects pooled together, a finding which strongly confirmed the usefulness of our approach.

If we look at the results of meta-analysis for pain-specific activation (Fig. 5c), we found overlapping cerebellar areas withclustersVandVIbut notfortheclusterVII.This could be explained by a greater difficulty in the detection of this cluster as proven by the threshold we hadto use in this work orwithabiasoftheBrainMapdatabasethatincluded toofew studies with a complete mapping of the lowest part of the cerebellum that often is cut away. Interestingly, we couldobserve amygdala activity,linkedtoclusterVII network, onlyin the pain condition.

Limits

Alimitation of this studyis a common bias of almost allthe fMRI pain studies: the paradigm was based on a condition of unavoidable pain. Generally speaking, it is quite difficult to create an ecologicpainparadigmintheMRIsetting:thewithdrawal motor reflex is impossible, and the action inhibition andthe antinociception response maximized. In these conditions, we stress the importance of not limitingthe analysis to the standard GLM because an important part of the brain activity could be neglected if we do not include corrections or use more sophisticated models that could capture all the pain time locked variation of signal inside the brain. The cerebellarsignal analysisofthispaperisagooddemonstrationof this assertion.

An importantunresolved question aboutthe cerebellar role inpainprocessingisifthecerebellumisapassiveintegrator or anactiveparticipator.Inouropinion,thisworkcouldcontribute tofuture experimenttoclarifythisquestion.Weproposeto use the delineated cerebellar clusters as targets in virtual lesions Transcranial Magnetic Stimulation experimental paradigm during conscious nociception to determine a possible active or passive role of the cerebellum.

Conclusions

Amongthe multiple dimensions of pain, evidencedby many studies, the more common and consistent are the sensorial/intensive, cognitive/evaluative, and emotional dimensions [5–8]. These three dimensions matched well with the functions that we hadattributed to the networks that synchronized with the independentcerebellar clusters: a sensory-motor network, a cognitive network, and an emotional network.

Speculatively, the clusterization of the cerebellum into different systems, one localizedin the paleocerebellum andtwo in the neocerebellum, one intermediate, and one more posteriorandphylogenetically newer, couldbeindicativeof functions specializedin different phases of evolution of these structures.

The question of if there exists a single specific brain network for pain processing perhaps is illposed; rather, it could be argued if some areas with very different specialization could be synchronized by pain as it is, intrinsically, a multi-factorial construct. The simultaneous cerebellum activations of independentareas linked to different functional networks is a perfect representation of an integrated view of the pain processing.

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Conflictof Interest The authors declare no conflict of interest.

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