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Cerebellar Clustering and Functional Connectivity During Pain Processing

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Abstract The cerebellum has been traditionally considered a sensory-motor structure, but more recently has been related to other cognitive and affective functions. Previous research and meta-analytic studies suggested that it could be involved in pain processing. Our aim was to distinguish the functional networks subserved by the cerebellum during pain processing. We used functional magnetic resonance imaging (fMRI) on 12 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 of the FC networks. We identified three significant clusters: cluster V, involving the culmen and

M. Diano and F.D'Agata contributed equally to this work.

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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), and cluster VII, involving the inferior semilunar lobule (VIIb, crus 1, crus 2). Cluster V was more connected during pain with sensory-motor areas, cluster VI with cognitive areas, and cluster VII 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 . Fuzzy clustering . Functional connectivity

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, spatial processing, executive functions, emotion processing, 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 in the posterior cerebellar vermis [2], and in

humans, cerebellar lesion altered pain perception[3]. As shown in a large functional magnetic resonance imaging–positron emission tomography (fMRI-PET) meta-analysis[4], the cerebellum is activated during pain-related stimulation in conjunction with the areas typically associated with pain: the bilateral insula (with a right dominance), anterior cingulate cortex (ACC), bilateral primary motor/sensory cortex (MI/SI), secondary somatosensory cortex (SII), posterior parietal cortex, and prefrontal cortex. Specifically, the nociceptive-related activation was observed in the deep cerebellar nuclei and anterior vermis IV-V, bilaterally in hemispheric lobules VI, VIIb, crus 1, and crus 2. Noxious stimuli elicit other processes related to pain, but not exclusive to it. In fact, pain is a multidimensional construct[5–8] and includes many aspects that could be related to the cerebellum: motor reactions, orienting of attention, evaluation processes, anticipation and negative emotions, and consciousness[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 memory recall[9]. Although there are many different functions, some authors have postulated that the cerebellum develops only a single computational function: being able to learn/predict the outcome of an action using internal stored models (see [10] for a general description of the internal model idea). Some data[9] suggested that computational power of the cerebellum could be used for different tasks, depending on 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 impending pain and the bilateral anterior cerebellum during the actual pain. Taking into account the different spatial locations of the anticipatory and pain activations, multiple parts of the cerebellum seem to be involved together with different large-scale networks that process painful events in different ways[9]. In particular, the cerebellum is directly and indirectly connected with some cortical and sub-cortical areas which are part of a central pain modulation network: the periaqueductal gray (PAG) and periventricular gray (PVG), the rostroventral medulla (RVM), the medial frontal and orbital cortices, the thalamus, the hypothalamus, and the brainstem[11–17]. This pattern of multiple connections could be the reason of the heterogeneity of functions carried out by the cerebellum.

The aim of this study was to distinguish the main cerebrocerebellar networks subserved by the cerebellum during mechanical punctuate stimuli processing. The nociceptive stimulus was delivered by a pinprick stimulator that is composed by a flat contact area (edge curvature) of 0.25 mm in diameter and the punctuate stimulus can be administered with different standardized forces. We used a fuzzy clustering technique on fMRI data to find cerebellar areas exhibiting different and independent brain dynamics. We choose this analysis instead of canonical Hemodynamic Response Function (HRF), implemented in a general linear model (GLM), because the latter only allows identifying the areas with a standard response to stimuli. Recently, different authors demonstrated that the pain-related blood oxygenation level dependent (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 fuzzy clustering technique, we determined, using functional connectivity (FC) techniques, to which cerebello-cortical networks they were connected, correlating clusters time courses with the whole brain voxel's temporal profile. Furthermore, we compared the obtained pain-related FC maps with the ones derived from resting state data from the same cerebellar clusters. This step could help us to understand if the brain activity and connectivity are normally present, or if this is specifically related to mechanical 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 statistical technique, to determine if the cerebellar independent clusters that we found are prevalently observed in literature in pain studies or can be also observed for other passive sensorial conditions, such as tactile stimulation.

Materials and Methods

Meta-analyses were conducted with Sleuth 2.2 and Ginger Ale 2.3 (<http://www.brainmap.org/>).

All other statistical analyses were conducted using Brain Voyager QX 2.3.1 (Brain Innovation, Maastricht, the Netherlands, <http://www.brainvoyager.com>).

Participants

Twelve healthy volunteers (5 females and 7 males, mean age \pm SD = 27 \pm 4 years) took part in this study. The volunteers were recruited from the staff and students of the University of Turin and all gave written informed consent. All the volunteers were right-handed according to the Edinburgh Assessment Scale [21], with no histories of neurological or psychiatric disorders. This study conformed to the standards required by the Declaration of Helsinki and was approved by the local ethics committee.

Experimental Procedure

During the imaging acquisition, the subjects were asked to remain still with their eyes closed. The paradigm (slow event-related design) was divided into four runs, with 12 mechanical punctuate stimuli applied during each run. The stimulation was delivered on the back of the hands with a MRI-compatible 256 mN pinprick.

The pinprick is a hand-held device that has been shown to selectively activate type I A-delta fibers which is included in the common clinical evaluation of nociception in the German protocol. It activates areas related to the elaboration of noxious inputs [22] and therefore constitutes a 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 pinprick stimulus duration of about 1 s to correctly study the complete BOLD response generated by the painful stimulation.

The side and the stimulus location on the hand were chosen using a pseudorandom strategy, maximum three times consecutively on the same hand, counterbalanced within subjects and avoiding stimulating twice the same point. The stimuli had a variable inter-stimulus interval of 18–22 s. To assess the intensity of the stimulation without introducing any modification of the pain-induced BOLD response, the subjects were asked to indicate the mean of the pain intensity only at the end of each run, using a scale from 0 to 10, with 0 corresponding to no pain intensity and 10 to the highest pain intensity imaginable (group mean 3.3 with standard deviation ± 2.2).

For resting state acquisition, the participant was instructed to lay down with their eyes closed and asked to not fall asleep during the scan. The resting state acquisition was set up prior to the mechanical punctuate stimulus task.

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. Functional T2*-weighted images were acquired using echoplanar (EPI) sequences, with a repetition time (TR) of 2000 ms, an echo time (TE) of 50 ms, and a 90° flip angle. The acquisition matrix was 64×64 ; the field of 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 consisted of 19 axial slices, parallel to the anterior-posterior (AC-PC) commissure line and covering the whole brain; the slice thickness was 5 mm with a 1-mm gap ($4 \times 4 \times 5$ mm voxels). Three dummy scans were added at the beginning of functional scanning and the data discarded to reach a steady-state magnetization before acquisition of the experimental data.

In the same session, a set of 3D high-resolution T1-weighted structural images was acquired for each participant. This data set was acquired using a Fast Field Echo (FFE) sequence, with a TR of 8100 ms, ultra-short TE 25 ms, and a 30° flip angle. The acquisition matrix was 256×256 , and the FoV 256 mm. The set consisted of 107 contiguous sagittal images covering the whole brain. In-plane resolution was 0.9×0.9 mm and slice thickness 1.5 mm ($0.9 \times 0.9 \times 1.5$ mm voxels).

fMRI Pre-processing

BOLD images were pre-processed in order to reduce the noise and remove the artefacts for all the functional runs: (1) slice scan time correction using a sinc interpolation algorithm; (2) the whole volumes were adjusted by mean intensity [23]; (3) 3D motion correction using a trilinear/sinc interpolation algorithm (all volumes were spatially aligned to the first volume); (4) whole volumes were spatially smoothed using a Gaussian kernel of 4 mm full width at half maximum (FWHM); (5) temporal filtering (linear trend removals) and a high pass filter to reduce cardiac and respiratory noise.

We performed a series of pre-processing steps to ease the coregistration of the functional data into a Talairach template, allowing inter-subject comparison. First, each subject's functional scans were coregistered on their structural scan. Second, the structural data were skull stripped (removal of the skull and scalp) and transformed into the standard Talairach space [24]. Third, the functional data of each subject were coregistered using the transformation matrix derived from the previous steps. The Talairach transformation was performed using this procedure implemented in Brain Voyager software: (1) the brain was rotated and aligned into the anterior and posterior commissure plane; (2) the cerebrum borders were identified to allow the scale transformation of the 3D structural data into the Talairach and Tournoux standard brain using an affine transformation.

Clustering Cerebellum 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 them by their temporal similarity.

Fuzzy c-mean clustering is a data-driven method that compares the time courses of voxels, divides them into clusters, and labels them with a value derived from the distance from the clusters' centroids. This technique is named fuzzy because instead of assigning every element to exactly one cluster, it produces for each voxel multiple cluster memberships with different probabilities [25].

All the centroids and memberships are constantly updated following the mathematical procedure described by Bezdek [26], which terminates when the interactions do not significantly change, established via 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 to the cerebellum with an exclusive mask, hand-drawn on the mean of all functional data.

We decided to use a slow event-related paradigm because this design optimizes the clustering techniques, leaving the time necessary to BOLD response to expire between stimuli, so every event was decoupled to the others. On the other side, the slow event-related paradigm entailed less signal respect to other paradigms (i.e., block design) and penalized the standard GLM approach with canonical HRF. We also employed a little spatial smoothing (4 mm) in the pre-processing because of the smallness of cerebellar structures.

We used Self-organizing group-level ICA (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 extracted from fuzzy clustering ($p < 0.01$), cluster-level corrected for multiple comparisons ($p < 0.05$), using a Monte Carlo simulation [31, 32]. We used classTAL (doi:10.1038/npre.2011.6142.2), a Matlab script that automatically classifies Brain Voyager 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, obtained from the group analysis, were carefully examined to keep only the significant clusters unrelated to residual physiological or movement artefacts.

Functional Connectivity of the Clusters

Functional connectivity maps were computed as in [33]. BOLD time courses were extracted, both during nociception and resting state conditions, from each significant cerebellar cluster by averaging over voxels within each region previously derived from fuzzy clustering.

To reduce the noise derived from physiological processes (cardiac and respiratory rhythms), we included eight additional covariates that modeled nuisance signals sampled from the white matter (WM) and cerebrospinal fluid (CSF), as well as from six motion parameters (three rotations and three translations as saved by the 3D motion correction). We derived the WM/CSF nuisance signals averaging the time courses of the voxels in each subject's WM/CSF masks, and hand-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 each cerebellar seed ROI and each subject, an FC map was computed on a voxel-wise basis for each condition: mechanical stimulation, resting state, and comparison between them. For each subject, the GLM [34] for multiple regression analysis resulted in ROI-based t-maps (SPM_t). RFX group-level maps were computed on SPM_t with a threshold of $p < 0.01$ cluster-level corrected ($p_{\text{corr}} < 0.05$) for multiple comparisons [31, 32] and classified with classTAL Matlab script (doi:10.1038/npre.2011.6142.2).

Meta-analytic Behavioral Profile Analysis

BrainMap is a database of published functional neuroimaging studies (mainly PET and fMRI) that contains both metadata descriptions of experimental design and activation locations in the form of stereotactic coordinates [35]. At the point of analysis, BrainMap contained 2390 neuroimaging papers that analyze 11,353 experiments using 99 unique paradigm classes, yielding to 91,039 locations or foci (February 28, 2014).

We extracted the behavioral profiles [36] of the FC contrasts nociception > resting state results to enrich the functional 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_behavioral_analysis.html. The plugin performed regional behavior analysis based on selected brain ROIs. The analysis was coordinate based, and results were presented for BrainMap's five main behavioral domains (Action, Cognition, Emotion, Interoception, and Perception) and 51 subdomains (e.g., Emotion-Fear). Only z-scores ≥ 3.0 are considered significant ($p \leq 0.05$ with Bonferroni correction for multiple comparisons).

Meta-analysis

The ALE analysis is a quantitative meta-analytic method that can be used to estimate consistent activation across different imaging studies [37]. ALE maps of coactivations are derived based on patterns of foci of interest, where multiple studies have reported statistically significant peak activation. To limit the intersubject and interlaboratory variability, we used an algorithm that estimates the spatial uncertainty of each focus, taking into account the possible differences among the neuroimaging studies [37]. The advantage of such an algorithm is that it comprises a method to calculate the above-chance clustering 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>).

We sent queries to the database with the Sleuth 2.2 software [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:

Somesthesis 102 papers (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 supplementary doc file.

Statistical maps with the regions of convergence were calculated using GingerAle 2.3 software [37,39] in the Talairach space with the more conservative mask size and FWHM values subject-based.

For all analyses, the selected p threshold was selected using the False Discovery Rate with positive dependence assumption, $FDR p < 0.05$ and minimum clusters extent $K_e > 500$ mm³. We used Chris Rorden's MRIcron software (<http://www.nitrc.org/projects/mricron>) to visualize and save images, overlaying the GingerAle maps onto a Talairach brain template (created by [40]).

To compare the pain and somesthesia maps (pain > touch; touch > pain), all experiments contributing to either analysis were then pooled and randomly divided into two groups of the same size as the two original sets of experiments. That is, if 102 experiments in BrainMap featured activation in ALE somesthesia 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 was then tested against this null distribution yielding a p value 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 the independent clusters that we found in the 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 contained WM voxels; three clusters were retained (see supplementary Fig. S1).

We observed three significant clusters (Figs. 1, 2, 3) using fuzzy clustering RFX and we named them 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 $x, y, z = -1$ mm, -50 mm, -11 mm; total volume $10,592$ mm³; involving the culmen (vermis IV-V), anterior and posterior quadrangular lobules (hemi-

spheres IV-V-VI);

□ Cluster VI (colored red, Figs. 1, 2, 3) involving the posterior quadrangular lobule (hemisphere VI) and superior semilunar lobule (crus1, crus2), with two distinct activation clusters: one to the left (geometric center of mass coordinates $x, y, z = -29$ mm, -57 mm, -30 mm; total volume $1,318$ mm³) and one to the right of midline (geometric center of mass coordinates $x, y, z = 30$ mm, -58 mm, -27 mm; total volume 137 mm³);

□ Cluster VII (colored yellow, Fig. 3) involving the inferior semilunar lobule (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 bilateral posterior localization, situated more in the right hemisphere (60% right lateralized). We refer to this network as a B sensory-motor network[^] because the FC analysis shows an affinity with cerebral areas typically associated with sensory-motor function (i.e., bilateral primary and secondary sensory-motor areas MI, SI, SII).

The cluster V FC also showed a negative correlation (Fig. 1a, supplementary Fig. S2, supplementary Table S2) in the bilateral middle and superior frontal gyrus, bilateral inferior parietal lobule, bilateral supramarginal gyrus, angular gyrus, and precuneus.

Regarding cluster VI (Fig. 2a, supplementary Fig. S3, supplementary Table S3), the FC showed a positive correlation in the bilateral middle frontal gyrus, superior and medial frontal gyrus, bilateral cingulate cortex (middle cingulate), bilateral inferior parietal lobule, bilateral premotor areas (BA6), bilateral supramarginal gyrus, angular gyrus and precuneus, bilateral anterior insula, and right thalamus. In this case, the anterior insula activation was situated more in the left hemisphere (60% left lateralized). We refer to this network as the

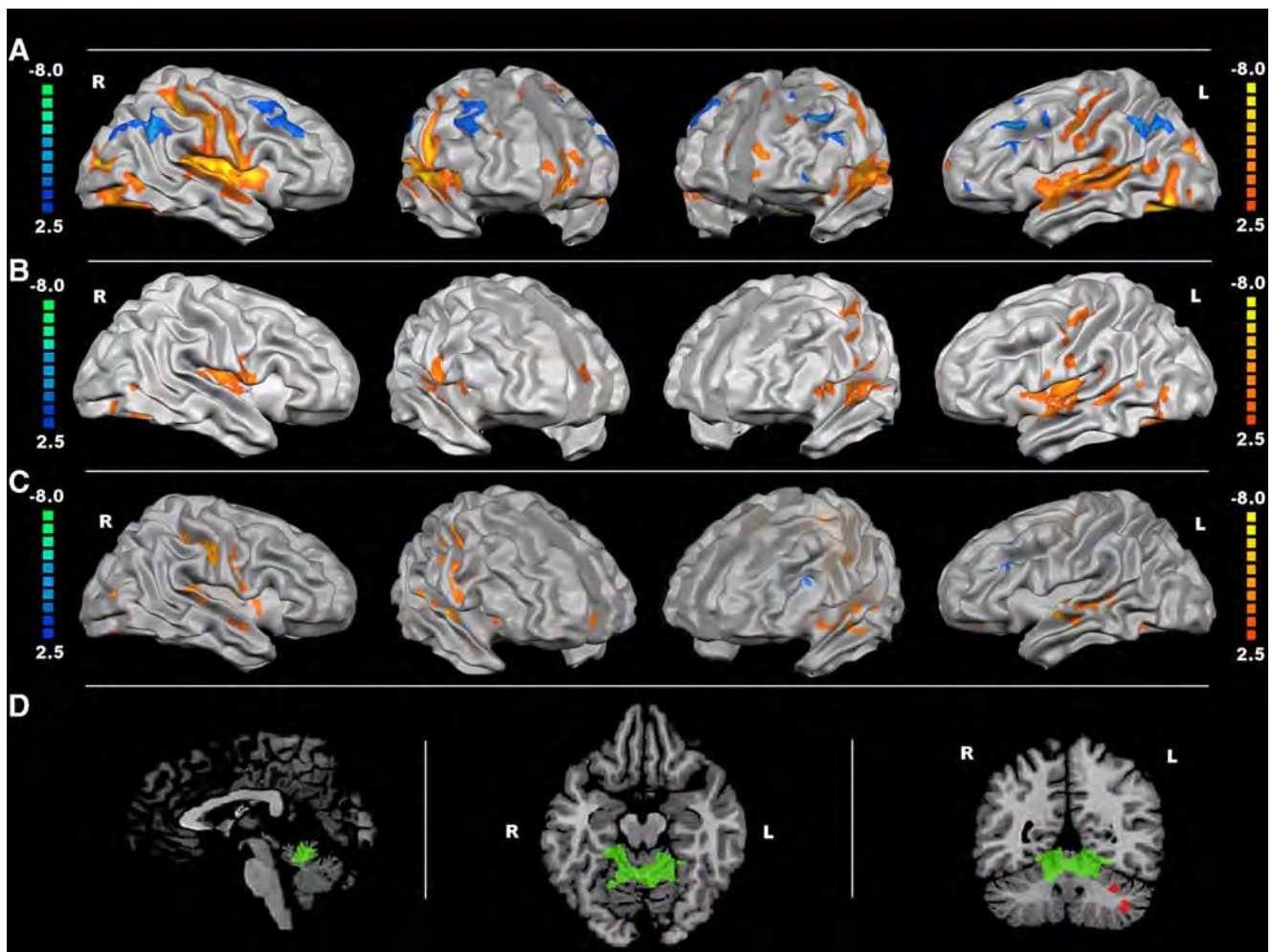


Fig.1 Pain-processing clustering: cluster V. a Functional connectivity of cerebellar cluster V (green) during mechanical punctuate stimulation projected on a 3D template. Positive correlations included the bilateral MI, SI, SII, posterior insula, anterior cingulate cortex, midbrain, and hippocampus; negative correlation included the bilateral frontal gyrus and parietal lobule. b Functional connectivity of the cerebellar cluster V during resting state condition. c Comparison between pain and rest

network, conditions. Positive correlations included the right postcentral gyrus, bilateral superior temporal gyrus, and posterior insulae. d Fuzzy clustering, map of cluster V projected on a 2D template. The cluster extended into the culmen (vermis IV-V) and anterior and posterior quadrangular lobules (hemispheres IV-V-VI). The maps were computed with Brain Voyager QX2.3, RFX $p < 0.01$ cluster-level threshold corrected for multiple comparisons ($p < 0.05$)

B attentional network[^] because the FC analysis shows an affinity with cerebral areas typically associated with attentional functions (i.e., dorsolateral prefrontal cortex, DLPFC, inferior parietal lobule).

Cluster VI showed negative correlation (Fig. 2a, supplementary Fig. S3, supplementary Table S4) in the bilateral primary and secondary sensory-motor areas (MI, SI), bilateral middle and superior temporal gyrus, bilateral visual areas (occipital cortex, cuneus, lingual gyrus), bilateral posterior insula, cingulate cortex (ACC and posterior cingulate), putamen, and parahippocampal gyrus.

Many areas that were positively correlated with cluster V were negatively correlated with cluster VI, and many areas negatively correlated with cluster V were positively correlated with cluster VI, so globally cluster V was correlated with motor and negatively correlated with the attentional

while cluster VI was correlated with attentional and negatively correlated with the sensory-motor network (Figs. 1a vs. 2a, supplementary Figs. S2 vs. S3, supplementary Tables S1 vs. S4, S3 vs. S2). In fact, the timecourses of clusters V and VI were inversely correlated ($r = -0.31$, $p = 0.031$).

For cluster VII, no FC survived the multiple comparison correction, so we used an uncorrected threshold of $p < 0.01$ to examine the connected network (see discussion below). Cluster VII (Fig. 3a, supplementary Fig. S4, supplementary Table S5) showed a positive correlation in the bilateral inferior, middle and superior frontal gyrus, bilateral amygdala, and parahippocampal gyrus. We refer to this network as the B emotional network[^] due to its FC with bilateral amygdala and parahippocampal gyri. Relating to negative correlation, the FC showed cingulate cortex (ACC, middle, and posterior) and some other small sparse clusters, although this pattern was

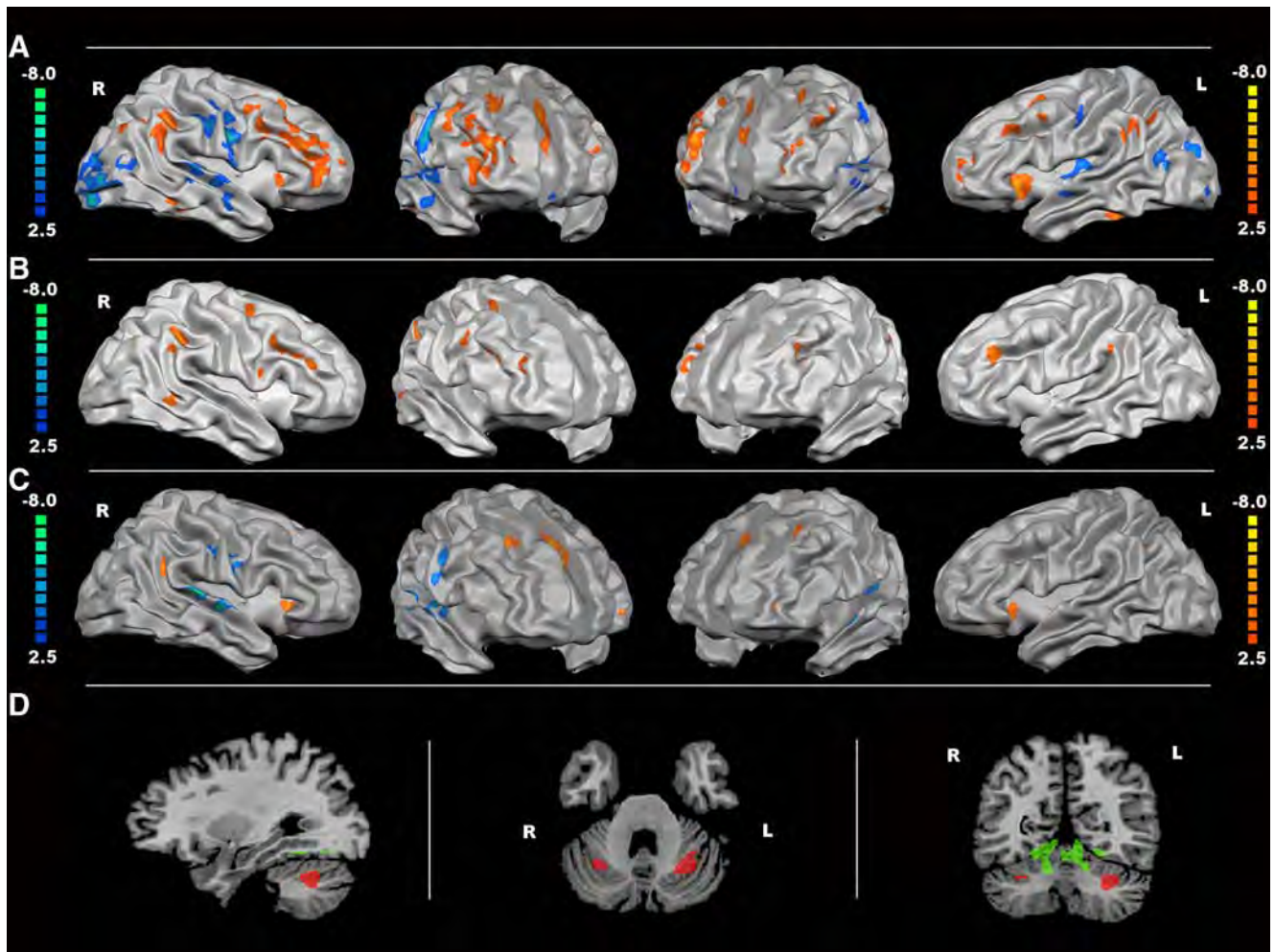


Fig.2 Pain-processing clustering: cluster VI. a Functional connectivity of cerebellar cluster VI (red) during mechanical punctuate stimulation projected on a 3D template. Positive correlations included the bilateral frontal gyrus and parietal cortex, middle cingulate, and bilateral anterior insula; negative correlations included the bilateral MI, SI, posterior insula, anterior cingulate cortex, and parahippocampal gyrus. b Functional connectivity of the cerebellar cluster VI during resting state condition. c

not clear-cut or very meaningful (supplementary Fig. S4, supplementary Table S6).

Functional Connectivity During Resting State

FC of the cerebellar clusters V and VI during resting state showed positive correlations to different networks partially overlapping to that of the FC during pain and generally composed by smaller subsets (also less connected, $p_{\text{pain}} < p_{\text{rest}}$) 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 V and VI during rest were absent or nearly absent (Figs. 1b, 2b, supplementary Figs. S5, S6, supplementary Tables S9).

FC of cluster VII was not significant outside the cerebellum (Fig 3b, supplementary Fig. S7, supplementary Table S10), Comparison between pain and rest condition. Positive correlations included anterior cingulate cortex, anterior insula, and bilateral inferior parietal lobule. d Fuzzy clustering, map of cluster VI projected on a 2D template. The cluster extended into the posterior quadrangular lobule

(hemisphere VI) and superior semilunar lobule (crus 1, crus 2). The maps were computed using BrainVoyager QX 2.3, RFX $p < 0.01$ cluster-level threshold corrected for multiple comparisons ($p < 0.05$)

whereas a large network containing including areas from both the motor and the attentional networks were negatively correlated with this cluster (supplementary Fig. S7, supplementary Table S11).

Difference Between Functional Connectivity During Pain and Resting State

We contrasted the FC during mechanical punctuate stimulation and resting state. The BOLD activity of cluster V during nociception task was increased in the postcentral gyrus (primary sensory cortex, mainly located in the right hemisphere), bilateral superior temporal gyrus, and posterior insulae (Fig. 1c, supplementary Fig. S8, supplementary Table S12). The BOLD activity of cluster VI during nociception task was increased in the anterior cingulate cortex, anterior insula

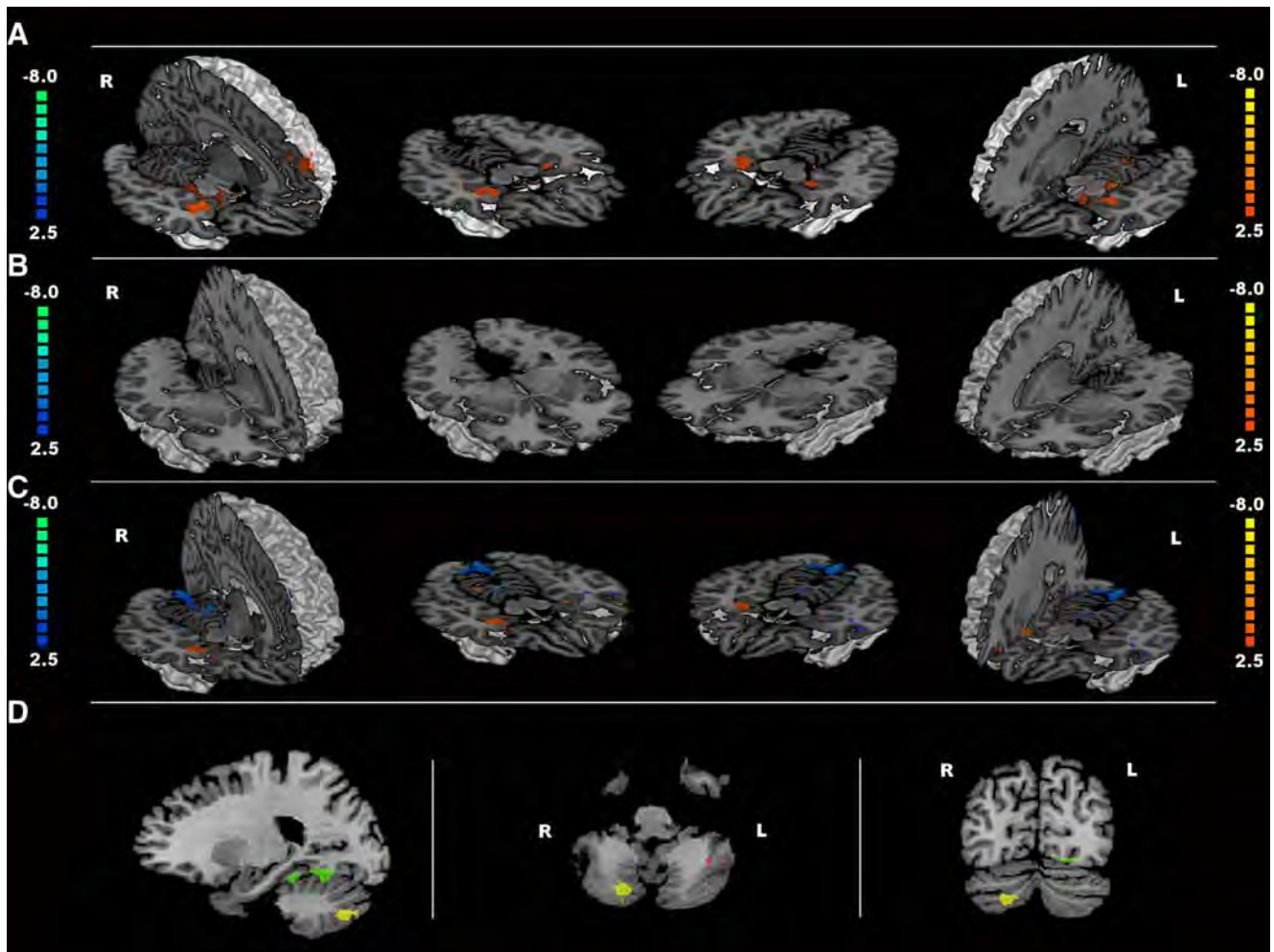


Fig.3 Pain-processing clustering: cluster VII. a Functional connectivity of cerebellar cluster VII (yellow) during mechanical punctuate stimulation projected on a 3D template. Positive correlations included the bilateral inferior, middle, and superior frontal gyrus, amygdala, and parahippocampal gyrus. b Functional connectivity of the cerebellar cluster VII during resting state condition. c Comparison between pain

BrainVoyagerQX2.3, RFX $p < 0.01$ uncorrected threshold

(mainly located on the left hemisphere), and bilateral inferior parietal lobule (Fig. 2c, supplementary Fig. S9, supplementary Table S13).

The BOLD activity of cluster VII during nociception task was increased in the amygdala (mainly located on the right hemisphere), right anterior insula, left putamen, and left inferior frontal gyrus (Fig. 3c, supplementary Fig. S10, supplementary Table S14).

Meta-analytic Behavioral Profile Analysis

According to the BrainMap database, brain regions, we identified to be connected to cluster V, have been typically found to be active in tasks involving behavioral functions such as Action and Perception, while they are not usually involved in Cognition and Emotion (Fig. 4a).

and rest condition. Positive correlations as in a. d Fuzzy clustering, map of cluster VII projected on a 2D template. The cluster extended into the inferior semilunar lobule (VIIb, crus1, crus2). The maps were computed using

In the Perception domain, it was noteworthy that Audition ($z=6.16$), Vision Shape ($z=3.08$), and Somesthesia Other ($z=3.01$) subdomains were particularly involved while Somesthesia Pain was not.

Brain regions connected to cluster VI showed a behavioral profile involving Cognition functions (Fig 4b), in particular the subdomains of Memory Working Memory ($z=7.39$), Attention ($z=5.64$), and Reasoning ($z=3.66$). Tasks involving Somesthesia Pain subdomain ($z=5.14$) activated the areas, in contrast with tasks of the general Perception domain (Fig 4b).

Brain regions connected to cluster VII showed a behavioral profile involving Action and Emotion, in particular in the subdomain of Fear ($z=3.56$), while Perception and Cognition tasks did not usually activate these regions (Fig 4c). Again, tasks involving Somesthesia Pain subdomain ($z=3.49$) activated the areas in contrast with the general factor (Perception).

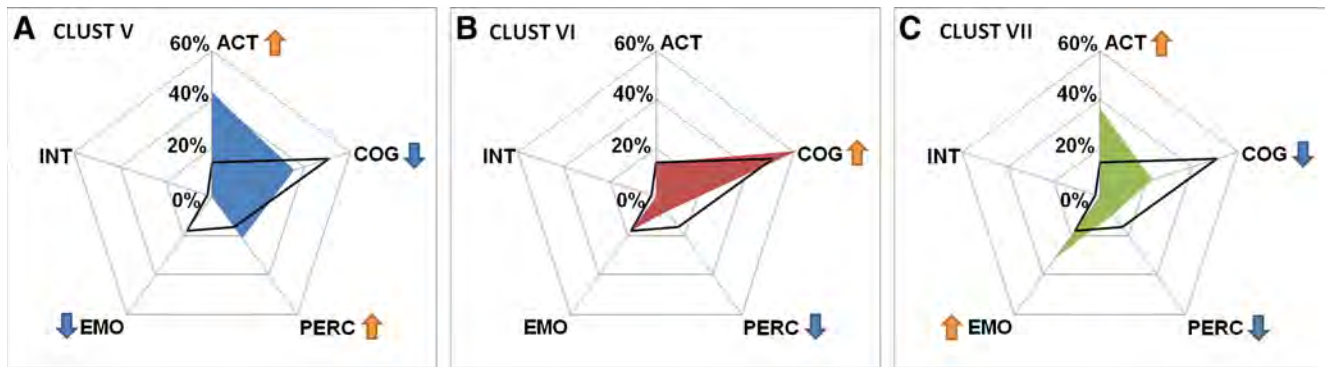


Fig.4 Behavioral profiles of areas with greater FC in pain compared to rest. a Pain>rest FC behavioral profile of cluster V (blue area). b Pain> rest FC behavioral profile of cluster VI (red area). c Pain>rest FC behavioral profile of cluster VII (green area). All profiles were plotted on radar graphs with frequencies of the main behavioral domains of

arrows indicated increased specialization, blue arrows decreased specialization compared to BrainMap mean frequencies

Meta-analysis

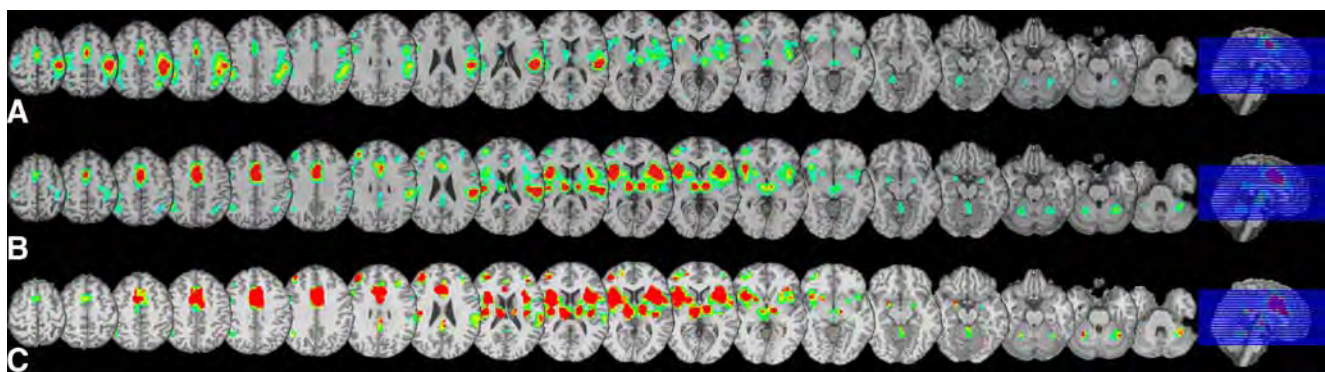
The meta-analysis showed that in touch (Fig. 5a, supplementary Table S15) and pain (Fig. 5b, supplementary Table S16), our pain and touch conditions led to overlapping activation patterns (Fig. 5a vs. 5b, supplementary Table S15 vs. S16): bilateral insula, bilateral postcentral gyrus, bilateral supramarginal gyrus, bilateral inferior parietal lobule, bilateral thalamus, bilateral precentral gyrus, bilateral putamen, medial frontal gyrus, and paracentral lobule. Some of these areas, included in the pain neuromatrix, were more strongly activated for pain (e.g., insula, putamen, thalamus, see Fig. 5c) and some were more strongly activated for touch (e.g., sensory-motor cortex, see Fig. 5a). Some areas were observed only for the pain condition (Fig. 5c, supplementary Table S17), in particular bilateral amygdala, cingulate gyrus (BA 23), anterior (BA 24, 32) and posterior cingulate cortex (BA 31).

In the touch condition, the cerebellum showed significant activation in two activation clusters, one left and one right, in culmen/declive [IV-V-VI]. In the pain

in the vermis of the culmen [vIV-V], and two symmetrically, similar to the ones related to the touch condition, but more posterior, in the declive [VI, crus 1]. The medial one had geometric center of mass coordinates x, y, z = 0 mm, -52 mm, -15 mm, total volume 1384 mm³; the left had geometric center of mass coordinates x, y, z = -30 mm, -53 mm, -29 mm, total volume 3136 mm³; and the right had geometric center of mass coordinates x, y, z = 25 mm, -56 mm, -26 mm, total volume 1464 mm³. These clusters were significantly more active in pain (Fig. 5c, supplementary Table S17), and in particular, the vermal activation was evident only in pain (Fig. 5b, 5c).

Discussion

The cerebellar activation during pain stimulation is often detected in neuroimaging studies and it has been mostly reported in the anterior vermis and posterior lobules [9], although the significance of these activations has been rarely commented upon and often neglected. Initially, the cerebellar activity was considered as



condition, there were three activation clusters: one in the midline of the cerebellum,

unspecific and linked to motor behavior or

BrainMap database (blackline, ACT = Action, PERC = Perception, INT = Interoception, COG = Cognition, EMO = Emotion). Yellow

Fig.5 Meta-analysis: pain and somesthesia comparison. a ALE meta-criteria: [Diagnosis = Normals] AND [Behavioral Domain = Perception]. b ALE meta-analysis with these 0.05 and minimum clusters extent $K_e > 500 \text{ mm}^3$. c ALE meta-analysis difference Perception Domain = Perception Somesthesia AND NOT [Behavioral Domain = Somesthesia (Pain) > Perception Somesthesia]. Threshold was $FDR_{pN} < \text{Perception Somesthesia (Pain)}$.

attentive processing [41], but recently the hypothesis of a direct involvement of the cerebellum in pain modulation has gained more consideration. In our experiment, we described the independent cerebellar contribution during nociception. We used a fuzzy c-mean-based decomposition of the BOLD signal acquired in 12 subjects during mechanical pain stimulation to extract three cerebellar functional independent clusters. In the following paragraphs, we comment upon the results based on the localization of cerebellar activity both in our experiment and in the meta-analytic analyses, deepening our discussion with the aid of the intrinsic cerebellar FC during different conditions.

Clusters V and VI

Cluster V was situated mainly in the sensory-motor cerebellum, localized in the IV, VI lobules and vermis (Fig. 1). The FC cerebral network related to this cluster included the bilateral primary and secondary sensory-motor areas, so we decided to call this cluster the **B** sensory-motor network; however, this network also included the bilateral posterior insula (more activated on the right side), cuneus, posterior cingulate cortex, superior temporal gyrus and PAG (Fig. 1a). This network is consistent with the major cerebral areas associated with pain processing as described in a recent review by Duerden and Albanese [4], but we also observed areas not so commonly observed in pain studies (the cuneus and PCC) that could be linked with the self-relevance/awareness of the intensity of pain sensation [42], or with a specific affinity for A-delta fiber information processing, elicited by the pinprick, in multi-sensory integration regions [43]. We found the PAG only in the sensory-motor network and it seems to be linked to this part of the cerebellum and to the ascending pain/sensory and descending limbic/emotional/motor cerebello-cortical loops [9, 12, 17]. A pattern similar to this network, including the cluster V, was also found in the fMRI-based neurologic signature of pain recently reported in the *New England Journal of Medicine* [44]: a pattern extracted with multivariate techniques whose mean activity could well discriminate between noxious and harmless heat stimuli.

Cluster VI was localized in the cerebellum lobule VI, crus 1 and 2. This cluster was functionally connected with a network (Fig. 2a) including the right thalamus, dorso-frontal parietal areas (with the DLPFC more localized in the right hemisphere), cingulate cortex, and anterior insula (left lateralized). In agreement with our results, the DLPFC, in particular in the right hemisphere, has been suggested as having specific pain evaluation features as shown by some studies [9, 20, 45, 46]. Additionally, this network is similar to the network called the **B** how 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 connection to the cerebellum is relevant, given that this region is implicated in cognitive control as well as in pain modulation [9]. We found parietal areas together with

frontal areas, both of which normally coactivate in attentional tasks, in particular with the spatial representation or ability to plan required for such tasks and so we decided to refer to this cluster as **B** attentional [48]. The cerebellar clusters of the sensory-motor and attentional networks were negatively correlated ($r = -0.31$): the increase of BOLD (derived from the event-related average analysis) in one corresponded to the decrease in the other. This is also a typical finding in the correlation between interoception and exteroception systems studied with functional connectivity, e.g., in the anterior and posterior insula [33] or in the Task Positive and Task Negative networks [49]. Baliki [47] suggests that the subjective pain rating depends on the transfer of information from the multi-sensory system to the anterior insula and lateral prefrontal cortex, from the sensory-motor to attentional network.

Alternatively, this negative correlation could be linked to the shifting between attention and automatic behavior or between anticipation and sensation of pain [20]. Mobbs and colleagues [50] investigated spatial imminence of threat in an fMRI active avoidance paradigm in which volunteers were pursued through a maze by a virtual predator with the ability to inflict pain. They found that as the virtual predator grew closer (the pain was unavoidable, as it was in our study), brain activity shifted from the ventromedial prefrontal cortex to the periaqueductal gray, a pattern that is very similar to prior observations reporting that as electric shock grew closer, brain responses shifted from rACC to PAG and the surrounding areas, including the thalamus, striatum, pallidum, hypothalamus, and cerebellum.

The relationship of these networks (sensory-motor and attentional) could be supported by the observation of Moulton and colleagues [20] who found two distinct phases in pain processing: an **B** early phase, involving the anterior insula, frontal, and cingulate cortex associated with attentional, threat-detection, and evaluative processes; and a **B** late 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 evaluative and sensory-motor networks, with a right lateralization of the posterior insula (sensory-motor network) and a left lateralization of the anterior insula (evaluative network). This is in line with the schema proposed by Craig where body feelings are transmitted from 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 **B**the 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 using fuzzyclustering 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. 1 and 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 moregen-

eral 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 terminalis, 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 refer to this cluster as 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, crusI 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 uncorrected threshold, because cluster VII had a good overlap with meta-analytic results of emotional activation of the cerebellum [1,64]. Also, it has been demonstrated that lobule VIIb is functionally connected with the vermis VII [53]. The cerebellar vermis is an area that participates during emotional memory and modulates the typical physiological response to adverse stimuli as demonstrated by many experiments in humans and animals [62]. Therefore, we could expect major activity of this area during nociception in human as due to the fact that the pain has a high impact on biological preserving of life creating fear memory conditioning and visceral primal aversive emotion. In animals (e.g., mice and rodents), the posterior vermis or limbic vermis [62] plays a central role in fear conditioning, mediated by pain; in humans, the cerebellar areas involved could also include posterior cerebellar hemispheres together with the vermis as suggested by our results. Adamaszek et al. provided preliminary evidence of that in a group of cerebellar stroke patients with a specific impairment for emotional processing 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 lobe development, well represented by this cerebellar network.

We did not observe any significant FC during resting state for this cluster (Fig. 3b). The areas recruited by cluster VII network during nociception belonged to Action and Emotion, in particular to Fear. This is suggestive of fear conditioning where an action pattern could be linked to an aversive stimulus. Pain could be extremely effective in conditioning and cerebellum is a key area in animals to store fearful memories [62]. The subdomain Somesthesia/Pain tasks, included in Perception domain, activated this network in contrast with the general Perception factor, supporting a specific effect of nociception in the promotion of associative learning processing in the cerebellum.

Meta-analysis

We compared pain and somesthesia perception to look if a simple tactile stimulation could elicit the same cerebellar areas. The results (Fig. 5) showed that three activation clusters (Fig. 5c) had more activation specifically in pain condition. The first was inside the vermis IV-V and it was completely inside in the larger clusters V that also included the cerebellar lateral lobules IV-V (supplementary Fig. S11). The second and third activation cluster of the meta-analysis overlapped completely with cluster VI from our pain condition. We could argue that a simple passive sensorial stimulation like touch did not elicit these cerebellar clusters.

We reliably detected with a nonstandard technique (fuzzy c-mean clustering and SogICA) the results of a meta-analytic approach with

200 papers and 2688 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 with clusters V and VI but not for the cluster VII. This could be explained by a greater difficulty in the detection of this cluster as proven by the threshold we had to use in this work or with a bias of the BrainMap database that included too few studies with a complete mapping of the lowest part of the cerebellum that often is cut away. Interestingly, we could observe amygdala activity, linked to cluster VII network, only in the pain condition.

Limits

A limitation of this study is a common bias of almost all the fMRI pain studies: the paradigm was based on a condition of unavoidable pain. Generally speaking, it is quite difficult to create an ecologic pain paradigm in the MRI setting: the withdrawal motor reflex is impossible, and the action inhibition and the antinociception response maximized. In these conditions, we stress the importance of not limiting the 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 cerebellar signal analysis of this paper is a good demonstration of this assertion. An important unresolved question about the cerebellar role in pain processing is if the cerebellum is a passive integrator or an active participant. In our opinion, this work could contribute to future experiment to clarify this question. We propose to 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

Among the multiple dimensions of pain, evidenced by 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 had attributed to the networks that synchronized with the independent cerebellar clusters: a sensory-motor network, a cognitive network, and an emotional network.

Speculatively, the clusterization of the cerebellum into different systems, one localized in the paleocerebellum and two in the neocerebellum, one intermediate, and one more posterior and phylogenetically newer, could be indicative of functions specialized in 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 independent areas linked to different functional networks is a perfect representation of an integrated view of the pain processing.

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Conflict of Interest The authors declare no conflict of interest.

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