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A probabilistic template of human mesopontine tegmental nuclei from in vivo 7 Tesla MRI

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Abstract

Mesopontine tegmental nuclei such as the cuneiform, pedunculotegmental, oral pontine reticular, paramedian raphe and caudal linear raphe nuclei, are deep brain structures involved in arousal and motor function. Dysfunction of these nuclei is implicated in the pathogenesis of disorders of consciousness and sleep, as well as in neurodegenerative diseases. However, their localization in conventional neuroimages of living humans is difficult due to limited image sensitivity and contrast, and a stereotaxic probabilistic neuroimaging template of these nuclei in humans does not exist. We used semi-automatic segmentation of single-subject 1.1 mm-isotropic 7 Tesla diffusion-fractional-anisotropy and T₂-weighted images in healthy adults to generate an *in vivo* probabilistic

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neuroimaging structural template of these nuclei in standard stereotaxic (Montreal Neurological Institute, MNI) space. The template was validated through independent manual delineation, as well as leave-one-out validation and evaluation of nuclei volumes. This template can enable localization of five mesopontine tegmental nuclei in conventional images (e.g. 1.5 Tesla, 3 Tesla) in future studies of arousal and motor physiology (e.g. sleep, anesthesia, locomotion) and pathology (e.g. disorders of consciousness, sleep disorders, Parkinson's disease). The 7 Tesla magnetic resonance imaging procedure for single-subject delineation of these nuclei may also prove useful for future 7 Tesla studies of arousal and motor mechanisms.

Keywords

Human mesopontine tegmental nuclei; ascending arousal and motor systems; *in vivo* neuroimaging template; multi-contrast 7 Tesla MRI; cuneiform; pedunculotegmental; oral pontine reticular; paramedian raphe; caudal linear raphe nuclei

1. Introduction

Mesopontine tegmental nuclei such as the cuneiform (CnF), pedunculotegmental (PTg, also known as pedunclopontine), oral pontine reticular (PnO, also known as pontis oralis), paramedian raphe (PMnR) and caudal linear raphe (CLi, also known as raphe linearis, caudal part) nuclei, are critical for arousal (e.g. wakefulness and REM sleep) and motor functions (e.g. locomotion) (Alam et al., 2011; Goetz et al., 2016; Ikemoto, 2007; Mori, 1987; Paxinos et al., 2012; Sandoval-Herrera et al., 2011). They are involved in the pathogenesis of disorders of consciousness (Edlow et al., 2012), as well as of sleep disorders (Boeve et al., 2007) and neurodegenerative diseases (Braak et al., 2003; Mazzone et al., 2016). However, the inability to precisely localize these nuclei *in vivo* coupled with the absence of a stereotaxic probabilistic template of these nuclei in living humans, has limited our understanding of their role in these diseases, a prerequisite for new surgical and pharmaceutical interventions. Note, that a large part of the subcortex (about 93% according to recent estimates (Alkemade et al., 2013; Forstmann et al., 2017) - including mesopontine tegmental nuclei - is not captured in standard anatomical atlases. For instance, the PTg is a promising target for deep brain stimulation in Parkinson's disease (Mazzone et al., 2016; Zrinzo et al., 2008); however, variability in patient outcome after this novel surgical approach has been ascribed to uncertainty in localizing the PTg and the resulting possible concomitant stimulation of other nuclei (e.g. CnF) (Mazzone et al., 2016; Zrinzo et al., 2008).

Identification of mesopontine tegmental nuclei (as well as of most of the subcortex as noted above) in conventional MRI has been hampered by limited gray-white matter contrast in the brainstem compared to the cortex, and by low MRI sensitivity in deep brain regions (e.g. distal from the MR receiver array). Notably, spatial resolution has not been the main limiting factor for the identification of these nuclei. In fact, a 1–1.5 mm isotropic MRI is expected to resolve the borders of these structures, which have a volume greater than about 20 mm³ (see Result section); see also (Edlow et al., 2016). Currently, the identification of these nuclei in single subject MRI is based on visual extrapolation of anatomical landmarks from *ex vivo*

histological atlases (Naidich et al., 2009; Olszewski and Baxter, 1954; Paxinos et al., 2012; Paxinos and Huang, 1995), which suffers from limited accuracy, reproducibility, and the lack of an error metric. Previous work has shown the feasibility of identifying the caudal and rostral tip of the PTg using the contrast provided by proton density images (Zrinzo et al., 2008) at a conventional magnetic field strength (1.5 Tesla). However, currently, a stereotaxic probabilistic template of the PTg, as well as of the CnF, PnO, PMnR, and CLi in living humans does not exist. Such a template, coupled with precise co-registration of the template to single subject MRIs, would allow the automatic identification of these nuclei in individual subjects, complementing existing probabilistic templates in stereotaxic (Montreal Neurological Institute, MNI) space that are currently used to identify the location of other brain regions (Desikan et al., 2006; Destrieux et al., 2010; Tzourio-Mazoyer et al., 2002).

The aim of this study was to create a stereotaxic probabilistic neuroimaging structural template of the left and right CnF (CnFl, CnFr), left and right PTg (PTgl, PTgr), left and right PnO (PnOl, PnOr), left and right PMnR (PMnRl, PMnRr), and CLi by the use of: (i) cutting-edge acquisition technology (7 Tesla scanner, 32-channel receive coil-array), which enabled us to push the current limits of MRI sensitivity; and (ii) a high-resolution (1.1 mm isotropic) multi-contrast (diffusion fractional anisotropy (FA) and T₂-weighted) echo-planar imaging (EPI) approach, which provided exquisite complementary anatomical contrasts for brainstem anatomy with precisely matched geometric distortions and resolution.

2. Material and methods

2.1 MRI data acquisition

Data were acquired in a previous study (Bianciardi et al., 2015). Data acquisition parameters are briefly reported here; more details can be found in (Bianciardi et al., 2015). Twelve healthy subjects (6m/6f, age 28 ± 1 years) underwent 7 Tesla MRI after giving written informed consent, under approval of the Massachusetts General Hospital Institutional Review Board. A custom-built 32-channel receive coil and volume transmit coil was used (Keil et al., 2010), which provides better coverage into the brainstem area than commercial coils. We adopted a common single-shot 2D EPI readout for 1.1 mm isotropic sagittal diffusion-tensor images (DTI), and T₂-weighted images, with matrix size/GRAPPA factor/nominal echo-spacing = $180 \times 240/3/0.82$ ms. This yielded T₂-weighted anatomical images with exactly matched resolution and geometric distortions to the DTI, and also allowed us to overcome the specific-absorption-rate limits of spin-warp T₂-weighted MRI at 7 Tesla. Additional MRI parameters for DTI and T₂-weighted images were: spin-echo EPI, 61 slices, echo-time/repetition-time = 60.8 ms/5.6 s, partial Fourier: 6/8, unipolar diffusion-weighting gradients (for DTI), 60 diffusion directions (for DTI, b-value ~ 1000 s/mm²), 7 interspersed “b0” images (non-diffusion weighted, b-value ~ 0 s/mm², which were also used as T₂-weighted MRI), 4 repetitions, acquisition time/repetition 6’43”. The total acquisition time for DTI and T₂-weighted MRI was $\sim 27'$. Note that the use of unipolar (Stejskal and Tanner, 1965) rather than bipolar (Reese et al., 2003) diffusion gradients enabled us to shorten the echo-time by ~ 30 ms and to considerably improve the sensitivity of high-resolution DTI (Bianciardi et al., 2015).

In the same subjects and session of the DTI/T₂-weighted MRI, we also acquired a GRE field map (employed in (Bianciardi et al., 2016) to correct for geometric distortions in fMRI data) with parameters: isotropic resolution/matrix size/bandwidth/N. slices/slice orientation/slice acquisition order/TEs/TR/FA/total acquisition time = 2.7 mm/ 270 × 270/1515 Hz-per-pixel/70/sagittal/interleaved/[2.79 3.81] ms/570 ms/36°/2°.

2.2 MRI data pre-processing and alignment to MNI space

For each subject, the diffusion FA map was computed from the DTI data concatenated across 4 repetitions and preprocessed (distortion and motion-corrected) with the –Diffusion Toolbox of the FMRIB Software Library (FSL, Oxford, UK) as in (Bianciardi et al., 2015). Distortion correction of DTI data included correction for geometric distortions mainly due to eddy-currents, and was performed with the FSL tool “eddy_correct”. For each subject, after motion correction, the 28 “b0” T₂-weighted images were averaged and co-registered to DTI data via affine transformation as in (Bianciardi et al., 2015).

Single-subject FA and T₂-weighted images were precisely co-registered to MNI space as previously described (Bianciardi et al., 2015). The coregistration procedure (Bianciardi et al., 2015) is summarized here. Single-subject FA images were aligned to a brain diffusion FA template (which we call “IIT space”) in MNI space (IIT human brain atlas, v.3, Chicago, USA) (Varentsova et al., 2014). We chose this FA template because it is a stereotaxic space useful for diffusion-based tractography analysis, it covers the whole brainstem (including the medulla), as well as it displays high contrast and several structural details in the brainstem. This was achieved by the use of the Advanced Normalization Tool (ANTs, Philadelphia, USA) (Avants et al., 2011) by concatenating a generic affine and a high-dimensional non-linear transformation computed for images having the same modality (FA maps) – as in (Bianciardi et al., 2015). The generic affine transformation was computed by concatenating center-of mass alignment (degrees of freedom, dof = 3), rigid (dof = 6), similarity (dof = 7) and fully affine (dof = 12) transformations with smoothing sigmas: 4, 2, 1, 0 voxels – fixed image space. The high-dimensional non-linear transformation used a symmetric diffeomorphic normalization transformation model with smoothing sigmas: 3, 2, 1, 0 voxels – fixed image space –, and histogram matching of images before registration. Additional parameters used in both the affine and non linear transformations were: cross correlation metric, regular sampling, gradient step size: 0.2, four multi-resolution levels: shrink factors 6, 4, 2, 1 voxels – fixed image space –, data winsorization – quantiles: 0.005, 0.995 –, convergence criterion: slope of the normalized energy profile over the last 10 iterations < 10⁻⁸. The combined transformation was then applied to both single-subject FA and T₂-weighted images, using a single-interpolation step (interpolation method: linear). Single-subject FA and T₂-weighted images were also aligned to MNI152 standard (non linear 6th generation MNI152_T1_1mm available for instance in FSL) space (which we call “MNI152_1mm space”), which is commonly used as a stereotaxic space for functional MRI analysis. The MNI152_1mm space and the IIT space are well aligned in most of the brain, but they display a small misalignment in the brainstem (especially in pontine and medullary regions). Thus, single-subject FA and T₂-weighted images were aligned to MNI152_1mm space by applying two concatenated transformations, using a single-interpolation step (interpolation method: linear): first, the single-subject to IIT space transformation computed

above; and second, the IIT to MNI152_1mm non-linear transformation computed in (Bianciardi et al., 2015), using the same parameters as above. Finally, in order to provide a template of mesopontine tegmental nuclei with higher spatial resolution, we also used as a reference template the 2009b nonlinear asymmetric 0.5×0.5×0.5mm MNI template (<http://www.bic.mni.mcgill.ca/ServicesAtlases/ICBM152NLin2009>), which we call the MNI152_0.5mm space. As for the (1 mm isotropic) MNI152 space, we computed the IIT to MNI152_0.5mm non-linear transformation with ANTs, using the same parameters as above; thus, we aligned single-subject FA and T₂-weighted images to MNI152_0.5mm space by applying the latter transformation concatenated to the single-subject to IIT space transformation computed above.

2.3 Single-subject semi-automatic labeling and probabilistic template generation

On a single-subject basis, M.B. performed semi-automatic segmentation of multi-contrast (FA maps and T₂-weighted) images in IIT space to yield single-subject labels (i.e. masks) of mesopontine tegmental nuclei (CnF/l/r, PTg/l/r, PnO/l/r, PMnR, CLi), using a procedure similar to (Bianciardi et al., 2015). For each subject, the procedure consisted of multiple steps. First, to exclude CSF, a mask (generated by identifying the voxels with mean diffusivity values greater than 0.001 mm²/s) was applied to the FA map and to T₂-weighted images before segmentation of mesopontine tegmental nuclei. Second, after CSF masking, the FA map (used to segment the CnF, PTg, PMnR, CLi) or the T₂-weighted image (used to segment PnO) was supplied to an automatic, single-modality intensity-based clustering algorithm implemented in Matlab 8.0 (The MathWorks, Natick, USA) (method: k-means clustering; distance function: normalized squared Euclidean distance; maximum number of iterations: 300, which always obtained algorithm convergence; randomized initialization, with cluster re-ordering across the group to provide consistent inter-individual clustering; number of clusters: four for PTg and PMnR; five for CnF, CLi, PnO). Note that, to delineate each nucleus, we used only the image modality (either FA or T₂-weighted MRI – for instance FA for CLi) that displayed the nucleus boundaries with good contrast, and did not employ the other modality (which had poor contrast for that nucleus – e.g. T₂-weighted MRI for CLi). Third, the cluster containing the nucleus of interest was manually identified (cluster with the second lowest intensity value for PTg, PMnR, CLi, PnO; cluster with the lowest intensity value for CnF) and labels for 3D connected neighborhood components were automatically extracted using Matlab; within these labels, the label for nuclei of interest were identified by centroid matching (centroid location defined manually); when distinct neighboring anatomical structures (for instance a nucleus of interest and an adjacent gray matter region) displayed similar intensity values, a “disconnection-mask” (obtained by manually delineating the shared border between the nucleus of interest and the neighboring region) was applied before extraction of the 3D connected components to exclude the neighboring region from the nucleus label of interest (for instance, this procedure was used to “disconnect” the CnF from the periaqueductal gray, or the CLi from the dorsal raphe). A schematic diagram of the entire semi-automatic segmentation procedure can be found in Figure 2 of (Bianciardi et al., 2015).

For each nucleus, a probabilistic neuroimaging template in IIT space was created in the form of an average probability map of the nuclei labels across subjects (highest probability =

100 % overlap of nuclei labels across subjects, $n = 12$). Similarly, after aligning the single-subject labels to MNI152_1mm and MNI152_0.5mm spaces (by applying the IIT to MNI152 transformations described above, interpolation method: nearest neighbors), a probabilistic neuroimaging template in MNI152_1mm and MNI152_0.5mm spaces of these nuclei was also created. The template was developed in both IIT and MNI152 spaces (the latter at two different resolutions) to facilitate its use in both diffusion and functional MRI studies.

For each subject and label (coregistered to single-subject native space using the inverse of the transformation described in 2.2) we also computed the label volume in native space, and reported the mean (s.e.) volume across subjects. Specifically, volumes were computed on labels in native space after correction (fugue, FSL, Oxford, UK) for possible residual (after eddy-current correction) geometric distortions (e.g. due to off-resonance B_0 effects) using the acquired field map. To evaluate the impact of off-resonance B_0 effects on the evaluation of the volumes of mesopontine tegmental nuclei, label volumes were also computed on labels in native space before the application of the field map.

2.4 Atlas validation

C.S. manually segmented (fslview, FSL, Oxford, UK) the same regions (CnFl/r, PTgl/r, PnOl/r, PMnR, CLi) in each subject, yielding single-subject manual labels of these nuclei. The manual segmentation was based on the image contrast and on the identification of mesopontine tegmental anatomical landmarks (Paxinos et al., 2012) as follows. The CnF was identified as a wedge-shaped region hypointense in FA maps at the pontomesencephalic border neighboring the periaqueductal gray and the inferior colliculus (Paxinos et al., 2012). The PTg was identified in the ventrolateral part of the caudal mesencephalic tegmentum, as a nucleus hypointense in FA maps bounded medially by the superior cerebellar peduncle (Mesulam et al., 1989; Paxinos et al., 2012). The PnO was identified as a region hypointense in T_2 -weighted MRI located in the oral pontine tegmentum (Paxinos et al., 2012) lateral to the median/PMnR raphe and bounded ventro-laterally by white matter tracts (superior cerebellar peduncle, central tegmental tract and medial lemniscus). The PMnR was identified in the oral part of the pontine tegmentum and mesopontine junction (Paxinos et al., 2012) as an area hypointense in FA compared to adjacent white matter fibers (for example the medial longitudinal fasciculus positioned dorsal to it) and hyperintense compared to the neighboring median raphe (medial to the PMnR). CLi was identified as a hypointense FA area medial to the superior cerebellar peduncle and anterior to the dorsal raphe (Paxinos et al., 2012).

The probabilistic nuclei template was validated by computing, for each nucleus, the spatial overlap between each single-subject label (derived from semi-automatic segmentation) and a reference label. As reference label, we adopted: (i) the label derived from manual segmentation; (ii) the probabilistic template label (thresholded at 35%) generated by averaging the labels across the other 11 subjects (leave-one-out cross validation). For both (i) and (ii), the spatial overlap between two labels (the label derived from semi-automatic segmentation and the reference label) was computed using the modified Hausdorff distance (Dubuisson and Jain, 1994) (commonly used in neuroimaging (Augustinack et al., 2013;

Fischl et al., 2008; Ghosh et al., 2010; Klein et al., 2010; Yendiki et al., 2011)), as follows: the minimum distance of each point on one label from the other label was averaged across all points on each label, yielding two distance values; the maximum value of these two distance values was computed. For each nucleus, the spatial overlap of (i) and (ii) was then averaged across subjects and displayed.

As a further atlas validation, we compared the mesopontine tegmental nuclei volumes with the nuclei volumes precisely computed from the Paxinos atlas (Paxinos et al., 2012). To compute the (Paxinos et al., 2012) nuclei volumes, we took a snapshot (Adobe Acrobat Reader) of brainstem plates ranging from +20 mm to +39 mm (Figure 8.37–8.56) of (Paxinos et al., 2012) (.pdf document), and converted them to single-slice nifti images (with the proper spatial resolution) using Matlab. The slice thickness was set to 1 mm. The in plane isotropic spatial resolution of each slice (varying between .0294 mm and .0474 mm in the examined plates) was determined (Matlab) by manually computing the number of pixels between adjacent coordinates (coordinate system provided for each plate by (Paxinos et al., 2012)). Then, we manually delineated (fslview, FSL, Oxford, UK) CnF, PTg, PnO, PMnR, CLi following the borders drawn by (Paxinos et al., 2012). Finally, for each slice we multiplied the number of delineated voxels in each label by the voxel volume, and, for each label, we added the resulting number across slices to get a gold standard label volume.

3. Results

The probabilistic neuroimaging structural labels in MNI space of CnF/r, PTg/r, and PnO/r are shown in Figure 1. CnF/r and PTg/r appeared as regions of hypointensity compared to the white matter in FA maps (as expected for gray matter regions). PnO/r was hypointense in T₂w MRI compared to the median raphe and surrounding white matter tracts (e.g. the medial lemniscus), possibly indicating a higher iron concentration compared to neighboring areas. The probabilistic neuroimaging structural labels in MNI space of PMnR/r and CLi are shown in Figure 2. In FA maps, PMnR/r and CLi appeared as regions of hypointensity compared to surrounding white matter. The PMnR/r surrounded a darker area in FA, which was previously identified as the median raphe (Bianciardi et al., 2015).

The spatial overlap computed to validate the template labels with manually segmented labels, as well as using the leave-one-out cross validation approach, is displayed in Figure 3A)-B). The labels of mesopontine tegmental nuclei displayed very good spatial accuracy (average modified Hausdorff distance < 0.65 mm for both approaches), thus validating the generated probabilistic nuclei template.

The volume (mean $\pm$ s.e. across subjects) of each semi-automatic label in native space (after correction of residual geometric distortion due to off-resonance B₀ effects) and of gold standard labels computed from (Paxinos et al., 2012) is shown in Figure 3C). For CnF/r, PTg/r, CLi, the mean volume of semi-automatic labels did not differ from the volume of gold standard labels ($p < 0.05$). For PnO/r, the mean volume of semi-automatic labels was smaller ($p < 0.05$) than the volume of gold standard labels. However, we noticed that the extent of the delineated PnO in the z-direction was ~ 9 mm, while in the Paxinos atlas (Paxinos et al., 2012) it is ~ 14 mm. Thus, for PnO/r, we compared the mean volume of

semi-automatic labels with the volume of only the superior 9mm-thick part of the gold standard label computed from (Paxinos et al., 2012) Figure 8.42–8.50, and we did not find any significant difference ($p < 0.05$). For PMnRl/r, the mean volume of semi-automatic labels was larger ($p < 0.05$) than the volume of gold standard labels computed from (Paxinos et al., 2012).

For each nucleus, correction of residual geometric distortion (due to off-resonance B_0 effects) of labels in native space did not produce significant differences ($p < 0.05$) in label volumes (changes smaller than 3% of the label volume).

4. Discussion

4.1 On the template creation and its use

Our findings demonstrate the feasibility of delineating, on a single-subject basis, five mesopontine tegmental nuclei of the motor (CnF (Alam et al., 2011; Mori, 1987; Olszewski and Baxter, 1954), PTg (Goetz et al., 2016; Olszewski and Baxter, 1954), PnO (Sandoval-Herrera et al., 2011)) and arousal (PTg (Goetz et al., 2016; Olszewski and Baxter, 1954), PnO (Sandoval-Herrera et al., 2011), PMnR (Paxinos et al., 2012), CLi (Ikemoto, 2007; Olszewski and Baxter, 1954)) systems. This methodological advance was enabled by semi-automatic segmentation of high-contrast and high-sensitivity multi-contrast MR images acquired at 7 Tesla with a coil that extended coverage to this region (Keil et al., 2010). Our findings build upon those of a few previous reports (Edlow et al., 2012; Mazzone et al., 2016; 2013; Zrinzo et al., 2008) of single-subject, neuroimage-based manual localization of these nuclei. For instance, Edlow et al. (Edlow et al., 2012) demonstrated the feasibility of performing a manual delineation of the CnF, PTg, and PnO on *ex vivo* MRI guided by histological sections of the same brainstem specimen. Moreover, the caudal and the rostral tip of the PTg have been identified using *in vivo* proton density MRI by (Zrinzo et al., 2008). Further, in surgical procedures of deep brain stimulation electrode implantation in Parkinson's disease (Mazzone et al., 2016; 2013) the PTg has been localized using *ex vivo* atlas schematics (Paxinos et al., 2012; Paxinos and Huang, 1995) along with the single-subject *in vivo* MRI identification of anatomical reference markers (e.g. the pontomesencephalic junction line, the ventricular floor line and the obex). Our 7 Tesla procedure showed that ~1 mm isotropic diffusion-based images (FA maps) and T_2 -weighted images are key contrasts for the *in vivo* identification of CnF, PTg, PnO, PMnR, and CLi. This procedure will prove useful in 7 Tesla clinical and research single-subject studies of arousal and motor mechanisms in healthy conditions (e.g. sleep, anesthesia) as well as in disease (e.g. disorders of consciousness (Edlow et al., 2012), sleep disorders (Boeve et al., 2007), Parkinson's disease (Braak et al., 2003; Mazzone et al., 2016; 2013)).

Crucially, our work also demonstrated the feasibility of generating a validated *in vivo* stereotaxic (in MNI space) probabilistic neuroimaging template of these structures after precise coregistration of single-subject labels to MNI space. This template will be publicly released on the same website as our previous template of 11 brainstem nuclei (Bianciardi et al., 2015), as well as on public repositories of neuroimaging data and tools (such as neurovault.org, nitrc.org, github.com, sourceforge.net). This template will complement existing *in vivo* neuroimaging atlases of other brain structures (Desikan et al., 2006;

Destrieux et al., 2010; Tzourio-Mazoyer et al., 2002). We foresee the use of the generated probabilistic template of CnF, PTg, PnO, PMnR and CLi to aid the localization of these nuclei in *conventional* imaging (e.g. 1.5 Tesla and 3 Tesla) in future studies of arousal and motor function. Further, upon coregistration to clinical MRI, our template may improve the accuracy of interventions (e.g. placement of deep brain stimulation electrodes, dosage and types of anesthetic drugs), the evaluation of lesions and the assessment of connectivity pathways underlying arousal and motor mechanisms in a broad set of disease populations (e.g. disorders of consciousness (Edlow et al., 2012), sleep disorders (Boeve et al., 2007) and neurodegenerative diseases (Braak et al., 2003; Mazzone et al., 2016)).

4.2 On the template validation

For each nucleus label, the modified Hausdorff distance indicated a very good agreement (< 0.65 mm, on average across subjects) between the semi-automatic and the manual segmentations, as well as a very good performance of the leave-one-out cross-validation (< 0.65 mm, on average across subjects). Considering that mesopontine tegmental nuclei are tiny structures, the modified Hausdorff distance (Dubuisson and Jain, 1994) was used to evaluate spatial overlap rather than other metrics such as the Jaccard or Dice index. The latter (sometimes employed to validate the segmentation of cortical and larger subcortical regions (Destrieux et al., 2010; Xiao et al., 2014)) perform better for larger structures, whereas the Hausdorff distance (often used in neuroimaging (Augustinack et al., 2013; Fischl et al., 2008; Ghosh et al., 2010; Klein et al., 2010; Yendiki et al., 2011)) is mostly unaffected by structure size.

We employed two different procedures using as metric the Hausdorff distance to validate our neuroimaging-based template of mesopontine tegmental nuclei: validation (i) using reference manual labels was employed to assess the precision of the semi-automatic nuclei delineation against an independent delineation; validation using reference labels (ii) was employed to estimate the across-subject variability (i.e. the internal consistency) in the label location. Note that, in light of their different meaning and significance, any direct comparison between the spatial overlap figures obtained in (i) and (ii) should be interpreted with caution.

Further, we validated our template of mesopontine tegmental nuclei by comparing the label volumes (mean/s.e. across 12 subjects) with gold standard labels computed from (Paxinos et al., 2012). The agreement between the two sets of labels was very good for CnF, PTg and CLi, thus further validating our label delineations. PnO underestimated the volume of the gold standard label, and PMnR overestimated it. We discuss this mismatch in the next section entitled “Limitations”.

Interestingly, the label volumes in native space did not change after correcting for residual geometric distortions due to off-resonance B_0 effects. This was in line with visual inspection of the field maps: the latter revealed that off-resonance B_0 effects in the brainstem were mainly present anterior to the mid-pons, and crucially, they did practically not extend to the tegmentum (the dorsal part of the brainstem), where mesopontine tegmental nuclei are located. Note, that off-resonance B_0 effects anterior to the mid-pons are smaller (about 1/3)

than those in orbitofrontal regions and of opposite sign (possibly due to a paramagnetic source, such as a venous sinus/large vein anterior to the mid-pons).

4.3 Limitations

The qualitative agreement (performed by visual comparison) and the label volume comparison between our template and the Paxinos atlas drawings derived from histology (Paxinos et al., 2012) was quite good for CnF, PTg, CLi, but there was some mismatch for PnO and PMnR. For instance, the extent of the delineated PnO in the z-direction was ~ 9 mm, while in the Paxinos atlas (Paxinos et al., 2012) it is ~ 14 mm. This difference could be ascribed to limitations in the resolution and contrast of our images, as well as to intrinsic differences in brainstem anatomy due to biological and methodological variables (lower age of our sample compared to the sample of (Paxinos et al., 2012); manipulations of the *ex vivo* specimen, which might have caused distortions along the long axis of the brainstem, and misalignment between our MRI z-direction and the direction of the long brainstem axis used to cut the brainstem slices in (Paxinos et al., 2012)). Interestingly, the mismatch between the volume of the delineated PnO and the gold standard volume computed from (Paxinos et al., 2012) could be reconciled when a similar extent in the z-direction was considered (upper 9mm-thick label derived from (Paxinos et al., 2012), rather than using the full 14mm-thick label); this suggested that our delineation most probably did not include the caudal part of the pontine reticular nucleus displaying a diminished T₂-weighted contrast with respect to the oral part.

Moreover, a delineated nucleus (PMnR) partially overlapped (the mean $\pm$ s.e. - across subjects - ratio of the volume of the common part divided by the volume of the union label was $.16 \pm .01$) with a neighboring nucleus (PnO). This (together with the biological and methodological-related variability, as explained above for the PnO) might partially explain why the estimated PMnR volume was larger than the gold standard PMnR volume from (Paxinos et al., 2012); however, further validation is needed to clearly disambiguate this common area. Labels overlap was not found for other nuclei, including, interestingly, the CnF and PTg, which are targets for deep brain stimulation. Note that the *ex vivo* atlas delineation of some mesopontine tegmental nuclei has evolved over time (for example the location and shape of CnF and PTg has slightly changed from (Paxinos and Huang, 1995) to (Paxinos et al., 2012)); nevertheless, we used the most up-to-date atlas (Paxinos et al., 2012) as guidance for our delineations. Histology and *ex vivo* imaging - using a procedure similar to the one employed in this *in vivo* work- of the same brainstem specimen might further elucidate these small discrepancies in location with respect to previous work (Paxinos et al., 2012), and further validate the proposed delineation of CnF, PTg, PnO, PMnR and CLi.

Limited accuracy in the coregistration of this template to conventional MRI is a potential barrier to implementation of this template in future work. Nonetheless, coregistration of deep brain structures (“volumes”) is less demanding than coregistration of cortical foldings (Fischl et al., 1999; Greve and Fischl, 2009). Further, the accuracy of coregistration (for example from native single-subject space to MNI space) has considerably improved in recent years by the development of new registrations tools (Avants et al., 2011; Klein et al., 2009) and of multi-modal atlases in MNI space (Varentsova et al., 2014) (including

diffusion-based contrast and T₂-weighted contrast, beyond the original MNI T₁-weighted contrast). These novel tools and atlases can facilitate the use of more accurate same-modality coregistration of diffusion and functional based single-subject images to target stereotaxic images (e.g. of single-subject FA maps to the IIT FA map, as used here). In this and previous work (Bianciardi et al., 2016; 2015), the use of same-modality coregistration using an advanced coregistration tool (Avants et al., 2011), has demonstrated the feasibility of generating a template in MNI space and connectome of tiny brainstem structures, thus proving the feasibility of accurately coregistering these small nuclei across subjects to a common template.

To delineate each mesopontine tegmental nucleus, we employed a single-channel segmentation method that used only one image modality (either FA or T₂-weighted MRI), and discarded the other image modality, which did not clearly display the nucleus boundaries. Nevertheless, future work, with additional image modalities (e.g. T₂^{*}, quantitative susceptibility mapping, magnetization transfer, proton density) or further optimization of FA and T₂-weighted MRI, might employ multi-channel segmentation methods (Visser et al., 2016a; 2016b) and exploit the enhanced contrast and information provided by multiple images.

5. Conclusions

We developed a neuroimaging procedure to delineate mesopontine tegmental nuclei such as CnF, PTg, PnO, PMnR, and CLi on a single-subject basis, as well as a probabilistic structural template of these nuclei in stereotaxic (MNI) space. These structures are vital for arousal and motor function, yet their localization *in vivo* has been limited by a multitude of methodological barriers. Here, we overcome these barriers and demonstrate the feasibility of localizing CnF, PTg, PnO, PMnR, and CLi in neuroimages of living subjects. The resulting template of mesopontine tegmental nuclei has the potential to facilitate precise *in vivo* localization of brainstem lesions, as well as mapping the structural and functional arousal and motor connectivity pathways in health and disease.

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Highlights

- A procedure for identification of 5 mesopontine tegmental nuclei is proposed
- It is based on semi-automatic segmentation of single-subject multi-contrast 7T MRI
- It is validated by manual delineation, a leave-one-out method and volume evaluation
- A validated probabilistic human template of these nuclei is created in MNI space
- This template can be aligned to conventional MRI to identify the nuclei location

Figure 1. Probabilistic (n = 12) template labels in MNI space of the left (blue-lightblue) and right (red-yellow) cuneiform nucleus (CnFl, CnFr), pedunculotegmental nucleus (PTgl, PTgr), and oral pontine reticular nucleus (PnOl, PnOr)

Nuclei labels are overlaid on the group average contrast that was used for segmentation (indicated as FA or T₂w). Very good spatial agreement of labels across subjects was observed indicating the feasibility of delineating probabilistic labels of these mesopontine tegmental nuclei.

Figure 2. Probabilistic (n = 12) template labels in MNI space of left (blue-lightblue) and right (red-yellow) paramedian raphe (PMnRl, PMnRr), and caudal linear raphe (CLi, green)
 Very good spatial agreement of labels across subjects was observed indicating the feasibility of delineating probabilistic labels of these mesopontine tegmental nuclei.

Figure 3. Template validation and label volumes

We show the spatial overlap (modified Hausdorff distance, bar/errorbar = mean/s.e. across 12 subjects) of semi-automatic labels of CnFl/r, PTgl/r, PnOl/r, PMnRl/r, CLi with: A) manually defined labels; B) the probabilistic atlas label (thresholded at 35%) generated averaging the labels across the other 11 subjects (leave-one-out cross validation). In C) the volume of semi-automatic labels (dark gray, bar/errorbar = mean/s.e. across 12 subjects) and the gold standard label volumes (light gray) computed from (Paxinos et al., 2012) is displayed (for PnOl/r, the intermediate bar at ~ 70 mm³ indicates the volume of the superior

9mm-thick part of the gold standard label computed from (Paxinos et al., 2012) Figures 8.42–8.50). Significant differences ($p < 0.05$) between semi-automatic labels and gold standard labels are indicated with an asterisk (*).

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