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Human Brainstem and Cerebellum Atlas- Chemoarchitecture and Cytoarchitecture Paired to MRI

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39 **Abstract**

40 Lesion localization is the basis for understanding neurological disease, which is predicated on
41 neuroanatomical knowledge carefully cataloged from histology and imaging atlases. However, it
42 is often difficult to correlate clinical images of brainstem injury obtained by MRI scans with the
43 details of human brainstem neuroanatomy represented in atlases, which are mostly based on
44 cytoarchitecture using Nissl stain or a single histochemical stain, and usually do not include the
45 cerebellum. Here, we report a high resolution (200 μ m) 7T MRI of a cadaveric male human
46 brainstem and cerebellum paired with detailed, coregistered histology (at 2 μ m single cell
47 resolution) of the immunohistochemically stained cholinergic, serotonergic, and
48 catecholaminergic (dopaminergic noradrenergic and adrenergic) neurons, in relationship to each
49 other and to the cerebellum. These immunohistochemical findings provide novel insights into the
50 spatial relationships of brainstem cell types and nuclei, including subpopulations of melanin and
51 tyrosine hydroxylase⁺ neurons, and allows for more informed structural annotation of cell
52 groups. Moreover, the coregistered MRI-paired histology helps validate imaging findings. This is
53 useful for interpreting both scans and histology, and to understand the cell types affected by
54 lesions. Our detailed chemoarchitecture and cytoarchitecture with corresponding high resolution
55 MRI builds on previous atlases of the human brainstem and cerebellum, and makes precise
56 identification of brainstem and cerebellar cell groups involved in clinical lesions accessible for
57 both laboratory scientists and clinicians alike.

58

59

60

61 **Significance Statement:**

62 Clinicians and neuroscientists frequently use cross-sectional anatomy of the human brainstem
 63 from MRI scans for both clinical and laboratory investigations, but they must rely upon brain
 64 atlases to neuroanatomical structures. Such atlases generally lack both detail of brainstem
 65 chemical cell types, and the cerebellum which provides an important spatial reference. Our
 66 current atlas maps the distribution of key brainstem cell types (cholinergic, serotonergic, and
 67 catecholaminergic neurons) in relationship to each other and the and cerebellum, and pairs this
 68 histology with 7T MR images from the identical brain. This atlas allows correlation of the
 69 chemoarchitecture with corresponding MRI, and makes the identification of cell groups that are
 70 often discussed, but rarely identifiable on MRI scan, accessible to clinicians and clinical
 71 researchers.

72

73 **ABREVIATIONS**

74 A1-A14, catecholaminergic groups (nomenclature from nomenclature from Dahlstrom and Fuxe 1964)
 75 acc V/VII, accessory trigeminal and accessory facial neurons
 76 AMB, nucleus ambiguus
 77 AP, area postrema
 78 Aq, cerebral aqueduct
 79 C1, catecholaminergic group
 80 C2, catecholaminergic group
 81 CbF, cerebellar flocculus
 82 CbH, cerebellar hemisphere
 83 CbV, cerebellar vermis
 84 CbVg, cerebellar vermis granular cell layer
 85 CbVm, cerebellar vermis molecular layer
 86 CbVp, cerebellar vermis Purkinje cell layer
 87 CDPMn, caudal dorsal paramedian nucleus.
 88 CGRP, calcitonin gene-related peptide
 89 ChAT, choline acetyl transferase
 90 Cu, nucleus cuneatus
 91 DCN, dorsal cochlear nucleus
 92 DMX, dorsal motor nucleus of vagus
 93 DR, dorsal raphe
 94 DRd, dorsal subdivision of dorsal raphe
 95 DRv, ventral subdivision of dorsal raphe

96	DT, dentate nucleus
97	DTg, dorsal tegmental nucleus
98	Emb, emboliform nucleus
99	EW, Edinger-Westphal nucleus
100	Fas, fastigial nucleus
101	Glo, globose nucleus
102	Gr, nucleus gracilis
103	ICol, inferior colliculus
104	icp, inferior cerebellar peduncle
105	III, oculomotor nucleus
106	IIIIn, oculomotor nerve/ third cranial nerve
107	IO, inferior olive
108	IPN, interpeduncular nucleus
109	IRt, intermediate reticular zone
110	ISN, inferior salivatory nucleus
111	IV, trochlear nucleus
112	IVn, trochlear nerve/ fourth cranial nerve
113	IVv, fourth ventricle
114	IX, glossopharyngeal nerve nucleus
115	IXn, glossopharyngeal nerve, ninth cranial nerve
116	LC, locus coeruleus
117	LDT, laterodorsal tegmentum
118	LL, lateral lemniscus
119	LPGi, lateral paragigantocellular reticular nucleus
120	LRT, lateral reticular neurons
121	LRTm, lateral reticular neurons magnocellular part
122	LRTpc, lateral reticular neurons parvicellular part
123	mcp, middle cerebellar peduncle
124	Me5, mesencephalic trigeminal nucleus
125	me5, mesencephalic trigeminal tract
126	ml, medial lemniscus
127	mlf, medial longitudinal fasciculus
128	MnR, midline median raphe
129	NTS, nucleus of the solitary tract
130	PAG, periaqueductal gray
131	PBel, external lateral parabrachial nucleus
132	PBG, parabigeminal nucleus
133	PBl, lateral parabrachial nucleus
134	PBm, medial parabrachial nucleus
135	PnB, pontobulbar nucleus
136	PnO, pontis oralis
137	PPT, pedunculopontine tegmentum
138	Pr, prepositus nucleus
139	PRF, pontine reticular formation
140	PSV, principle sensory trigeminal nucleus
141	py, pyramidal tract
142	pyx, pyramidal tract decussation
143	RMg, raphe magnus
144	ROb, raphe obscurus
145	SCol, superior colliculus
146	scp, superior cerebellar peduncle

147 SN, substantia nigra
 148 sol, solitary tract
 149 SOC, superior olivary complex
 150 SpV, spinal trigeminal nucleus
 151 spV, spinal trigeminal tract
 152 SpVes, spinal vestibular nucleus
 153 SSN, superior salivatory nucleus
 154 SuL, suprallemniscal/B9
 155 TH, tyrosine hydroxylase
 156 TpOH, tryptophan hydroxylase
 157 V, trigeminal motor nucleus
 158 VCN, ventral cochlear nucleus
 159 Vn, trigeminal nerve/ fifth cranial nerve
 160 VI, abducens nucleus
 161 VII, facial nucleus
 162 VIIIIn, vestibulocochlear nerve
 163 VIIIn, facial nerve/ seventh cranial nerve
 164 VIn, abducens nerve/sixth cranial nerve
 165 VLM, ventrolateral medulla
 166 Vn, trigeminal nerve/ fifth cranial nerve
 167 VN, vestibular nuclei
 168 VTA, ventral tegmental area
 169 X, vagal motor neurons
 170 XI, spinal accessory nucleus
 171 XII, hypoglossal nucleus
 172 XIIIn, hypoglossal nerve, twelfth cranial nerve
 173 XIn, XI, spinal accessory nerve, eleventh cranial nerve

174 **Introduction**

175 Lesion localization is the basis for understanding neurological disease, which is predicated on
 176 neuroanatomical knowledge carefully cataloged from histology and imaging. As functionally
 177 connected networks become further characterized, brain atlases have become essential reference
 178 resources for both clinical and scientific inquiry. Among human brainstem atlases, previous work
 179 was mostly based on cytoarchitecture (which limits the delineation of cell groups to Nissl-stained
 180 neuronal morphology and packing) or a single histochemical stain (often acetylcholinesterase;
 181 Buttner-Ennever & Horn, 2014; Paxinos, 1995; Sabin, 1901). Therefore, a detailed map of
 182 neurons expressing key brainstem neurotransmitters has yet to be documented in a single human
 183 brainstem resource. Additionally, many human brainstem atlases do not include the cerebellum,

184 neglecting the spatial relationship between the cerebellum and brainstem nuclei. This
185 relationship is often important when considering infratentorial pathology such as tumors, and the
186 consequences of neurosurgical intervention. Furthermore, many histological atlases are difficult
187 to correlate with the less detailed MRI used in clinical settings. While work delineating cell
188 groups onto MRI cross-sections has been performed, these have largely been limited to drawing
189 nuclei onto MRI scans informed by prior, separate histological work (Soria et al., 2011). Only
190 recently has 7 Tesla (7T) imaging, which provides ultra-high spatial resolution, been performed
191 on cadaveric specimens (Adil et al., 2021; Augustinack & van der Kouwe, 2016; Edlow et al.,
192 2019).

193 A goal of this current work was to map the distribution of key brainstem cell types (cholinergic,
194 serotonergic, and catecholaminergic neurons) in relationship to each other and the cerebellum.
195 Additionally, we aimed to pair this detailed histology with 7T MRI. Our detailed chemo- and
196 cytoarchitecture with corresponding high resolution MRI builds on previous atlases of the human
197 brainstem, and makes precise identification of brainstem and cerebellar cell groups involved in
198 clinical lesions accessible for both laboratory scientists and clinicians alike.

199

200 **Materials and methods**

201 **1. Brain Tissue History**

202 An adult brain was provided by the Iowa Neuropathology Department per research protocol. All
203 donors or donor next of kin consented to use of tissue for research under the applicable sections

204 of Iowa state law. This study was reviewed by the University of Iowa Institutional Review Board
 205 and determined not to constitute human subjects research under the Revised Common Rule
 206 (determination # 201706772). We picked a case without known brainstem pathology, and
 207 compared the brainstem to others in our lab to pick a representative case. The brain was from a
 208 68 year old male patient with a past medical history of multiple myeloma, type 2 diabetes
 209 mellitus, hypertension, peripheral vascular disease and tobacco use; the cause of death was
 210 complications secondary to atherosclerotic cardiovascular disease. The brainstem and cerebellum
 211 were sharply separated from the diencephalon. There was a post-mortem interval of 36 hours.
 212 The brain was removed from the skull and the brainstem and cerebellum were preserved and
 213 fixed by cannulating the left vertebral artery (right vertebral was ligated at its most distal aspect)
 214 and basilar artery with 24 gauge angiography catheters and then arterially perfused with 10%
 215 formalin followed by submersion fixation in formalin for approximately 14 days. The subsequent
 216 7T MRI scan and serial section histology of the entire brainstem and cerebellum showed no
 217 evidence of vascular infarct or other pathology.

218

219 **2. 7T MRI Imaging Technique**

220 **Brain Preparation for Imaging**

221 To minimize motion and wave artifacts of a liquid-based medium, the brainstem was embedded
 222 in 3% agar in an MRI compatible container. A lumbar drain catheter was inserted into the
 223 cerebellomedullary fissure oriented toward the cerebral aqueduct and 1% agar was injected to fill
 224 the 4th ventricle to minimize air artifacts in the 4th ventricle, as the high resolution susceptibility

225 weighted imaging (SWI) sequence is highly sensitive to the presence of air. The container
226 holding the tissue and agar was then refrigerated at 4C° overnight, before being equilibrated to
227 ambient temperature the following morning prior to imaging.

228 **Image Acquisition:**

229 Images were acquired on a 7T GE Discovery MR950 scanner using a 32-channel head coil with
230 a set of three anisotropic, susceptibility-weighted scans. Each scan was isotropic and had higher
231 resolution in the acquisition plane (0.1758 x 0.1758mm), but lower resolution out of this plane
232 (0.4mm); other scan parameters were as follows: (TR=66.3ms, TE=23.276ms, flip angle=15
233 degrees, FOV=1024x1024, number of slices=258, voxel resolution=0.1758 x 0.1758mm, slice
234 thickness=0.4mm, slice spacing=0.4mm). In between each acquisition, the field of view was
235 shifted in the plane perpendicular to the plane of acquisition by 1mm. Since the slice thickness
236 and slice gap total 0.8 mm, this 1 mm shift means that slices between acquisitions are staggered
237 by 0.2 mm, such that across the three scans the physical space is fully sampled at $\sim 0.2 \text{ mm}^3$ and
238 can be reconstructed at this resolution (**Fig. 1**).

239 **Image Reconstruction:**

240 After acquisition, images were processed using image processing procedures available in
241 Advanced Normalization Tools (ANTs) (Avants et al., 2011). First, images were preprocessed
242 independently to remove noise, using an adaptive non-local procedure and a Rician noise model
243 and non-uniform intensity due to regional inhomogeneity in the magnetic field, using the N4
244 procedure (Manjon, Coupe, Marti-Bonmati, Collins, & Robles, 2010; Tustison et al., 2010).
245 After images were cleaned in their native, anisotropic space, they were each resampled to exhibit

246 isotropic resolution at the smallest voxel dimension ($\sim 0.2\text{mm}^3$), using a 3rd order B-spline
247 interpolation. Images were then coregistered using a 4-stage registration in the following order:
248 1) translation, 2) rigid with 6 degrees of freedom 3) low-resolution affine with 12 degrees of
249 freedom, 4) high resolution affine. Coregistration was initialized by generating an average of all
250 resampled images and using this as the fixed target for registration. For each subsequent
251 coregistration step, the output coregistered images of the prior step were averaged to generate a
252 new fixed target for registration; this iteratively improves the average for each step. The averages
253 of the final coregistration step constitute the isotropically reconstructed image. If acquisitions are
254 perfect, translation or rigid registration would be all that was necessary; however affine
255 registration adds in correction for differences in image distortion that may be present due to the
256 different field of view.

257

258 **3. Histology**

259 After MRI imaging, the tissue was then sectioned axially in the agar into 4 mm tissue blocks. We
260 then selected the first 10 blocks for further dissection, since they contained the majority of the
261 brainstem. These blocks were incubated in a 20% sucrose solution for approximately 48 hours or
262 until the blocks sunk. Then, using a freezing microtome, we sectioned the tissue into 40 μm
263 slices, and we collected at least 4 sections from each block. One section from each block was
264 mounted on either a 2" x 3" or 5" x 7" gelatinized slide for Nissl staining. The rest of the tissue
265 was placed in a cryoprotection solution and stored at -20 °C for later immunohistochemistry
266 staining.

267 To fully assess each block, four adjacent sections of each block were stained to visualize either
268 Nissl, catecholaminergic, cholinergic, or serotonergic neurons. To visualize the cytoarchitecture,
269 we used the first section cut from each block to perform a Nissl stain with thionin. These slide
270 mounted sections were submerged in water for 1 minute followed by 3 minutes in thionin. Slides
271 were then rinsed in water until the water ran clear, and then we dehydrated sections in a graded
272 ethanol series for 3 minutes each, and cleared in xylenes before coverslipping with a toluene-
273 based mounting media (Cytoseal; Thermo Scientific).

274 We then took the next three adjacent sections from each block and prepared them for
275 immunohistochemistry. First, we performed antigen retrieval by boiling the sections in a solution
276 of 0.1M sodium citrate for 10 minutes and then rinsing in PBS. We then took the second, third,
277 and fourth section from each block and incubated it overnight in either rabbit anti-tyrosine
278 hydroxylase (TH) (1:1,000; Millipore; AB152), goat anti-choline acetyltransferase (ChAT)
279 (1:100; Millipore, AB144P), or mouse anti-tryptophan hydroxylase (TpOH) (1:1,000; Millipore,
280 MAB5278) to label catecholaminergic, cholinergic, and serotonergic cells, respectively. We then
281 placed sections in biotinylated donkey anti-goat secondary antiserum (1:500; Jackson
282 ImmunoResearch; 705-065-147), biotinylated donkey anti-goat IgG secondary antiserum (1:500;
283 Jackson #705-065-147), or biotinylated donkey anti-mouse (1:500; Jackson; 715-066-150)
284 followed by 1 hour in avidin-biotin complex (Vectastain ABC Elite Kit; Vector Laboratories,
285 Burlingame, CA). We made DAB (3,3'-diaminobenzidine) solution in Tris-buffered saline (TBS)
286 containing 0.024% hydrogen peroxide, and modified the DAB to produce a black stain by adding
287 0.2% ammonium nickel (II) sulfate (Sigma) to the DAB solution. After immunolabeling, we
288 mounted and dried sections on 5" by 7" or 2" by 3" slides. We dehydrated sections in graded

289 ethanols for 3 min each and cleared in xylenes before coverslipping with a toluene-based
 290 mounting media (Cytoseal; Thermo Scientific).

291

292 **4. Microscope Imaging and Mapping**

293 We acquired whole-slide images using a slide-scanning microscope (Olympus VS120, Shinjuku,
 294 Tokyo, Japan) using 10x and 20x objectives and extended focal imaging (EFI). EFI makes a z-
 295 series of images through the tissue and combines them into one in-focus image. We reviewed
 296 images in Olympus' OlyVIA software. We placed images of sections from OlyVia into Adobe
 297 Illustrator, and for each block, we layered the images from adjacent tissue sections containing the
 298 immunohistochemical stains on top of the corresponding Nissl section. We then graphically
 299 marked each DAB-stained neuron with different color dots (ChAT stain= orange, TpOH stain=
 300 pink, TH stain= lime green, melanin-pigmented= dark green), to map the spatial relationship of
 301 different cell types. These dots are larger than the actual cells to allow for visibility in the
 302 zoomed out images. Additionally, these dots usually represent a single neuron, but in a few cases
 303 when neurons were too densely packed, a single dot might overlay 2-3 neurons. We used dashed
 304 lines to represent rough outlines of nuclei, and solid lines for the more well-defined white matter
 305 of axons/ fiber tracts (that was well-defined in the Nissl or immunohistochemical stains). To
 306 highlight cranial nerves, we traced ChAT+ axons with solid, transparent grey lines. In general,
 307 we labeled nuclei with capital letters and fiber tracts with lower case. Cranial nerve nuclei are
 308 labeled with roman numerals, and their corresponding nerves are labeled with the roman numeral

309 + n (for example, VII for facial motor nucleus and VIIIn for facial nerve or cranial nerve 7).

310

311 **5. Imaging Histologic Coregistration**

312 After final MRI reconstruction using ANTs, the original block slabs were coregistered to the
313 MRI using manual registration. Then the MRI was coregistered to each individual axial
314 histologic section from each of the 10 blocks in ItK-SNAP (Yushkevich et al., 2006),
315 www.itksnap.org, version 3.8.0) (Yushkevich et al., 2006). These axial histology levels are
316 demonstrated on a sagittal image to help show the rostro-caudal distribution (**Fig. 5**). We then
317 identified the rostral tip of the inferior olive as point zero, and then calculated the distance of
318 each histologic section from the inferior olive zero point. This MRI image of each histologic
319 section was then exported for imaging segmentation and structure labeling.

320

321 **Results**

322 Overall, we were able to robustly label cholinergic, serotonergic, and catecholaminergic neurons,
323 axons, and terminal fields throughout the human brainstem (**Fig. 2-4**). For each level, we mapped
324 all of these cell types and tracts onto one Nissl stained section to visualize their spatial
325 relationships (**Fig. 6-25**).

326

327 Briefly, TpOH robustly and consistently labels serotonergic neurons including fibers (**Fig 2 B**,
 328 C). This allows for easy visualization of the raphe nuclei and surrounding white matter tracts.

329 The catecholaminergic neurons (described further in the Discussion- “Catecholaminergic
 330 Groups”) are marked by either TH stain, melanin pigment, or both (**Fig. 2D, 4C**). We marked the
 331 cells lime green if they were TH+, regardless of whether they were also pigmented with melanin,
 332 but we labeled them darker green if they were pigmented and TH-. Immunofluorescence is
 333 typically used to view colocalization, but when looking at this tissue under a microscope, this
 334 was easily visualized. The melanin pigment is brown, granular, and mostly concentrated in only
 335 a portion of the neurons’ cytoplasm; this contrasts to the more translucent greyish-black TH stain
 336 in the cytoplasm that surrounds the melanin (filling up the entire cytoplasm) in those neurons in
 337 which the two markers colocalize (**Fig 2D**). In general, we found that the TH+ neurons that are
 338 nonpigmented stain more darkly for TH than the TH+ cells that contain pigmented melanin, and
 339 the most darkly pigmented melanin neurons appear to be TH- (or their TH levels are too low to
 340 detect). The best example of the darkest melanin-pigmented neurons is in the substantia nigra,
 341 and most of these pigmented neurons are TH- (**Fig 6, 7**). The NTS/C2 is an example of robustly
 342 stained TH+ neurons that lack melanin, and ventrolaterally, there are two large melanin-
 343 pigmented neuron without TH staining (**Fig 4C**).

344 Robustly labeled ChAT+ cholinergic neurons are found in motor nuclei, preganglionic
 345 parasympathetic nuclei (preganglionic Edinger-Westphal nucleus, salivatory nuclei), the
 346 pedunculopontine tegmentum (PPT), the laterodorsal tegmentum (LDT), the lateral parabrachial
 347 nucleus, and the reticular formation. The largest and most numerous cholinergic cells were
 348 located within the motor nuclei of cranial nuclei, particularly those of cranial nuclei 3, 5, 6, 7, 10,

349 and 12 (**Fig. 3B, 3C, 4B**). Robustly labeled ChAT+ fibers were useful in identifying cranial
350 nerves, for example, the facial nerve wrapping around the abducens nucleus (**Fig. 3**).

351 The location of each axial brainstem level is mapped in **Figure 5**. The levels are approximately
352 4mm apart. We paired each histologic level with the corresponding level on MRI (**Fig. 6-25**).
353 These 7T MRI sequences have 200 μ m resolution. The MRI SWI images represent axonal white
354 matter as dark grey (example, superior cerebellar peduncle (scp) in Levels D-F or medullary
355 pyramids (py) on Level F) and grey matter/ nuclei as lighter grey (inferior olive, Levels G-H).

356 In terms of mapping the different cell types, we hesitate to draw circles around cell groups since
357 many do not have obvious, crisp borders such as the diffuse serotonergic neurons across the
358 lateral pons. Additionally, some cell groups overlap more than we expected, such as the
359 cholinergic neurons of the laterodorsal tegmentum (LDT) intermingling with the
360 catecholaminergic neurons of the locus coeruleus (LC) (**Fig 10, 11**). Therefore, we limited the
361 dashed line drawings to more obvious nuclei, and used dots representing neurons to demarcate
362 the less defined regions. Furthermore, the boundaries between some nuclei identified by
363 cytoarchitecture are less obvious given the lack of distinct chemogenic markers, retrograde
364 tracing, or functional data in post mortem human brains, such as between the superior and
365 inferior colliculi (**Fig 8, 9**). In this case we identified the superior colliculus (SCol) by the clear
366 laminations (as in other species), with smaller, densely packed cells in the outer layer, medium
367 sized cells in the middle layer, and larger cells in the deeper layer, while the inferior colliculus
368 (ICol) was identified by the central nucleus surrounded by a fibrous lamina. The exact boundary
369 between these nuclei is unclear with cytology alone.

370 We subdivided the cerebellar cortex into the cerebellar vermis (CbV) in the center, and
 371 cerebellar hemisphere (CbH) laterally using cytoarchitecture (**Fig. 10, 12, 14, 16, 18, 20, 22, 24**),
 372 and further identify the flocculus (CbF) on the ventral surface (**Fig. 18**). Within the vermis, we
 373 subdivided the parallel layers to show the outer, moderately stained molecular layer, the narrow
 374 middle layer containing Purkinje cells (CbVp), and the inner, densely packed and deeply stained
 375 granular cell layer (CbVg) (**Fig. 16**). We delineated the deep cerebellar nuclei based on
 376 cytoarchitecture. From medial to lateral, they include: fastigial (Fas), globose (Glo), emboliform
 377 (Emb), dentate (DT) nuclei (**Fig. 20, 22, 24**).

378 Here we will briefly review the main structures on each Level A-J (**Fig. 6-25**), but the Discussion
 379 Section provides details about how each delineation was determined and comparison to prior
 380 literature. The caudal midbrain is located in the dorsal half of Levels A and B, and the ventral
 381 half contains the rostral portion of the basis pontis and corticospinal tract. Levels C-G contain the
 382 bulk of the pons and the rostral cerebellum. Levels H-J contain the majority of the medulla and
 383 the widest portion of the cerebellum.

384
 385 Level A: The *upper midbrain* (**Fig. 6, 7**) contains the catecholaminergic (dopaminergic)
 386 substantia nigra laterally, and ventral tegmental area (VTA) medially. Dorsal to these structures
 387 are adrenergic A8 cells that surrounds the superior cerebellar peduncles (scp) more laterally. In
 388 the midline lies the rostral portion of the serotonergic dorsal raphe (DR) neurons. Dorsal to the
 389 DR is the third cranial nucleus (III). The oculomotor nucleus (III) is identified by large
 390 cholinergic somata in the midbrain with ChAT+ fibers exiting ventrally (III_{in}), as they eventually
 391 exit out of the base of the midbrain. The parasympathetic neurons of III, which form the

392 preganglionic Edinger-Westphal nucleus (EW), are identified by the smaller ChAT+ neurons

393 situated just dorsally to the more tightly clustered IIIB

394

395 Level B: The *caudal midbrain level* (**Fig. 8, 9**) contains the superior colliculus (SCol)

396 dorsomedially, and inferior colliculus (ICol) laterally. Ventral to the to the colliculi, the

397 periaqueductal grey surrounds the aqueduct. Along the midline is the largest portion of the

398 serotonergic dorsal raphe (DR) and the more ventral median raphe (MnR). Ventral to the DR are

399 the cholinergic fibers of cranial nerve 4, the trochlear nerve (IVn). Additionally, this level

400 contains the cholinergic pedunculopontine tegmentum (PPT), and the cholinergic parabigeminal

401 nucleus (PBG) laterally.

402

403 Level C: The *midbrain-pontine junction level* (**Fig. 10, 11**) contains the rostral portion of the

404 adrenergic locus coeruleus (LC), which intermingles with the cholinergic neurons of the

405 laterodorsal tegmental nucleus (LDT) ventrally. Lateral to the LDT is the caudal portion of the

406 cholinergic PPT. Further dorsally, the cholinergic fibers of IVn can be seen decussating over the

407 fourth ventricle (IVv), ventral to the inferior colliculus (IColl). In the midline are a narrower

408 portion of the serotonergic DR, and a larger portion of the MnR ventrally. Lateral to the MnR is

409 the more diffuse pontine reticular formation (PRF) and suprallemniscal/B9 nucleus (SuL).

410

411 Level D: The *rostral pontine level* (**Fig. 12, 13**) contains the middle of the adrenergic locus

412 coeruleus (LC) which contains tightly clustered and robustly stained TH+ neurons. Along the

413 midline is the caudal serotonergic DR and MnR. Lateral to the MnR is the more diffuse

414 serotonergic PRF and SuL which run along the medial lemniscus (ml). The rostral cerebellum is
 415 present at this level.

416

417 Level E: At the *mid- pontine level* (**Fig. 14, 15**) contains the densely clustered adrenergic
 418 neurons of the LC. The caudal portions of the serotonergic groups are present (DR and MnR
 419 midline, with the more diffuse PRF and SuL laterally). The medial parabrachial nucleus (PBm) is
 420 medial to the crescentic superior cerebellar peduncle (scp). Lateral to the scp is the external
 421 lateral parabrachial nucleus (PBel), which contains a few cholinergic neurons. Additionally, the
 422 rostral portion of the cholinergic trigeminal nerve/ cranial nerve 5 (Vn) can be seen traveling
 423 laterally through the middle cerebellar peduncle (mcp). There cerebellar vermis (CbV) and
 424 hemispheres (CbH) are present.

425

426 Level F: The *caudal pontine level* (**Fig. 16, 17**) is marked by the large cholinergic motor neurons
 427 of the trigeminal motor nucleus (V), and the cholinergic axons of cranial nerve 5 can be seen
 428 exiting the nucleus laterally (Vn). The principle sensory trigeminal nucleus (PSV) is seen lateral
 429 to V. Ventral to V are the cholinergic accessory trigeminal and accessory facial neurons (acc
 430 V/VII), and the cholinergic superior salivatory nucleus (SSN). Dorsal to V are the caudal
 431 portion of the adrenergic LC and the PBm, while immediately ventral to V are the adrenergic A5
 432 neurons. At this level, there are only a few serotonergic neurons of the DR, MnR, and PRF. At
 433 this level and one caudal, the cerebellum is attached to the brainstem via the middle cerebellar
 434 peduncle (mcp).

435

436 Level G: The *ponto-medullary junction level* (**Fig. 18, 19**) contains the large cholinergic motor
 437 neurons of cranial nuclei 6 and 7, the abducens nucleus (VI) and the facial nucleus (VII). The
 438 cholinergic fibers of the facial nerve (VIIIn) can be seen exiting the facial nucleus dorsally and
 439 wrapping around VI. This level also contains the parasympathetic cholinergic neurons of the
 440 superior salivatory nucleus (SSN), and dorsally, scattered cholinergic cells were found in the
 441 vestibular nuclei (VN) with the cholinergic fibers of the vestibulocochlear nerve (VIIIIn). Lateral
 442 and ventral to VIIIIn are the cholinergic neurons of the ventral cochlear nucleus (VCN) and
 443 pontobulbar nucleus (PNB), respectively. Serotonergic neurons are only located in the ventral
 444 portion of the brainstem at this level, in the raphe magnus (RMg) medially and ventrolateral
 445 medulla (VLM) laterally. This level also contains the largest portion of the inferior olive (IO),
 446 with the medial lemniscus (ml) and pyramidal tract(py) just medial. The cerebellar flocculus
 447 (CbF) is located on the ventral aspect of the cerebellum.

448

449 Level H: The *rostral medulla level* (**Fig. 20, 21**) contains the cholinergic cranial nerves 9 and 10
 450 (IXn, Xn) exiting the brain laterally, and cranial nerve 12 (XIIIn) exiting ventromedially.
 451 Additional cholinergic groups include the dorsal cochlear nucleus (DCN) and PnB dorsolaterally,
 452 the prepositus nucleus (Pr) dorsomedially, the nucleus ambiguus (AMB) located within the
 453 catecholaminergic intermediate reticular zone (IRt), and the lateral reticular neurons (LRT) just
 454 dorsolateral to the IRt. The rostral nucleus of the solitary tract (NTS) and solitary tract (sol) are
 455 at this level, with the cholinergic inferior salivatory nucleus (ISN) just medial to them.
 456 Additionally, the caudal midline serotonergic nuclei, including raphe magnus (RMg) and raphe
 457 obscurus (ROb) are present at this level, with the serotonergic neurons of the ventrolateral
 458 medulla (VLM) extending laterally. The ventral brainstem at this level contains the IO laterally,

459 and the ml and py medially. The cerebellar nuclei are prominent at this level (Fas, Glo, EMb, and
460 DT).

461

462 Level I: The *mid-medulla level* (**Fig. 22, 23**) contains the densely packed adrenergic neurons of
463 the NTS and C2, with the serotonergic (and to a lesser extent, adrenergic) area postrema (AP)
464 just medial. Along the midline of the brainstem are the serotonergic neurons of the ROb.
465 Extending diagonally is the IRt, and at this level it contains a mixture of serotonergic and
466 adrenergic neurons, with few cholinergic AMB neurons. The medial cholinergic neurons are the
467 motor neurons of the dorsal motor nucleus of vagus (DMX) and hypoglossal nucleus (XII), with
468 the vagus nerve (Xn) extending laterally and XIIIn exiting ventrally. The lateral cholinergic
469 neurons are the LRT. Dorsolaterally are the spinal trigeminal nucleus (SpV) and spinal
470 trigeminal tract (spV).

471

472 Level J: The *caudal medulla* (**Fig. 24, 25**) contains the decussation of the pyramids (pyx). The
473 caudal portion of the IRt and AMB are at this level, extending laterally to the adrenergic neurons
474 of A1. The cholinergic vagal motor neurons and possible spinal accessory neurons (X/XI) can be
475 seen just dorsal to the cholinergic XIIIn that exits the ventral surface of the brain. Laterally, this
476 level also has a more dense SpV and dorsal column nuclei (nucleus gracilis, Gr, and nucleus
477 cuneatus, Cu).

478

479 **Discussion**

480 Clinicians are familiar with cross-sectional anatomy of the human brainstem from MRI scanners,
 481 but generally have had to rely upon atlases which either a) lack a detailed view of brainstem cell
 482 groups as they appear in a Nissl stain, b) lack the cerebellum for spatial reference, or c) were not
 483 cut in the same plane (Buttner-Ennever & Horn, 2014; Paxinos, 1995; Sabin, 1901). We chose to
 484 cut the brainstem in the axial plane of section (and map these axial sections along the rostro-
 485 caudal extent of a sagittal view, **Figure 5**) because the axial plane is most frequently used to
 486 view clinical MRI scans of human brainstems. The goal of this current atlas is to map the
 487 distribution of key brainstem cell types (cholinergic, serotonergic, and catecholaminergic
 488 neurons) in relationship to each other and to the main cell groups within the human brainstem
 489 and cerebellum, and also to pair this histology with 7T MR images. This project allows
 490 correlation of the chemoarchitecture with corresponding MRI of the brainstem, and makes the
 491 identification of cell groups that are often discussed, but rarely identifiable on MRI scan,
 492 accessible to clinicians and clinical researchers.

493 **1. Cholinergic groups**

494 We identified robustly labeled ChAT+ cholinergic neurons in motor nuclei, preganglionic
 495 parasympathetic nuclei (preganglionic Edinger-Westphal nucleus, salivatory nuclei), PPT, LDT,
 496 lateral parabrachial nucleus, and reticular formation. We found these cholinergic neurons in a
 497 similar distribution to previous reports in rat (Armstrong, Saper, Levey, Wainer, & Terry, 1983),
 498 cat (Jones & Beaudet, 1987; Kimura, McGeer, Peng, & McGeer, 1981), and human brainstems

499 (Mesulam, Geula, Bothwell, & Hersh, 1989), but our staining appears more extensive and the
 500 differences are described below.

501 **1a. Cranial Nuclei and parasympathetics:**

502 The cranial nerve motor nuclei were identified by large, densely grouped ChAT+ motor neurons.
 503 There is not a clear separation between the cholinergic neurons of cranial nuclei III and IV, but
 504 the direction of the cholinergic fibers exiting from the cell bodies is informative. The oculomotor
 505 nucleus (III) was identified by large cholinergic somata in the midbrain periaqueductal region
 506 with ChAT+ fibers exiting ventrally (III_v), as they eventually exit out of the base of the midbrain
 507 (**Fig. 6, 7**). The parasympathetic neurons of III, the preganglionic Edinger-Westphal nucleus
 508 (EW), were identified as the smaller ChAT+ neurons situated just dorsal to the more tightly
 509 clustered III. The trochlear nerve (IV_n) was identified just caudally, as it coursed dorsally along
 510 the medial longitudinal fasciculus (mlf), and around the periaqueductal gray (PAG), and
 511 eventually above the fourth ventricle (IV_v) where it decussated caudal to the midbrain (**Fig. 8-**
 512 **11**).

513 The motor trigeminal nucleus (V) was identified by the large cluster of cholinergic somata in the
 514 lateral pons (**Fig. 16, 17**) that gave rise to dense ChAT+ fiber bundles exiting ventrolaterally
 515 through the middle cerebellar peduncle (mcp) (**Fig. 12-17**). The principle sensory trigeminal
 516 nucleus (PSV) was identified by the patchy ChAT+ neuropil just lateral to V and just rostral to
 517 the spinal trigeminal nucleus (SpV).

518 The abducens nucleus (VI) and the facial nucleus (VII) contain large, ChAT+ motor neurons,
 519 and can be seen on the same level, Level G (**Fig. 3, 18, 19**). The abducens nucleus is comprised

of both ChAT+ cholinergic neurons and neurons that are not ChAT+ (**Fig. 3**). The latter are presumably the abducens internuclear neurons that project contralaterally through the ChAT-medial longitudinal fasciculus (mlf) to the opposite III motor neurons and medial rectus to yoke the eyes in horizontal movement. The axons of the facial nerve are also robustly ChAT+ and can be seen exiting the facial nucleus dorsally, approaching the abducens nucleus ventromedially and wrapping over and around it, and traveling ventrolaterally before exiting the brain (**Fig. 3, 18, 19**).

Between the motor trigeminal nucleus (V) and facial nucleus (VII) is a continuum of neurons that comprise the accessory trigeminal and accessory facial neurons (acc V/VII, **Fig. 16, 17**). In rodents, the rostral part of this accessory continuum (acc V) innervates the tensor tympani, mylohyoid and anterior belly of the digastric, while the caudal portion (acc VII) innervates the stapedius, stylohyoid and posterior belly of the digastric (Shohara & Sakai, 1983; Szekely & Matesz, 1982). The neurons in the accessory nuclei are large like the primary trigeminal and facial motor neurons, and likely extend caudally as the unlabeled ChAT+ neurons within the facial nerve between the facial and abducens nuclei (**Fig. 18, 19**).

We delineated the salivatory nuclei based on ChAT+ staining and the spatial relationship to other known nuclei based on previous tract tracing studies (Brown & Howlett, 1968; Contreras, Gomez, & Norgren, 1980; Hiura, 1977; Satomi, Takahashi, Ise, & Yamamoto, 1979; Spencer, Sawyer, Wada, Platt, & Loewy, 1990). The superior salivatory nucleus (SSN) is comprised of preganglionic cholinergic neurons that project via the facial nerve (some via the greater petrosal nerve) to the pterygopalatine ganglion, which innervates submandibular, sublingual, and lacrimal glands, and cerebral blood vessels (Contreras et al., 1980; Nakai, Tamaki, Ogata, Matsui, &

542 Maeda, 1993; Spencer et al., 1990). The SSN has been located in rodents and cats by
 543 retrogradely labeling neurons from the pterygopalatine ganglion and the facial nerve (Brown &
 544 Howlett, 1968; Contreras et al., 1980; Hiura, 1977; Satomi, Takahashi, et al., 1979; Spencer et
 545 al., 1990). They consist of cholinergic neurons dorsolateral to the facial nucleus along the
 546 ascending facial nerve (**Fig. 18, 19**). These parasympathetic SSN cells have smaller somata than
 547 the motor neurons of the facial nucleus proper.

548 The inferior salivatory nucleus (ISN, **Fig. 20-21**) contains cholinergic preganglionic
 549 neurons that project via the glossopharyngeal nerve (IXn) (lesser petrosal nerve) to the otic
 550 ganglion, which in turn innervates the parotid gland. The location of the inferior salivatory
 551 neurons have been worked out in rat and cat via retrograde tracing from the
 552 glossopharyngeal and vagus nerves, and appear to intermingle with the retrogradely
 553 labeled neurons from the facial nerve branches that define the SSN (Contreras et al., 1980;
 554 Satomi, Takahashi, et al., 1979; Satomi, Yamamoto, Ise, & Takahashi, 1979). At least in
 555 the rat, the inferior salivatory nucleus is adjacent to the lingual-tonsillar branch of IXn
 556 caudally at the level of the rostral NTS (Contreras et al., 1980); we labeled the cholinergic
 557 neurons in this area as ISN in the human brainstem section at this level (**Fig. 20-21**).
 558 Furthermore, in human studies, the ISN was labeled as AChE+ cells at the rostral tip of the
 559 dorsal motor nucleus of vagus, and dorsomedial to the NST (Braak, 1972; McRitchie &
 560 Tork, 1993); we found ChAT+ neurons in a similar location just medial to the rostral NTS.
 561 These robustly stained ChAT+ neurons are tightly clustered with a dense ChAT+ neuropil,
 562 and have very prominent TpOH fiber staining specifically limited to the region of
 563 cholinergic neurons. By contrast, TpOH is not present over the cholinergic neurons
 564 rostrally in the SSN (**Fig. 18, 19**).

565 Lateral to the ISN is the nucleus of the solitary tract (NTS) and the solitary tract (sol) (Fig.
 566 **20, 21**). At this level, the NTS is marked by a ChAT+ neuropil indicating the medial
 567 subdivision of the NTS. For more details about the NTS, see the catecholaminergic/
 568 adrenergic section.

569 The nucleus ambiguus (AMB) contains cholinergic motor neurons that project to the larynx and
 570 pharynx, and also contains smaller preganglionic parasympathetic neurons which decrease heart
 571 rate via cranial nerves 9 and 10. We identified AMB neurons within the medullary reticular
 572 formation as the more compact ChAT+ neurons within a ChAT+ neuropil (**Fig. 20, 21**). These
 573 neurons are larger, more robustly labeled, and more tightly clustered than the other surrounding
 574 cholinergic neurons. We hypothesize that these AMB neurons likely represent the compact part
 575 of the AMB, which according to a detailed study in rat, mainly contains cells innervating the
 576 esophagus (Bieger & Hopkins, 1987). AMB extends caudally, though it is less densely clustered
 577 at these levels (**Fig. 22-25**).

578 In the medial medulla, the dorsal motor nucleus of the vagus nerve (DMX) and the hypoglossal
 579 nucleus (XII) can be seen at the same level (**Fig. 4, 22, 23**). The robustly ChAT+ fibers of cranial
 580 nerve 12 (XIIIn) can be seen traveling ventrally, just lateral to the midline. The large, ChAT+
 581 somata of DMX can be seen in **Figure 4**. Additionally, some of the unlabeled ChAT+ neurons
 582 marked at the ventral aspect of the prepositus nucleus (PR, **Fig. 20, 21**) are likely neurons of the
 583 rostral hypoglossal nucleus.

584 We marked the spinal accessory nucleus (XI), the nucleus of cranial nerve 11, as the cholinergic
 585 neurons located lateral to the pyramids, and dorsal to the cholinergic fibers of XIIIn exiting the
 586 brain (**Fig. 24-25**). However, a previous study in monkeys found that the vagal motor neurons

587 innervating the infrahyoid muscles (the sternohyoid and sternothyroid muscles, rostrally, and the
 588 omohyoid, caudally) form the rostral end of a continuum with the neurons that innervate the
 589 spinal accessory muscles (sternocleidomastoid and trapezius muscles via CN 11) (Ueyama et al.,
 590 1990). This continuum of cells begins in the medulla rostral to the decussation of the pyramids,
 591 and continues caudally into the cervical spinal cord. The medullary section at Level J (**Fig 24,**
 592 **25**) appears relatively rostral in the continuum of cells given that the pyramidal decussation and
 593 cholinergic neurons are adjacent to the hypoglossal nerve, suggesting these rostral cholinergic
 594 neurons may be mostly vagal motor neurons. Without retrograde tracing studies we cannot be
 595 sure which of these cholinergic neurons innervate infrahyoid muscles or spinal accessory
 596 muscles, so we labeled them all as X/XI to indicate that they may include both vagal motor
 597 neurons (X) and spinal accessory neurons (XI).

598 **1b. PPT and LDT:**

599 We found a distribution of cholinergic neurons in the pedunculopontine tegmentum (PPT) and
 600 laterodorsal tegmentum (LDT), similar to that previously reported in human, cat, and rat
 601 brainstems (Armstrong et al., 1983; Jones & Beaudet, 1987; Manaye et al., 1999; Mesulam et al.,
 602 1989). The majority of cholinergic neurons in this region were localized to the more lateral PPT
 603 (**Fig. 8, 9**, with the sparse yet caudalmost portion of the PPT on **Fig. 10, 11**), and fewer
 604 comprised the LDT in the central grey matter (**Fig. 10, 11**). Dorsal LDT neurons intermingle
 605 with adrenergic locus coeruleus (LC) neurons (**Fig.10, 11**).

606 **1c. Parabrachial nucleus:**

607 The parabrachial nuclei are caudal to the decussation of the scp, which appears as a crescent in
 608 the dorsolateral pons, lateral to the fourth ventricle (IVv). At this level of the caudal scp, we
 609 labeled the lateral (PBl) and medial parabrachial nuclei (PBm) as the neurons that are lateral and
 610 medial to the caudal scp, respectively (**Fig. 10-17**). The ventrolateral portions of the PBl
 611 contained cholinergic neurons caudally (**Fig. 14, 15**), and we labeled this the external lateral
 612 parabrachial nucleus (PBel). This region also contains calcitonin gene-related peptide (CGRP+)
 613 neurons (de Lacalle & Saper, 2000), and we know from rat studies that the CGRP+ neurons are
 614 adjacent to (but separate from) the ChAT+ neurons in the PBel.

615 **1d. Reticular formation:**

616 Within the reticular formation, we labeled the swath of intermingled cholinergic, serotonergic,
 617 and catecholaminergic neurons as the intermediate reticular zone (IRt) (**Fig. 20-25**). We found a
 618 similar distribution of TH+ labeling to what had previously been described in this region
 619 (Arango et al., 1988; Halliday et al., 1988; Pearson, Goldstein, Markey, & Brandeis, 1983).
 620 Along the IRt, we found darkly labeled TH+ neurons intermixed with lightly labeled ChAT+
 621 neurons, and their relationship to serotonergic neurons varies along the rostro-caudal extent.
 622 Rostrally in the IRt, we found that cholinergic and TH+ neurons were just dorsal and adjacent to
 623 the separate and robustly labeled serotonergic neurons of the ventrolateral medulla (VLM, **Fig.**
 624 **20, 21**). This distinct distribution is different than the arrangement of overlapping adrenergic and
 625 serotonergic neurons presented by Halliday who used phenylalanine hydroxylase+ (PH8) to label
 626 serotonergic neurons (Halliday et al., 1988). However, we found that these separate populations
 627 merged more caudally in the IRt/VLM where serotonergic neurons intermixed with TH+ and
 628 ChAT+ neurons (**Fig. 22, 23**).

629 Within the IRt, we designated the more compact distribution of ChAT+ neurons within a ChAT+
 630 neuropil as nucleus ambiguus (AMB) (**Fig 20, 21**). These neurons are larger, more robustly
 631 labeled, and more tightly clustered than the other surrounding cholinergic neurons. We
 632 hypothesize that these AMB neurons likely represent the compact part of the AMB, which
 633 according to a detailed study in rat, may contain cells innervating the esophagus (Bieger &
 634 Hopkins, 1987).

635 **1e: Precerebellar nuclei**

636 The lateral reticular neurons (LRT) are cholinergic precerebellar neurons, and are difficult to
 637 pinpoint in a human brain because they are defined by their efferent projections to the
 638 cerebellum. Since we do not have tracing data in our human brains, we estimated the location of
 639 these neurons using cholinergic staining, cytology, and landmarks. We marked the LRT neurons
 640 as the small, tight clusters of generally larger, robustly ChAT+ neurons that avoided C1, and are
 641 above and below the IRt and VLM (**Fig. 17 -22**). Previously, Walberg detailed the
 642 cytoarchitecture of the LRT across numerous mammals, and he generally used the nomenclature
 643 “main portion” (pars principalis) and “subtrigeminal portion” (Walberg, 1952). Within the main
 644 portion, he noted that many species have a magnocellular (LRTm) and parvicellular (LRTpc)
 645 part where the cells are predominantly larger or smaller, respectively. Adapting Walberg’s
 646 nomenclature, we labeled the oval/elliptical neuropil with occasional small ChAT+ somata as
 647 “LRTpc” in the lateral pons (**Fig. 20, 21**). Ventral to the LRTpc, there are much larger and more
 648 darkly stained ChAT+ neurons that we have labeled as magnocellular, LRTm.

649 The pontobulbar nucleus (PnB) was identified on the lateral edge of the medulla by its dense
 650 ChAT+ neuropil that obscures the view of the small ChAT+ somata (**Fig. 18-21**). The PnB was

distinguished from the ventral cochlear nucleus (VCN) which had weaker ChAT+ somata and faint ChAT+ neuropil (**Fig. 18, 19**). In contrast to the VCN, the dorsal cochlear nucleus (DCN) superiorly had more robust ChAT+ somata and neuropil (**Fig. 20, 21**). A similar pattern has been described in cats (Godfrey, Williams, & Matschinsky, 1977), but the VCN of rat might have more robust cholinergic staining than its human or cat counterparts (Godfrey & Matschinsky, 1981).

2. Catecholaminergic (dopaminergic and adrenergic) Groups

Catecholaminergic neurons (dopaminergic and adrenergic) have been described in the brains of many species. We used the A1-A14 nomenclature from Dahlstrom and Fuxe especially in cases where there is not a more widely accepted name, for example the large A4 neurons found in the lateral wall and roof of the fourth ventricle (**Fig. 16-18**) (Dahlstrom & Fuxe, 1964).

Catecholaminergic neurons can be identified by both the presence of the endogenous pigmented melanin and immunohistochemical staining for the enzyme tyrosine hydroxylase (TH). Prior reports have estimated that only 50-65% of medullary catecholaminergic neurons produce pigment, implying that quantifying only pigmented neurons would underestimate the number of catecholaminergic cells (Halliday et al., 1988; Huang, Paxinos, Halasz, McRitchie, & Tork, 1993). Additionally, we found many melanin-pigmented neurons that are TH-, suggesting that analyzing TH+ neurons alone would also underestimate the number of catecholaminergic neurons. Therefore, we decided to utilize both TH staining and endogenous pigmented melanin to detect the maximal number of catecholaminergic neurons in our study.

672 We are currently unaware of functional differences between neurons that are marked by either
 673 TH or melanin alone, or those that contained both. Given that melanin is a byproduct of
 674 catecholamine synthesis (Graham, 1979), one would expect to see melanin in TH+ neurons. We
 675 are currently unsure why some neurons are only either TH+ or melanin-pigmented (but TH-);
 676 perhaps they no longer produce high enough levels of the TH enzyme for IHC detection (as a
 677 neuron fills with melanin, its synthetic ability for making more catecholamines might become
 678 impaired), or they do not yet contain a visibly detectable amount of melanin since adult brains
 679 seem to develop melanin in these regions over a lifetime. More research is required in this area.

680 However, we did notice patterns between TH and melanin pigmentation in both staining quality
 681 and spatial distribution. In general, we found that the TH+ neurons that are nonpigmented stain
 682 darker for TH than the TH+ cells that contain pigmented melanin, and the darkest pigmented
 683 melanin neurons appear to be TH- (or their TH levels are too low to detect).

684 Overall, some brainstem catecholaminergic neurons contained both pigment and TH, such as in
 685 the adrenergic locus coeruleus (LC), as only a few pigmented cells in the LC seemed to be TH-.
 686 This is demonstrated in **Fig. 2D**, where the brownish melanin is surrounded by blackish grey
 687 TH+ stain, and in **Figures 10-17** where there are mostly lime green dots representing TH+ stain,
 688 rather than dark green representing melanin pigment alone. In the A8 group (**Fig. 6, 7**), most of
 689 the cells were both pigmented and TH+, but especially dorsally, there were some neurons that
 690 stained darker with TH than were nonpigmented.

691 Additionally, there are a few nuclei with cells that are either predominately TH+ or pigmented,
 692 but the two labels are not colocalized. For example, the lateral and ventral parts of the substantia
 693 nigra (SN) contains mostly robustly pigmented neurons that are not labeled by TH (**Fig. 6-9**),

694 which has also been reported by an earlier study (Pearson et al., 1983). Additionally, all of the
 695 TH+ cells superior to A8 are robustly labeled with TH (**Fig. 6, 7**), but are without pigment.
 696 Perhaps this pattern is related to the different catecholaminergic cell types, given the SN is
 697 dopaminergic, while neurons dorsal to A8 are likely adrenergic.

698 We found clusters of TH+ neurons in the ventrolateral portion of the IRt, which we have labeled
 699 C1 rostrally (Fig. 17, 18) and A1 caudally (Fig. 19-22), as previously described (Arango et al.,
 700 1988; Saper & Petito, 1982). In the IRt and C1 of the rostral medulla, the TH+ neurons do not
 701 appear to be pigmented (**Fig. 20, 21**). However, more caudally where we labeled A1, the TH+
 702 neurons of the IRt are pigmented, although there also are pigmented neurons that are TH- (**Fig.**
 703 **22-25**). See the “Cholinergic Groups” section for further discussion of TH+ neurons in the IRt.

704 In the medial medulla around the dorsal motor nucleus of the vagus nerve (DMX), there is a
 705 mixed population of catecholaminergic neurons (**Fig. 4, 22, 23**). Dorsally, in the NTS/C2, the
 706 neurons are medium-sized, robustly TH+, and either weakly pigmented or nonpigmented, with a
 707 few robustly pigmented neurons on the lateral border (**Fig. 4C**). More ventrally, in the dorsal and
 708 medial aspects of the DMX, there are smaller, more weakly TH+ neurons that are also either
 709 weakly pigmented or nonpigmented (**Fig. 23**). Lateral to the DMX and NST, there are robustly
 710 pigmented TH- neurons that we labeled as A2. This finding of TH+ neurons (A2) dorsal and
 711 medial to DMX and smaller, robustly melanin-pigmented neurons lateral to DMX is consistent
 712 with prior work (Huang et al., 1993; Saper & Petito, 1982).

713

714 3. Serotonergic Groups

715 An earlier study using Nissl stain on human brainstem parsed out subdivisions of the dorsal
 716 raphe nucleus (DR) (Baker et al., 1991). Based on cell morphology, this study identified four DR
 717 subgroups. While we also observed some of their findings (such as that fusiform-shaped cells are
 718 more often found in the midline of the DR), we found that after staining for tryptophan
 719 hydroxylase (TpOH), these four subgroups were less obviously delineated. We also did not
 720 observe their finding that lipofuscin pigmented cells were limited to the dorsal DR. In fact, we
 721 found the ventral portion of the DR to be distinguished by slightly smaller cells with weaker
 722 TpOH staining, but darker lipofuscin pigment and a slightly denser neuropil. Thus, for
 723 simplicity, we subdivided the large middle level into DRd and DRv where we saw this
 724 distinction (**Fig. 8, 9**), but we labeled the rostral and caudal most portions of the DR with just
 725 “DR,” given less obvious differences at these levels (**Fig. 6, 7, 10, 11**). Additionally, we found
 726 the lipofuscin pigment to be almost exclusively in the serotonergic DR cells as opposed to the
 727 non-serotonergic raphe cells.

728 Outside of the DR, we tried to keep the nomenclature of pontine serotonergic groups similar to
 729 previous work. Consistent with prior reports, we labeled the smaller midline neurons as median
 730 raphe (MnR) (**Fig. 6-17**), and laterally, we labeled the larger, ventral neurons along the lateral
 731 lemniscus as suprallemniscal/B9 (SuL) (**Fig. 10-15**) (Baker et al., 1991; Dahlstrom & Fuxe, 1964;
 732 Hornung, 2003). Lateral to the MnR there are more diffusely scattered serotonergic neurons that
 733 have been called nucleus pontis oralis or nucleus reticularis pontis oralis, but due to the
 734 heterogenous and diffuse nature of this region, we referred to them as the pontine reticular
 735 formation (PRF) (**Fig. 10-17**).

736 We found the caudal midline serotonergic nuclei, including raphe magnus (RMg), raphe pallidus
737 (RP), and raphe obscurus (ROb), to be a continuum of neurons with indistinct borders, rather
738 than discrete nuclei (**Fig. 8-23**). However, to preserve prior nomenclature, we labeled the large
739 portion of midline neurons at the level of the rostral and middle inferior olive as RMg, and the
740 more caudal midline groups dorsal to the medial lemniscus as ROb (dorsally) and RMg
741 (ventrally), and RPa ventral to the lemniscus (Halliday et al., 1988; Hornung, 2003).

742 The ventrolateral medulla (VLM) contains a robustly stained group of large serotonergic neurons
743 mixed with a few TH+ neurons and sparse cholinergic neurons (**Fig. 18-21**). These serotonergic
744 VLM neurons are also referred to as the lateral paragigantocellular reticular nucleus (LPGi) in
745 some texts (Buttner-Ennever & Horn, 2014; Hornung, 2003).

746

747 **Conclusion**

748 Our atlas provides a unique resource in co-registering a high resolution MRI scan of the human
749 brainstem with immunohistochemistry for three key neurotransmitter systems (acetylcholine,
750 serotonin, and catecholamines including dopamine and norepinephrine), with an atlas of the cell
751 groups of the brainstem. This set of data will allow future investigators and clinicians to register
752 their MRI scans to this atlas, and improve segmentation of specific cell groups in MR studies.

753

754

755 **Limitations**

756 Although this study has novel features, there are also limitations worth discussing. As the work
757 was completed in cadaveric tissue, air artifact became a variable to address with MR imaging,
758 particularly with the use of SWI sequences. Although SWI provides some of the highest
759 resolution in distinguishing gray-white matter differentiation at high resolution, it is particularly
760 affected by air artifact. As such, we utilized agar as a medium to submerge the tissue into and
761 occupy the 4th ventricle and cerebral aqueduct to displace air inside and around the tissue. While
762 the agar largely minimized air artifact, it did introduce some distortion of tissue, particularly of
763 the obex and central canal more caudally. This presented some inaccuracy in the coregistration
764 between the histology and MRI. Future work to adapt non-linear registration techniques to
765 population-based neuroanatomy may provide means to correct these distortions.

766 Next, this brainstem and cerebellum atlas was derived from a single specimen, which provides
767 both benefits and limitations. We find our single subject to be a relative strength because all the
768 sections are in register, the cells marked are of exact neurons and not an estimation, and
769 consistent with the practice of most classic atlases, the data come from a single case of
770 continuous, serial sections. However, given that the brainstem is one specimen, the histology
771 could only be cut in one plane of section (while the MRI can be virtually cut in any and multiple
772 planes). We chose the axial plane of section for histology given this is the most frequently
773 viewed plane clinically. Additionally, new MRI studies are using large data sets, which was out
774 of the scope of our study given we did not want the MRI to be an estimation but rather a
775 representation of our exact histology. We also picked a case without known brainstem pathology,
776 and compared the brainstem to others in our lab to pick a representative case.

777 Additionally, in some cases the brainstem was satisfactorily connected to the cerebellum via the
 778 peduncles on the 40 μ m section; in others (particularly rostral pons and caudal medulla), the
 779 brainstem is not structurally connected to the cerebellum. When mounting the tissue on the slide,
 780 every effort was used to maintain the anatomic spatial relationship between the brainstem and
 781 cerebellum that was not structurally connected; however, in some cases this was not identically
 782 executed. This therefore introduced some error in the MRI image exactly aligning with the
 783 histological counterpart.

784 Lastly, this atlas is unfortunately missing the anterior portion of the midbrain as a consequence
 785 of the autopsy protocol, which severed the fresh brainstem from the forebrain by a knife cut
 786 caudal to the mammillary bodies. We included the most rostral portion of the midbrain that we
 787 had as Level A. However, the dorsal part of the brainstem was not available at that level.

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915 **Figure legends**

916 **Figure 1: Schematic of the voxel-shift scanning procedure.** Colored checkerboard blocks
 917 represent slices during separate image acquisitions, acquisition 1 in red, 2 in blue, and 3 in green.
 918 Slices are thicker in the slicing plane (0.4mm) than the acquisition plane (0.2mm). Each
 919 acquisition is offset in the slice plane such that the slice planes differ by an amount similar to the
 920 acquisition plane resolution (0.2mm). In this example, an offset of 1mm between acquisitions
 921 results in a 0.2mm slice offset between acquisitions given the 0.4mm slice thickness and 0.4mm
 922 slice spacing. Across the full set of acquisitions the volume is measured with high in-plane
 923 resolution (gray checkerboard cube, displays the effective reconstructed voxel spacing).

924 **Figure 2. Example of staining serotonergic and catecholaminergic neurons. (A)** Level C
 925 Nissl stain with dots representing each cell type (further shown in **Figures 7, 8**). Black boxes
 926 represents panels B-C. **(B)** Serotonergic neurons (TpOH) in the dorsal raphe (DR) are robustly
 927 labeled. Scalebar = 100 um. **(C)** Serotonergic neurons are smaller in the median raphe (MnR) are
 928 and larger laterally in the pontine reticular formation (PRF). **(D)** Most of the brown pigmented
 929 adrenergic neurons of the locus coeruleus (LC) also stain black with tyrosine hydroxylase (TH)
 930 (solid black arrows), but some melanin pigmented neurons do not stain with TH (hollow arrows).
 931 Scalebars are 50 um.

932 **Figure 3. Example of ChAT stain used to identify cranial nerves and nuclei. (A)** Level G
 933 Nissl stain with dots representing each cell type (further shown in **Figures 18, 19**). Red box
 934 represents panel B. **(B-C)** ChAT stain revealing cholinergic fibers of cranial nerve 7, facial
 935 nerve, wrapping around motor neurons and axons of cranial nerve 6, abducens nerve. Scalebar is
 936 200um and 100mm in B and C, respectively.

937 **Figure 4: Individual ICH stains were used to map different cell types. (A)** Example section
 938 (Level I, further shown in **Figures 22, 23**) Nissl section with dots used to represent each cell type
 939 (orang= ChAT, lime green = TH, dark green= melanin, pink= TpOH). Boxes show location of
 940 panels B-D. **(B-D)** Adjacent section stained blackish purple with DAB for ChAT, TH, or TpOH,
 941 respectively. Brown cells are endogenous pigmented melanin. **(D)** Most of the TH+ NTS
 942 neurons do not contain melanin (examples marked with green arrows), but one cell contains both
 943 melanin and TH (solid black arrow), and some cells with melanin are TH- (hollow arrows).
 944 Scalebars are 100um.

945

946 **Figure 5. Sagittal MRI image demonstrating the location of each histological Level, and**
 947 **their distances from the anterior inferior olive (IO) (blue line).** Level A is referenced in
 948 Figures 6-7, Level B is referenced in Figures 8-9, Level C is referenced in Figures 10-11 and
 949 Figure 2, Level D is referenced in Figures 12-13, Level E is referenced in Figures 14-15, Level F
 950 is referenced in Figures 16-17, Level G is referenced in Figures 18-19 and Figure 3, Level H is

951 referenced in Figures 20-21, Level I is referenced in Figures 22-23 and Figure 4, Level J is
 952 referenced in Figures 24-25.

953 **Figure 6. The upper midbrain.** Level A is comprised of MRI image on left and Nissl stain on
 954 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark
 955 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
 956 level.

957 **Figure 7. The upper midbrain, a zoomed in view.** This is a zoomed in view of the brainstem
 958 of Level A from **Figure 6**.

959

960 **Figure 8. The caudal midbrain.** Level B is comprised of MRI image on left and Nissl stain on
 961 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark
 962 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
 963 level.

964

965 **Figure 9. The caudal midbrain, a zoomed in view.** This is a zoomed in view of the brainstem
 966 of Level B from Figure 8.

967

968 **Figure 10. The midbrain-pontine junction.** Level C is comprised of MRI image on left and
 969 Nissl stain on right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright
 970 green= TH, dark green= melanin-pigmented). Dashed line through sagittal section represents the
 971 rostro-caudal level.

972

973 **Figure 11. The midbrain-pontine junction, a zoomed in view.** This is a zoomed in view of the
 974 brainstem of Level C from Figure 10.

975

976 **Figure 12: The rostral pons.** Level D is comprised of MRI image on left and Nissl stain on
 977 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark

978 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
979 level.

980

981 **Figure 13. The rostral pons, a zoomed in view.** This is a zoomed in view of the brainstem of
 982 Level D from Figure 12.

983

984 **Figure 14: The mid- pons.** Level E is comprised of MRI image on left and Nissl stain on right.
 985 Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark green=
 986 melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal level.

987

988 **Figure 15. The mid- pons, a zoomed in view.** This is a zoomed in view of the brainstem of
 989 Level E from Figure 14.

990

991 **Figure 16. The caudal pons.** Level F is comprised of MRI image on left and Nissl stain on
 992 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark
 993 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
 994 level.

995

996 **Figure 17. The caudal pons, a zoomed in view.** This is a zoomed in view of the brainstem of
 997 Level F from Figure 16.

998

999 **Figure 18. The ponto-medullary junction.** Level G is comprised of MRI image on left and
 1000 Nissl stain on right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright
 1001 green= TH, dark green= melanin-pigmented). Dashed line through sagittal section represents the
 1002 rostro-caudal level.

1003

1004 **Figure 19. The ponto-medullary junction, a zoomed in view.** This is a zoomed in view of the
 1005 brainstem of Level G from Figure 18.

1006

1007 **Figure 20. The rostral medulla.** Level H is comprised of MRI image on left and Nissl stain on
 1008 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark

1009 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
1010 level.

1011

1012 **Figure 21. The rostral medulla, a zoomed in view.** This is a zoomed in view of the brainstem
1013 of Level H from Figure 20.

1014

1015 **Figure 22: The mid-medulla.** Level I is comprised of MRI image on left and Nissl stain on
1016 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark
1017 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
1018 level.

1019

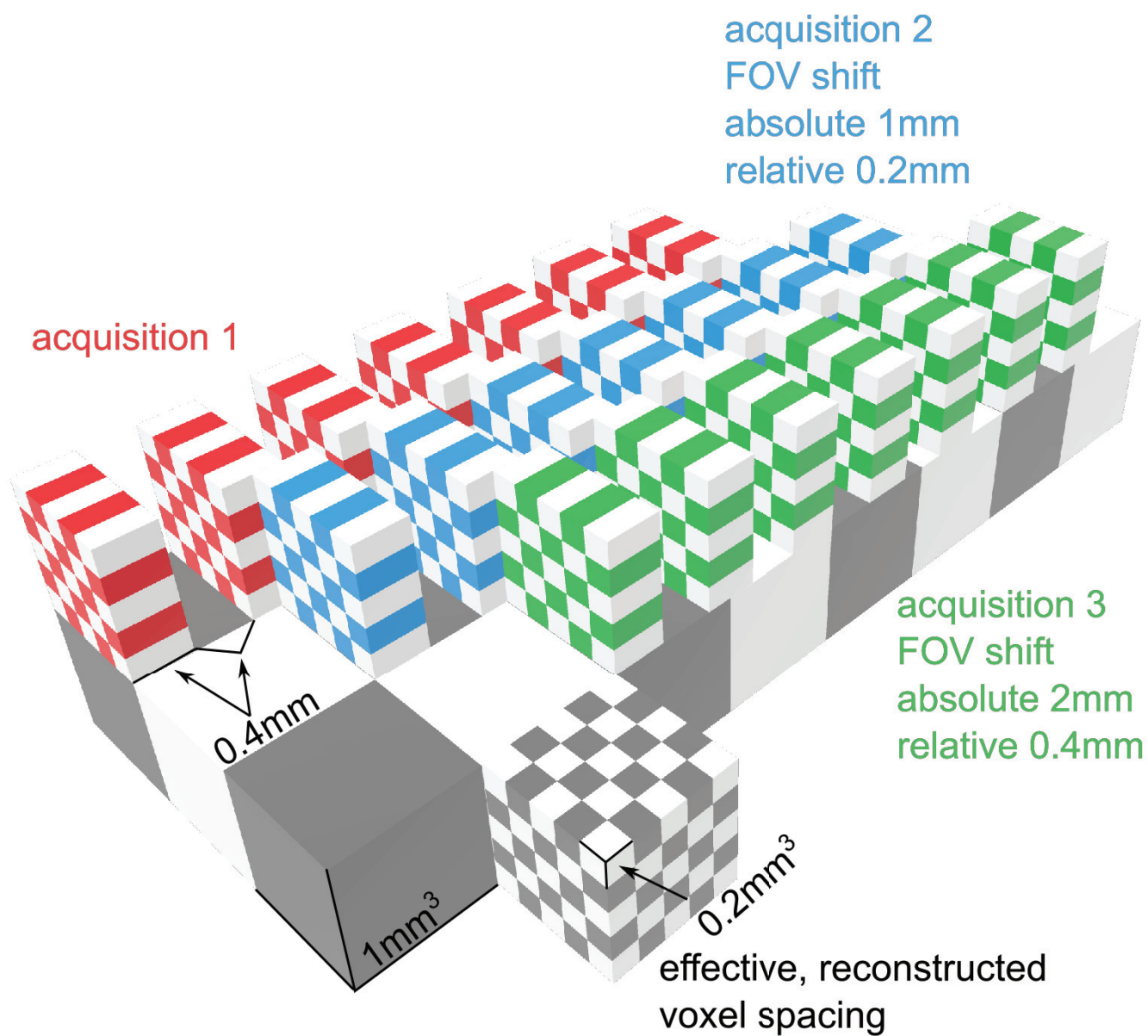
1020 **Figure 23. The mid-medulla, a zoomed in view.** This is a zoomed in view of the brainstem of
1021 Level I from Figure 22.

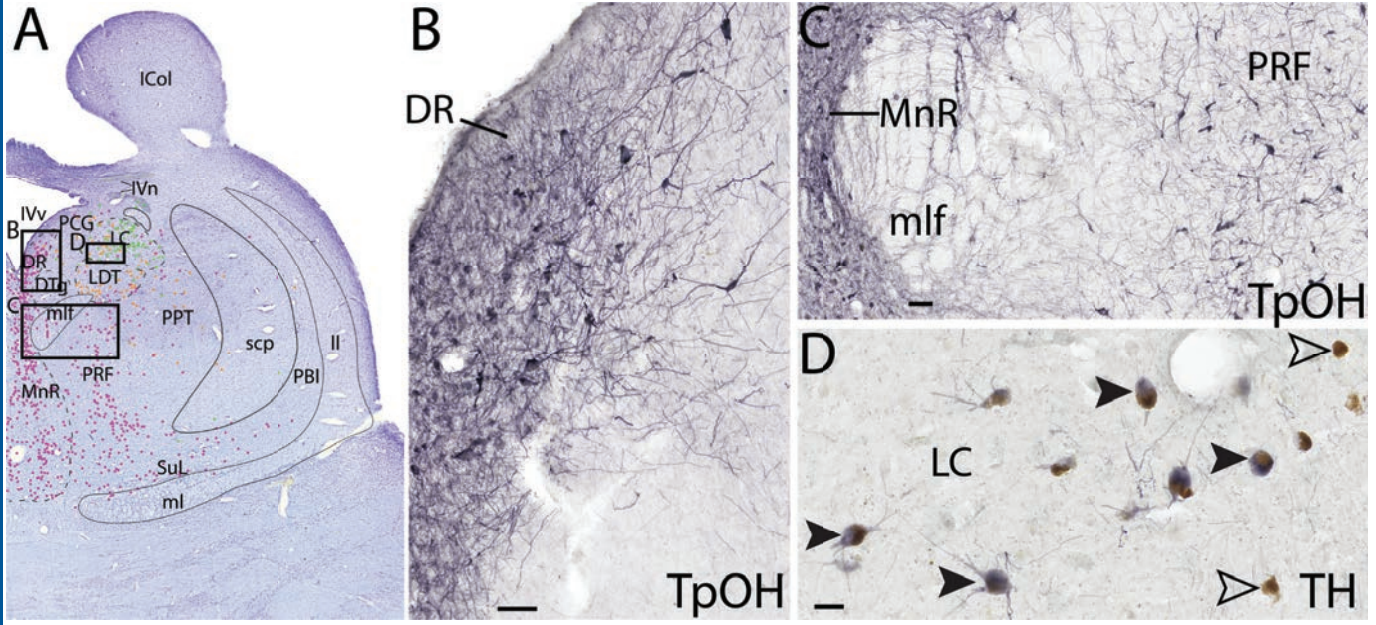
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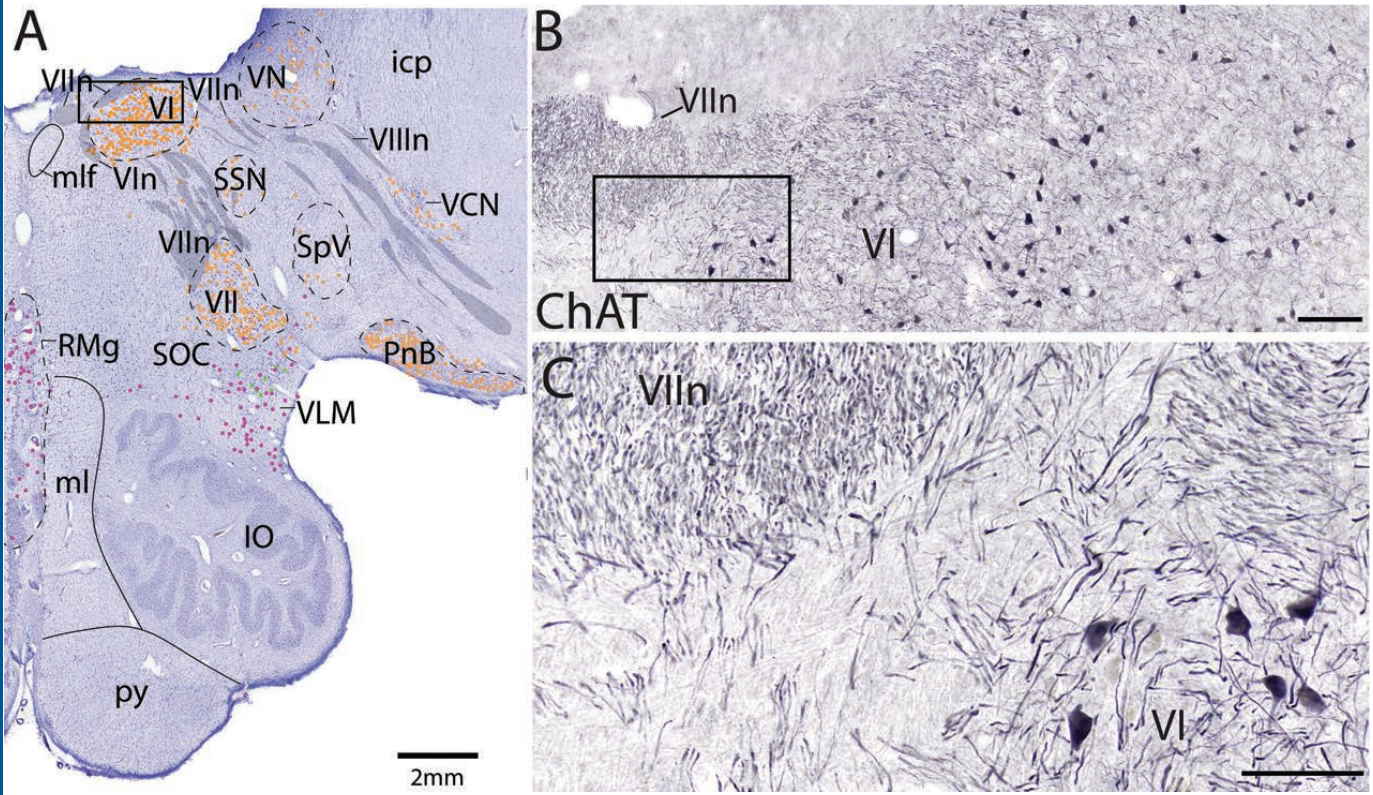
1023 **Figure 24: The caudal medulla.** Level J is comprised of MRI image on left and Nissl stain on
1024 right. Dots represent different cell types (orange= ChAT, pink= TpOH, bright green= TH, dark
1025 green= melanin-pigmented). Dashed line through sagittal section represents the rostro-caudal
1026 level.

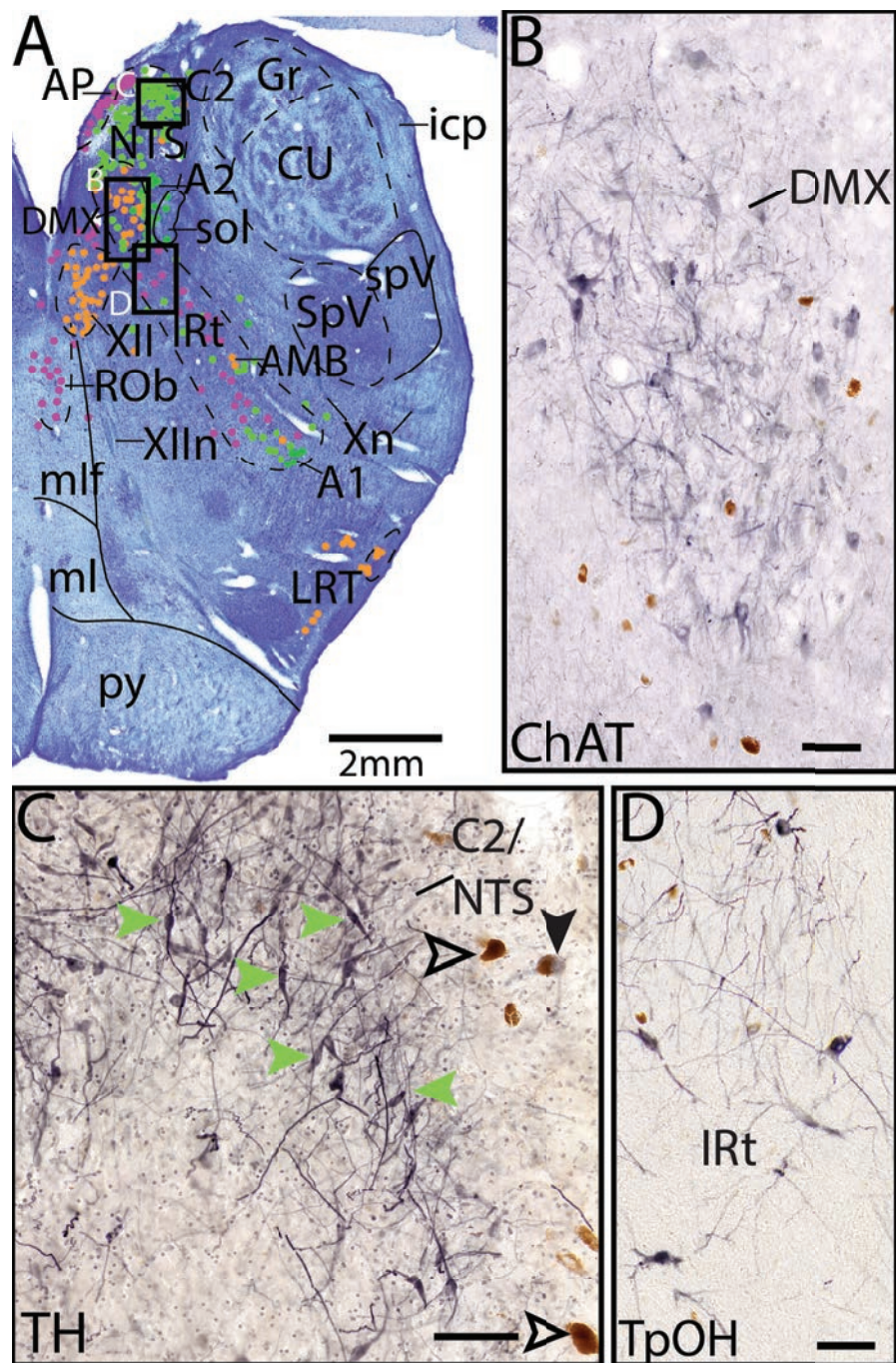
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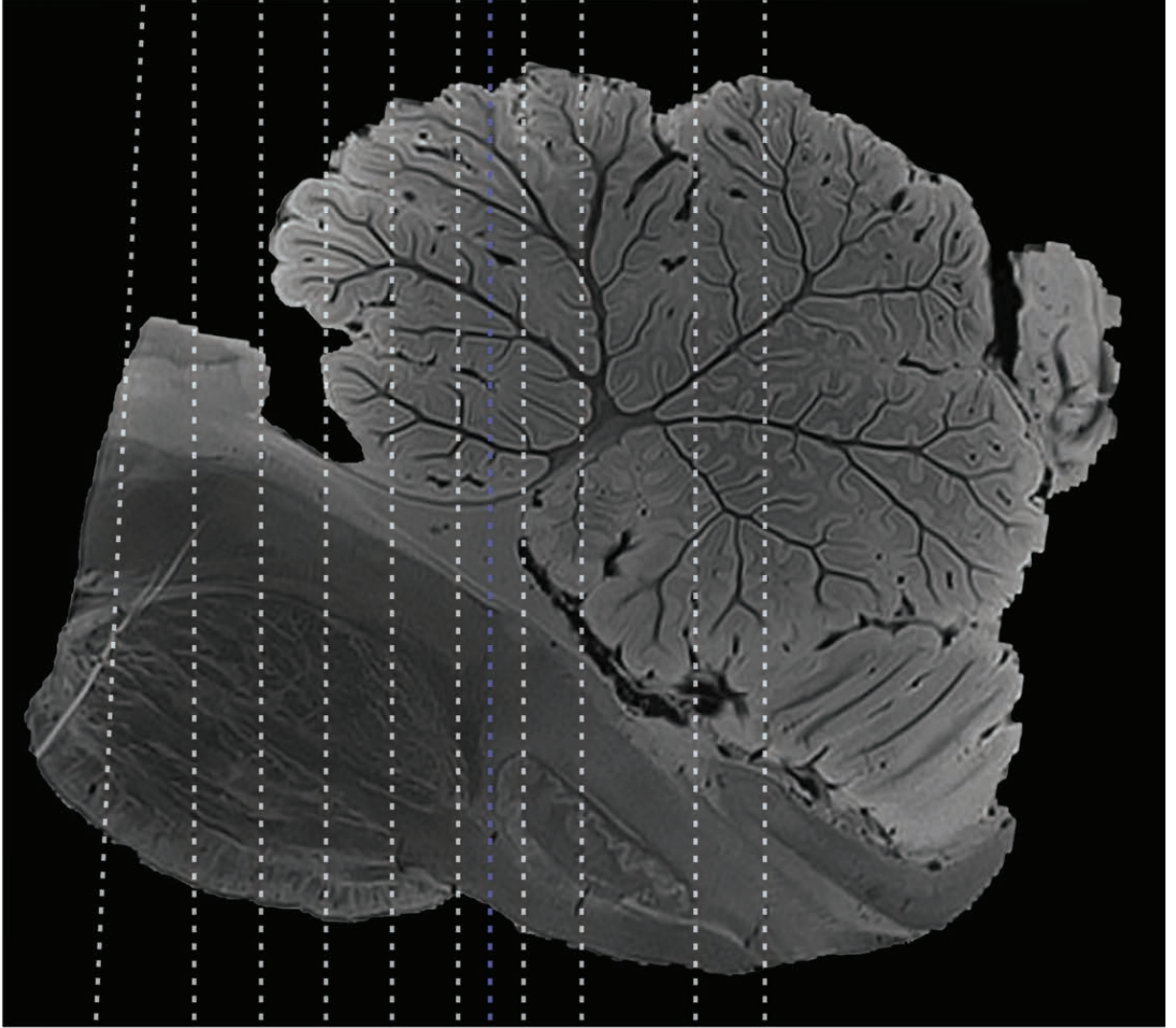
1028 **Figure 25. The caudal medulla, a zoomed in view.** This is a zoomed in view of the brainstem
1029 of Level J from Figure 24.





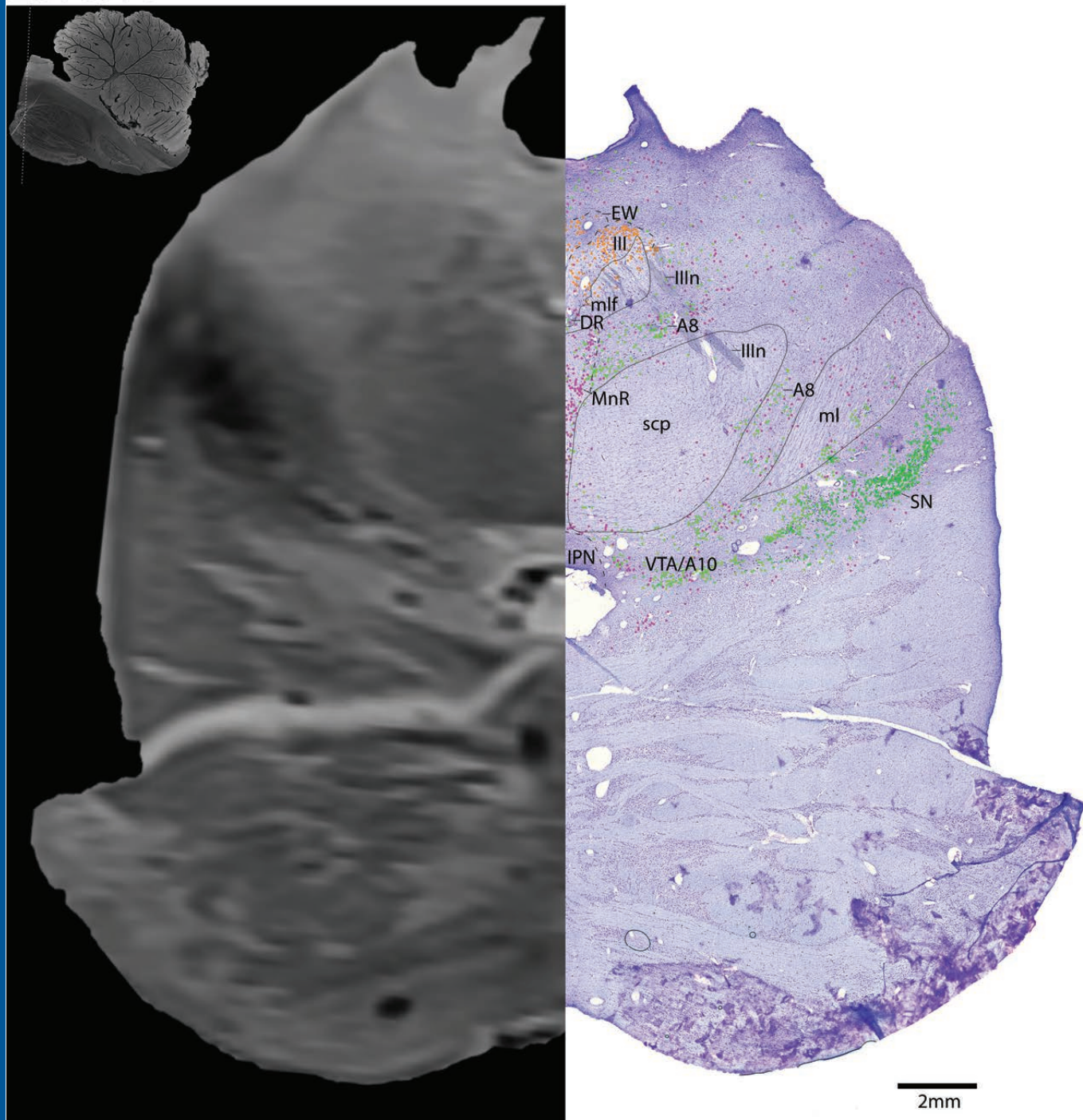


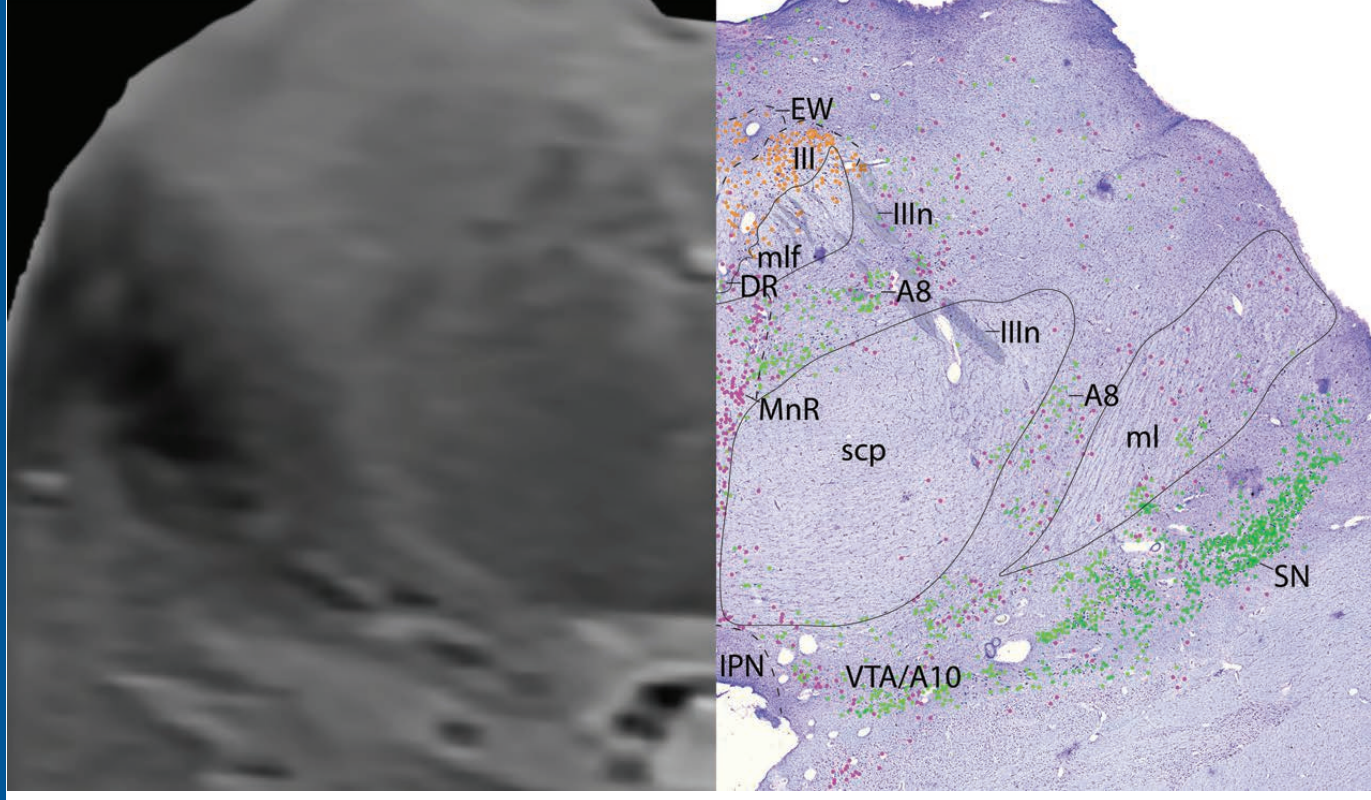




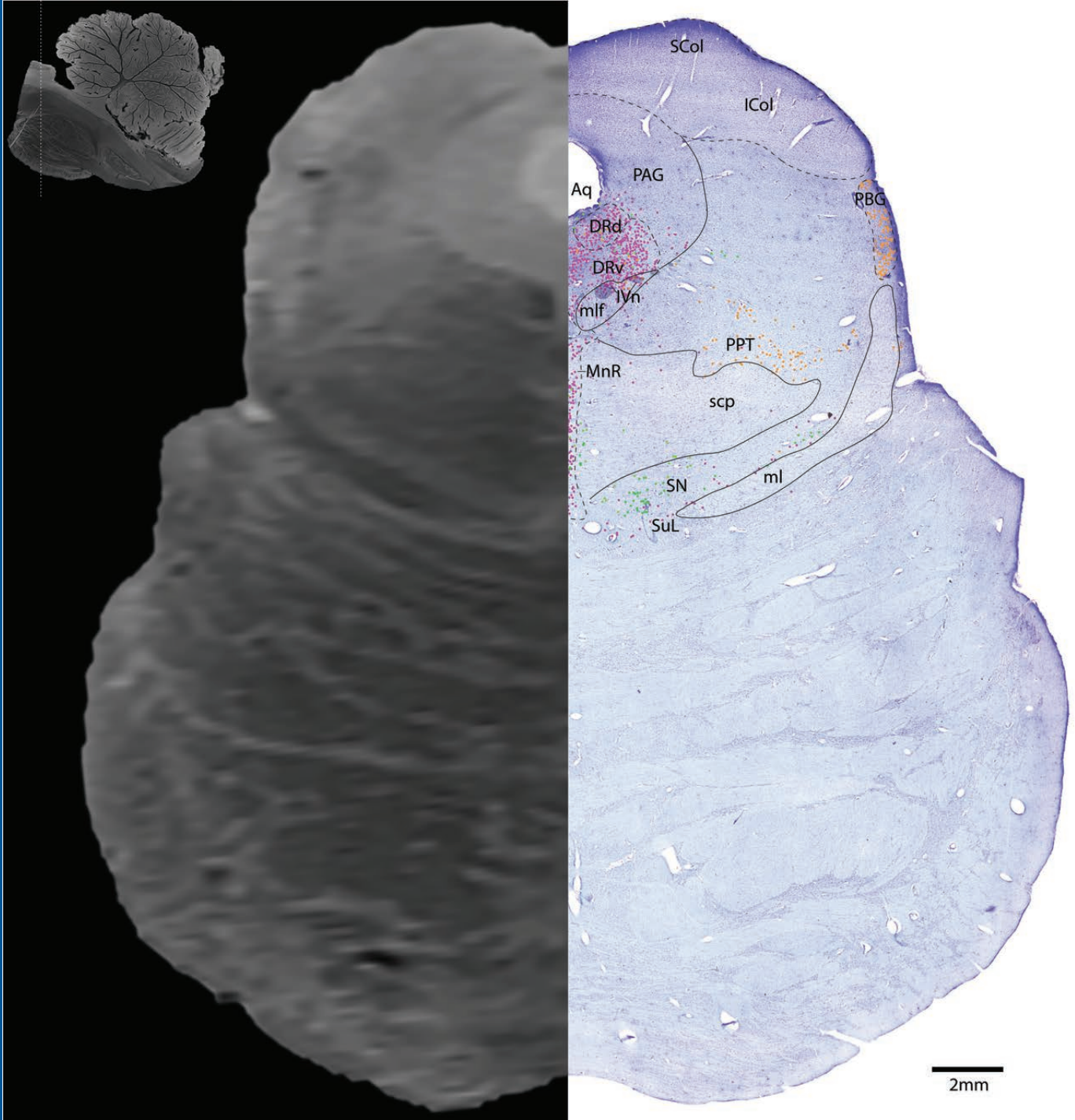
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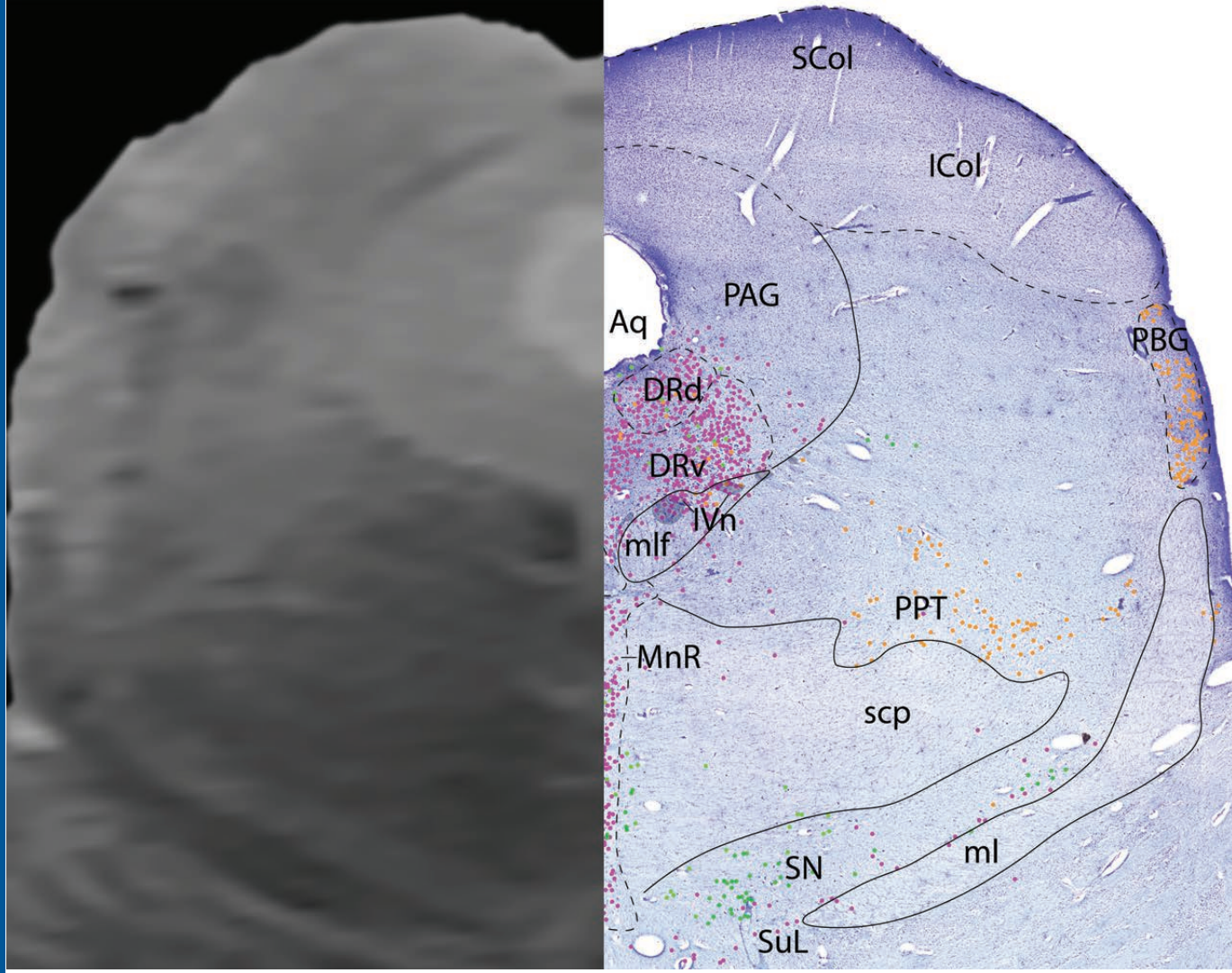
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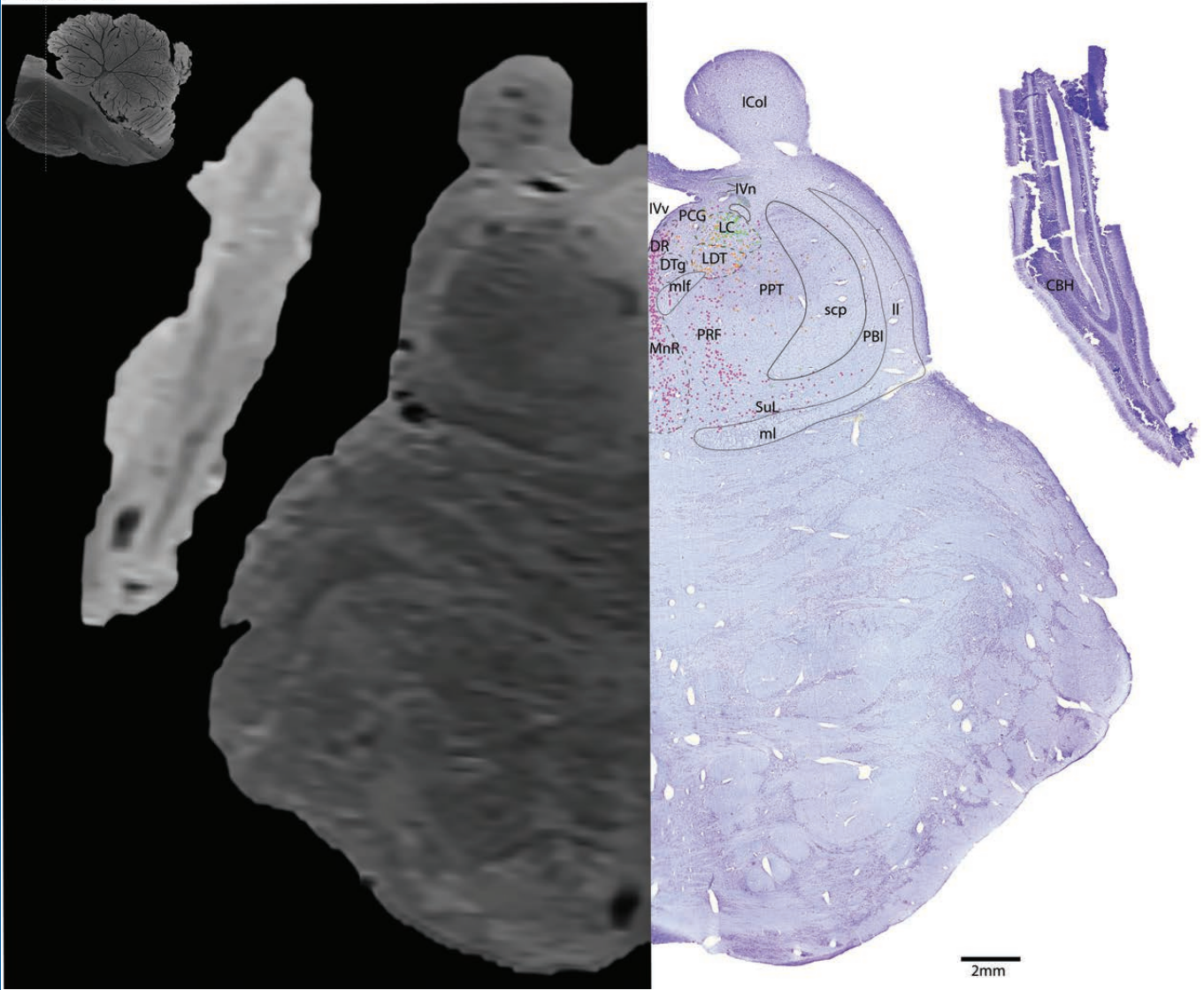


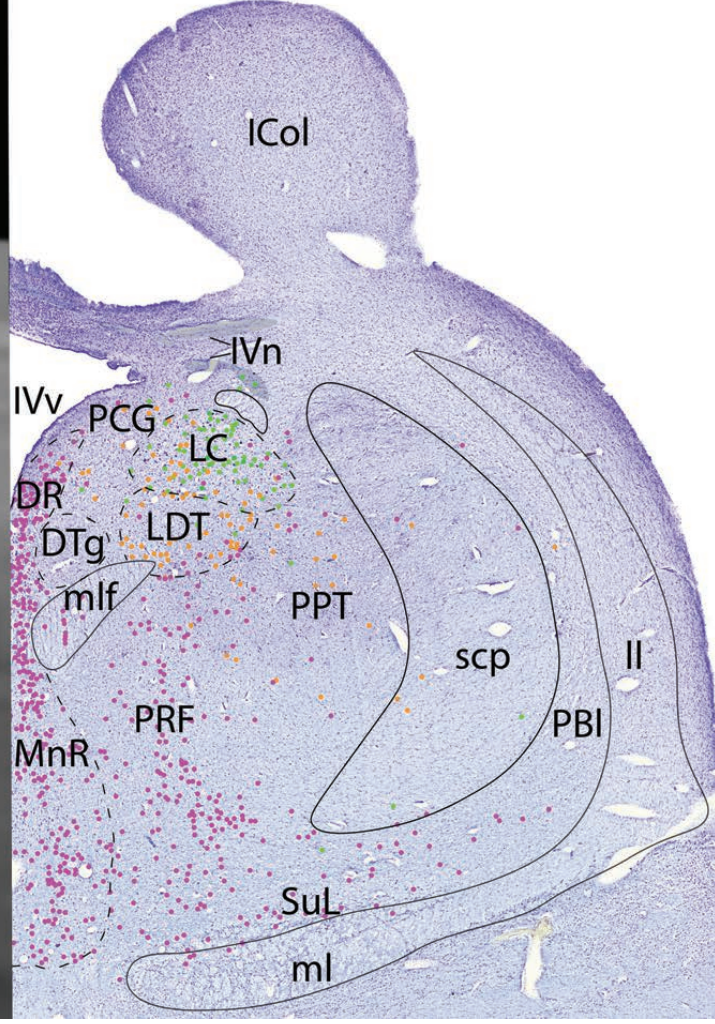
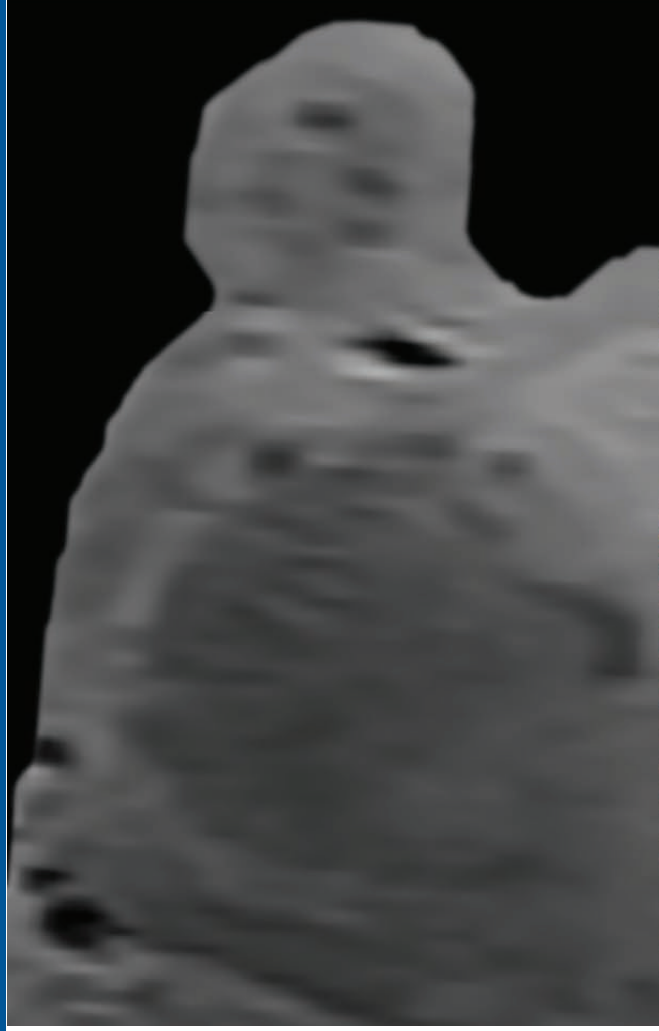
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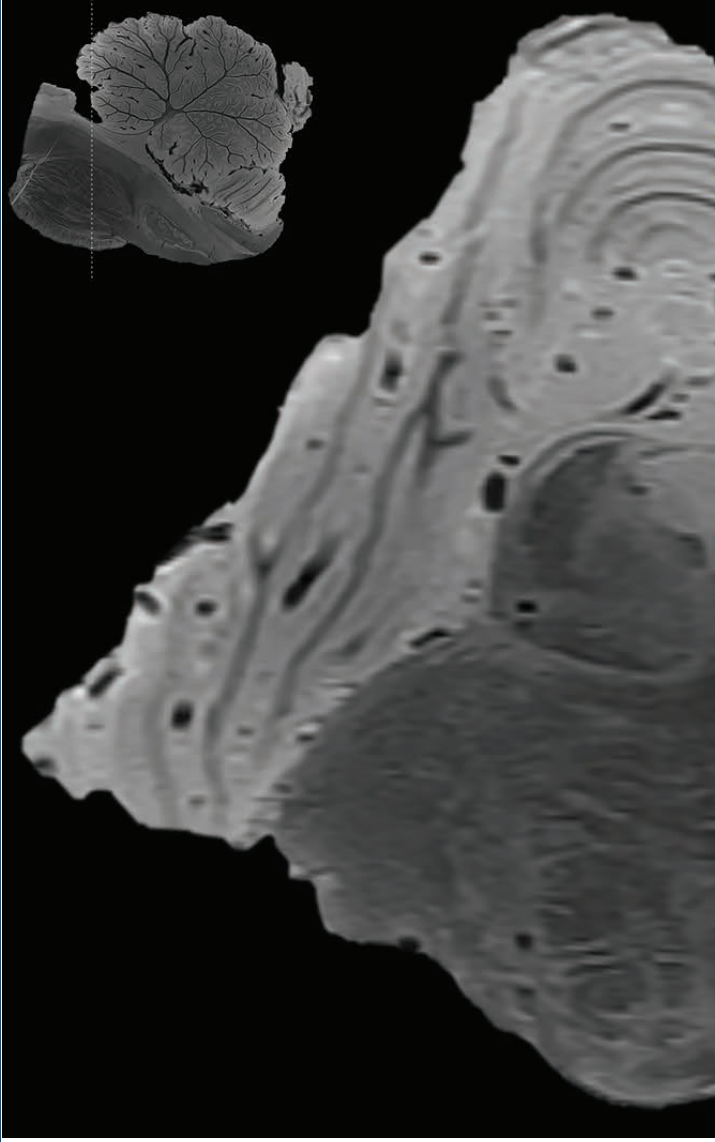


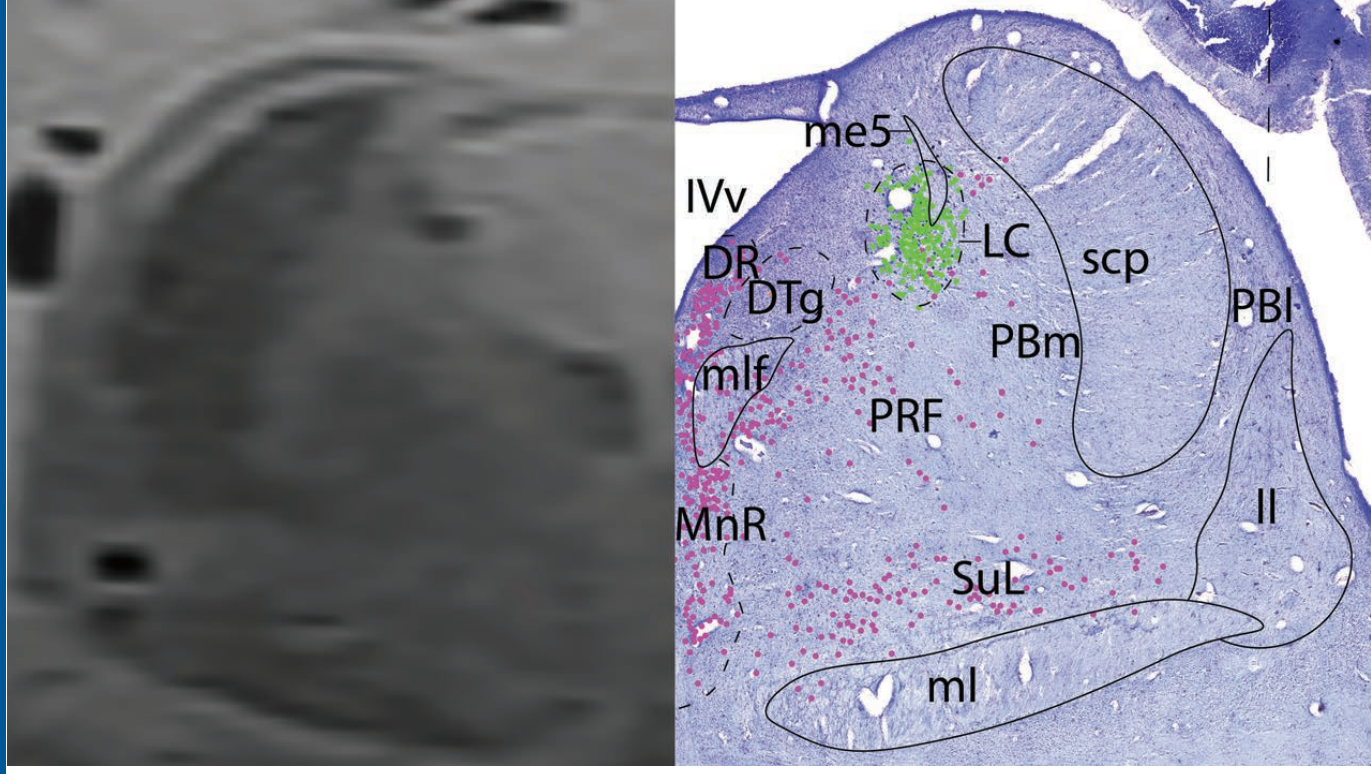
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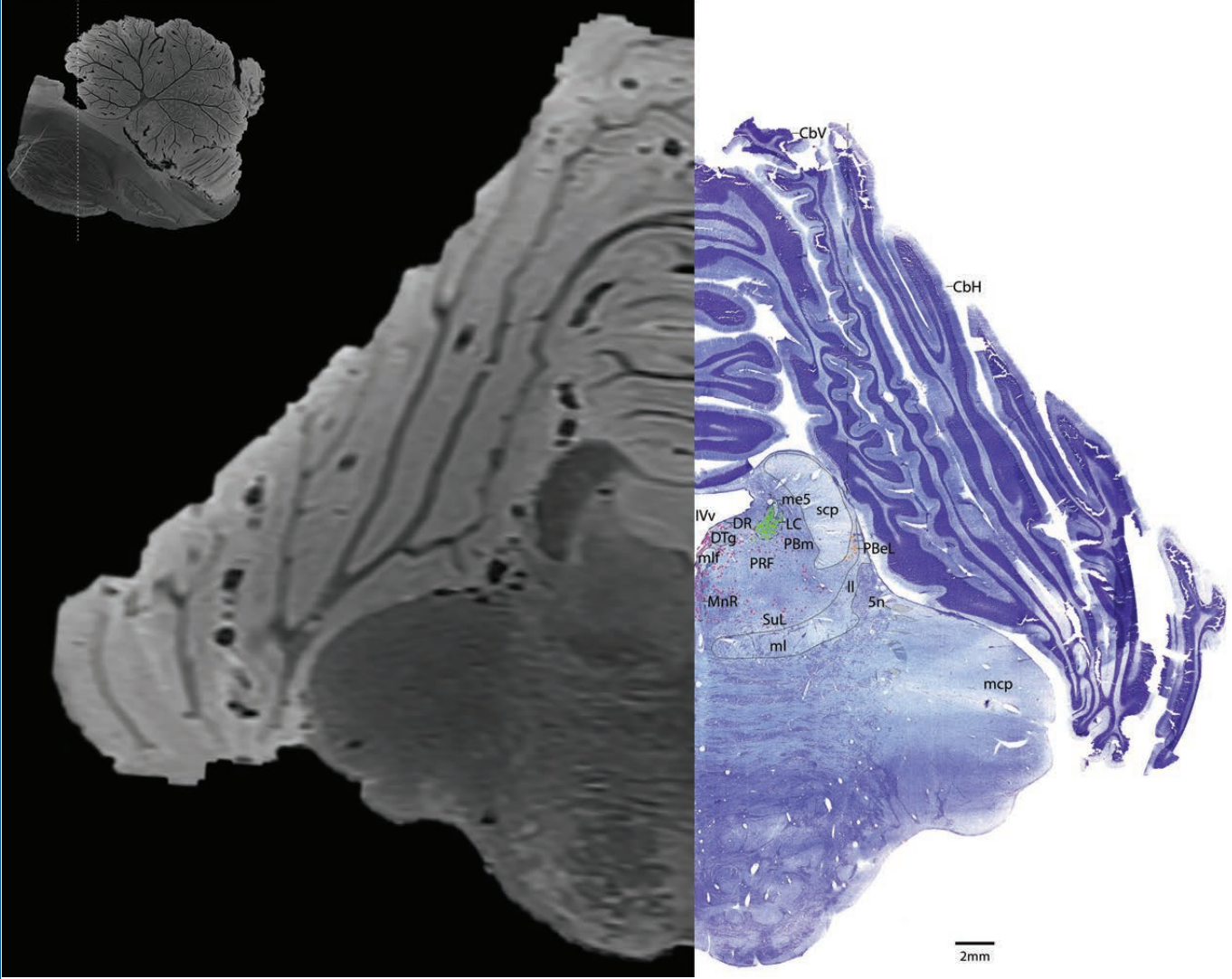


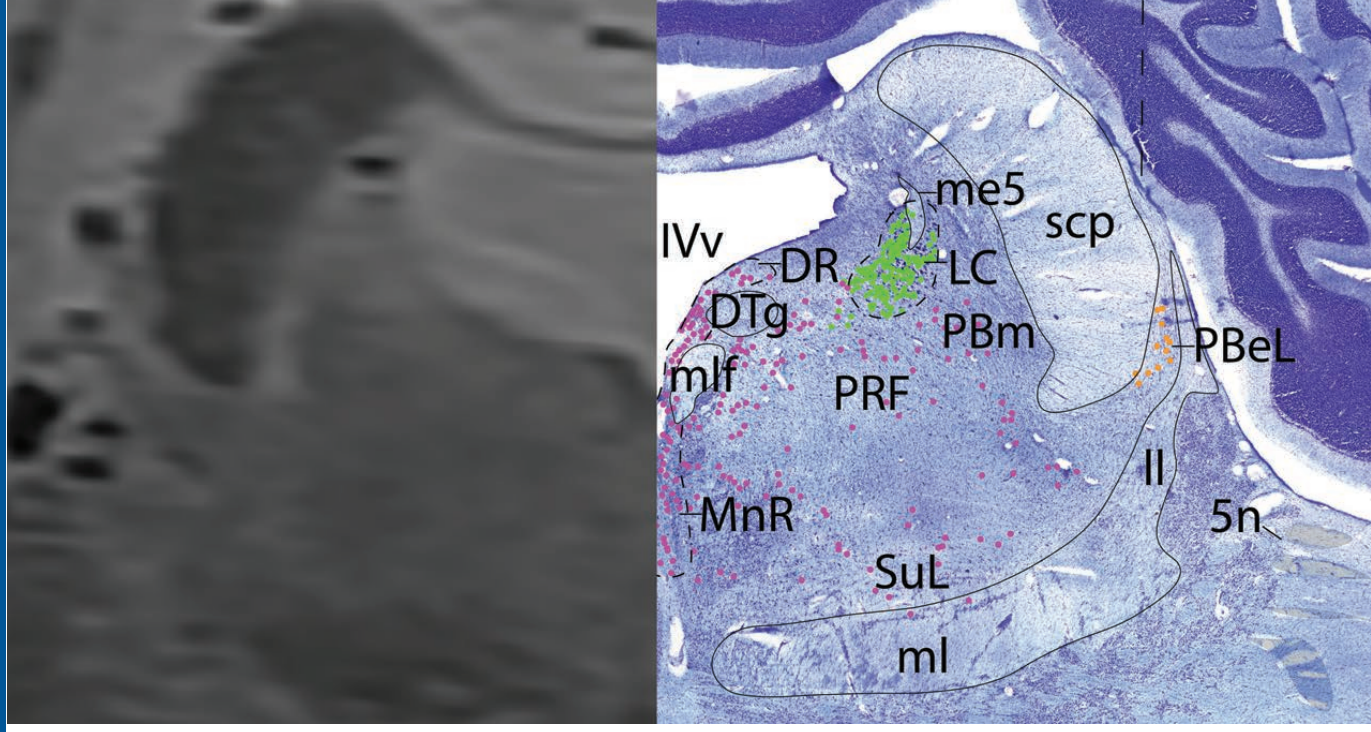
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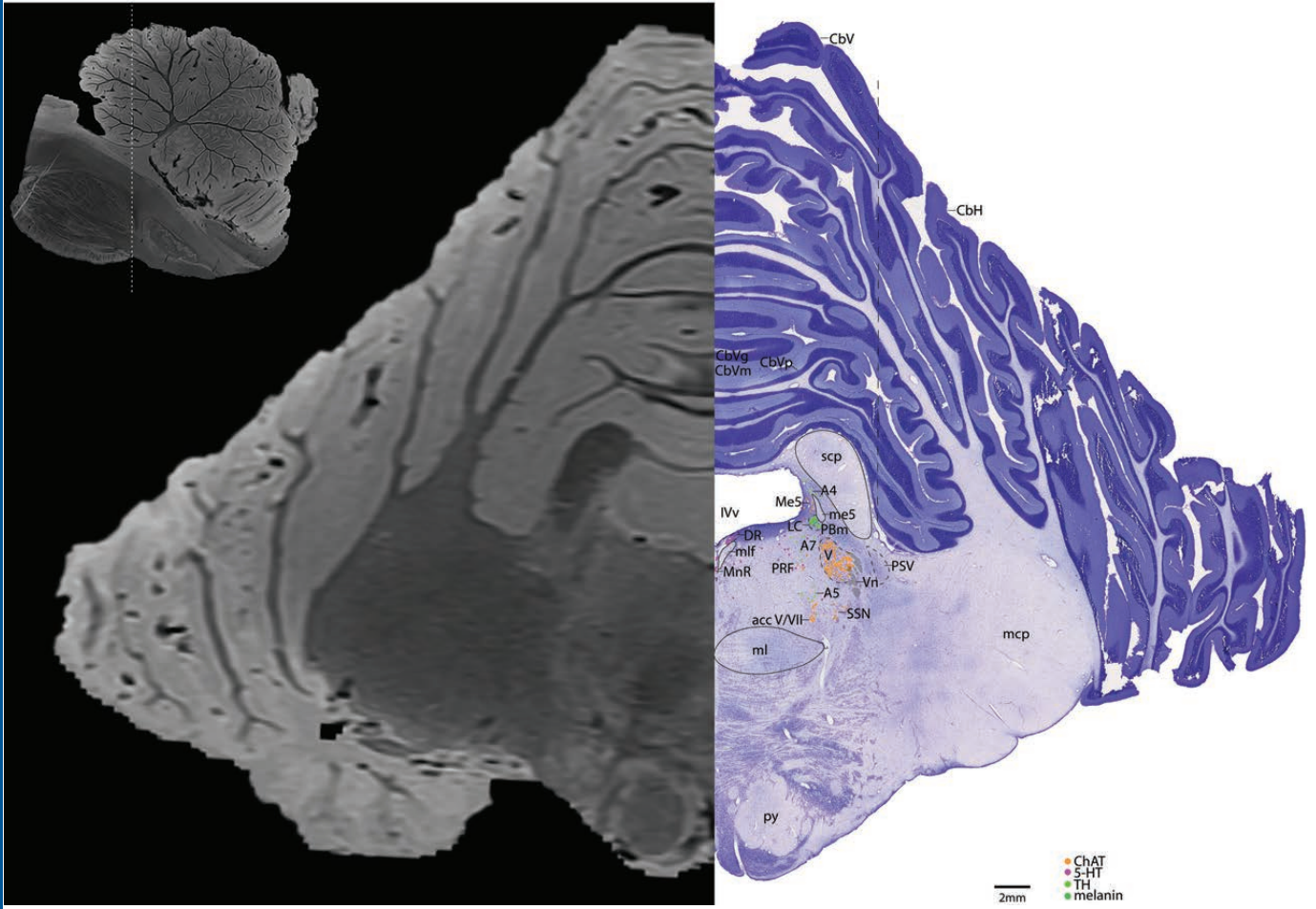


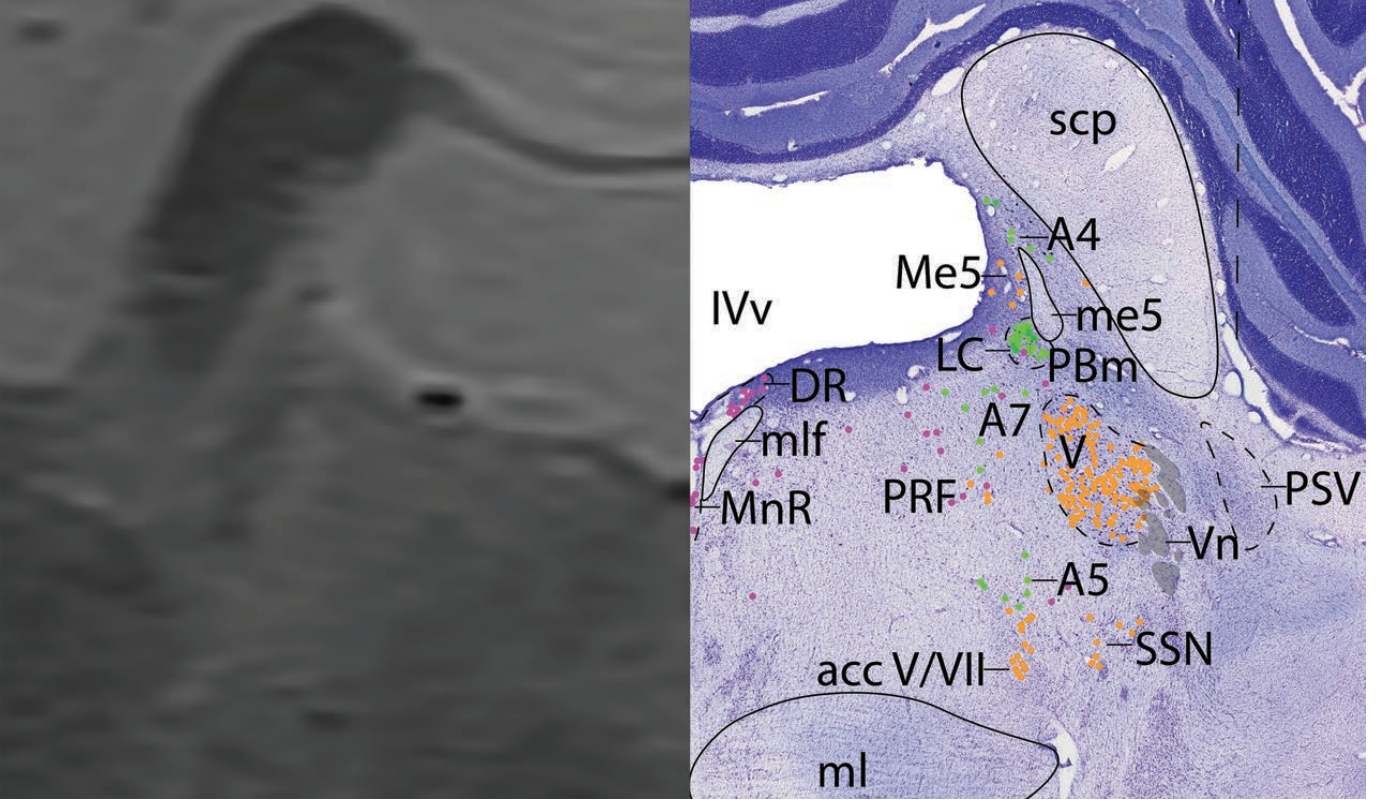
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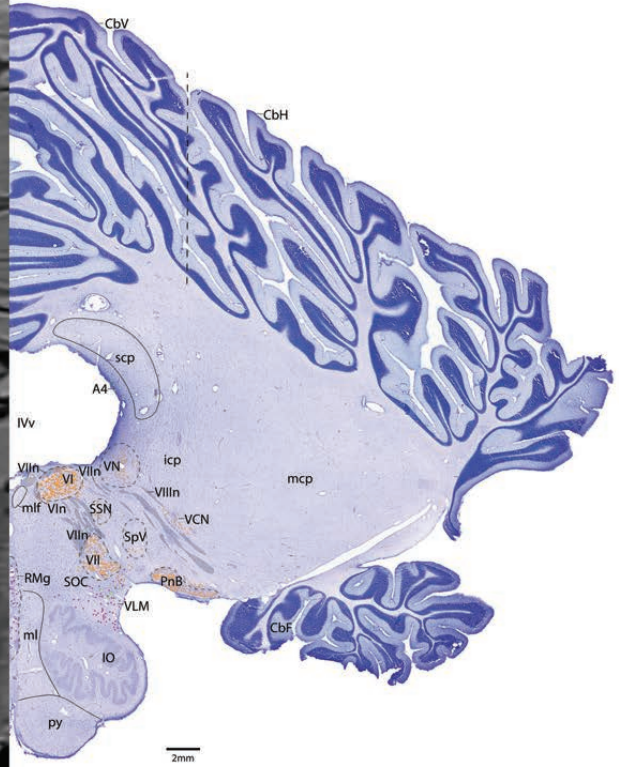
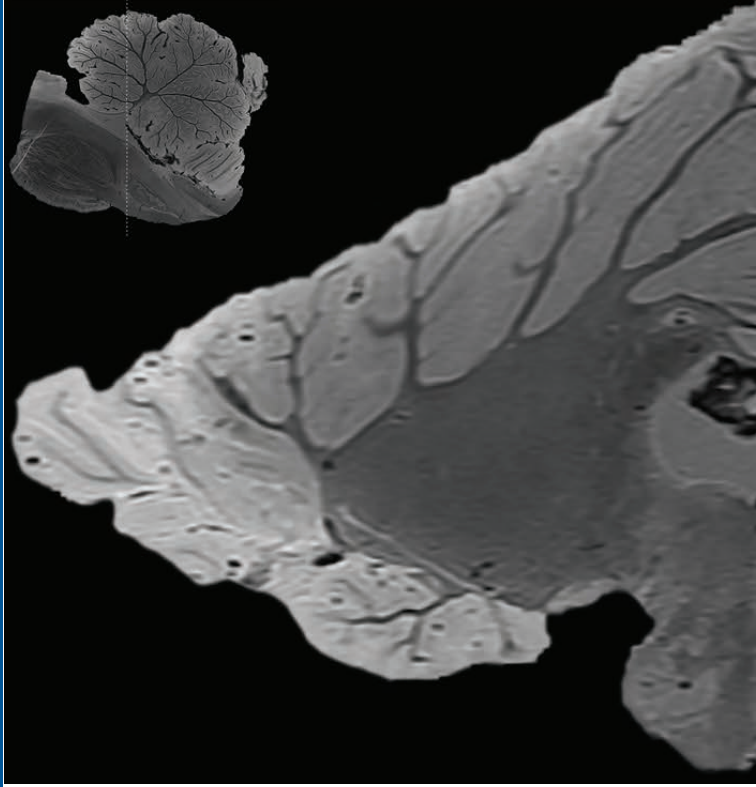


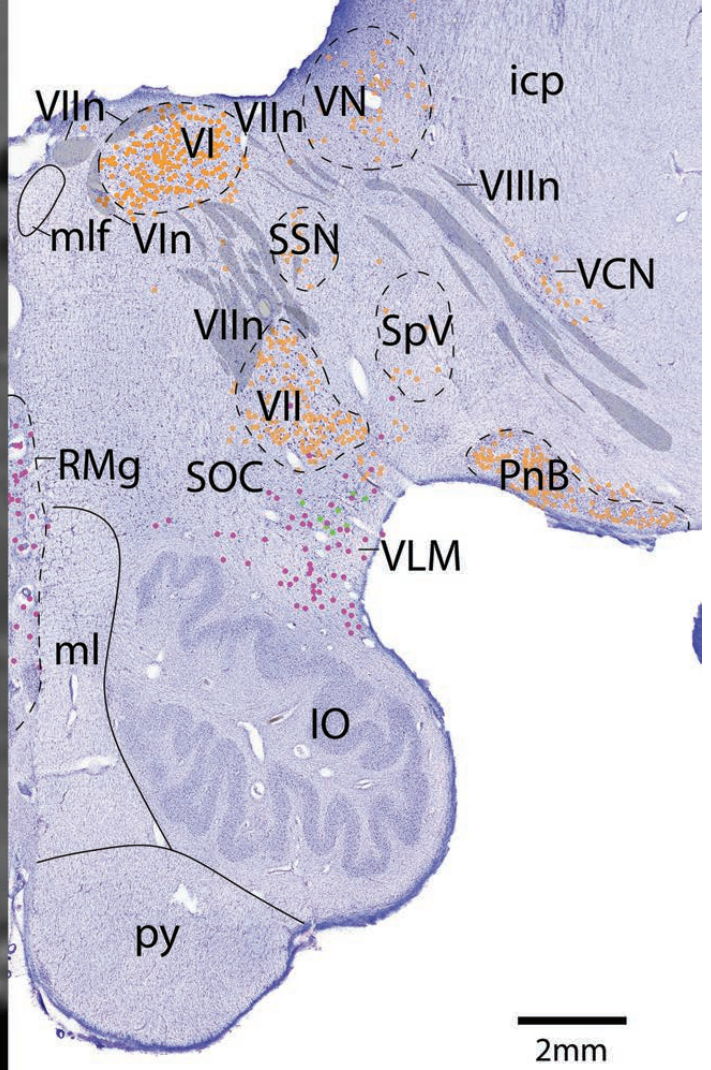
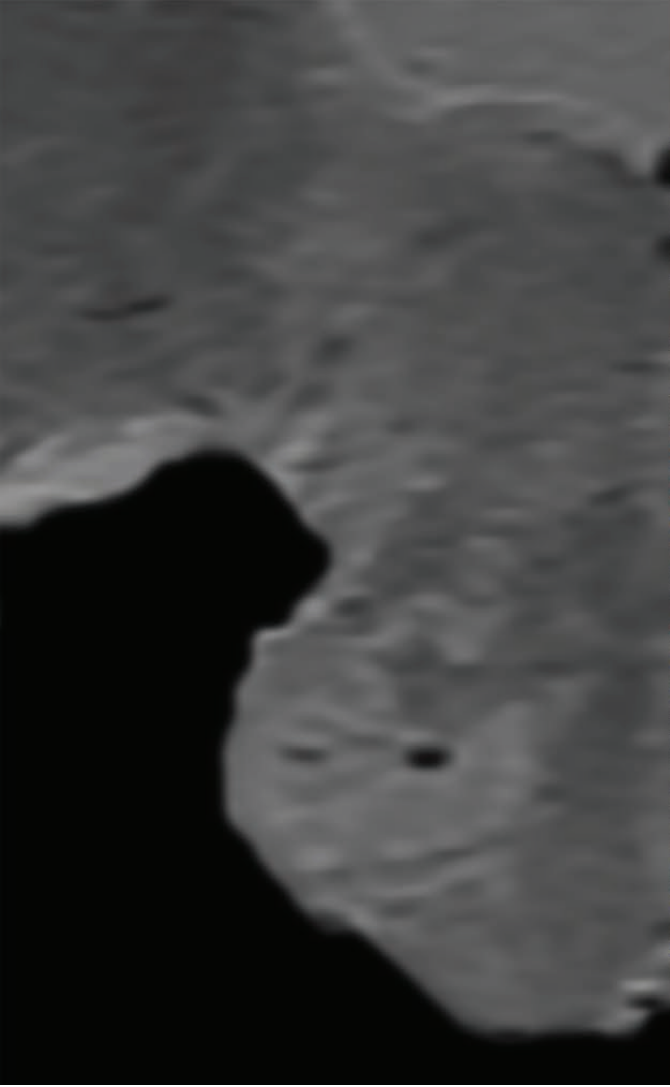
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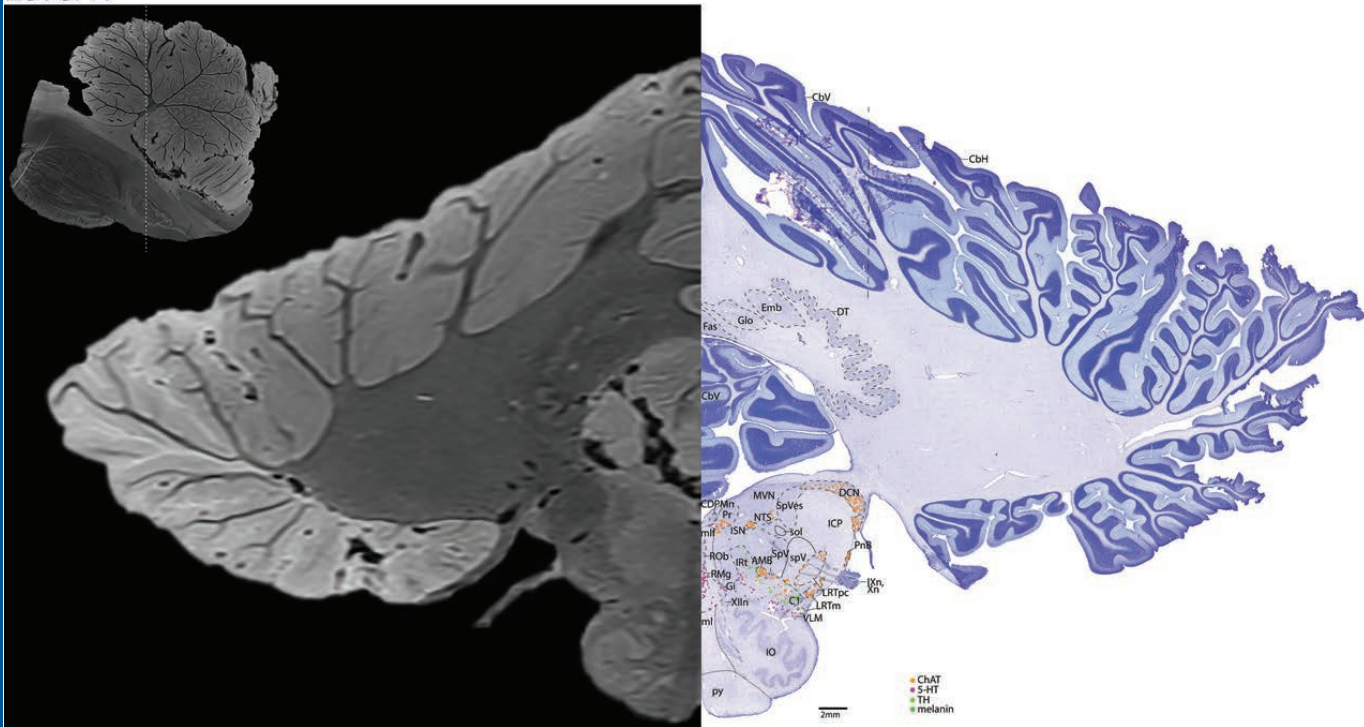


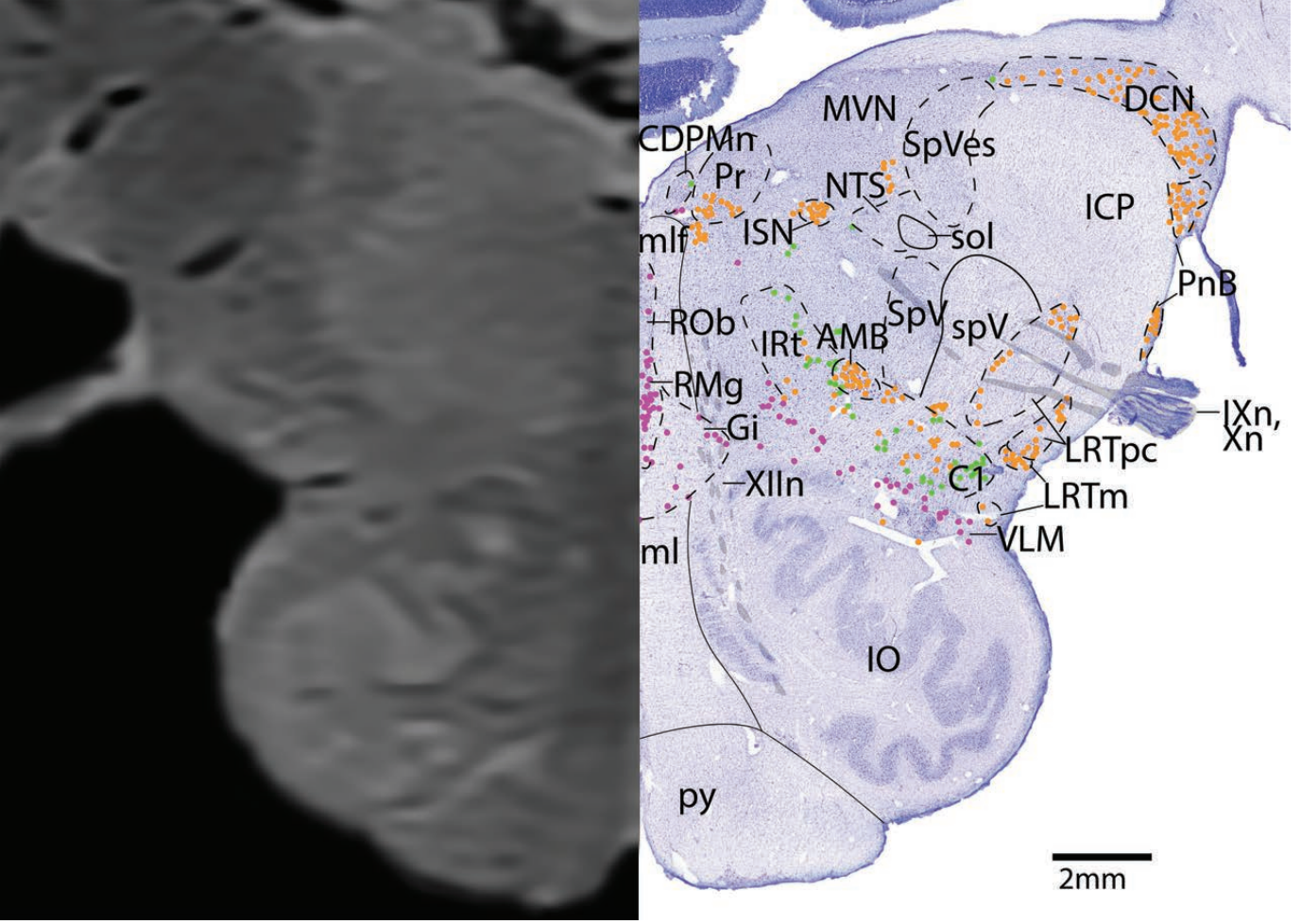
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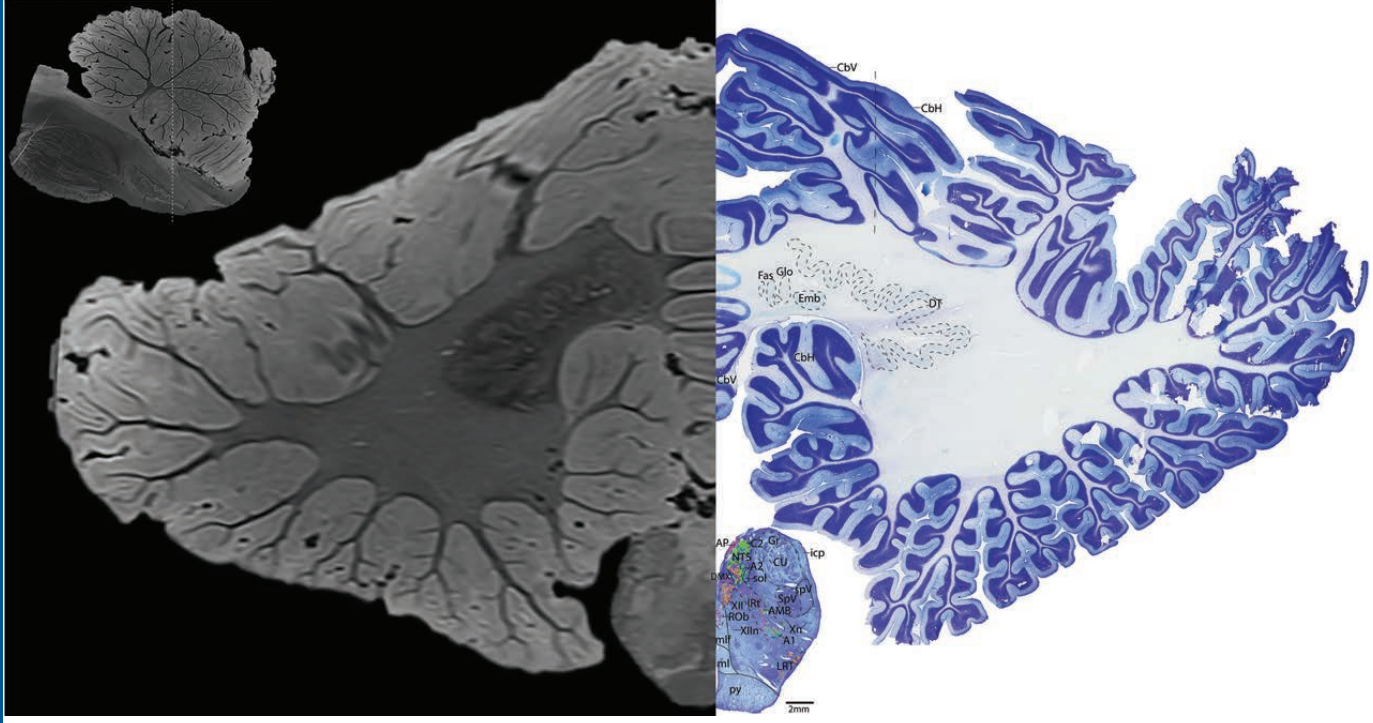


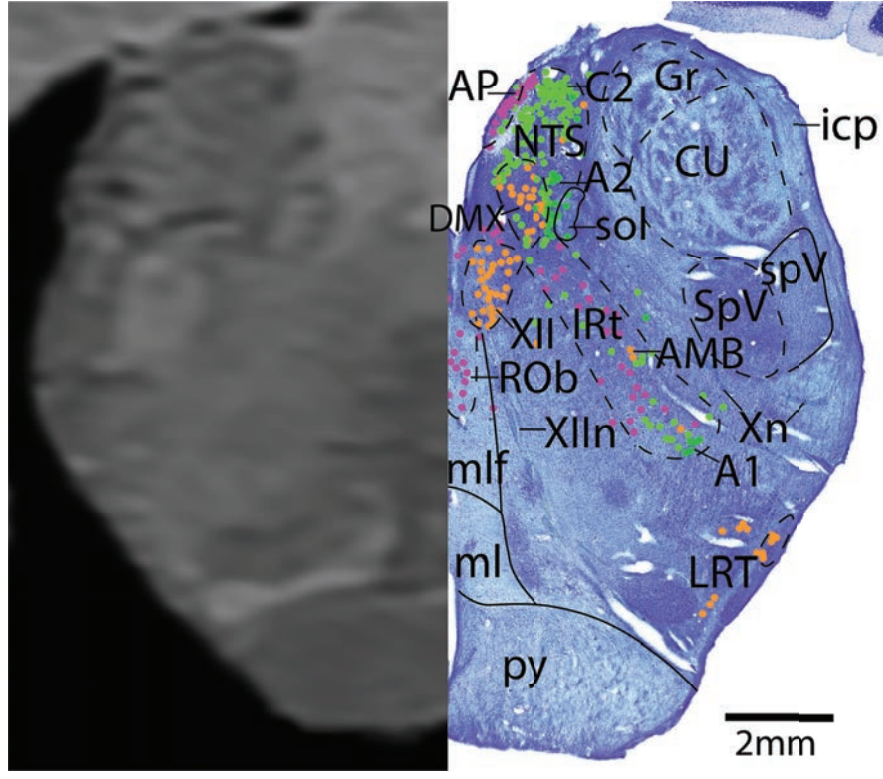
Level H





Level I





Level J

