Supplement - Neuroanatomy of the grey seal brain: bringing pinnipeds into the neurobiological study of vocal learning

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1. Extended Methodology

1.1 Animal information

The brains of two grey seals were collected at the Sealcentre Pieterburen (the Netherlands), a seal rehabilitation center successfully treating and returning to health an estimated number of 500 phocids (family Phocidae) every year [1,2]. The two grey seals examined in the current study were either euthanized due to serious injuries incompatible with survival in the wild (Grey Seal 1) or died of natural causes (Grey Seal 2). No animal was hurt or sacrificed for the purpose of this study. For both seals, age was estimated by experienced veterinarians based on the presence and appearance of the umbilical cord. *Table S1* contains the details of age, weight, and health condition for each of the two seals. The brains were extracted from the skulls shortly after death (maximum 2 hours) and were suspended in vats of regularly changed 10% formalin for approximately 6 weeks.

1.2 MRI

1.2.1 MRI acquisition

On the day before scanning, the brains were transported from the Sealcentre Pieterburen to the University of Groningen (Groningen, the Netherlands), where they were suspended in a plastic

Table S1 - Details of the two grey seals whose brains were analyzed in the present study.

	R17-517 - "Grey Seal 1"	**R18-047 - "Grey Seal 2"**
Sex	f	f
Age at rescue	1 month (pup)	3 months (weaner)
Age at death	2.5 months (weaner)	3 months (weaner)
Weight at rescue	17 kg	16 kg
Weight at death	27 kg	16 kg
Day of rescue	December 30, 2017	February 14, 2018
Day of death	February 9, 2018	February 17, 2018
Rescue site	Oosterend, Terschelling (NL)	IJmuiden, North Holland (NL)
Cause of rescue	Emaciation	Trauma, emaciation
Cause of death	Euthanized for an inoperable flipper abnormality incompatible with survival in the wild	Possible septicemia

Table S2. MRI scanning parameters. The table describes the scan duration, repetition time (TR), echo time (TE), flip angle, acquisition matrix, field of view (FOV), slice number, slice thickness, and voxel size for each of the MR image contrasts that were acquired and analyzed.

	T2 FLAIR transverse	**T2 FLAIR sagittal**	**T2 FLAIR coronal**	**T2 WTSE transverse**	**T2 STIRTSE transverse**	**T1 3D**
Acquisition time (s)	825	418	418	53	390	25
TR (ms)	11000	11000	11000	2947	5000	9
TE (ms)	107.0	99.0	99.0	80.0	20.0	3.6
Flip angle (degrees)	90	99	99	90	90	8
Acquisition matrix	256 x 256	224 x 224	224 x 224	512 x 512	512 x 512	256 x 256
FOV (mm)	220 x 220	190 x 190	190 x 190	230 x 230	200 x 200	256 x 256
Num	69	45	45	36	29	170
Slice thickness (mm)	2.00	2.00	2.00	3.98	2.98	1.00
Voxel size (mm)	0.86 x 0.86 x 2.00	0.85 x 0.85 x 2.00	0.85 x 0.85 x 2 .00	0.45 x 0.45 x 3.98	0.39 x 0.39 x 2.98	1.00 x 1.00 x 1.00

container (volume: 1.8 l) filled with 2% agar to reduce image artifacts [3]. Care was taken to avoid any bubbles in the agar. To avoid MR signal artifacts due to temperature fluctuations [4], the brains were kept at a constant room temperature overnight and then transported to the University of Groningen Medical Center (Groningen, the Netherlands). Here, the brains were scanned in a 3-Tesla MRI scanner (Philips Intera) equipped with a 32-channel head coil for approximately 2 hours each. For both seal brains, the following image contrasts were acquired and analyzed in the current study: T2 weighted fluid inversion recovery (T2 FLAIR; in the transverse direction, in the sagittal direction, and in the coronal direction), T2 weighted turbo spin echo (T2WTSE; in the transverse direction), T2 weighted short tau inversion recovery turbo spin echo (T2STIRTSE; in the transverse direction), and T1 (T1 3D; acquired in 3D; *Table S2*).

1.2.2 MRI pre-processing and template creation

The PAR/REC files were converted into NIfTI files using dcm2niiGUI (MRIcron [5]),

reoriented using FSLUTILS (FSL, 6.0; FMRIB Analysis Group [6]), and resliced to an isotropic voxel size of 1 x 1 x 1 mm for Grey Seal Brain 1 and 0.9 x 0.9 x 0.9 mm for Grey Seal Brain 2 in 3D Slicer (4.10.1 [7]). For Grey Seal Brain 1, the T1 image was set as the reference image and rotated in 3D Slicer. For Grey Seal Brain 2, due to the presence of formalin gradients in T1 [8], the transverse T2 FLAIR image was set as reference and rotated in 3D Slicer. The remaining images for each seal were then registered to the reference via FSL FLIRT, using a 3D Rigid Body transformation with 6 degrees of freedom. To separate brain voxels from the surrounding agar, an initial brain mask was created in ITK-SNAP (Version 3.6.0 [9]) and refined through a combination of manual editing (ITK-SNAP) and automatic segmentation (FSL FAST). Bias field corrections were applied to each image contrast via FSL FAST to remove inhomogeneity gradients in the MR images. The resulting pre-processed transverse T2 FLAIR images of Grey Seal Brain 2 were rotated to match the resting position of the anatomical atlas and centered approximately to the anterior commissure to serve as an anatomical brain template of a grey seal brain (*Figure 1*).

1.2.3. MRI analysis: segmentation and 3D model

The automatic segmentation using FSLFAST failed to correctly classify subcortical nuclei and structures along the midline. Therefore, separate masks for white and grey matter were created from the pre-processed T2 FLAIR images with FSLMaths, manually edited in ITK-SNAP, and over imposed to the whole brain mask. The cerebral and cerebellar hemispheres, brain stem, caudate nucleus, and thalamus were segmented in ITK-SNAP and overlaid on the anatomical brain template described above. The resulting whole-brain volumetric information, retrieved from ITK-SNAP, is presented in *Table S4-S5* and was used to calculate the relative percentage, compared to the whole brain, of each brain structure, and its composition in terms of grey and white matter.

1.3. Dissection, histology, and immunohistochemistry

1.3.1 Brain dissection

After scanning, Grey Seal Brain 2 was kept at 4°C for 24 hours and then transported to the Max Planck Institute for Psycholinguistics in Nijmegen, the Netherlands. Here, the brain was sliced in eight approximately equidistant 1 cm slices in the coronal plane (*Figure S1*). These slices were then placed and photographed on a 1 x 1 cm background grid. Three cortical sections were randomly selected and subsequently dissected from grey seal brain 2, embedded

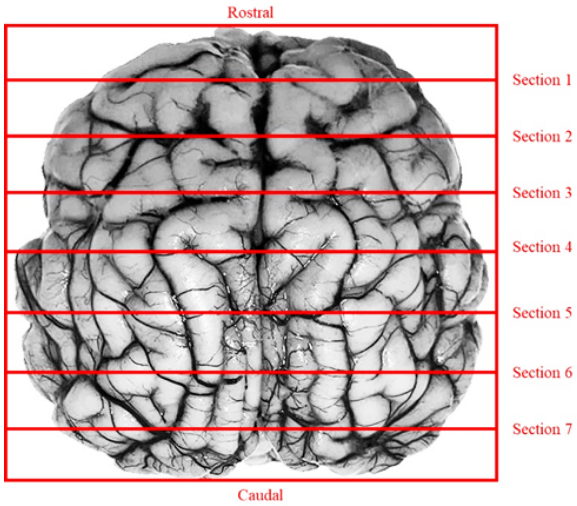

Figure S1. Indication of the slicing depth of the coronal atlas images found in *Figures S2-S8* on a dorsal view of Grey Seal Brain 2. Sections were each approximately 10 mm thick.

in paraffin, and slices at a thickness of 4 microns. The sections were transferred to Superfrost plus glass slides (Menzel Gläser) and dried overnight at room temperature, after which they were placed at 57 °C for 1 hour, and then stored in slide boxes at room temperature.

1.3.2. Image processing and analysis dissection photographs

The photographs were digitally corrected (Adobe Photoshop CC 2015, Adobe Systems, Inc.) with the help of the 1 x 1 cm background grid. A custom measurement scale was then set for each photograph, which was used to make a total of 100 cortical thickness measurements, as well as brain (structure) size measurements.

1.3.3. Histology

Slides with paraffin-embedded tissue were dewaxed by washing them twice in xylene (Sigma) for 10 minutes. The slides were rehydrated by washing them twice for 5 minutes each in 100% ethanol, for 3 minutes in 95% ethanol, and 3 minutes in 70% ethanol. The slides were briefly dipped first in tap water and then distilled water. The slides were placed in hematoxylin solution (Bio-Optica) for 1.5 minutes, and subsequently washed 3 minutes in tap water in 2 minutes and distilled water. They were then dipped 10 times in 95% ethanol and placed for 1 minute in the Eosin Y solution (Tissue-Tek). The slides were then dehydrated 3 minutes in 95% ethanol and twice in 100% ethanol for 5 minutes each. Lastly, the slides were washed in xylene twice for 5 minutes each and mounted with DPX mounting medium.

1.3.4. Immunohistochemistry

Immunohistochemistry experiments were performed with three different antibodies: FoxP2 (73A/8), FoxP2 (N16), and TLE4 (E-10), described in *Table S3*. Basic Local Alignment Search Tool (BLAST) analyses of the epitopes of the FoxP2 73A/8 antibody (raised against amino acids 1-86 of the human FOXP2 protein) and FoxP2 N16 antibody (raised against a region within amino acids 1-50 of the human FOXP2 protein) showed that these epitopes were 100% conserved in all full-length isoforms of the FoxP2 protein in grey seals (XP_035924278.1;XP_035924283.1;XP_035924284.1;XP_035924285.1;XP_035924286.1; XP_035924287.1;XP_035924290.1;XP_035924276.1). BLAST analysis of the epitope of the TLE4 E-10 antibody (which was raised against the amino acids 273-473 of the mouse TLE4 protein) revealed a 99% conservation of the epitope in the grey seal TLE4 protein (XP_035951725.1;XP_035951726.1;XP_035951734.1;XP_035951735.1;XP_035951729.1; XP_035951732.1;XP_035951727.1; XP_035951737.1;XP_035951728.1;XP_035951736.1; XP_035951733.1). The TLE4 antibody was used as a marker of the lower layers of the cortex (as used successfully in previous studies [10]).

The immunohistochemistry protocol was as per [11]. Namely, slides with paraffin-embedded tissue were dewaxed by washing them twice in xylene (Sigma) for 5 minutes. The slides were rehydrated by washing them 2 minutes each in 100%, 95%, 70%, and 50% ethanol, and distilled water. Antigen retrieval was performed by microwaving the slides in a pH 9 Tris-EDTA antigen retrieval buffer for 5 minutes at 850 Watt and 10 minutes at 180 Watt. The slides were washed and cooled in running distilled water for 3 minutes, followed by a 5-minute wash in distilled water on a shaker. To block endogenous peroxidase, the slides were placed in 0.3% H_2O_2 (Sigma) diluted in distilled water for 30 minutes, followed by a 5-minute wash in distilled water on a shaker. With a PAP pen, a rectangle was drawn around the tissue sections. The tissue sections were blocked with 10% normal animal serum from the species the secondary antibody was raised in diluted in 1x PBS for 1 hour at room temperature. The blocking solution was discarded from the slides and the slides were incubated with the relevant primary antibody diluted in blocking solution (10% animal serum from the species the secondary antibody was raised diluted in 1x PBS) overnight at 4 °C. The following day, the slides were washed three times in 1x PBS on a shaker, and then incubated with the relevant secondary antibody diluted in 1x PBS for 1 hour at room temperature. The slides were washed three times for 5 minutes on a shaker in 1x PBS. The slides that were incubated with the Biotinylated GAM or the Biotinylated HAG secondary antibody were then incubated with

avidin-biotin-horseradish peroxidase complex (ABC) from the Vectastain kit (Vector Laboratories). To do this, Reagent A and B were diluted together at a concentration of 1 in 100

Table S3. Immunohistochemistry conditions.

1ry antibody	name	FOXP2 (Clone 73A/8)	FOXP2 (N16)	TLE4 (E-10)
	Protein target	FOXP2	FOXP2	TLE4
	Epitope	N-terminus human protein FOXP2, within amino acids 1-50	N-terminus human protein FOXP2, amino acids 1-86	mouse protein TLE, amino acids 273-473
	Product #	MABE415	sc-21069	sc-365406
	LOT #	3146282	#E0715	#K2719
	Company	Millipore	Santa Cruz	Santa Cruz
	Raised in	Mouse	Goat	Mouse
	Concentration used	1:250	1:250	1:500
Antigen retrieval	Buffer	Tris-EDTA pH9	Tris-EDTA pH9	Tris-EDTA pH9
Blocking	Species	10% normal goat serum	10% normal horse serum	10% normal goat serum
	Company	Vector Laboratories	Vector Laboratories	Vector Laboratories
2ry antibody	Name	POLY HRP GAM/R/R	biotinylated Horse anti-goat	biotinylated Goat anti-mouse
	Product #	VWRKDPVO55-HRP	BA-92200	BA-9500
	Company	Immunologic	Vector Laboratories	Vector Laboratories
	Raised in	goat	horse	goat
	Concentration used	1:2	1:1000	1:1000
Amplification	Name	-	ABC Vectastain	ABC Vectastain
	Company	-	Vector laboratories	Vector laboratories
Color Reagent	Name	DAB	DAB	DAB
	Company	Immunologic	Immunologic	Immunologic

in 1x PBS and incubated for 30 minutes at room temperature; the slides were incubated with the ABC solution for 45 minutes at room temperature; and the slides were washed three times in 1x PBS on a shaker. These steps were not necessary for slides treated with the Poly-HRP-GAM/R/R antibody, as this antibody is directly conjugated to horseradish peroxidase. All slides were incubated with diaminobenzidine (DAB) solution (Immunologic) for 7 minutes at room temperature in the dark. After this, the slides were rinsed briefly in distilled water. The slides underwent dehydration by washing them 2 minutes each, in 50% ethanol, 70% ethanol, 95% ethanol, and twice in 100% ethanol. Lastly, the slides were washed twice for 5 minutes each in xylene (Sigma) and coverslipped with DPX (Sigma).

1.3.5. Microscope imaging

All slides were scanned with an AxioScan Z1 microscope at a 20x magnification and a 0.22x0.22 μm per pixel size with Zen pro 2.6 software (Zeiss).

2. Atlas of the Grey Seal brain

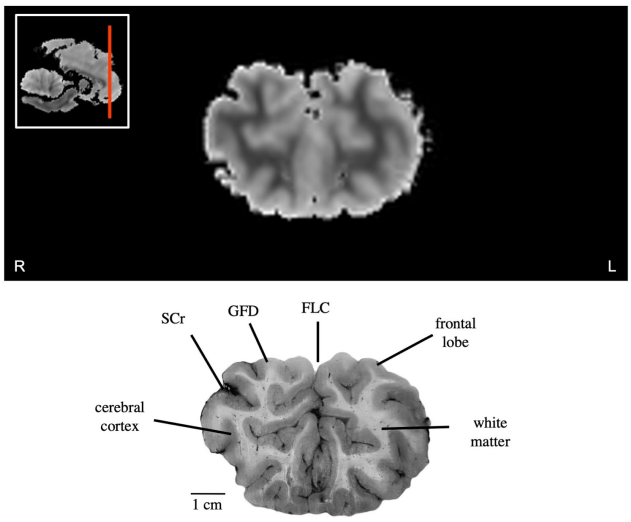

Figure S2. Atlas section 1. T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1.* Abbreviations: FLC = Fissura Longitudinalis Cerebri; GFD = Gyrus Frontalis Dorsalis; SCr = Sulcus Cruciatus. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

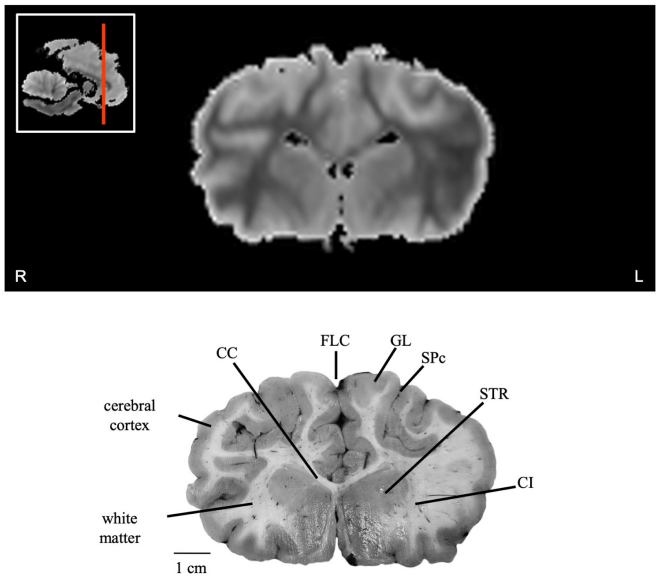

Figure S3. Atlas section 2, T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1.* Abbreviations: CC = Corpus Callosum; CI = Capsula Interna; FLC = Fissura Longitudinalis Cerebri; GL = Gyrus Lateralis; STR = Striatum; SPc = Sulcus Postcruciatus. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

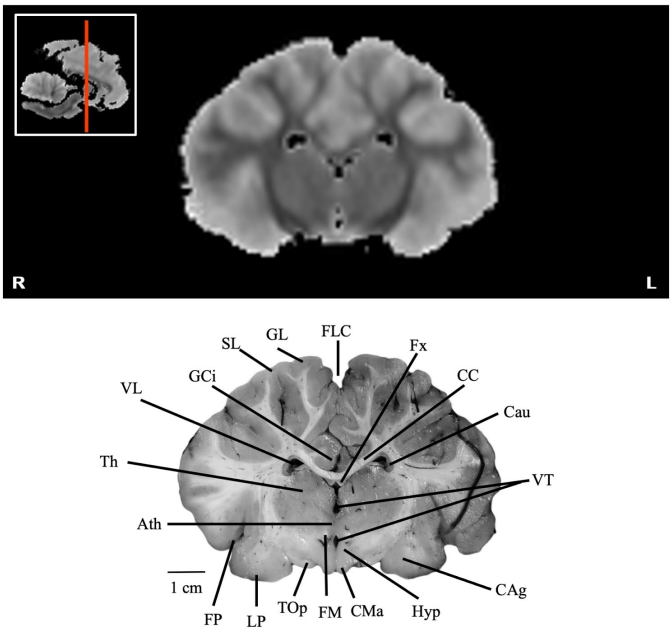

Figure S4. Atlas section 3. T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1*. Abbreviations: Ath = Adhaesio interthalamica; CAg = Corpus Amygdaloideum; CC = Corpus Callosum; Cau = Nucleus Caudatus; CMa = Corpus Mammillare; FLC = Fissura Longitudinalis Cerebri; FM = Fasciculus Mammillothalamicus; FP = Fissura Pseudosylvia; FX = Fornix; GCi = Gyrus Cinguli; GL = Gyrus Lateralis; Hyp = Hypothalamus; LP = Lobus Piriformis; SL = Sulcus Lateralis; Th = Thalamus; TOp = Tractus Opticus; VL = Ventriculus Lateralis; VT = Ventriculus Tertius. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

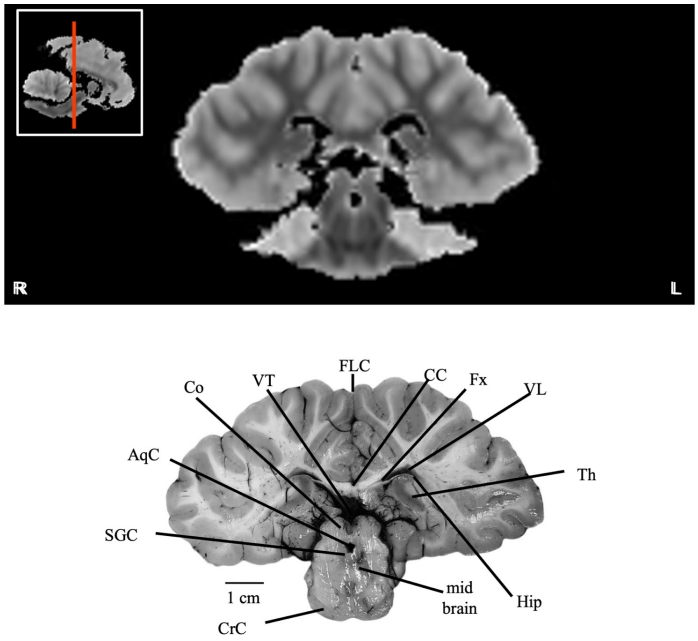

Figure S5. Atlas section 4. T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1*. Abbreviations: AqC = Aquaeductus cerebri; CC = Corpus Callosum; CrC = Crus Cerebri; Co = Colliculus (caudalis); FLC = FIssura Longitudinalis Cerebri; Fx = Fornix; Hip = Hippocampus; SGC = Substantia grisea centralis; Th = Thalamus; VL = Ventriculus Lateralis; VT = Ventriculus tertius. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

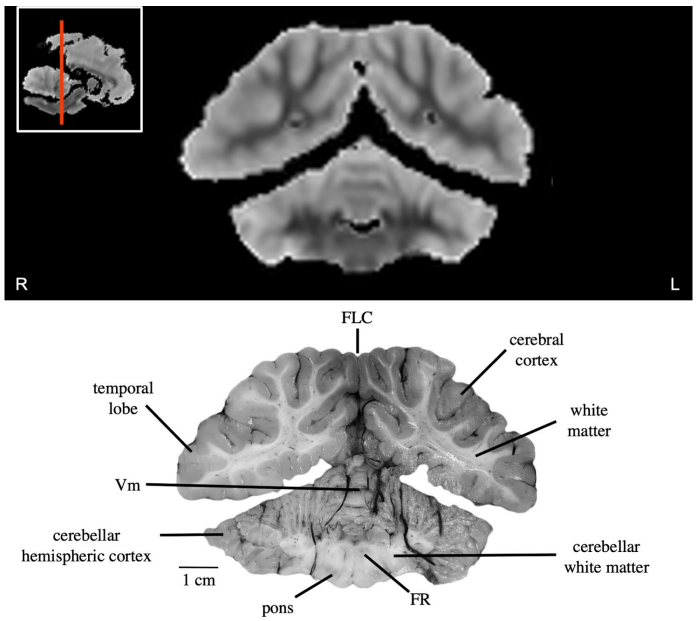

Figure S6. Atlas section 5. T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1* Abbreviations: FLC = Fissura Longitudinalis Cerebri; FR = Formatio Reticularis; Vm = Vermis. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

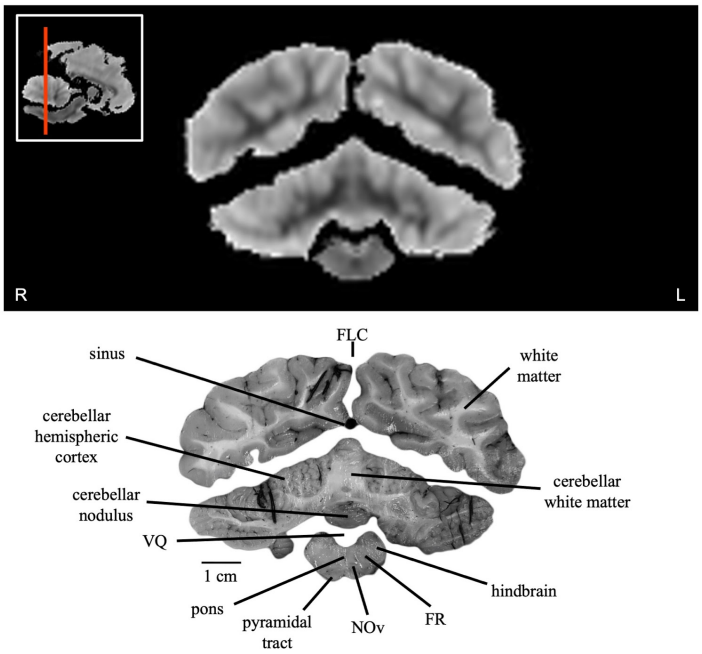

Figure S7. Atlas section 6. T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1*. Abbreviations: FLC = Fissura Longitudinalis Cerebri; FR = Formatio Reticularis; NOv = Nucleus olivaris; VQ = Ventriculus quartus. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

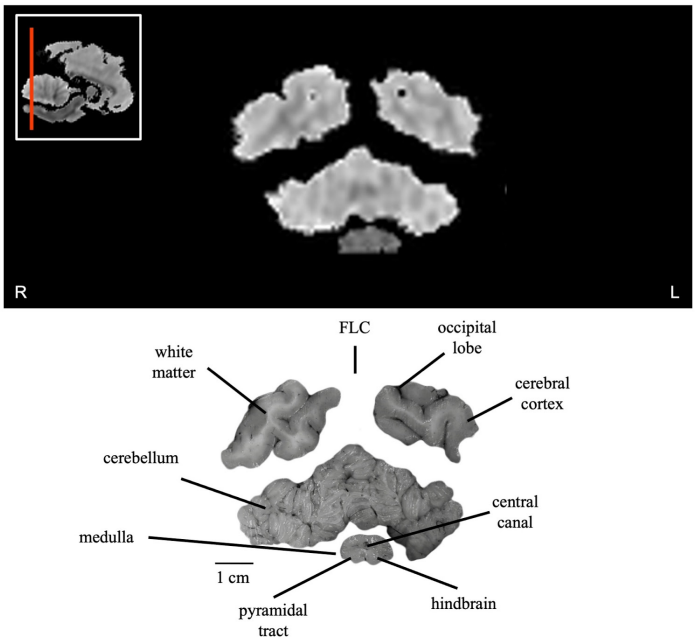

Figure S8. Atlas section 7. T2 FLAIR MRI (top) and labeled coronal section (bottom) of Grey Seal Brain 2. An indication of the slicing depth can be found in the sagittal MRI cut (top left corner) and in *Figure S1*. Abbreviations: FLC = Fissura Longitudinalis Cerebri. Both images are presented in radiological convention (i.e., the left hemisphere is on the right side of the image).

3. Volumetric Tables

Table S4. Volumetric information of the brain structures in Grey Seal Brain 1 and Grey Seal Brain 2. The columns underneath the heading 'Volume' give the absolute volumes of the brain structures, the columns underneath the heading '% of brain' give the relative percentage of a brain structure compared to the whole brain. The lateral geniculate nucleus was not included in the volume of the thalamus because the resolution of the MR images did not allow for a clear identification of its boundaries. It was hence classified within the surrounding grey or white matter.

		Volume (cm^3)			% of brain		
		Brain 1	**Brain 2**	**Average**	**Brain 1**	**Brain 2**	**Average**
Whole brain	Total	216.9	192.6	204.7	100.0	100.0	100.0
	Left	105.5	95.4	100.4	48.7	49.5	49.1
	Right	111.4	97.3	104.3	51.4	50.5	50.9
Forebrain	Total	173.0	155.1	164.0	79.8	80.5	80.1
	Left	85.7	77.1	81.4	39.5	40.0	39.8
	Right	87.3	78.0	82.6	40.2	40.5	40.4
Midbrain	Total	3.5	2.9	3.2	1.6	1.5	1.6
	Left	1.7	1.4	1.5	0.8	0.7	0.8
	Right	1.8	1.5	1.7	0.8	0.8	0.8
Hindbrain	Total	40.4	34.6	37.5	18.6	18.0	18.3
	Left	18.1	16.9	17.5	8.4	8.8	8.6
	Right	22.3	17.8	20.0	10.3	9.2	9.8
Caudate nucleus	Total	3.6	3.8	3.7	1.7	2.0	1.8
	Left	1.8	1.9	1.9	0.8	1.0	0.9
	Right	1.8	1.9	1.8	0.8	1.0	0.9
Thalamus	Total	4.0	4.0	4.0	1.9	2.1	2.0
	Left	2.0	2.1	2.1	0.9	1.1	1.0
	Right	2.0	1.9	2.0	0.9	1.0	1.0
Cerebellum	Total	33.1	28.3	30.7	15.2	14.7	15.0
	Left	14.6	13.9	14.2	6.7	7.2	7.0
	Right	18.5	14.4	16.4	8.5	7.5	8.0

Table S5. Volumetric information of white and grey matter distributions in Grey Seal Brain 1 and Grey Seal Brain 2. The columns underneath the heading 'Volume' give the absolute volumes of the white or grey matter, the columns underneath the heading '% of brain structure' give the relative percentage of white and grey matter within a specific brain structure.

Brain structure		**Volume (cm^3)**			**% of brain structure**		
		Brain 1	**Brain 2**	**Average**	**Brain 1**	**Brain 2**	**Average**
Whole brain white matter	Total	56.6	53.3	55.0	26.1	27.7	26.9
	Left	27.5	25.4	26.5	26.1	26.6	26.4
	Right	29.1	27.9	28.5	26.2	28.7	27.4
Whole brain grey matter	Total	160.2	139.4	149.8	73.9	72.3	73.1
	Left	78.0	70.0	74.0	73.9	73.4	73.6
	Right	82.2	69.4	75.8	73.9	71.3	72.6
Forebrain white matter	Total	46.6	41.8	44.2	26.9	27.0	27.0
	Left	22.5	19.9	21.2	26.3	25.8	26.0
	Right	24.1	22.0	23.0	27.6	28.2	27.9
Forebrain grey matter	Total	126.4	113.2	119.8	73.1	73.0	73.0
	Left	63.2	57.2	60.2	73.7	74.2	74.0
	Right	63.2	56.0	59.6	72.4	71.8	72.1
Cerebellum white matter	Total	5.7	4.5	5.1	17.1	15.9	16.5
	Left	2.8	2.2	2.5	19.1	15.8	17.5
	Right	2.9	2.3	2.6	15.5	15.9	15.7
Cerebellum grey matter	Total	27.4	23.8	25.6	82.9	84.1	83.5
	Left	11.8	11.7	11.7	80.9	84.2	82.5
	Right	15.6	12.1	13.9	84.5	84.1	84.3

4. List of abbreviations

Table S6. List of abbreviations for neuroanatomical structures.

Abbreviation	Latin term	English term	Figures
AqC	Aquaeductus Cerebri	Cerebral aqueduct	S5
Ath	Adhaesio Interthalamica	Interthalamic adhesion	S4
BO	Bulbus Olfactorius	Olfactory bulb	2
CAg	Corpus Amygdaloideum	Amygdaloid body	S4
Cau	Nucleus Caudatus	Caudate nucleus	S4
CC	Corpus Callosum	Corpus callosum	S3-S5
CI	Capsula Interna	Internal capsule	S3
CMa	Corpus Mammillare	Mammillary body	S4
Co	Colliculus (caudalis)	(caudal) colliculus	S5
CrC	Crus Cerebri	Cerebral crus	S5
FLC	Fissura Longitudinalis Cerebri	Longitudinal fissure	S2-S8
FM	Fasciculus Mammillothalamicus	Mammillothalamic tract	S4
FP	Fissura Pseudosylvia	Pseudo-sylvian fissure	S4
FR	Formatio Reticularis	Reticular formation	S6-S7
Fx	Fornix	Fornix	S4-S5
GCi	Gyrus Cinguli	Cingulate gyrus	S4
GEsC	Gyrus Ectosylvius Caudalis	Caudal ectosylvian gyrus	2
GEsR	Gyrus Ectosylvius Rostralis	Rostral ectosylvian gyrus	2
GFD	Gyrus Frontalis Dorsalis	Dorsal frontal gyrus	2, S2
GFM	Gyrus Frontalis Medius	Middle frontal gyrus	2
GFV	Gyrus Frontalis Ventralis	Ventral frontal gyrus	2
GL	Gyrus Lateralis	Lateral gyrus	2, S3-S4
GPc	Gyrus Postcruciatus	Postcrucial sulcus	2
GSC	Gyrus Sigmoideus Caudalis	Caudal sigmoid gyrus	2
GSR	Gyrus Sigmoideus Rostralis	Rostral sigmoid gyrus	2
GSsR	Gyrus Suprasylvius Rostralis	Rostral supra-sylvian gyrus	2
Hip	Hippocampus	Hippocampus	S5
Hyp	Hypothalamus	Hypothalamus	S4
LP	Lobus Piriformis	Piriform lobe	S4
NOv	Nucleus Olivaris	Olivary nucleus	S7

SA	Sulcus Ansatus	Ansiform sulcus	2
SC	Sulcus Coronalis	Coronal sulcus	2
SCr	Sulcus Cruciatus	Crucial sulcus	2, S2
SEL	Sulcus Entolateralis	Endolateral sulcus	2
SGC	Substantia Grisea Centralis	Central grey substance	S5
SL	Sulcus Lateralis	Lateral sulcus	2, S4
SPc	Sulcus Postcruciatus	Postcrucial sulcus	2, S3
SPs	Sulcus Praesylvius	Presylvian sulcus	2
SSsA	Sulcus Suprasylvius Anterior	Anterior suprasylvian sulcus	2
SSsP	Sulcus Suprasylvius Posterior	Posterior suprasylvian sulcus	2
SSsS	Sulcus Suprasylvius Superior	Superior suprasylvian sulcus	2
STR	Striatum	Striatum	S3
Th	Thalamus	Thalamus	S4-S5
TOp	Tractus Opticus	Optic tract	S4
VL	Ventriculus Lateralis	Lateral ventricle	S4-S5
Vm	Vermis	Vermis	S6
VQ	Ventriculus Quartus	Fourth ventricle	S7
VT	Ventriculus Tertius	Third ventricle	S4-S5

5. Extended gene expression and histological results

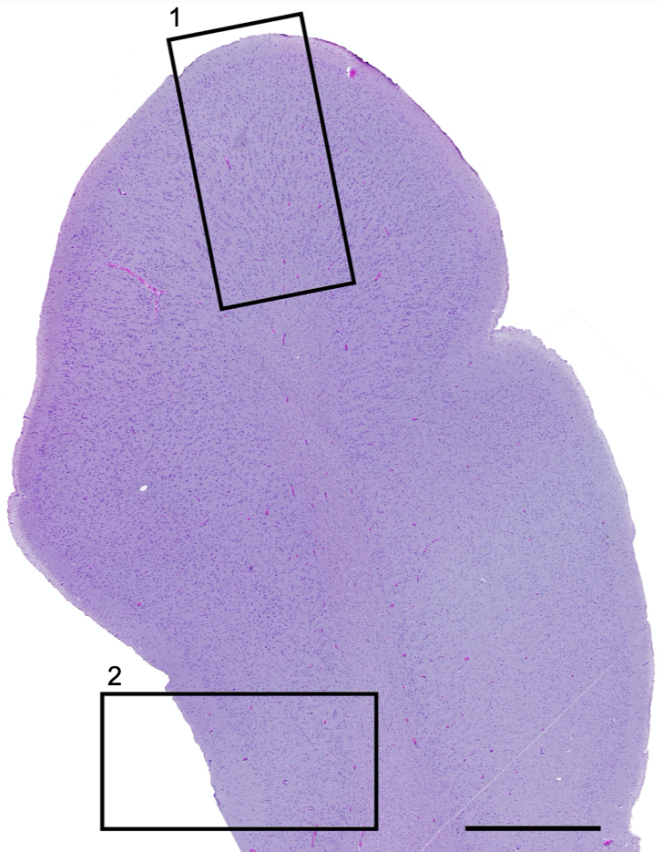

Figure S9. Structural overview of one of the three randomly collected cortical samples (each one of which included gyral and sulcal matter). Inset box 1 (gyral section) shows the anatomical location of panel A-C in *Figure S10* and inset box 2 (sulcal section) shows the anatomical location of panel D-F of *Figure S10*. Scale bar represents 1500 μm.

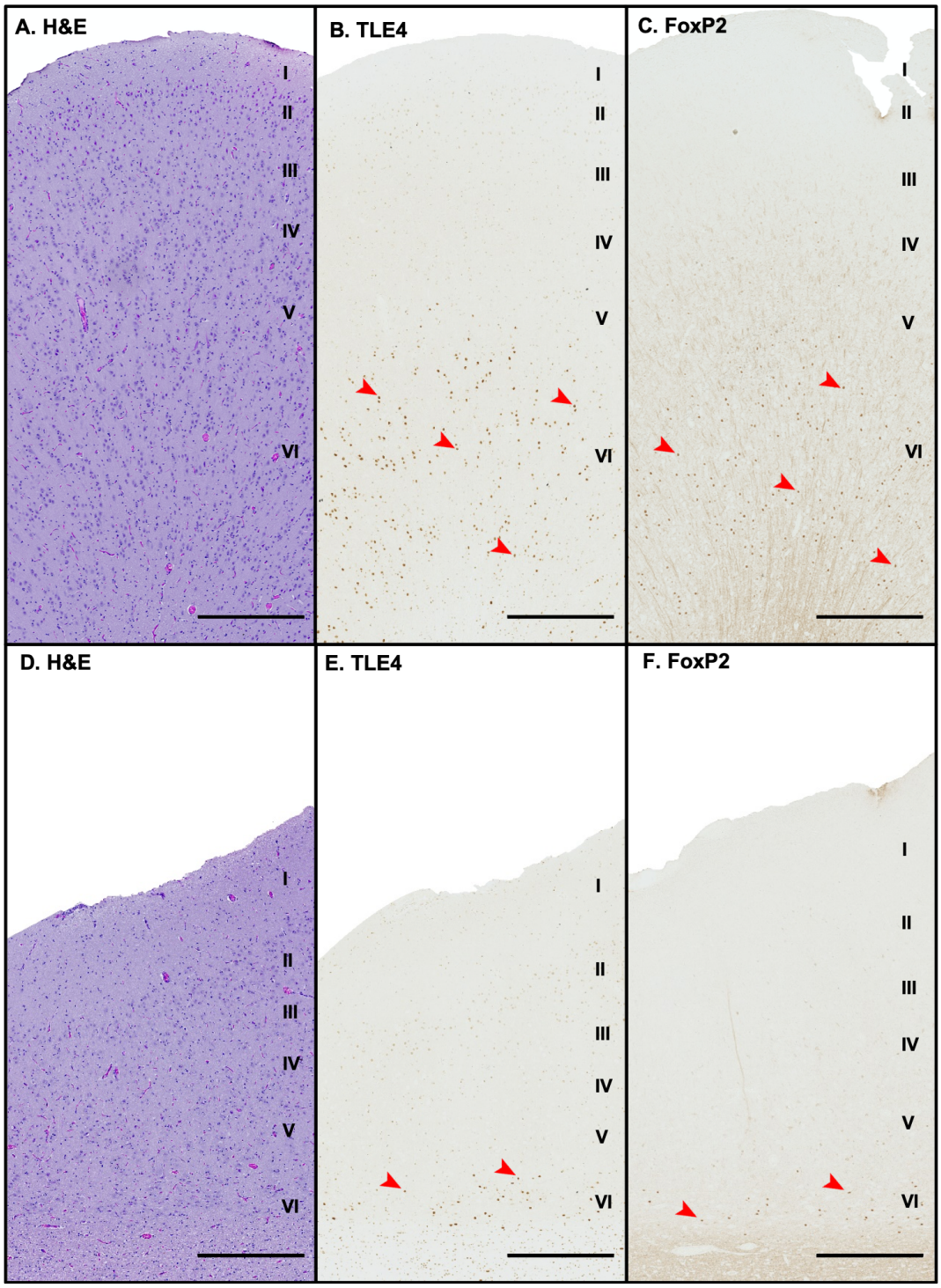

Figure S10. Laminar organization and FoxP2 expression in the grey seal cortex. Panels A-C show hematoxylin and eosin, TLE4, and FoxP2 staining taken from the gyral section (1) in *Figure S9*; panels D-F show hematoxylin and eosin, TLE4, and FoxP2 staining taken from the sulcal section (2) in *Figure S9*. The cortex is broader in the gyral section than in the sulcal section, and the lower layers of the cortex, specifically, are much reduced in size in the sulcal section, whereas Layer 1 size is increased in the sulcal section. A similar pattern was observed in harbor seals [12]. The TLE4 SC antibody (panel B and E) is commonly used as a layer 6 marker [13] and in all 3 cortical blocks tested from the same animal was seen to stain the deeper layers of the seal cortex. The FoxP2 N16 antibody (panel E and F) and the FoxP2 73A/8 antibody (not pictured) showed comparable signals in the deeper layers throughout the 3 cortex blocks interrogated from the same animal. Red arrows indicate examples of positively stained cells. Scale bars represent 500 µm.

6. Supplemental references

1. Osinga N, Hart PT, van Voorst Vader P. 2010 Albinistic common seals (Phoca vitulina) and melanistic grey seals (Halichoerus grypus) rehabilitated in the Netherlands. *Anim. Biol.* **60**, 273–281. (doi:10.1163/157075610X516493)
2. Rubio-García A, Contreras GJS, Acosta CJ, Lacave G, Prins P, Marck K. 2015 Surgical treatment of osteoarthritis in harbor seals (Phoca vitulina). *J. Zoo Wildl. Med.* **46**, 553–559. (doi:10.1638/2014-0229.1)
3. Pfefferbaum A, Sullivan E V, Adalsteinsson E, Garrick T, Harper C. 2004 Postmortem MR imaging of formalin-fixed human brain. *Neuroimage* **21**, 1585–1595. (doi:10.1016/j.neuroimage.2003.11.024)
4. D'Arceuil H, de Crespigny A. 2007 The effects of brain tissue decomposition on diffusion tensor imaging and tractography. *Neuroimage* **36**, 64–68. (doi:10.1016/j.neuroimage.2007.02.039)
5. Rorden C, Brett M. 2000 Stereotaxic display of brain lesions. *Behav. Neurol.* **12**.
6. Jenkinson M, Beckmann CF, Behrens TEJ, Woolrich MW, Smith SM. 2012 FSL. *Neuroimage* **62**, 782–790. (doi:10.1016/j.neuroimage.2011.09.015)
7. Fedorov A *et al.* 2012 3D Slicer as an image computing platform for the Quantitative Imaging Network. *Magn. Reson. Imaging* **30**, 1323–1341. (doi:10.1016/j.mri.2012.05.001)
8. Dawe RJ, Bennett DA, Schneider JA, Vasireddi SK, Arfanakis K. 2009 Postmortem MRI of human brain hemispheres: T2relaxation times during formaldehyde fixation. *Magn. Reson. Med.* **61**, 810–818. (doi:10.1002/mrm.21909)
9. Yushkevich PA, Gao Y, Gerig G. 2016 ITK-SNAP: An interactive tool for semi-automatic segmentation of multi-modality biomedical images. In *Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBS*, pp. 3342–3345. Institute of Electrical and Electronics Engineers Inc. (doi:10.1109/EMBC.2016.7591443)
10. Li L *et al.* 2019 The COMPASS Family Protein ASH2L Mediates Corticogenesis via Transcriptional Regulation of Wnt Signaling. *Cell Rep.* **28**, 698-711.e5. (doi:10.1016/j.celrep.2019.06.055)
11. Rodenas-Cuadrado PM, Mengede J, Baas L, Devanna P, Schmid TA, Yartsev M, Firzlaff U, Vernes SC. 2018 Mapping the distribution of language related genes FoxP1 , FoxP2 , and CntnaP2 in the brains of vocal learning bat species. *J. Comp. Neurol.* **526**, 1235–1266. (doi:10.1002/cne.24385)

12. Rioch DM. 1937 A physiological and histological study of the frontal cortex of the seal (phoca vitulina). *Biol. Bull.* **73**, 591–602. (doi:10.2307/1537617)
13. Yao J, Liu Y, Husain J, Lo R, Palaparti A, Henderson J, Stifani S. 1998 Combinatorial expression patterns of individual TLE proteins during cell determination and differentiation suggest non-redundant functions for mammalian homologs of Drosophila Groucho. *Dev. Growth Differ.* **40**, 133–146. (doi:10.1046/j.1440-169X.1998.00003.x)