**Supporting Information**

Mehdi Behroozi\*1, Brendon K. Billings\*2, Xavier Helluy1,3, Paul R. Manger2, Onur Güntürkün1,4, and Felix Ströckens1. Functional MRI in the Nile crocodile: A new avenue for evolutionary neurobiology. *Proceeding of the Royal Society B.* doi: 10.1098/rspb.2018.0178

**Supplementary methods**

***Preparation of Crocodiles:*** Five juvenile Nile crocodiles (*Crocodylus niloticus*, body length 61-73 cm), supplied by the crocodile farm “La ferme aux crocodiles” (Pierrelatte, France) and housed in the zoo of Bochum were used in this study. All animals were returned to the crocodile farm after the experiments. Crocodiles were housed together in a 200 cm x 250 cm terrarium containing both water and land sections, specifically designed to accommodate their natural environment to keep stress levels low. Air and water temperature within the terrarium were kept constant at 24˚C and 26˚C, respectively. Humidity was kept at 60%. Animals were fed two times a week with chicken or fish. Since animals never finished all of the food offered, we assume that they were fully sated when entering the experiment. Before the experiments, individual animals were transported to the scanner center of Bochum University in an insulated transport box. Before the scans, animals were mildly sedated with an intramuscular injection of medetomidine (0.5-1 mg/kg) on the ventral aspect of the tail. Previous studies have shown that medetomidine, an α-2 adrenergic agonist, has only a weak effect on BOLD signals and is thus widely used in fMRI studies [1]. The animal was then placed in a closed Styrofoam box on a heating blanket for 30 min to limit physiological stress and increase the efficacy of drug delivery. The animals were then secured in a custom made holding device which was placed in the scanner (Fig. 1A). Post scanning, animals received intramuscular injections of Atipamezol (Antisedan©, 1 mg/kg) in the forelimb to counteract the effects of sedation. After returning the animal to the zoo, it was kept in a separate terrarium for 24h to allow a complete recovery from sedation.

**MRI protocols**

Before running the anatomical and functional sequences, we measured the longitudinal (T1) and transverse (T2 and T2\*) relaxation times of crocodile brain tissue using exactly the same imaging parameters as in [2] in order to optimize the tissue contrast in the anatomical RARE images and the BOLD sensitivity of the GE-EPI sequence.

As in [2], T1 measurements were carried out using a multiple spin echo saturation recovery method with variable TR (RAREVTR). The following imaging parameters were used: effective echo time (TE) = 53.3 ms; TR-array = 0.300, 0.530, 0.800, 1.100, 1.460, 1.900, 2.460, 3.250, 4.570, 10s; number of average = 2; RARE factor = 14; matrix size = 256×256; field of view (FoV) = 40×40 mm2; slice thickness = 1 mm; number of slices = 1 coronal slice (to avoid interslice signal modulation); read orientation = Left-Right (L-R). The total acquisition time for T1 measurements was 15 min 50 s.

T2 values were measured using the Bruker MSME sequence. Imaging parameters were as follows: repetition time (TR) = 3s with an effective spatial bandwidth of 60 kHz; no averaging; number of slices = 1; number of echoes = 16 with first echo time = 10.73ms. The geometry was kept identical to the geometry in T1 measurements. The total acquisition time to measure T2 images was 12 min 48 s.

For T2\* measurements the multiple gradient echo (MGE) sequence of Bruker was used with TR=2.5 s; first echo time = 3 ms; echo spacing = 4 ms; number of echo images = 20; Excitation pulse angle = 60o. The geometry was kept identical to the geometry in T1 measurements.

While scanning animals, an infrared MRI compatible camera (MRC Systems GmbH, 12M-i) was used to monitor the behavior of the animal.

**Data Processing**

T1, T2, and T2\* relaxation times constant were estimated voxelwise by nonlinear least square data fitting using the image sequence analysis (ISA) toolbox of Paravision 5.1 (Bruker BioSpec, Germany). The T2 and T2\* values were estimated using the fit function:, where A is the absolute bias, C the signal intensity, T2 the transverse relaxation time and t the variable echo times. The T1 values were determined by fitting the saturation recovery (SR) fit function, y(t) = M0 (1-exp(-t/T1)), where M0 is the equilibrium magnetization and t the saturation recovery time. Figure S2 summarizes the in vivo T1 and T2 relaxation time maps for selected slices in the crocodile forebrain.

GE-EPI is the most common fMRI sequence used to investigate primate brain functions. Since, no data on the GE-EPI sequence behavior in cold blooded animal brains (in this case crocodiles) has been reported before, the spatial noise behavior and temporal stability of the MR signal was assessed by measuring time series of resting state scans for the duration of 10 minutes. These time series were analyzed at each voxel position for three metrics: signal-to-noise ratio (SNR), temporal SNR (tSNR), and signal-noise separation (SNS) [3]. To evaluate the quality of the data, the resting state data were processed in the same way as the task based data except that linear detrending and high-pass filtering (f>0.005) were done using the REST toolbox in MATLAB [4]. In order to calculate SNR and SNS, a region of interest (ROI) was defined in an artifact free region outside the brain. For each voxel, SNR was calculated as the mean signal of the time series divided by the standard deviation of all voxels of the background ROI (). Since the distribution of image intensity in magnetic resonance magnitude images is governed by a Rician distribution, the standard deviation of the background ROI was corrected using the relation to estimate the true noise power of the original complex data [5,6]. To evaluate the temporal stability of the MR signal, tSNR in each voxel was calculated as the ratio of the time-series mean by the time-series standard deviation (). SNS is defined as the Pearson correlation coefficient between the time series of single voxels within the brain and an average time series of voxels outside the imaged object.

Figure S1 shows a set of gray scale and color coded images depicting SNR, tSNR, and SNS maps used to assess the quality of the fMRI data. SNR and tSNR in the regions of interest were high and ranged from 300 to 500 and 200 to 300 respectively, after pre-processing. As illustrated in Figure S1e, there was no correlation between the chosen noise-ROI and the signals from voxels in brain tissue, indicating good SNS.

**Supplementary results**

**Temporal properties of the BOLD response**

The temporal properties of BOLD responses were not only modulated by stimulus type. During binocular visual stimulation there was a statistically significant difference between the onset latency of activation between cVsI and Rt. cVsI had a longer onset latency compared to Rt and a later offset persisting beyond the stimulation period by approximately 4s (Fig.2). Differences between the BOLD response of these two regions could be attributed to differences in neuronal density [7,8], anatomical topographic location [9] and vascularization, such as differences in the dynamic properties of the local regulation of cerebral blood flow and volume [10]. The peak time of BOLD responses (12-24s) in the visual ADVR of the crocodile brain differs considerably from studies in the visual cortex of monkey [11,12] in which BOLD signal reaches a peak at (7-10s) after stimulus onset.

The dynamic aspect of BOLD response in primary auditory regions of crocodiles are in agreement with the time lines established in the primary auditory system of birds [13]. In birds BOLD response peaks that range from 5-10s in Field L are very similar to those observed in the crocodile (6-10 s after stimulus onset for cAuI, cAuII). Data on the hemodynamic response of higher order auditory regions of birds [13] report BOLD response peaks after about 10-20s in NCM. These values are longer than the initial peak time in the comparable region of the crocodile brain (cAuIII, 4-7s). In terms of signal strength, the maximum BOLD signal change we observed in crocodiles was 0.7% using a gradient echo EPI sequence with TE=30ms at 7T scanner which is much lower than the signal strength in mammals like monkeys ([14] auditory stimulation, maximum BOLD change 3%) or humans ([15], auditory stimulation, maximum BOLD change 2%). In rats, strong BOLD responses (>1%) have been reported at 7T [16] and 11.5T [17] using EPI at echotimes of 15 to 19ms. In mice, Niranjan et al [18] reports max BOLD >1% at 9.4T with TE=19ms. Also in birds, BOLD signal changes after auditory stimulation are higher than in crocodiles (e.g. [13], European starling, auditory stimulation, change 3%). This difference can be likely attributed to the reptilian nature of the Nile crocodile. In contrast to homoeothermic mammals and birds, a reptile has a metabolic rate, breathing rate, cardiac output and blood pressure which are much more temperature dependent [19–25]. All these factors likely have an impact on BOLD signal strength. At a temperature level of approximately 23-24 degrees Celsius used in our study, all these characteristics were in comparison to mammals/birds reduced in our crocodiles which could be one explanation for the relatively low BOLD signal strength. Furthermore, the four chambered heart as well as the classic cardiac shunts common to reptiles with the consequent admixture of oxygenated and deoxygenated blood [24,26], are likely to have an effect on BOLD strength as well. Also, differences in hemoglobin binding mechanisms [27–29] blood viscosity [29] and oxidative energy requirements of tissue, all altering blood oxygen transport, between endotherms and ectotherms surely have an effect on BOLD signal strength. However, data on these factors in relation to BOLD signal composition are not available yet and trying to assign an effect size to each of the characteristics would be purely speculative at the current time point. Future fMRI studies in reptiles might consider using a higher spatial resolution or BOLD sensitive sequences based on Spin Echoes, which will result in more specific BOLD brain activation and for small regions in a higher BOLD signal strength.

Taken together, the latency of initial BOLD responses in the auditory and visual systems is much shorter in mammals compared to reptiles and birds. Differences in the temporal aspects of BOLD responses between mammals, birds, and reptiles might be related to a variety of physiological differences including vascular reactivity, neurovascular coupling, body temperature, brain size, neuronal processing, and also fMRI techniques employed. Thus, further cross class fMRI studies are required to understand these differences in detail.

In our study, we could not pick up any significant change in BOLD signal from the thalamofugal system in response to visual stimulation. This could have several reasons. In pigeons, the thalamofugal visual system is specialized for lateral visual field view while the tectofugal system mainly processes stimuli in the frontal visual field [30]. Since the light stimulus in the current study was positioned in the frontal visual field of the crocodile (Figure 1A), it is possible that we simply did not stimulate pathways that represent the lateral field of view sufficiently. Alternatively, the thalamofugal system could have been activated but due to the typical sparsity of spiking in the cortex, as demonstrated in turtles, visual responses might be rapidly adapting in this region (see e.g. [31,32]). Since the temporal resolution of fMRI is limited (in our case 2 sec) it might have not been sufficient to record any short lasting activity in the cortex. In this case, the expected activation of the lateral geniculate nuclei might have been overshadowed by the high activity of nucleus rotundus which lies in very close proximity to the lateral geniculate and might not be separable from it due to the relatively low spatial resolution of fMRI.

**Removal of further breathing artifacts**

Despite solving the motion artifact caused by respiration, whole brain signal modulation was observed as a result of the deep breathing bouts common to crocodiles (period of occurrence at ~20 min intervals, Figure S04). By detecting retrospectively the onsets and ends of those respiratory modulations in the whole brain BOLD signal, we could identify the time points compromised by deep respiration bouts. Because the amplitude of these signal distortions was unpredictable and larger than the BOLD signals investigated, we removed from the data analysis all the time points affected by these respiratory events. By precaution, we removed in addition all time points 8 seconds before the start and 8 seconds after the end of the deep breathing bouts signal modulations.

**Effect of Echo time on BOLD signal**

The BOLD contrast depends on the echo time (TE) and it is generally accepted that a TE close to the T2\* relaxation time is the desired value for most neuroimaging studies [33]. To test the validity of the BOLD signal, we also checked the effect of echo time on the evoked BOLD response in crocodilians. We scanned the animal brain with a short TE=18.6ms and long TE=30ms (which was close to our estimation of T2\* relaxation time of the crocodile brain, T2\* is between 30-45ms) when classical music was presented to the animals. The results showed that a short echo time increased signal to noise ratio, and reduced the susceptibility artifact but also reduced the amplitude of the BOLD signal (Figure S05) which is in line with the mammalian study conducted by Gorno-Tempini et al. [34]. Echo time of 30ms was used for ? all following fMRI experiments.

***Monocular visual stimulation***

The monocular visual stimuli were presented with optic fibers strategically positioned to present light to one eye only. The eyes were neither taped close nor were the eyelids taped open to reduce the amount of physiological stress.

To confirm our findings, we also performed monocular visual stimulation using the red 8 Hz stimulus in 4 subjects. Since the optic nerve of crocodiles decussates virtually completely at the optic chiasm [35,36] we only expected a contralateral activation to the stimulated eye. Indeed, monocular stimulation only evoked a BOLD response in the contralateral hemisphere, with the distribution of activated voxels within each hemisphere being identical to the binocular condition (Figure S06). In this monocular stimulation the BOLD response persisted after the stimulus period and reached a peak at 23.1±2.6s, identical to the binocular condition (two-sample t-test, p>0.05); however, in contrast to the binocular stimulation, no Rt activity was recorded during monocular visual stimulation.The lack of Rt responses in the monocular stimulation paradigm appears to be related to the observation that the Rt receives bilateral input from the optic tectum [37]. As monocular stimuli only activated the optic tectum contralateral to the stimulus, Rt will only receive unilateral input, resulting in lower Rt activation in comparison to a binocular stimulation paradigm, which is likely to be under the detection threshold of our protocol.

**References**

1. Pawela CP, Biswal BB, Hudetz AG, Schulte ML, Li R, Jones SR, Cho YR, Matloub HS, Hyde JS. 2009 A protocol for use of medetomidine anesthesia in rats for extended studies using task-induced BOLD contrast and resting-state functional connectivity. *Neuroimage* **46**, 1137–47. (doi:10.1016/j.neuroimage.2009.03.004)

2. Behroozi M, Chwiesko C, Ströckens F, Sauvage M, Helluy X, Peterburs J, Güntürkün O. 2017 In vivo measurement of T1 and T2 relaxation times in awake pigeon and rat brains at 7T. *Magn. Reson. Med.* (doi:10.1002/mrm.26722)

3. Shirer WR, Jiang H, Price CM, Ng B, Greicius MD. 2015 Optimization of rs-fMRI Pre-processing for Enhanced Signal-Noise Separation, Test-Retest Reliability, and Group Discrimination. *Neuroimage* **117**, 67–79. (doi:10.1016/j.neuroimage.2015.05.015)

4. Song X-W, Dong Z-Y, Long X-Y, Li S-F, Zuo X-N, Zhu C-Z, He Y, Yan C-G, Zang Y-F. 2011 REST: A Toolkit for Resting-State Functional Magnetic Resonance Imaging Data Processing. *PLoS One* **6**, e25031. (doi:10.1371/journal.pone.0025031)

5. Gudbjartsson H, Patz S. 1995 The rician distribution of noisy mri data. *Magn. Reson. Med.* **34**, 910–914. (doi:10.1002/mrm.1910340618)

6. Triantafyllou C, Hoge RD, Krueger G, Wiggins CJ, Potthast A, Wiggins GC, Wald LL. 2005 Comparison of physiological noise at 1.5 T, 3 T and 7 T and optimization of fMRI acquisition parameters. *Neuroimage* **26**, 243–250. (doi:10.1016/j.neuroimage.2005.01.007)

7. Olkowicz S, Kocourek M, Lučan RK, Porteš M, Fitch WT, Herculano-Houzel S, Němec P. 2016 Birds have primate-like numbers of neurons in the forebrain. *Proc. Natl. Acad. Sci.* **113**, 7255–7260. (doi:10.1073/pnas.1517131113)

8. Ngwenya A, Patzke N, Manger PR, Herculano-Houzel S. 2016 Continued Growth of the Central Nervous System without Mandatory Addition of Neurons in the Nile Crocodile (Crocodylus niloticus). *Brain. Behav. Evol.* **87**, 19–38. (doi:10.1159/000443201)

9. Schmolesky MT, Wang Y, Hanes DP, Thompson KG, Leutgeb S, Schall JD, Leventhal AG. 1998 Signal timing across the macaque visual system. *J. Neurophysiol.* **79**, 3272.

10. Kim S-G, Ogawa S. 2012 Biophysical and physiological origins of blood oxygenation level-dependent fMRI signals. *J. Cereb. Blood Flow Metab.* **32**, 1188–206. (doi:10.1038/jcbfm.2012.23)

11. Logothetis NK, Guggenberger H, Peled S, Pauls J. 1999 Functional imaging of the monkey brain. *Nat. Neurosci.* **2**, 555–62. (doi:10.1038/9210)

12. Logothetis NK, Pauls J, Augath M, Trinath T, Oeltermann A. 2001 Neurophysiological investigation of the basis of the fMRI signal. *Nature* **412**, 150–157. (doi:10.1038/35084005)

13. Van Meir V *et al.* 2005 Spatiotemporal properties of the BOLD response in the songbirds’ auditory circuit during a variety of listening tasks. *Neuroimage* **25**, 1242–1255. (doi:10.1016/j.neuroimage.2004.12.058)

14. Baumann S, Griffiths TD, Rees A, Hunter D, Sun L, Thiele A. 2010 Characterisation of the BOLD response time course at different levels of the auditory pathway in non-human primates. *Neuroimage* **50**, 1099–1108. (doi:10.1016/j.neuroimage.2009.12.103)

15. Backes WH, Van Dijk P. 2002 Simultaneous sampling of event-related BOLD responses in auditory cortex and brainstem. *Magn. Reson. Med.* **47**, 90–96. (doi:10.1002/mrm.10015)

16. Van Camp N, Verhoye M, De Zeeuw CI, Van der Linden A. 2006 Light stimulus frequency dependence of activity in the rat visual system as studied with high-resolution BOLD fMRI. *J Neurophysiol* **95**, 3164–3170. (doi:00400.2005 [pii]\r10.1152/jn.00400.2005)

17. Bailey CJ, Sanganahalli BG, Herman P, Blumenfeld H, Gjedde A, Hyder F. 2013 Analysis of time and space invariance of BOLD responses in the rat visual system. *Cereb. Cortex* **23**, 210–222. (doi:10.1093/cercor/bhs008)

18. Niranjan A, Christie IN, Solomon SG, Wells JA, Lythgoe MF. 2016 fMRI mapping of the visual system in the mouse brain with interleaved snapshot GE-EPI. *Neuroimage* **139**, 337–345. (doi:10.1016/j.neuroimage.2016.06.015)

19. Grigg GC, Cairncross M. 1980 Respiratory properties of the blood of Crocodylus porosus. *Respir. Physiol.* **41**, 367–380. (doi:10.1016/0034-5687(80)90083-3)

20. Glass ML, Johansen AK. In press. Periodic Breathing in the Crocodile, Crocodylus niloticus: Consequences for the Gas Exchange Ratio and Control of Breathing.

21. Smith EN. 1979 Behavioral and physiological thermoregulation of crocodilians. *Integr. Comp. Biol.* **19**, 239–247. (doi:10.1093/icb/19.1.239)

22. Perry SF. 1988 Functional Morphology of the Lungs of the Nile Crocodile, Crocodylus niloticus: Non-Respiratory Parameters. *J. Exp. Biol.* **117**, 99–117.

23. Perry SF. 1990 Gas exchange strategy in the Nile crocodile: a morphometric study. *J. Comp. Physiol. B* **159**, 761–769. (doi:10.1007/BF00691722)

24. Axelsson M, Franklin C, L&Ouml;Fman C, Nilsson S, Grigg G. 1996 Dynamic anatomical study of cardiac shunting in crocodiles using high-resolution angioscopy. *J. Exp. Biol.* **199**, 359–65.

25. Seebacher F, Franklin CE. 2004 Cardiovascular mechanisms during thermoregulation in reptiles. *Int. Congr. Ser.* **1275**, 242–249. (doi:10.1016/j.ics.2004.08.050)

26. Hicks JW. 2002 The Physiological and Evolutionary Significance of Cardiovascular Shunting Patterns in Reptiles. *Physiology* **17**, 241–245. (doi:10.1152/nips.01397.2002)

27. Bauers C, Forster M, Gros G, Mosca A, Perrella M, Rollema HS, Vogel D. 1981 Analysis of Bicarbonate Binding to Crocodilian Hemoglobin\*. *J. B~OLOOICAL Chem.* **256**, 8429–8435.

28. Brittain T, Wellst RM. 1991 AN INVESTIGATION OF THE CO-OPERATIVE FUNCTIONING OF THE HAEMOGLOBIN OF THE CROCODILE, CROCOD YLUS POROSUS. *Biochem. PhysioL* **98**, 641–646.

29. Wells RMG, Beard LA, Grigg GC. 1991 BLOOD VISCOSITY AND HEMATOCRIT ESTUARINE CROCODILE, CROWD YLUS. *Camp. B&hem. Physiol* **99**, 41–414.

30. Hellmann B, Güntürkün O. 1999 Visual-field-specific heterogeneity within the tecto-rotundal projection of the pigeon. *Eur. J. Neurosci.* **11**, 2635–2650. (doi:10.1046/j.1460-9568.1999.00681.x)

31. Naumann RK, Laurent G. 2017 1.25 – Function and Evolution of the Reptilian Cerebral Cortex. In *Evolution of Nervous Systems*, pp. 491–518. Elsevier. (doi:10.1016/B978-0-12-804042-3.00022-1)

32. Fournier J, Müller CM, Schneider I, Laurent G. 2018 Spatial Information in a Non-retinotopic Visual Cortex. *Neuron* **97**, 164–180.e7. (doi:10.1016/j.neuron.2017.11.017)

33. Triantafyllou C, Hoge RD, Krueger G, Wiggins CJ, Potthast A, Wiggins GC, Wald LL. 2005 Comparison of physiological noise at 1.5 T, 3 T and 7 T and optimization of fMRI acquisition parameters. *Neuroimage* **26**, 243–250. (doi:10.1016/j.neuroimage.2005.01.007)

34. Gorno-Tempini ML, Hutton C, Josephs O, Deichmann R, Price C, Turner R. 2002 Echo time dependence of BOLD contrast and susceptibility artifacts. *Neuroimage* **15**, 136–42. (doi:10.1006/nimg.2001.0967)

35. Bruce LL. 2007 Evolution of Nervous Systems in Reptiles. *Evol. Nerv. Syst.* , 125–156. (doi:10.1016/B0-12-370878-8/00130-0)

36. Derobert Y, Médina M, Rio JP, Ward R, Repérant J, Marchand MJ, Miceli D. 1999 Retinal projections in two crocodilian species, Caiman crocodilus and Crocodylus niloticus. *Anat. Embryol. (Berl).* **200**, 175–191. (doi:10.1007/s004290050271)

37. Güntürkün O, Stacho M, Ströckens F. 2017 The Brains of Reptiles and Birds. In *Evolution of Nervous Systems*, pp. 171–221. Elsevier. (doi:10.1016/B978-0-12-804042-3.00007-5)

**Supporting figures**

R:\Mehdi_PhD\Projects\Running Projects\Crocodile\text\Figures\FigureS2.tif

**Figure S01-** Assessment of data quality. (a) Representative anatomical images and (b) GE-EPI images from a single subject. Furthermore, (c) maps of SNR, (d) tSNR, and (e) SNS of resting state data were acquired using the same single shot- GE-EPI sequence as for task-fmri. Images were processed in the same way as the task based data except that linear detrending and high-pass filtering (f>0.005) were done using the REST toolbox in MATLAB. The SNS map demonstrated no correlation between the time course of the background noise ROI and the time course of single voxels within the brain.

R:\Mehdi_PhD\Projects\Running Projects\Crocodile_fMRI\text\Figures\Figure_S02.tif

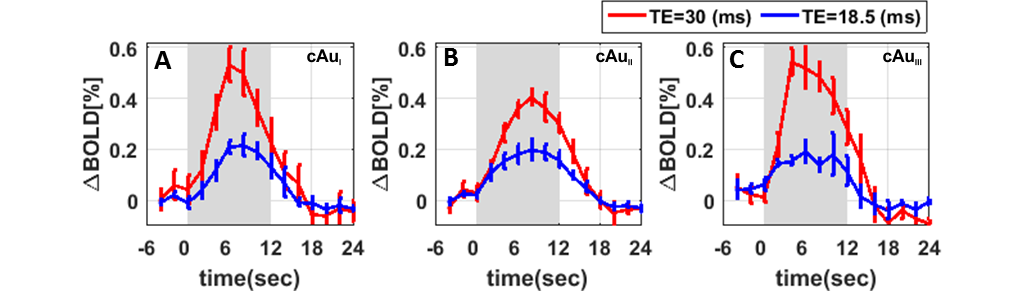
**Figure S02-** T1 and T2 relaxometry maps of the Nile crocodile brain. (a) A coronal image (left) acquired with the RARE sequence at 7T to indicate relevant ROIs. Furthermore, T1 (middle) and T2 relaxation maps (right) are depicted. Note the almost absent separation between gray and white matter in the forebrain. The selected ROIs were anterior dorsal ventricular ridge (ADVR), nucleus dorsomedialis anterior (Dma), general/dorsal cortex (DC), Hippocampus (Hipp), Hypothalamus (Hyp), posterior dorsal ventricular ridge (PDVR), Nucleus rotundus (Rt) and Ventricle (V). T1 relaxation times in these regions were (ADVR) 1.90±0.07, (Dma) 1.72±0.09, (DC) 1.85±0.29, (Hipp) 1.78±0.12, (Hyp) 164±0.12, (PDVR) 1.79±0.13, (Rt) 1.65±0.07 and (V) 3.22±0.31s (mean±SD). T2 relaxation times were 70.64+2.53, (Dma) 66.71+3.2, (DC) 70.55+3.09, (Hipp) 64.56±2.22, (Hyp) 67.83±1.96, (PDVR) 68.93±2.08, (Rt) 62.35±2.14 and (V) 336.14±11.14ms (mean±SD). Standard deviation represents variations within a selected ROI.

R:\Mehdi_PhD\Projects\Running Projects\Crocodile_fMRI\text\Figures\Figure_S03.tif

**Figure S03-** Plots of the 10x down-scaled translational (left) and rotational (right) motion parameters estimated by the toolbox MCFLIRT, FSL 4.0 for each of the five mildly sedated crocodiles used in this study (A-E). Data stability was estimated by a 3D rigid body model with six degrees of freedom for translation and rotation. Blue, green, and red lines correspond to the direction of movements in x, y, and z-direction. Mean motion across all animals in any direction was 0.018 ± 0.003mm (mean ± SD).

R:\Mehdi_PhD\Projects\Finished Project\Crocodile_fMRI\text\RoyalSociety\ProcB Submission\revision\Figure_S04.tif

Figure S04- BOLD signal intensity in (A) the auditory region (cAuI) during display of Bach music and (B) in the visual region (cVsI) during presentation of flashing red light at a 5Hz frequency. The red circles outline artifacts of the BOLD signal caused by the deep breathing bouts common to crocodiles. The affected volumes at the time of breathing were removed from the fmri analysis.



**Figure S05-** BOLD responses in three auditory regions of the Nile crocodile (A: cAuI, B: cAuII, C: cAuIII) in response to Bach music, measured with two different echo times TE=18.5ms (blue) and TE=30 ms (red). TE close to the T2\* relaxation time is the desired value for GE-EPI sequences to detect BOLD signal. The result depicts that short TE reduced the amplitude of the BOLD signal. To increase sensitivity, TE of 30ms was used in the following experiments.

R:\Mehdi_PhD\Projects\Finished Project\Crocodile_fMRI\text\RoyalSociety\ProcB Submission\revision\Figure_S06.tif**Figure S06-** Visual activation maps of the Nile crocodile brain during monocular visual stimulation. BOLD response patterns to monocular stimulation with red light at an 8 Hz flickering frequency of the left (A) and right eye (B) are depicted. Z score maps show group results for the stimulation period vs dark period comparison (mixed effect model, FLAME1+2, p<0.05, N=4). BOLD responses were only detectable in the hemisphere contralateral to the stimulated eye. Graphs in the lower right corner in A and B show changes in BOLD signal before, during and after the stimulus period (highlighted in grey). Error bars represent SEM.

R:\Mehdi_PhD\Projects\Finished Project\Crocodile_fMRI\text\RoyalSociety\ProcB Submission\revision\Figure_S07.tif

**Figure S07-** Image series acquired with a fast multiple spin-echo (RARE) sequence at 7T from an ex-vivo Nile crocodile brain. The spatial resolution is 80×80×98 µm3. Scan time was 51h. In the upper row, areas identified in this study are outlined. The lower row shows the measured BOLD signal mapped on the structural MRI images. The identified areas overlap well with fiber rich areas (darker image contrast) within the DVR, likely representing termination fields of diencephalic projection fibers. Also the diencephalic nucleus rotundus is well visible in the structural MRI picture and does overlap with the measured BOLD signal.

**Movie S01-** Real-time MRI movie of a crocodile head using a single-slice coronal 2D TrueFISP sequence with a frame duration of 326ms and total acquisition time of 10min 52s. Observe anatomical changes in the larynx during burst- and deep-breath events while the brain remains free of any motion. The dark bands outside of the brain are typical TrueFISP banding artifacts caused by off-resonant magnetization outside of the shimmed region.