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Supplemental Information

A Functionally and Anatomically Bipartite Vocal Pattern Generator in the Rat Brain Stem

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Transparent Methods

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All animal procedures were performed in strict accordance with the Humboldt-University Animal Care Committee's regulations. Procedures were approved by the State Office for Health and Social Affairs committee in Berlin (Protocol No. G0279/18). Male Long-Evans rats were provided by Janvier Laboratories (Le Genest-Saint-Isle, France). Experiments were done on adult animals (age $\geqslant$ five weeks).

METHOD DETAILS

Surgery

Animals (n=13) were anesthetized using intraperitoneal injections of urethane (1.5g/kg bodyweight). The skin covering the skull was shaved and the animals head was fixed in a stereotactic frame. Body temperature was maintained at 36°C using a rectal probe and a homeothermic blanket (FHC, 1201 Main St., Bowdoin, ME, 04287 USA). Before incision, we applied local anaesthesia with lidocaine 2%. The head was brought to level by using Bregma and Lambda measures. Four fixation screws were placed in the frontal bone and a head post was fixed to the screws and the bone using dental cement. For the cooling experiments, a first 2 x 1.5 mm craniotomy was placed along the longitudinal axis above the PAG (periaqueductal gray), centered 6.5 mm posterior and 0.75 mm lateral to Bregma. A 1 megaohm tungsten microelectrode (MicroProbes, 18247-D Flower Hill Way, Gaithersburg, MD, 20879 USA) was inserted into the left PAG 6.5 mm posterior and 0.75 mm lateral of Bregma in a depth of 4-4.5 mm depth. Ultrasonic vocalizations triggered by 1 second long, 100 Hz stimulation confirmed the correct placement of the electrode in the PAG. To expose the brainstem for the cooling and stimulation experiments, a craniotomy exposed the cerebellum by removing the

occipital bone 8 mm posterior and 4 mm bilateral of Bregma completely until the atlas (first vertebrae). Following a durotomy, the cerebellum was carefully removed using a vacuum pump (N86, KNF Neuberger GmbH, Alter Weg 3, 79112 Freiburg) connected to a glass pipet. The position of the obex was identified visually by the narrowing and surfacing of the 4th ventricle.

Brainstem cooling

Local cooling was achieved by a custom-build, fluid cooled Peltier element. A 4 x 4 mm Peltier element was outfitted with matching gold plate and an attached gold pin (0.5 mm diameter) on one side. The other side was cooled using a custom heatsink with chilled water running through it (Supplementary Figure 7). The cooling effect was measured with three thermistors on the contralateral side of the brainstem and during each trial, visual control ensured that cooling indeed reached the brainstem. Using three thermistors at different distances to the cooling probe allowed us to calculate the cooling strength. When applying 0.8 V and 0.825 A, we achieved a drop of 6°C at a distance of 2 mm to the tip after 45 seconds of cooling. These settings were used for all experiments to achieve the same tissue cooling.

The gold pin was then pseudo-randomly placed on the brainstem in a grid pattern ranging from 4 mm anterior of obex to 3 mm posterior of obex and from the midline up to 4 mm lateral of obex (Figure 1C). The grid spacing was 1 mm and we covered as many brainstem locations as possible. The gold pin was placed on the surface of the brainstem to prevent irreversible damage to the tissue.

At each cooling location, we initiated 29 electrical stimulations of the PAG to trigger calls. Stimulation consisted of a 1 second, 100 Hz stimulation of 220 ± 94 µA followed

by a 5 second inter-stimulus-interval and was controlled using PatchMaster (HEKA, Ludwigshafen/Rhein, HRB 41752). After stimulation 7, the Peltier element was activated and kept cooling the brainstem until after stimulation number 17 when it was deactivated and the brainstem rewarmed itself. Vocalizations were recorded with a condenser ultrasound microphone CM 16/CMPA (Avisoft-Bioacoustics e.K., Schoenfliesser Str. 83, 16548 Glienicke/Nordbahn, Germany) attached to an Avisoft Bioacoustic UltraSoundGate 416H and recorded using the Avisoft-RECORDER software.

Microstimulation of the brainstem

For stimulation experiments the cerebellum was removed as described above. We positioned the electrode in the same grid as described for the cooling above. Each position was chosen in a pseudorandom order to account for the duration of the experiment. At each location, we electrically stimulated the brainstem in 500 µm depth-steps starting dorsally. Stimulation was controlled using Spike2 (Cambridge Electronic Design Limited, 139 Cambridge Road, Milton, Cambridge, England) For each location, we determined if vocalizations could be triggered with a maximum current of 200 µA. After a mapping was completed, we placed electrolytic lesions (DC currents, tip neg, 8 µA, 10 s) the brainstem sites, at which we evoked the most robust vocalizations at the lowest thresholds. Then the animal was overdosed with 8 ml urethane followed by a transcardial perfusion (phosphate buffer followed by a 4% paraformaldehyde solution) and removal of the brain for histological processing.

Histology

To recover the lesion of the electrical stimulation experiments, the brains were sliced and stained as follows. After perfusion, the brains were fixed for 12-24h in 4% PFA in the fridge. Thereafter, they were sliced with a vibratome.

For Nissl staining, mounted brain sections (100 µm) were washed 2 minutes each in 90, 80 and 60% of ethanol in descending order, followed by 1 minute in aquadest kresyl-violet (1% kresyl-violet in NA-acetate buffer with a pH between 3.8 and 4.0) staining was applied for 40-50 seconds and then rinsed with aquadest for 30 seconds. For differentiation, we used 80% ethanol and 10% acetic acid, followed by 10-20 seconds in 96% ethanol. Before coverslips were added, brain sections were dehydrated with Isopropanol for 2 minutes and twice xylol for 5 minutes each. Pictures were taken on an Olympus BX51 microscope.

Myelin staining was performed on free-floating sections that were incubated for 2-4 hours in a 0.1% solution of gold in 0.02 M PB, ph7.4, and 0.9% sodium chloride. After staining, sections were rinsed for 5 minutes, fixed for 5 minutes in a 2.5% solution of sodium thiosulfate, and rinsed for 30 minutes before mounting with Mowiol.

For NeuN staining, floating brain sections (60 µm) were washed in 0.1 M phosphate buffer saline (PBS), followed by twice washing in 0.1 M PBS + 0.5% Triton-X. Blocking was done with 2.5% BSA + 0.75% Triton-X in 0.1 M PBS for one hour. Brain sections were then stored in 1% BSA + 0.3% Triton-X with NeuN (rabbit) 1:1000 concentration for 3 days on a shaker at 4°C. After 3 days, brain sections were washed 3 times with 0.1 M PBS. For the second antibody, we used Anti-NeuN (DAR rabbit polyclonal Fluor® 488 Conjugate LOT:3209767 by Millipore) with 1:200 concentration. The antibody was applied for 24 hours with 1% BSA in 0.1 PBS. After 3 washings with 0.1 PBS brain sections were mounted with Fluoromount® (Biozol, Eching, Germany)

mounting medium. Pictures were taken on a Leica DM5500B and subsequently adjusted with brightness and contrast.

Analysis of cell size was done manually with imageJ (Abràmoff et al., 2004). For each region, we sampled 600 cells, 50 per brain section in two animals. Overlapping and out of focus cells were not measured. Analyzed PCRtA ranged from Bregma -10.0 to -10.3 mm. VoPaRt, sp5, IrtA ranged from -10.5 to -11.2 mm. PCRt from -11.6 to -13.5 mm. Amb from -12.2 to -13.5 mm. MdD, MdV, Irt, NRA from -14.3 to -15.5 mm (all measures relative to Bregma).

Quantification of myelinization was done with imageJ as well. Measures were taken bilaterally of three brain sections in three brains each, resulting in 18 measures. We used a rectangle spanning from the lateral boarder of sp5 to the midline of the brainstem. The upper box limit was adjusted to be at the boarder of solitary tract (Sol) and the lower boarder touching the facial nucleus. We took the gray value profile along the horizontal axis. We interpolated each measure to a uniform length and normalized the values relative to the maximum myelinization of each brain section, resulting in a value between 0 and 1, where 1 always represented the maximum myelinization in the trigeminal tract and 0 the lowest measured myelinization elsewhere. The resulting normalized measures where then averaged and smoothed with a gaussian smoothing window of 10.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of Vocalizations

General analysis of cooling effects on calls was conducted with the automatic call detection and analysis tools of Avisoft SASLab Pro (Avisoft-Bioacoustics e.K., Schoenfliesser Str. 83, 16548 Glienicke/Nordbahn, Germany). For each cooling location, we analyzed calls evoked by three stimulations during the warm period, as well as the three last stimulations during the cooling period. Threshold for detection was set to -20 dB attenuation, call elements with less than 50 ms of interruption were considered one call. Number of evoked calls, maximum amplitude and maximum duration of calls was measured. For further analysis, we calculated the z-scores of the change between warm and cooled calls ((# calls cold - # calls warm)/(# calls cold - # calls warm)). Results were presented as a 2D map of the brainstem. For illustration purposes and to account for possible alignment differences between animals, the maps were smoothed with a Gaussian distribution. For Gaussian smoothing, we first increased the data matrix 18-fold and then applied a smoothing kernel with a standard deviation of 7. We included the raw data in unsmoothed maps in the supplementary section.

Maps depicting the cooling effects on high vs low frequency effects were scored manually. The scoring of the audio files was done blind, meaning that the cooling location of each file was unknown to the scoring person. Scoring done with Audacity (iWeb Media Ltd., Malta). The experimenters scored if high or low frequencies were affected by cooling in a subjective but repeatable fashion. The limits of this analysis are obvious and have thus been addressed in a second, objective analysis described below. As a threshold to discriminate between high and low frequencies we chose 30 kHz. This is a clear division between low frequency, fear calls and high frequency, positive calls (Brudzynski, 2005; Knutson et al., 2002; Panksepp and Burgdorf, 2003; Schwarting et al., 2007).

To extend upon this qualitative analysis, we conducted a second, quantitative analysis for our three focal locations, identical to the quantitative analysis of general call characteristics described above. The focus on a subset of trials and regions was necessary, since only trials were a single PAG stimulation induced both high and low frequency calls could be included in this analysis, greatly reducing the number of available trials (n=18). The number of high (>30 kHz) and low frequency (<30 kHz) calls was counted for 3 trials before cooling and the last three trials of cooling, identical to the general analysis of call effects. Next, the z-score between warm and cold periods was calculated and the data was grouped based upon their recording location (VoPaRt, NRA or central) and their frequency (high or low).

Analysis of brainstem stimulation evoked calls was done in a straightforward fashion. For each location, it was noted if a call could be evoked and if it was above of below 30 kHz. Generation of maps was done identical to the cooling results. If calls showed elements of both above and below 30 kHz, we omitted them from the frequency specific analysis. For analysis of the slope of calls, DeepSqueak (Coffey et al., 2019) was used.

Statistical analysis

We used MATLAB 2019b (Mathworks) for the statistical analysis of our data.

To test if the three defined regions are distinctly different from each other, we used the nonparametric Friedman test for paired data. For statistical testing, we used the raw, unsmoothed data. To obtain the specific differences between regions, we applied the Tukey-Kramer *post-hoc* test. For the analysis of the slope of calls upon brainstem stimulation, we used the Wilcoxon rank-sum test since some stimulation sites in the

center region did not evoke calls and thus, we could not measure the slope of the calls sufficiently.

Shuffling test

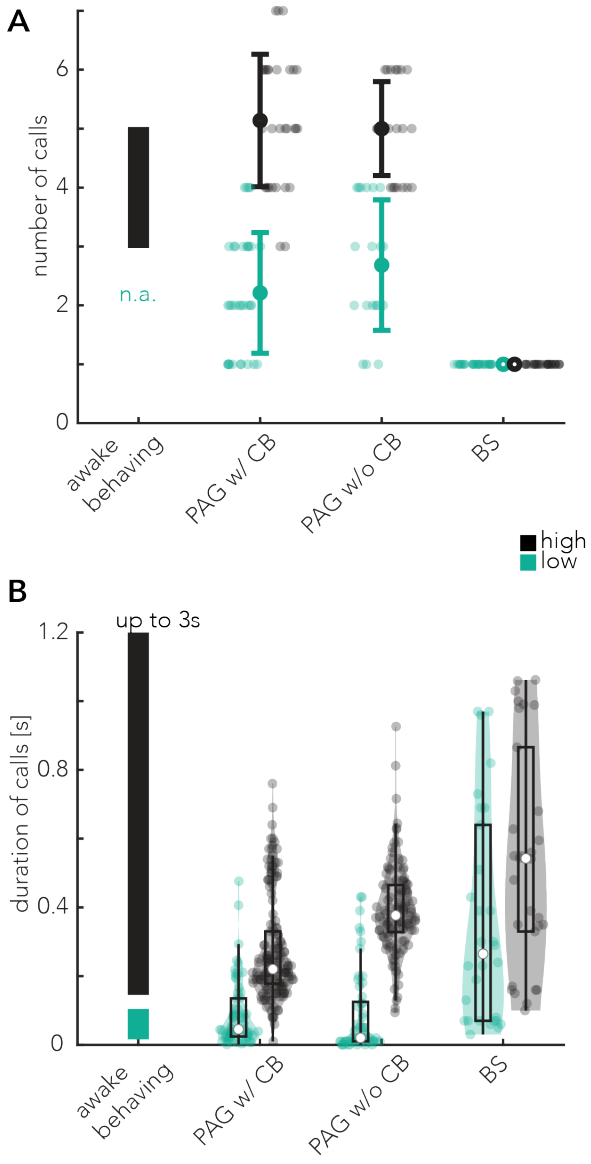
Potentially, one could assume that the distribution of measures and the resulting maps are occur out of a random distribution of the measures. To test whether the defined regions stem from such a random distribution or are indeed different from it, we applied a random shuffling test. We randomized the location ID of each measurement 10000 times and then compared the random distribution against the measured values at the same location. This method kept the obtained data and the spatial layout of our experiments intact and allowed to test if the observed results is different from random.

Cross-correlation

Cross-correlation between cooling effects and evoked calls was done with the Pearson Correlation.

Anatomical data

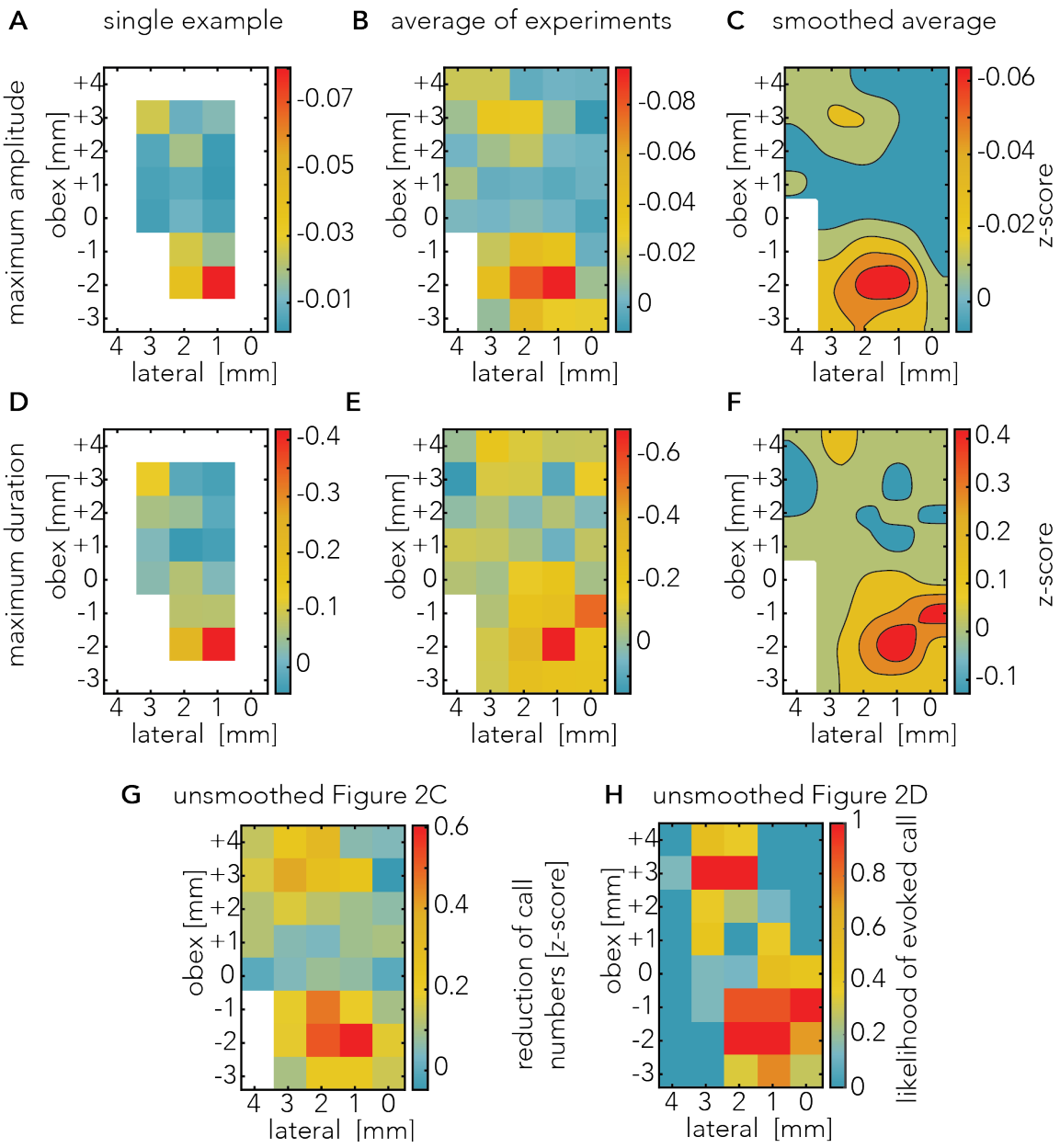
Comparison of cell sizes was performed with the non-parametric Kruskal-Wallis test since the data was not normally distributed. *Post-hoc* analysis of the Kruskal-Wallis tests was done with the Tukey-Kramer test. Normal distributed, paired myelinization data was analyzed with the Friedman test and the Tukey-Kramer *post-hoc* test.



Supplementary Figure 1, related to Figure 1: Comparison between evoked calls under different animal conditions.

A) Mean number of evoked calls in awake behaving animals, upon PAG stimulation with and without cerebellum and direct brainstem stimulation. Solid bars in the awake behaving column are taken from literature (Schwarting and Wöhr, 2012). High frequency calls are not quantifiable since there are no clear single stimuli triggering them. Whiskers depict the standard error, dots show single data points.

B) Duration of high frequency and low frequency calls. The distribution of the violin plot shows the probability density of the cell sizes overlaid with boxplots. Solid bars in the awake behaving column are taken from literature. Awake behaving condition taken from literature (Ishiyama and Brecht, 2016; Schwarting and Wöhr, 2012).



Supplementary Figure 2, related to Figure 2: Raw results of cooling and stimulation mapping experiments.

A-C) Maximum amplitude change after cooling, depicted as the z-score between cold and warm condition.

A) Single experiment, same as Figure 2A.

B) All experiments averaged, not smoothed.

C) Smoothed map of B.

D-F) Maximum duration of calls depicted as the z-score between cold and warm condition.

D) Single experiment, same as Figure 2A.

E) All experiments averaged, not smoothed.

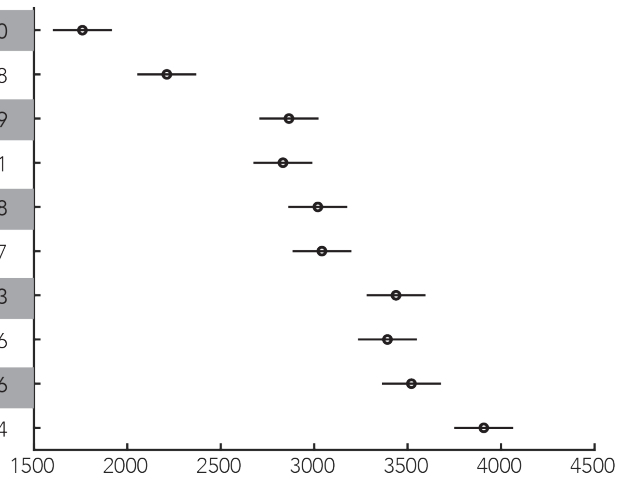
F) Smoothed map of E.

G) Unsmoothed map of Figure 2C.

H) Unsmoothed map of Figure 2D.

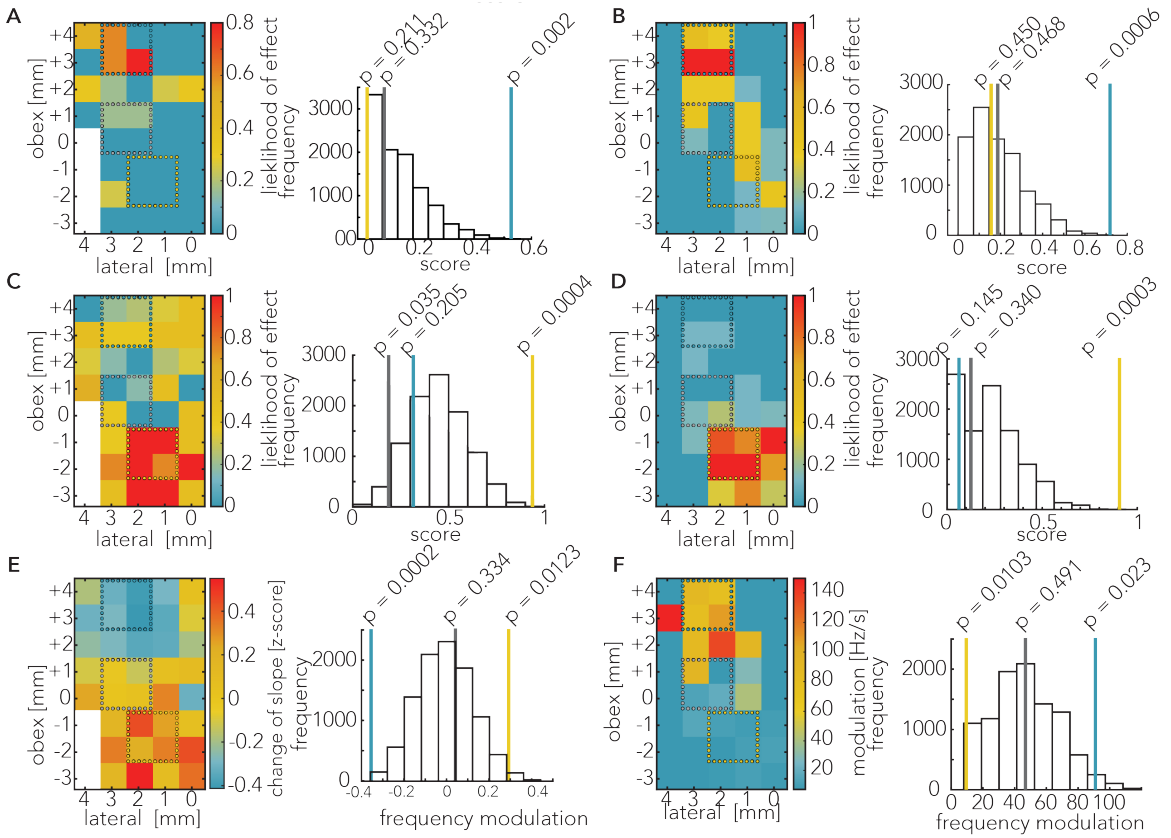
A

region	median	quartiles 25%	75%
NRA	166	122	230
VoPaRT	199	150	268
PCRt	242	149	379
IRt	245	171	341
MdD	255	181	358
sp5	257	171	387
PCRtA	293	192	483
Md	296	205	426
Amb	333	206	456
IRtA	338	254	464



Supplementary Figure 3, related to Figure 4:

A) Median cell cross sections in μm^2 as well as the lower and upper quartiles. The plot on the right is the result plot of the Tukey-Kramer with arbitrary units on the x-axis. If whiskers of two areas do not overlap, the difference is significant, see Table 1.



Supplementary Figure 4, related to Figure 5: Raw data for frequency and modulation specific effects and statistical analysis results.

A-F) On the left, the map shows the raw results of the cooling or stimulation results. On the right, the histogram shows the shuffled distribution of the results as well as the measured values of the target regions. Description of plots can be found along the description of the results shown in Figure 2 and 4.

A) Cooling effect on high frequency calls.

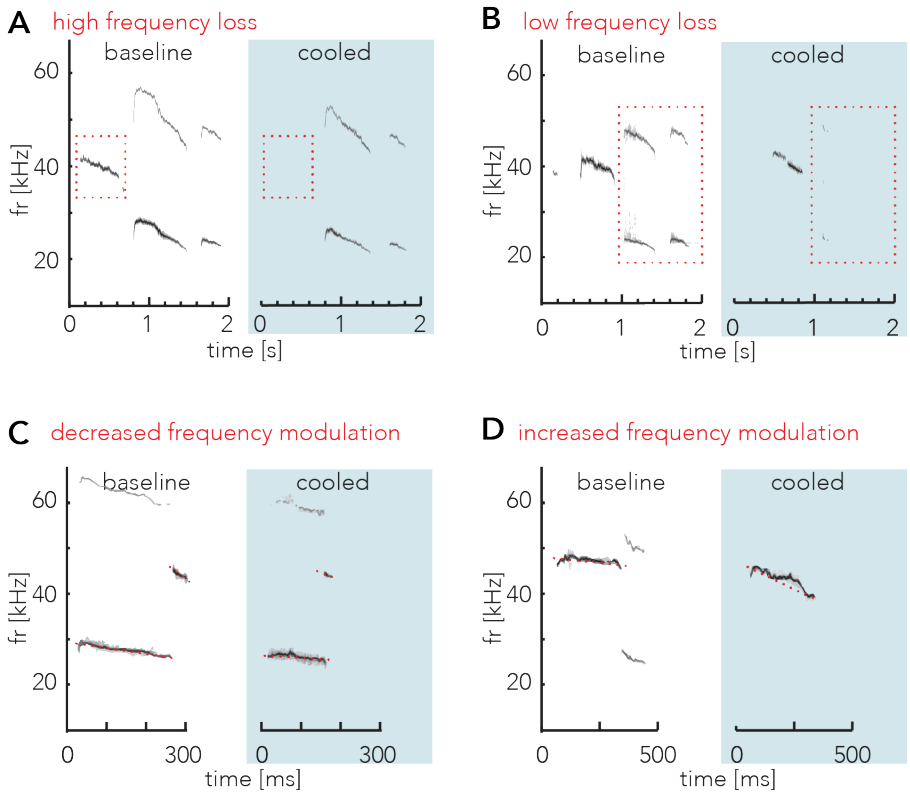
B) Stimulation evoked high frequency calls.

C) Cooling effect on low frequency calls.

D) Stimulation evoked low frequency calls.

E) Modulation (slope) of calls after cooling.

F) Modulation (slope) of stimulation evoked calls.



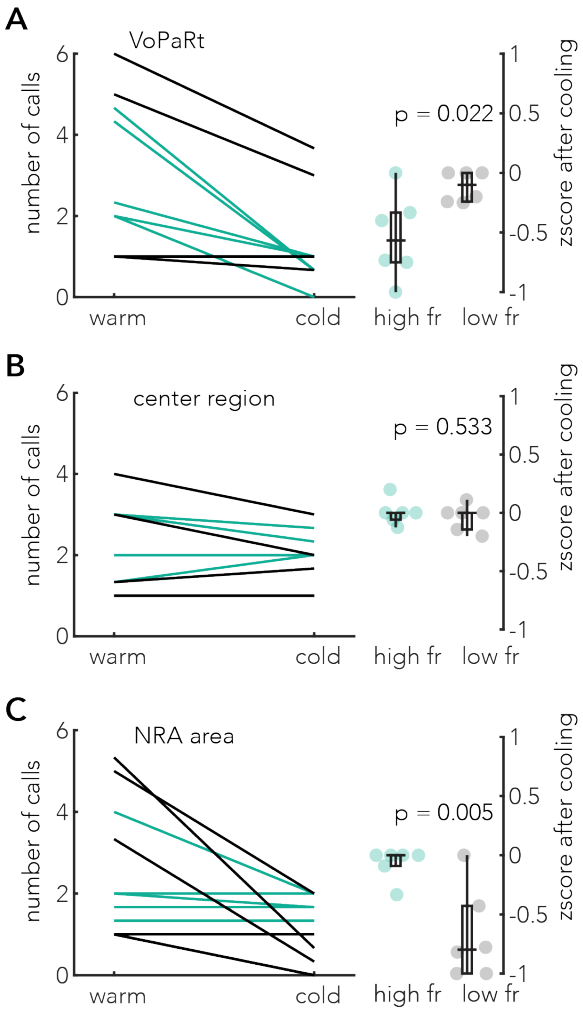
Supplementary Figure 5, related to Figure 5: Example cooling effects on high and low frequency calls and frequency modulation.

A) Second example of high frequency loss upon cooling of the brainstem in the VoPaRt.

B) Another example of low frequency loss upon cooling of the brainstem in the NRA region.

C) Decreased absolute frequency modulation when cooling the VoPaRt.

D) Increased absolute frequency modulation when cooling the NRA.

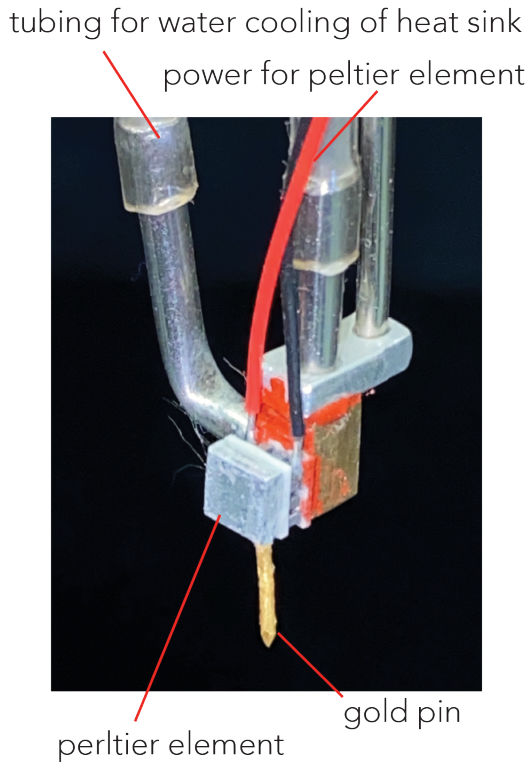


Supplementary Figure 6, related to Figure 5: Quantitative analysis of cooling of the VoPaRt region.

A) Black lines show the mean number of calls evoked by the last 3 trials before cooling and the mean number of calls evoked by the last 3 trials at the end of the cooling period. Cyan lines show the same results for the high frequency numbers (n=6 for each). The boxplot on the right side depicts the z-score for each frequency type after cooling.

B) Same as A, now for the central region.

C) Same as A and B, now for the NRA region.



Supplementary Figure 7, related to Methods and Figure 1: Cooling element used in this study.

Supplementary Table 1**, related to Figure 4**: Statistical results of the Tukey-Kramer test of cell sizes. Third and fifths column show the lower and higher 95% confidence interval. Fourths column the difference between the group ranks and the sixth column the associated p-value.

Area 1	Area 2	lower 95%	Group diff.	upper 95%	p-value
NRA	VoPaRt	-768.0287	-451.66022	-135.29174	0.0002692
NRA	PCRt	-1421.5537	-1105.1852	-788.81674	0.0000001
NRA	IRt	-1389.5204	-1073.02	-756.51965	0.0000001
NRA	MdD	-1576.1004	-1259.7319	-943.36341	0.0000001
NRA	sp5	-1597.882	-1281.5136	-965.14508	0.0000001
NRA	PCRtA	-1994.567	-1678.1986	-1361.8301	0.0000001
NRA	MdV	-1948.4954	-1632.1269	-1315.7584	0.0000001
NRA	Amb	-2077.078	-1760.5776	-1444.0772	0.0000001
NRA	IRtA	-2464.2254	-2147.8569	-1831.4884	0.0000001
VoPaRt	PCRt	-969.76152	-653.525	-337.28848	0.0000001
VoPaRt	IRt	-937.72829	-621.35981	-304.99133	0.0000001
VoPaRt	MdD	-1124.3082	-808.07167	-491.83514	0.0000001
VoPaRt	sp5	-1146.0899	-829.85333	-513.61681	0.0000001
VoPaRt	PCRtA	-1542.7749	-1226.5383	-910.30181	0.0000001
VoPaRt	MdV	-1496.7032	-1180.4667	-864.23014	0.0000001
VoPaRt	Amb	-1625.2859	-1308.9174	-992.54892	0.0000001
VoPaRt	IRtA	-2012.4332	-1696.1967	-1379.9601	0.0000001
PCRt	IRt	-284.20329	32.1651906	348.533671	0.9999994
PCRt	MdD	-470.78319	-154.54667	161.689856	0.8734727
PCRt	sp5	-492.56486	-176.32833	139.90819	0.7580039

PCRt	PCRtA	-889.24986	-573.01333	-256.77681	0.0000006
PCRt	MdV	-843.17819	-526.94167	-210.70514	0.0000061
PCRt	Amb	-971.76089	-655.39241	-339.02392	0.0000001
PCRt	IRtA	-1358.9082	-1042.6717	-726.43514	0.0000001
IRt	MdD	-503.08034	-186.71186	129.656623	0.6915050
IRt	sp5	-524.862	-208.49352	107.874957	0.5382060
IRt	PCRtA	-921.547	-605.17852	-288.81004	0.0000002
IRt	MdV	-875.47534	-559.10686	-242.73838	0.0000011
IRt	Amb	-1004.058	-687.5576	-371.05721	0.0000001
IRt	IRtA	-1391.2053	-1074.8369	-758.46838	0.0000001
MdD	sp5	-338.01819	-21.781667	294.454856	1.0000000
MdD	PCRtA	-734.70319	-418.46667	-102.23014	0.0011742
MdD	MdV	-688.63152	-372.395	-56.158477	0.0074643
MdD	Amb	-817.21422	-500.84574	-184.47726	0.0000242
MdD	IRtA	-1204.3615	-888.125	-571.88848	0.0000001
sp5	PCRtA	-712.92152	-396.685	-80.448477	0.0029033
sp5	MdV	-666.84986	-350.61333	-34.37681	0.0163780
sp5	Amb	-795.43255	-479.06407	-162.69559	0.0000725
sp5	IRtA	-1182.5799	-866.34333	-550.10681	0.0000001
PCRtA	MdV	-270.16486	46.0716667	362.30819	0.9999858
PCRtA	Amb	-398.74755	-82.379072	233.989408	0.9982393
PCRtA	IRtA	-785.89486	-469.65833	-153.42181	0.0001137
MdV	Amb	-444.81922	-128.45074	187.917742	0.9573058
MdV	IRtA	-831.96652	-515.73	-199.49348	0.0000110
Amb	IRtA	-703.64774	-387.27926	-70.910781	0.0042466