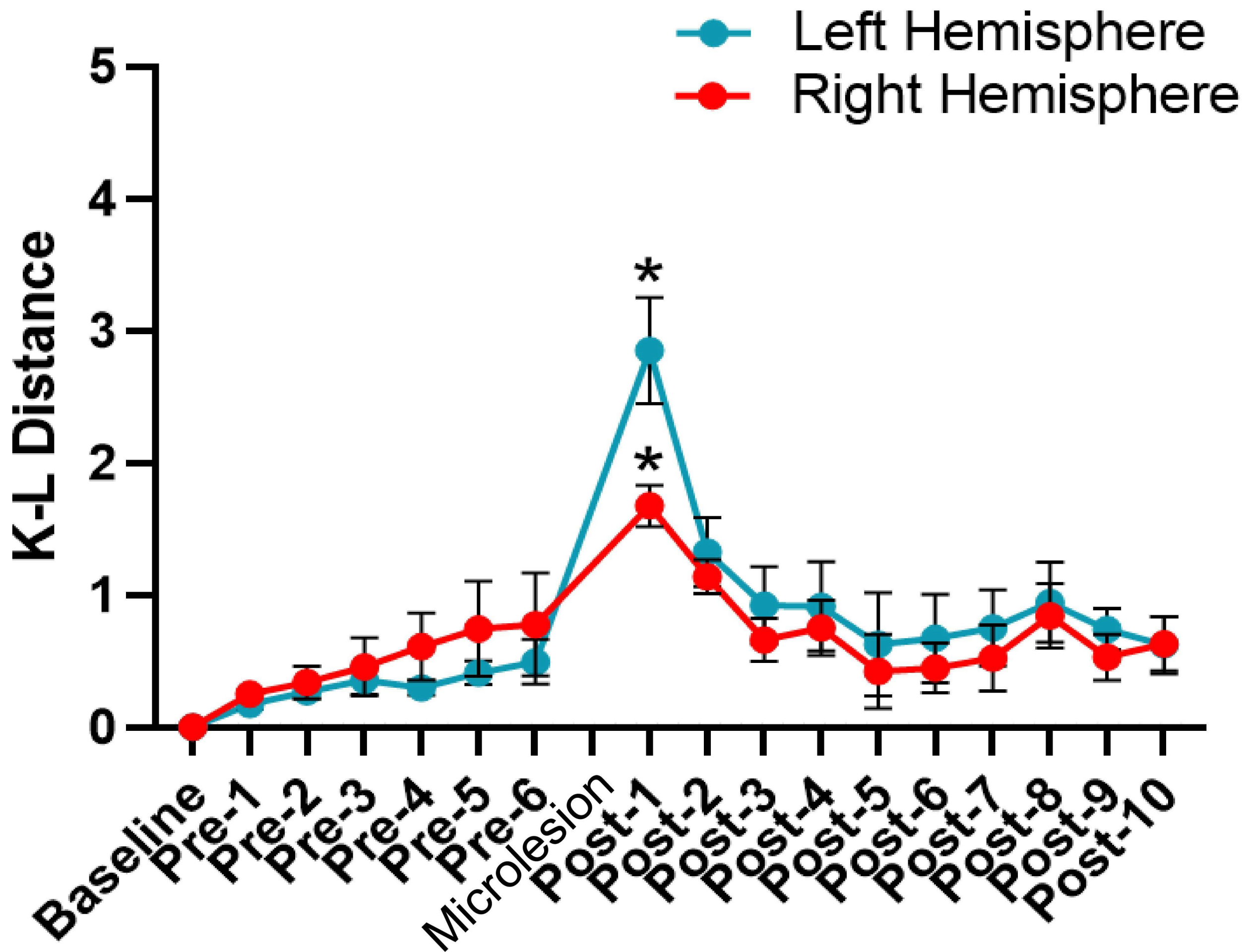
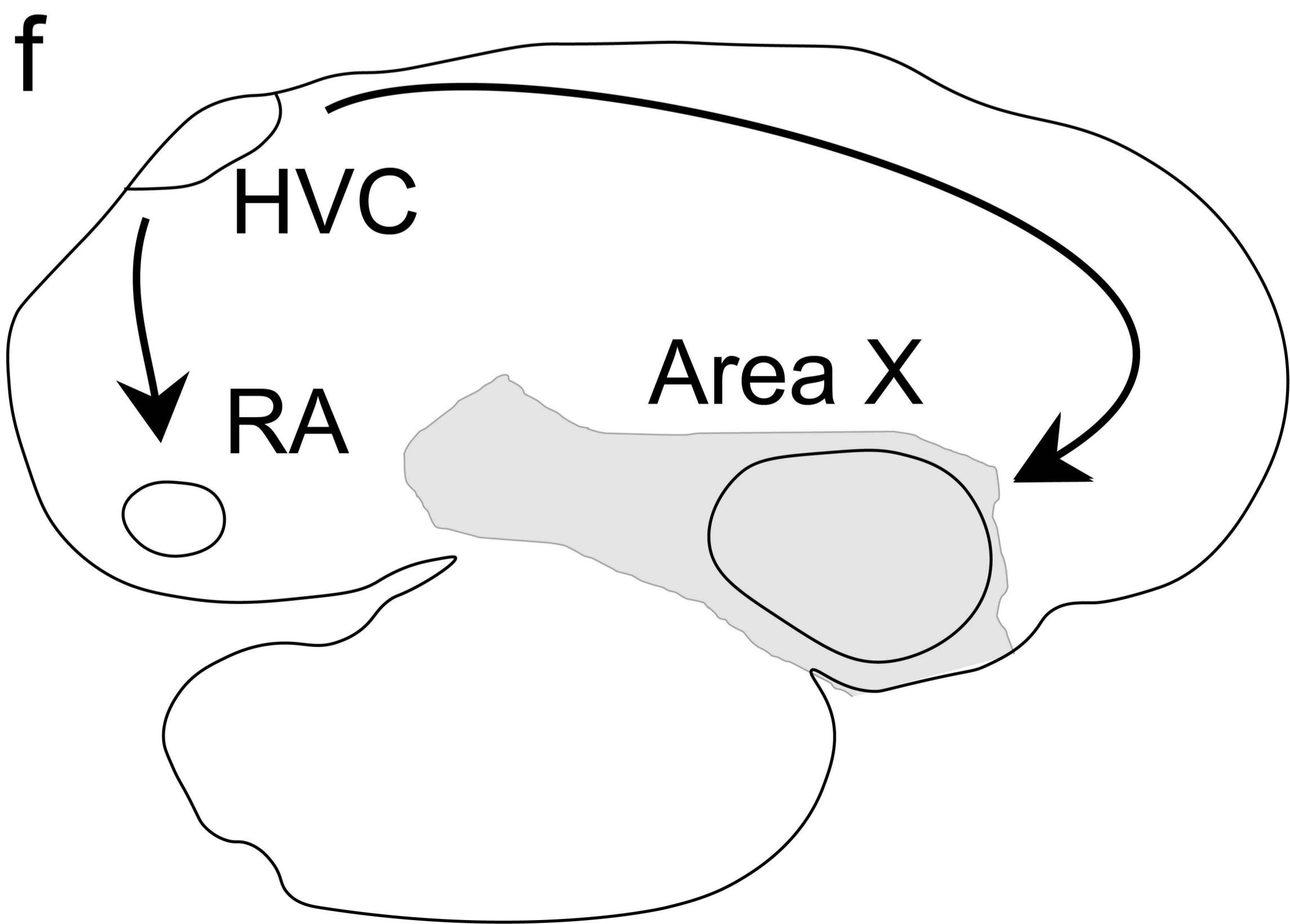
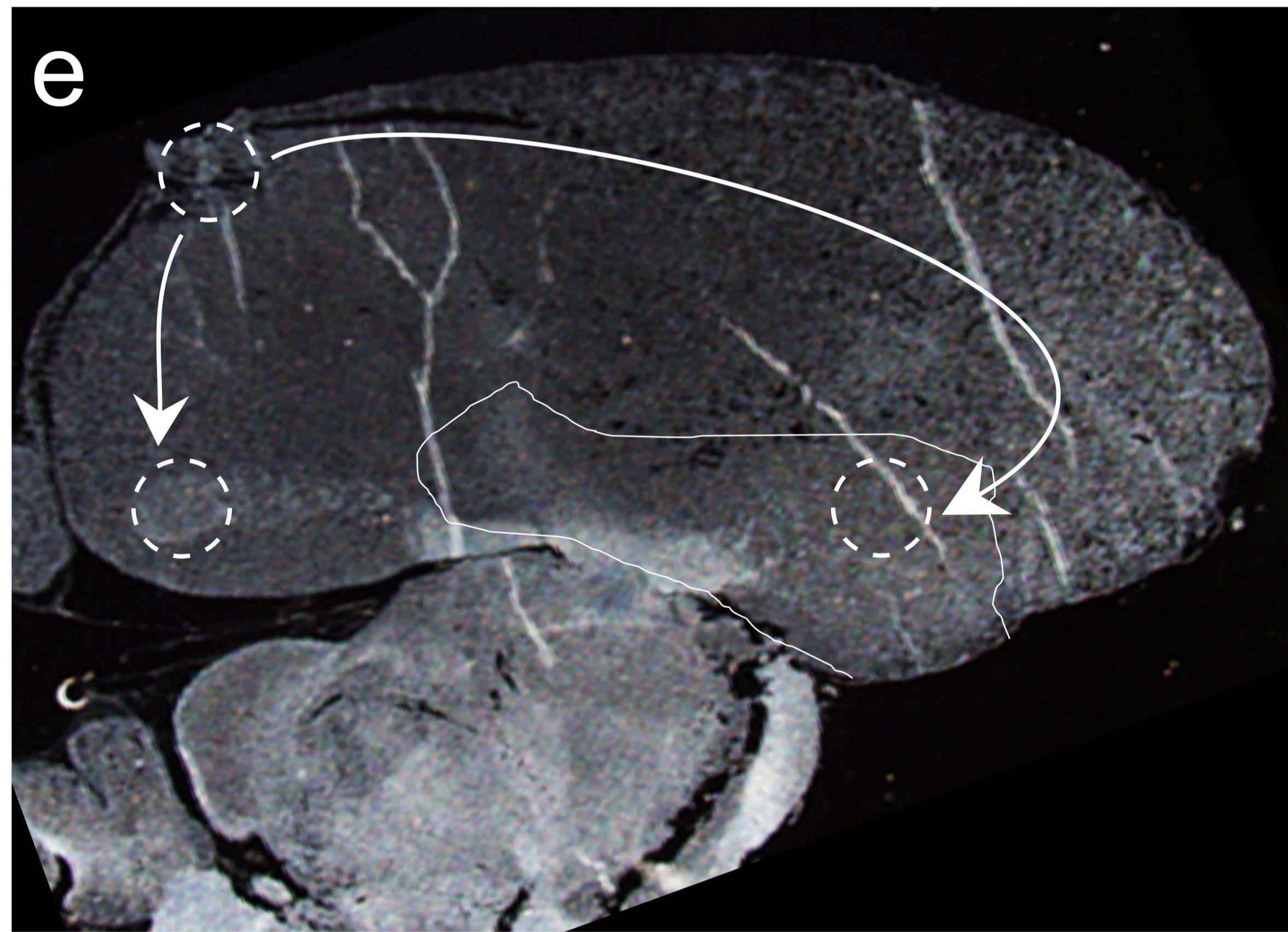
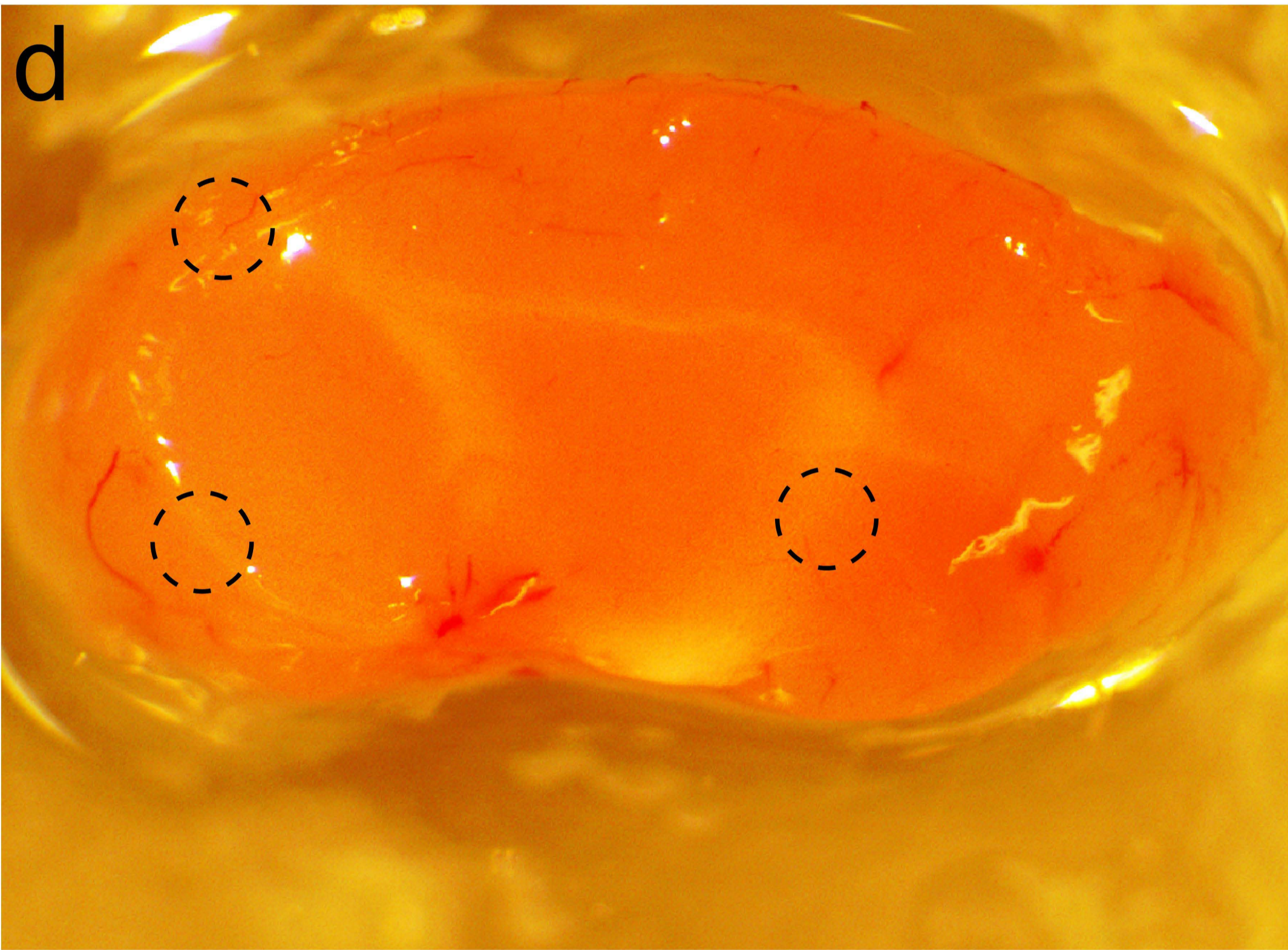
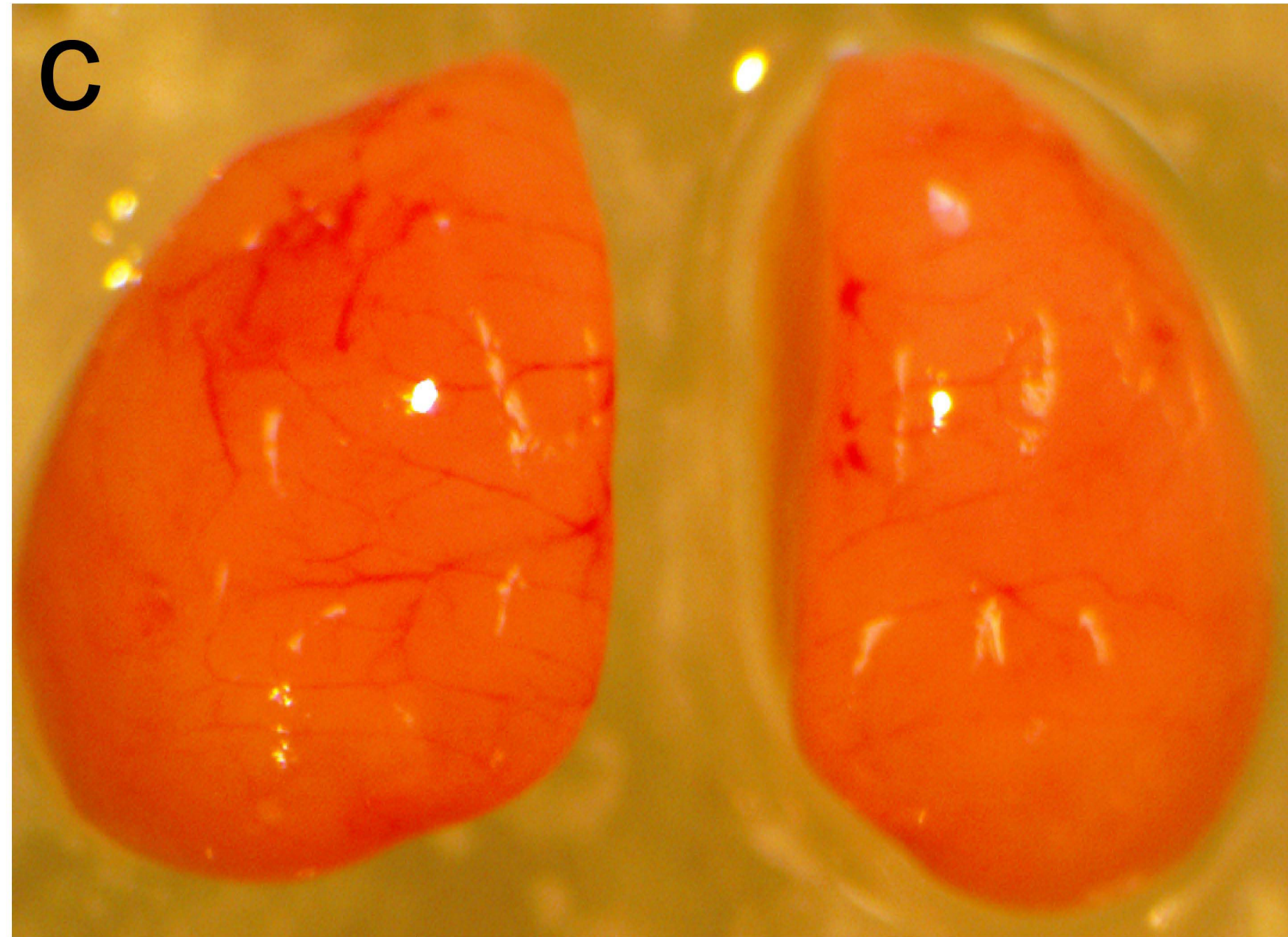
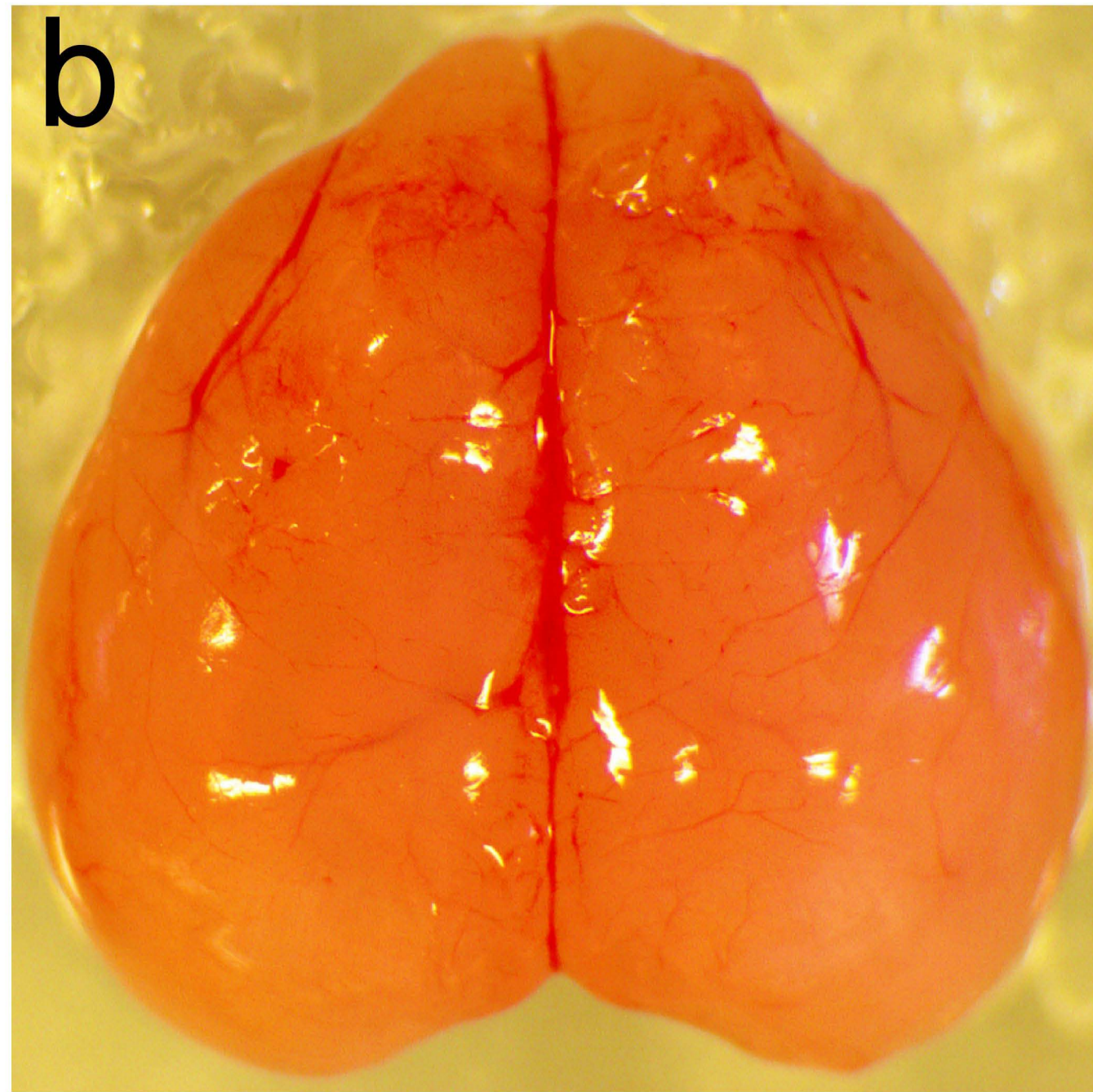
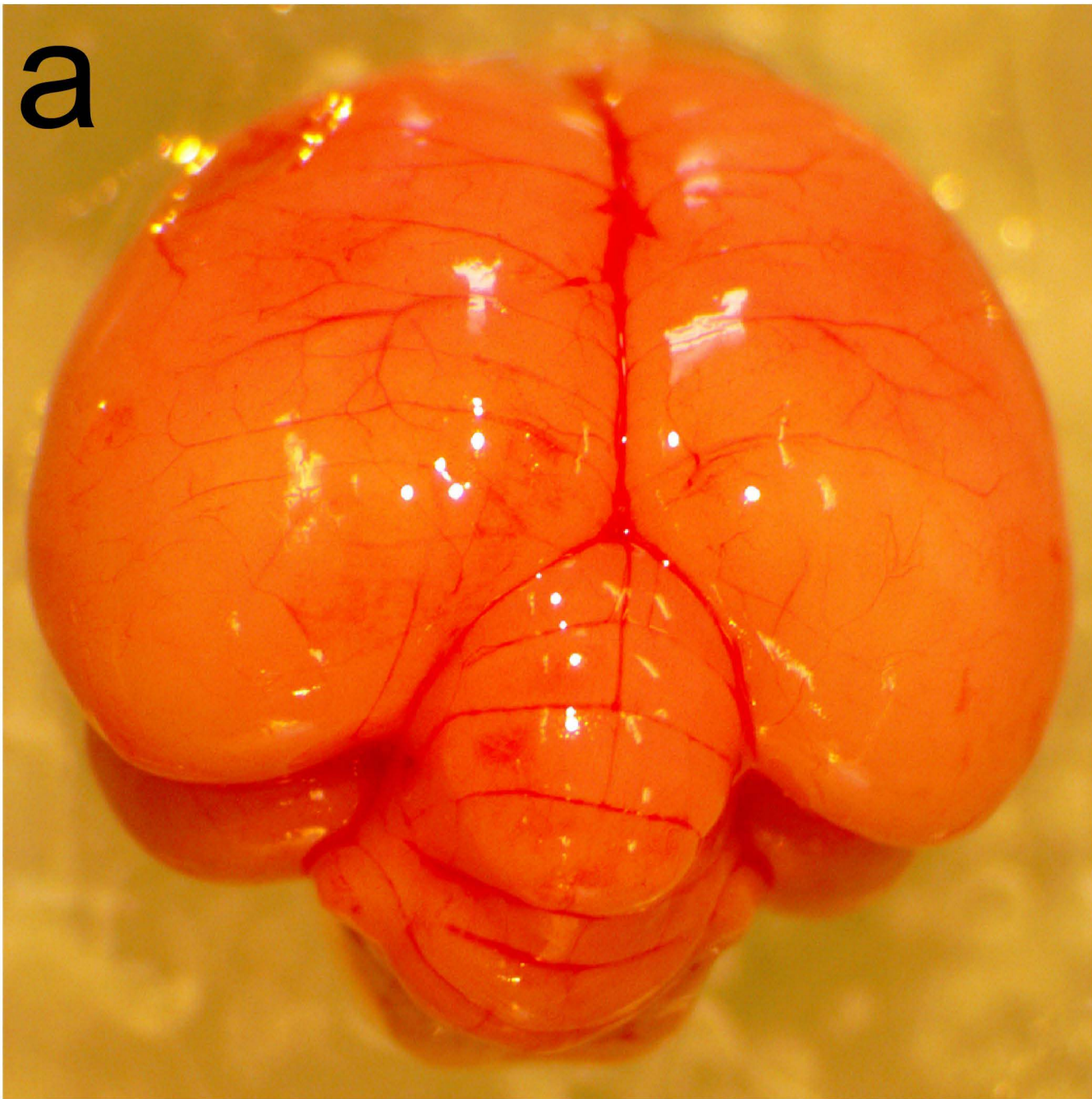


CANNABIDIOL INHIBITS NEUROINFLAMMATORY RESPONSES AND CIRCUIT-ASSOCIATED SYNAPTIC LOSS FOLLOWING DAMAGE TO A SONGBIRD VOCAL PRE-MOTOR CORTICAL-LIKE REGION

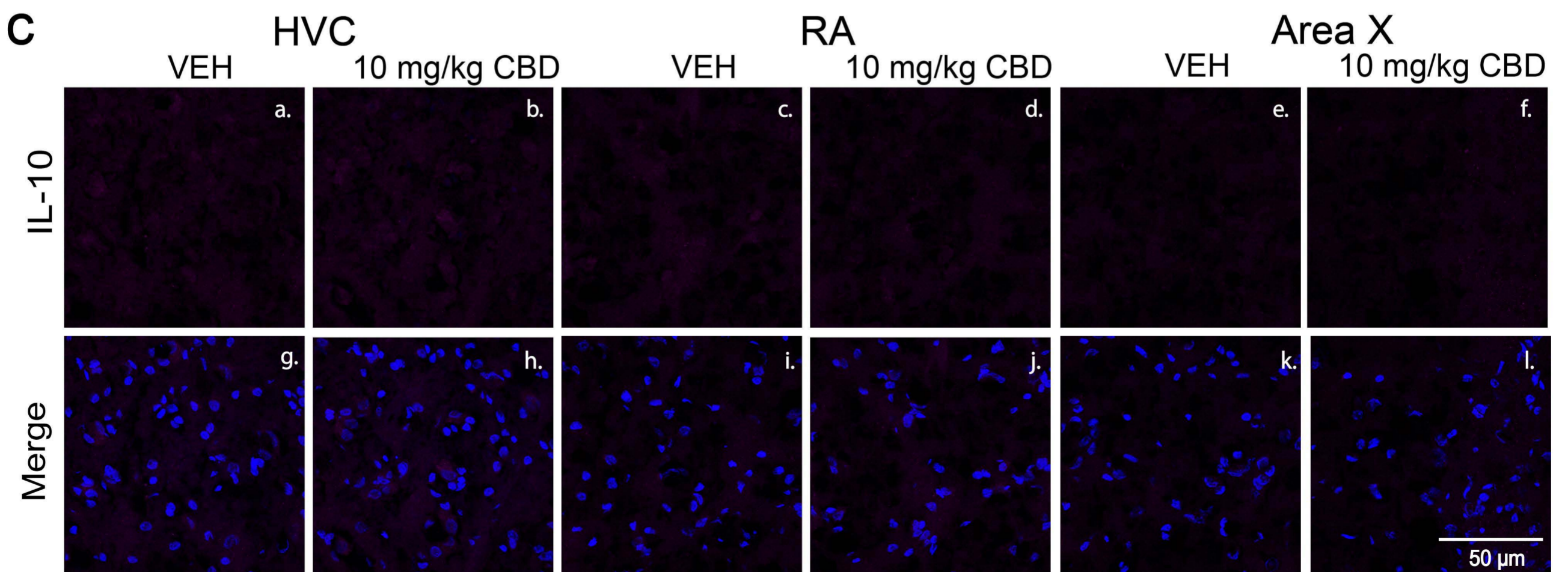
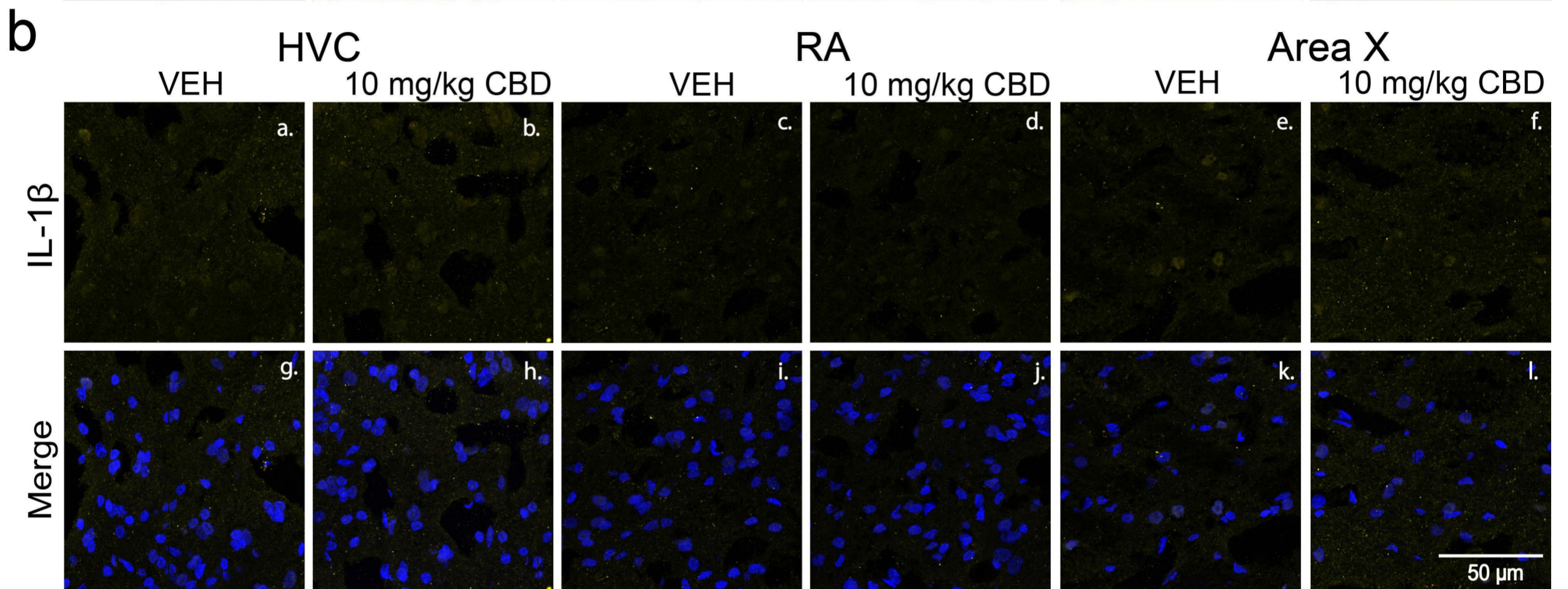
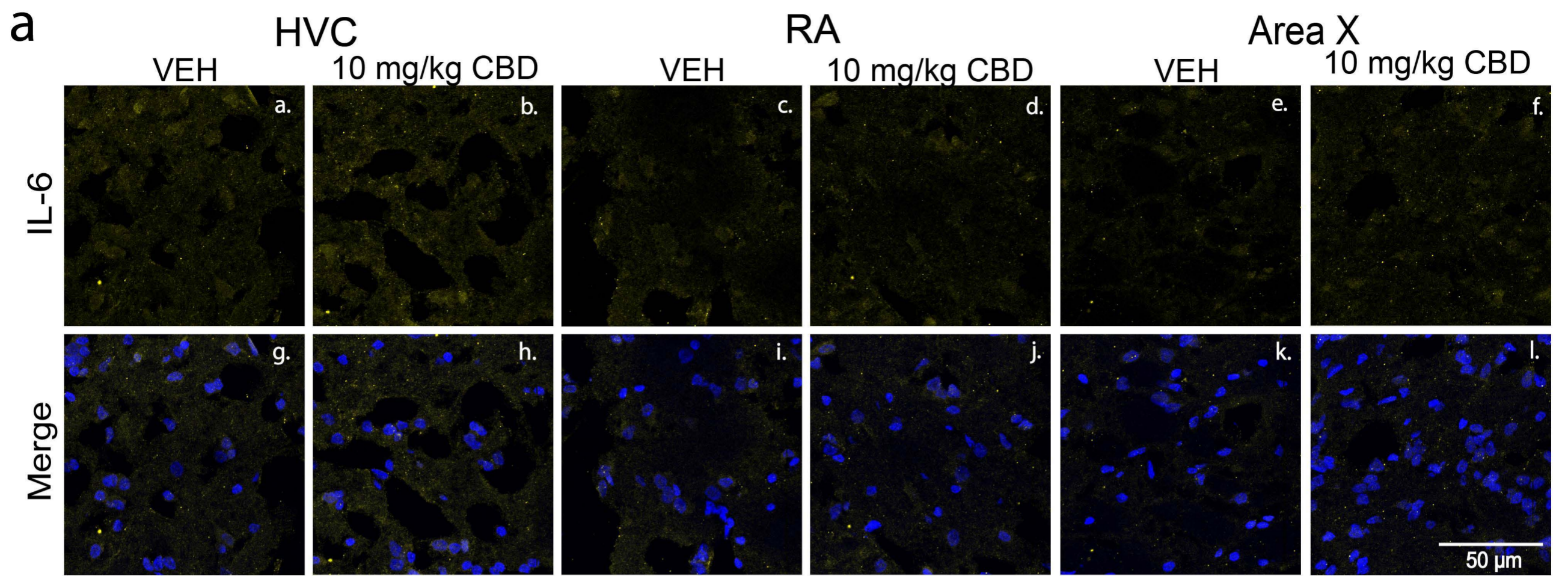
Mark Tripson, Karen Litwa, and Ken Soderstrom



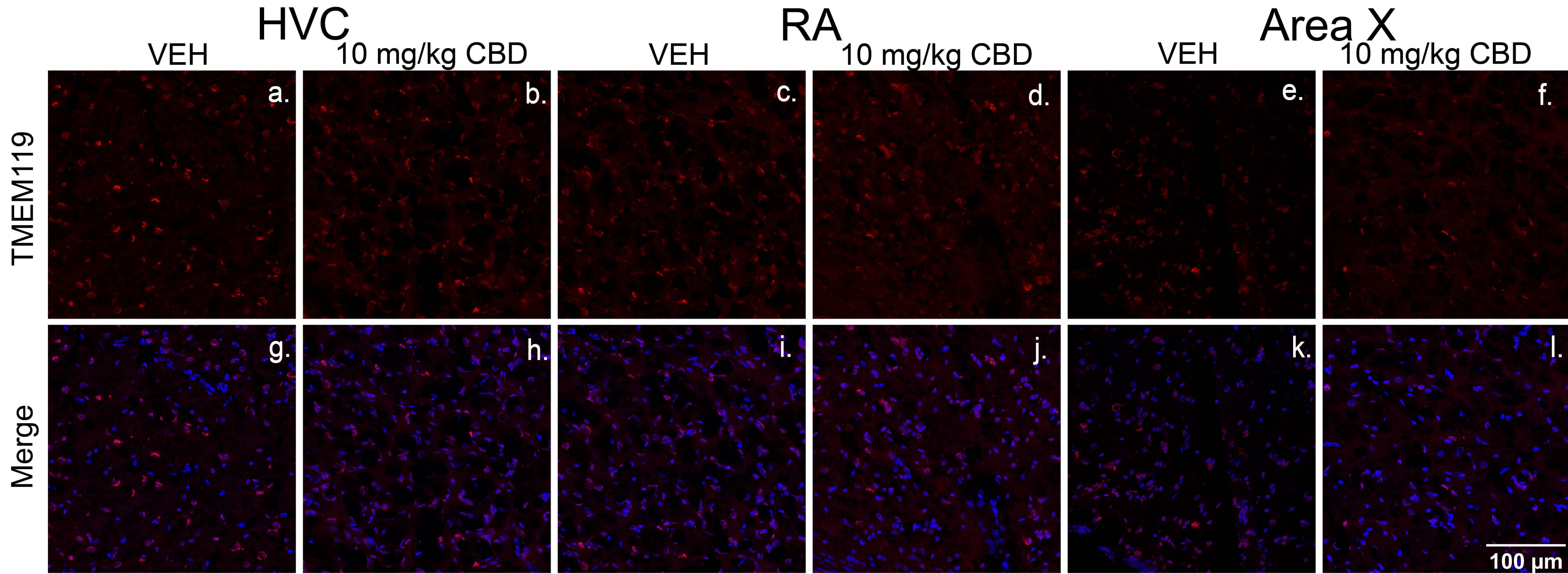
Supplemental Figure 1. Unilateral HVC microlesions temporarily disrupt zebra finch song. Y-axis are days relative to microlesions given on day 7. Using a measure of vocal variability, K-L distance^{13,64}, a unilateral HVC microlesion to either the right or left hemisphere, disrupts vocalization peaking 24 hours after surgery. Interestingly, left hemisphere lesions produced a greater change from baseline, although both were statistically significant. Birds were recorded continuously for the entire 20 day experimental period. Daily recordings from n = 3 birds/group were acoustically-analyzed and KL distance measures generated as previously described¹.



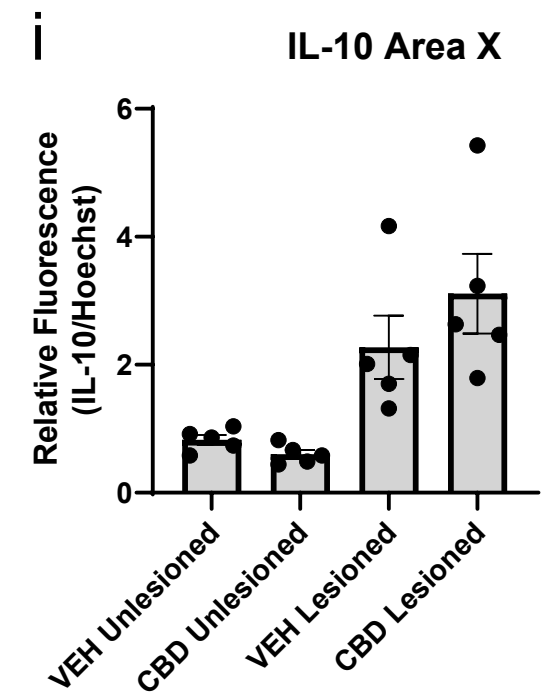
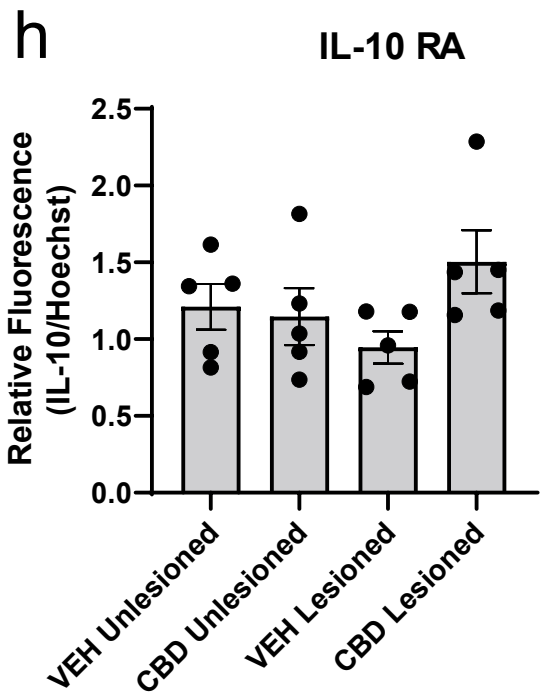
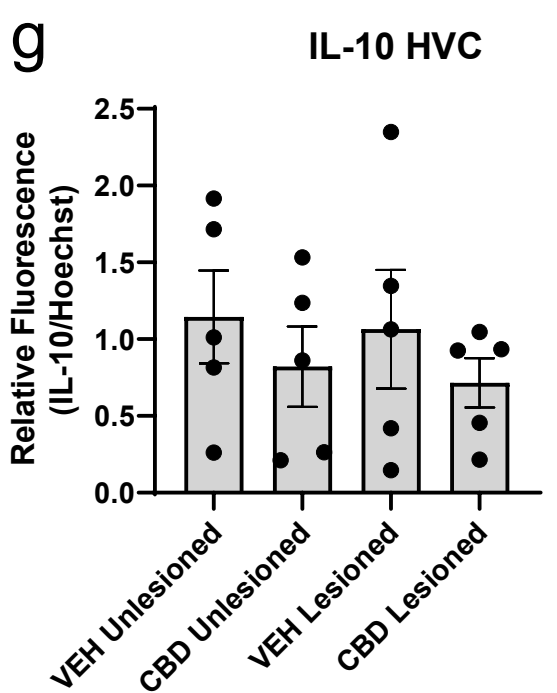
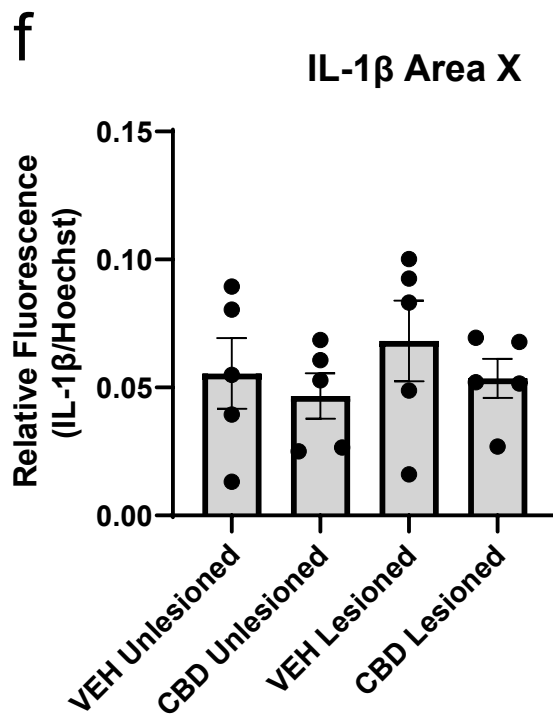
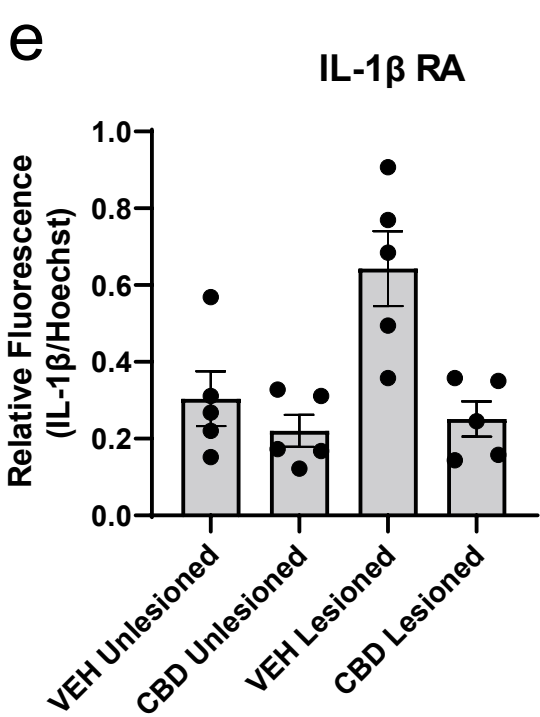
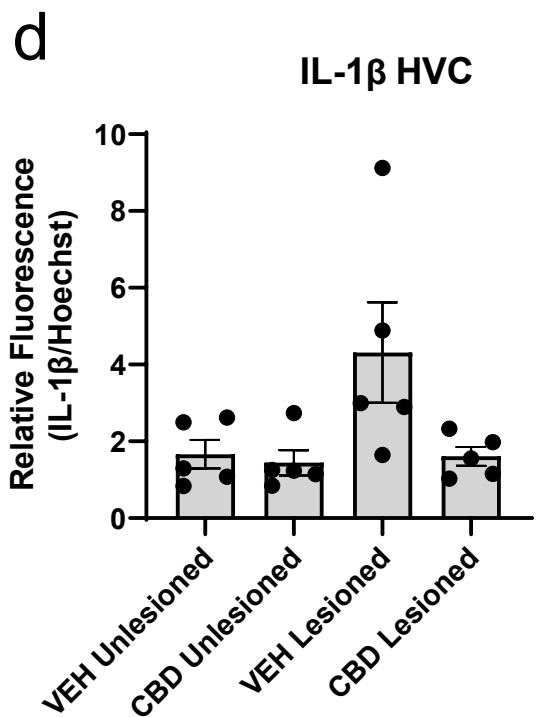
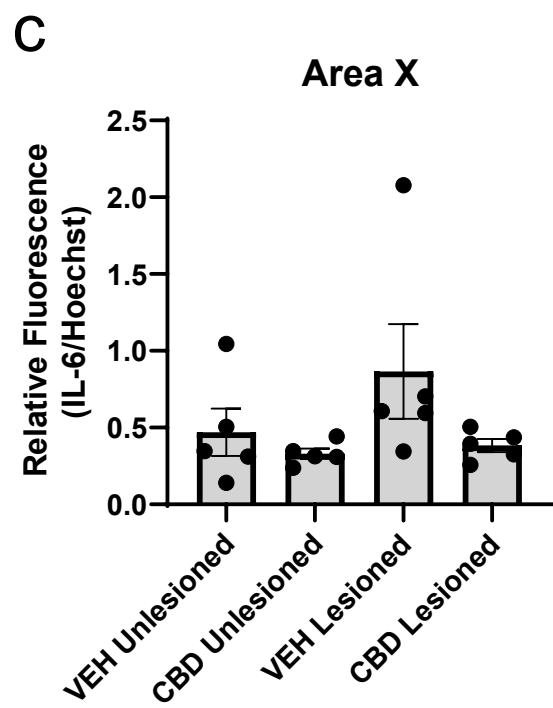
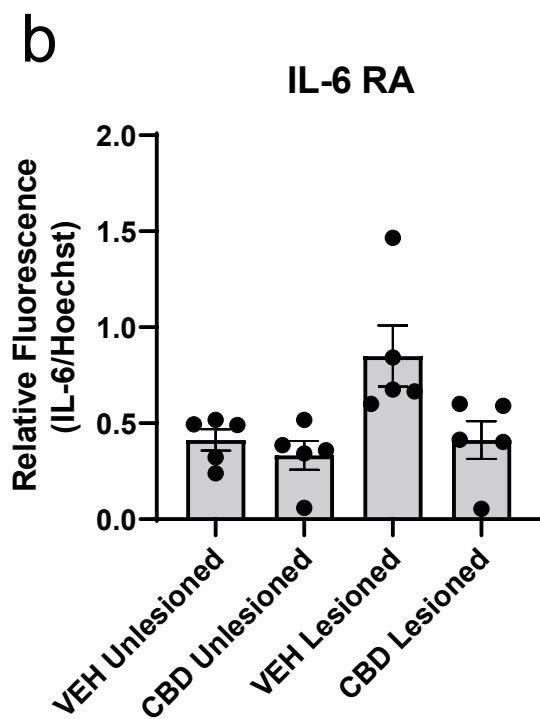
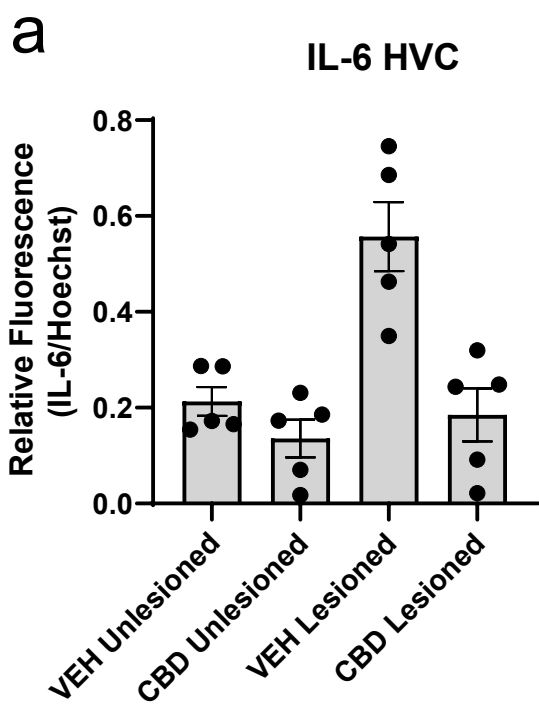
Supplemental Figure 2. Illustration of micro punch technique used to isolate regions of interest. A-D, Gross dissection of fresh zebra finch brain tissue and biopsy punch locations based on fiber tracts in a sagittal section. E, Dark-field image used to identify tissue sections with all regions of interest. Dense regions are circled, and arrows indicate circuits that connect them. F, Outline of sagittal section with areas of interest highlighted. Rostral is approximately right, dorsal up, and bar = 1 mm.



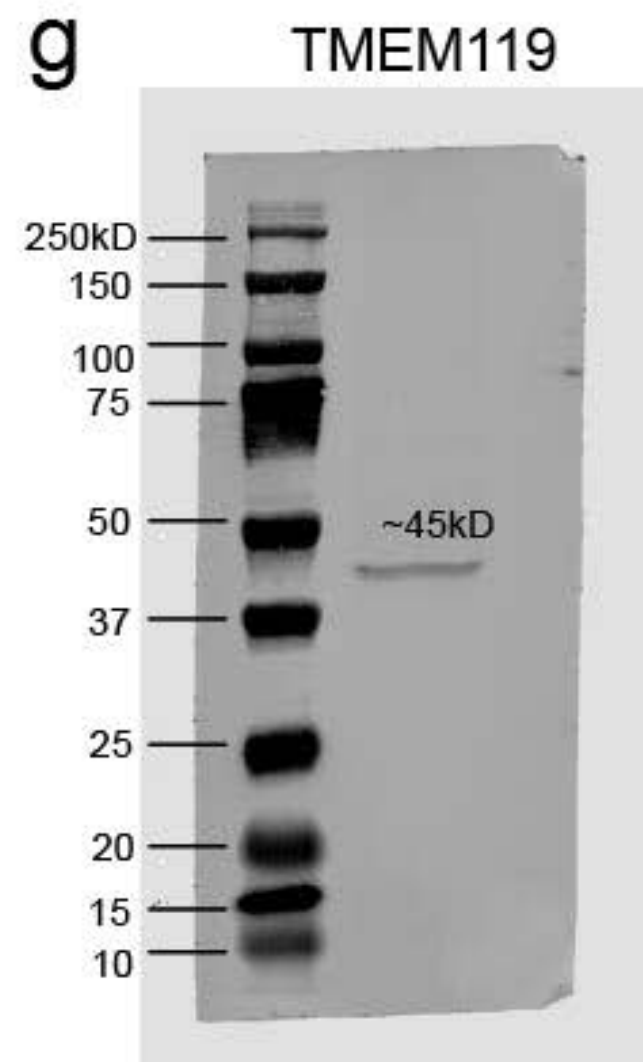
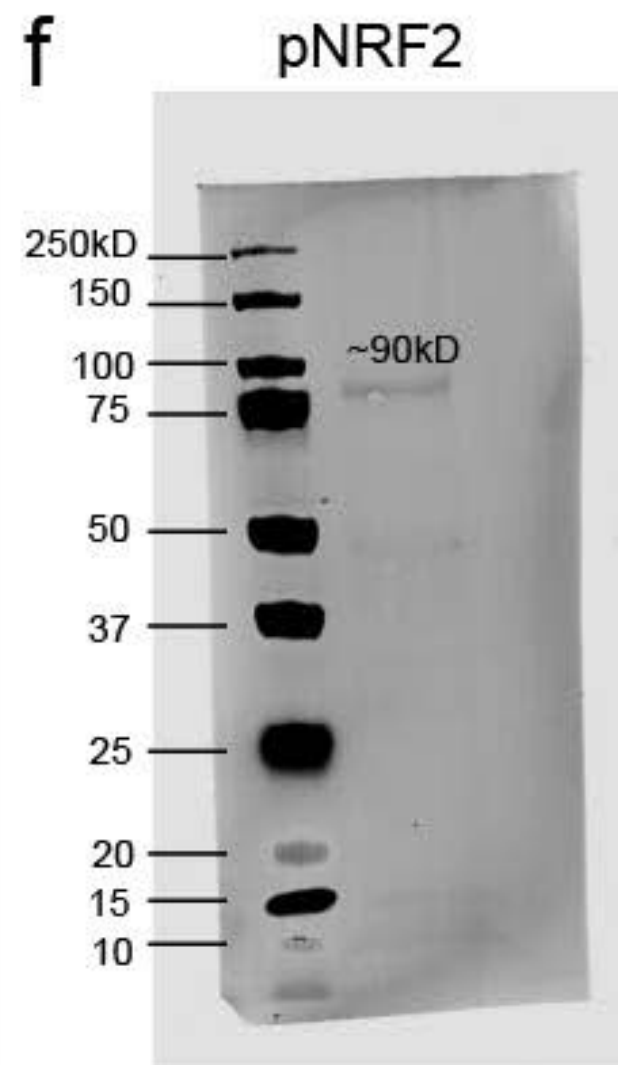
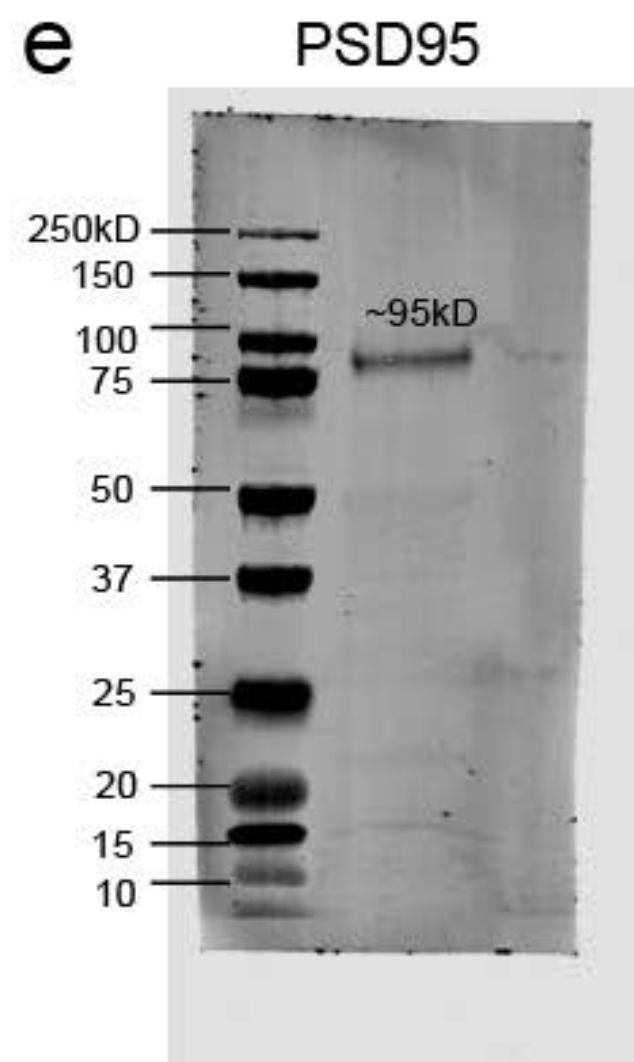
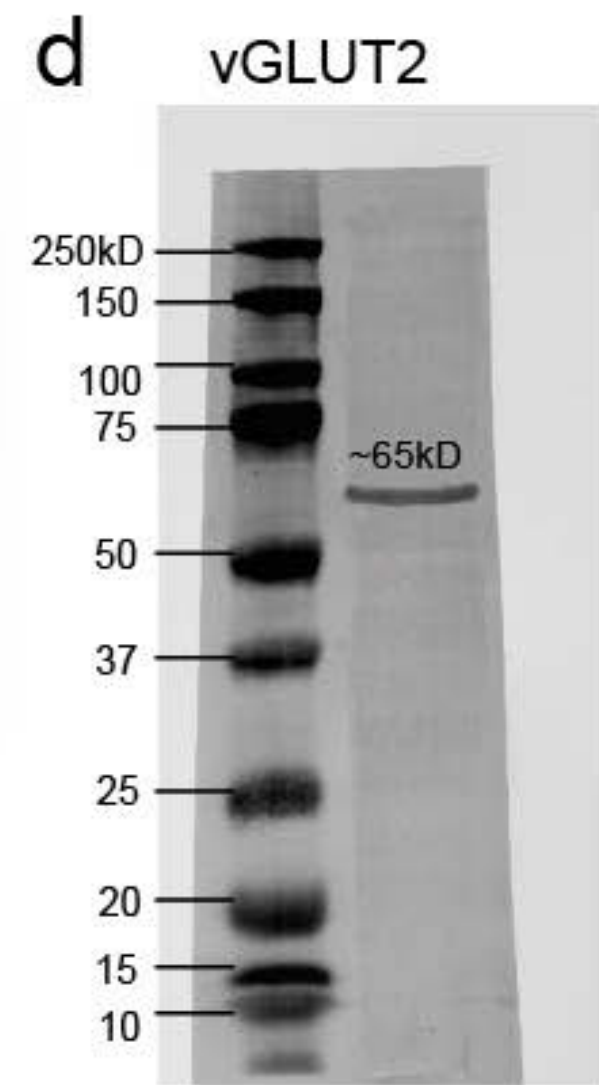
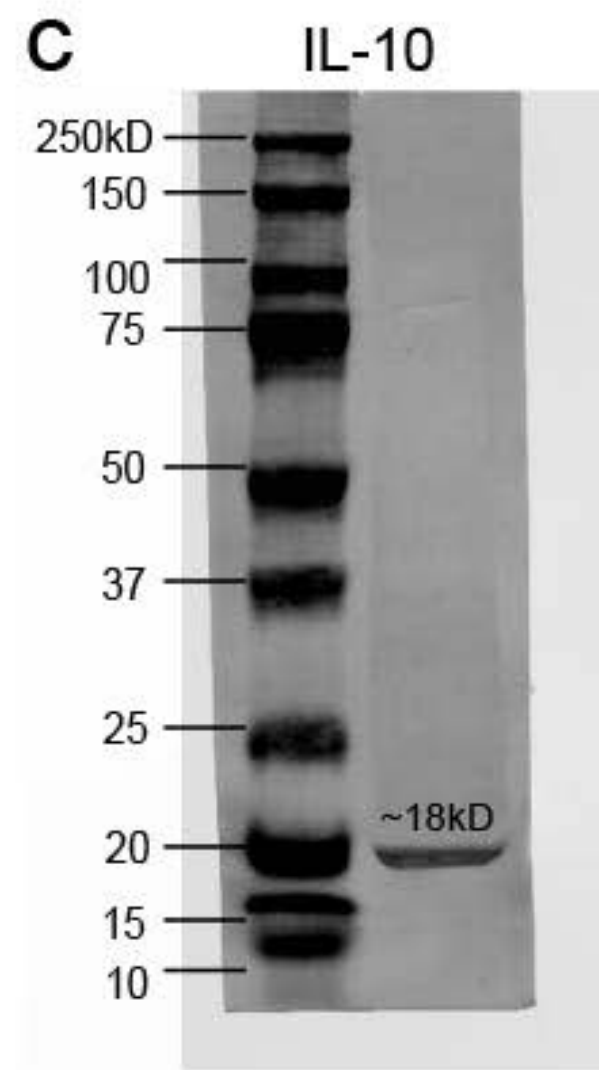
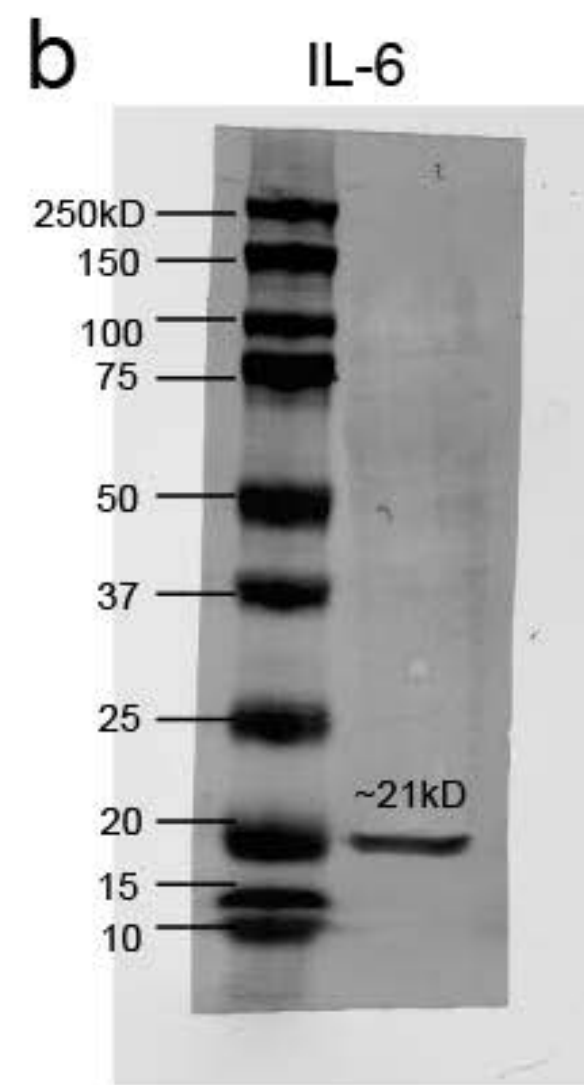
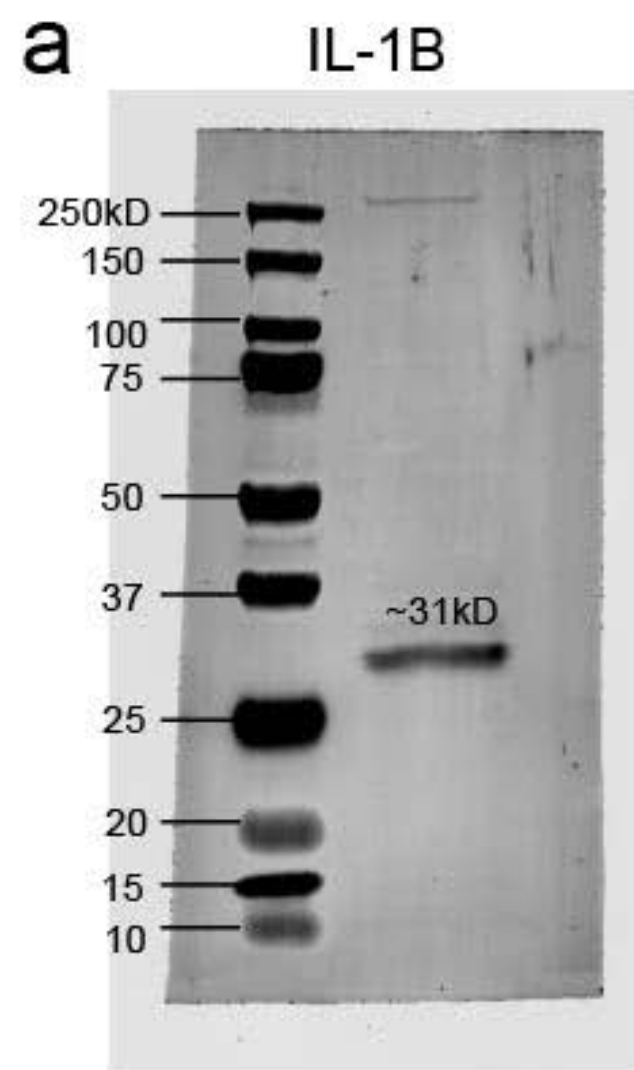
Supplemental Figure 3. Representative immunofluorescent confocal images of antibodies targeting IL-1B, IL-6 and IL-10 within motor (HVC & RA) and learning-essential (Area X) regions of contralateral (unlesioned) hemispheres. Note that little staining is observed. Aa-AI, Unlesioned hemisphere images showing representative regional distribution of IL-6 in vehicle- vs. CBD-treated birds. Ba-BI, images demonstrating regional distribution of IL-1B. Ca-CI, confocal images demonstrating regional distribution of IL-10.



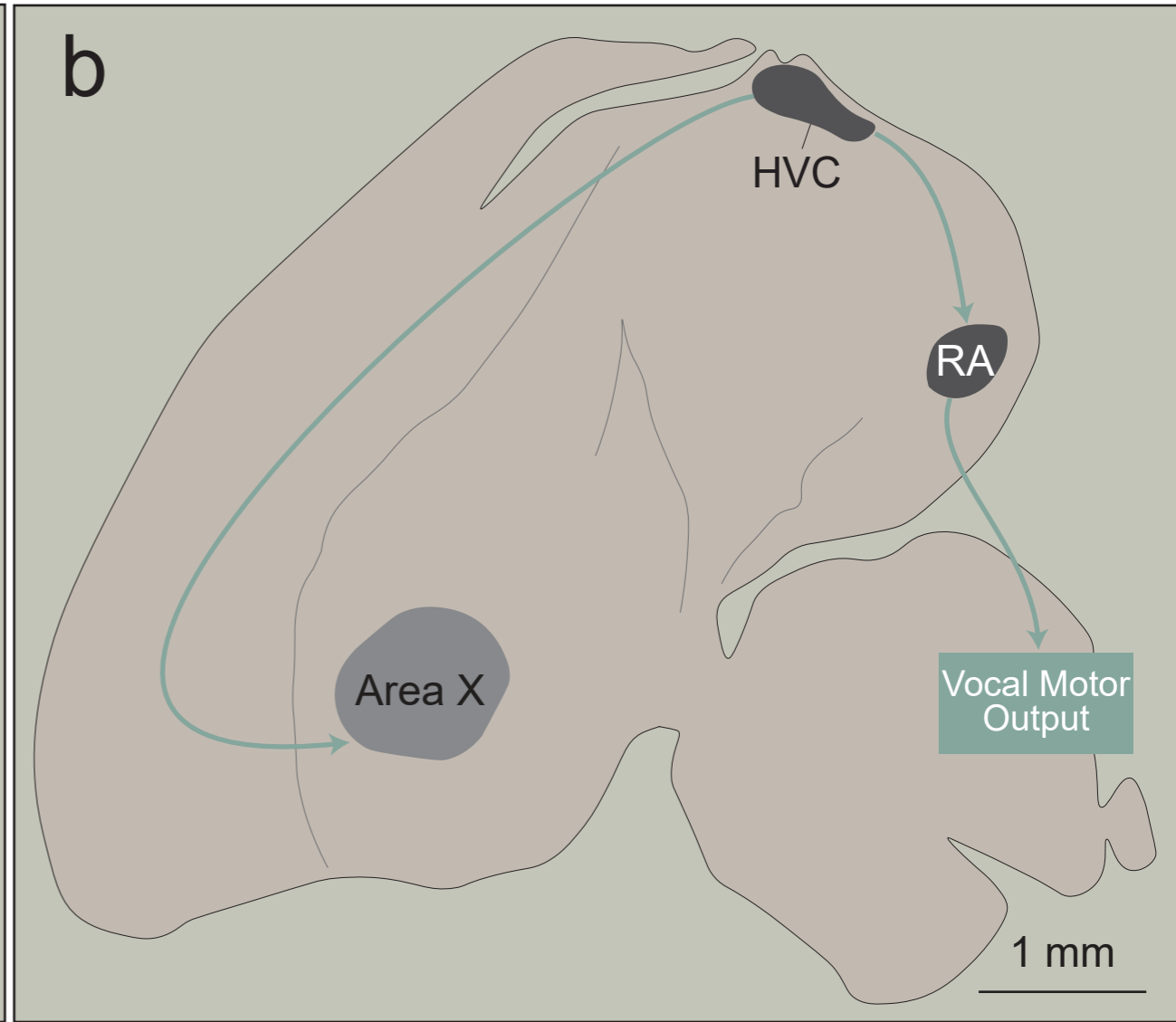
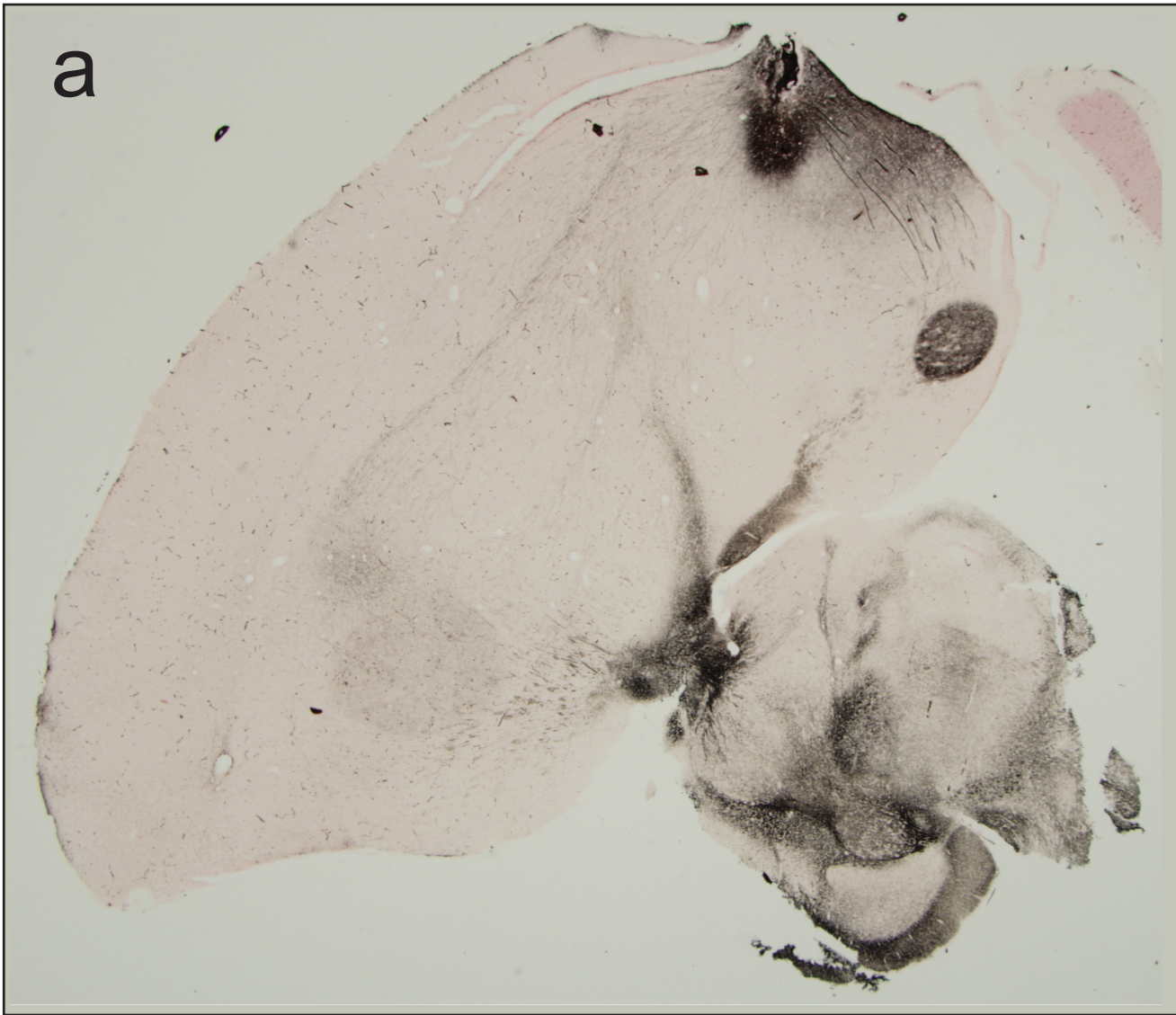
Supplemental Figure 4. Representative immunofluorescent confocal images of antibodies targeting TMEM119 within motor (HVC & RA) and learning-essential (Area X) regions of the contralateral (unlesioned) hemispheres. Aa – Af, TMEM119 immunofluorescence marks microglia. Ag – Al, Merge images of TMEM119 and Hoechst-stained nuclei.



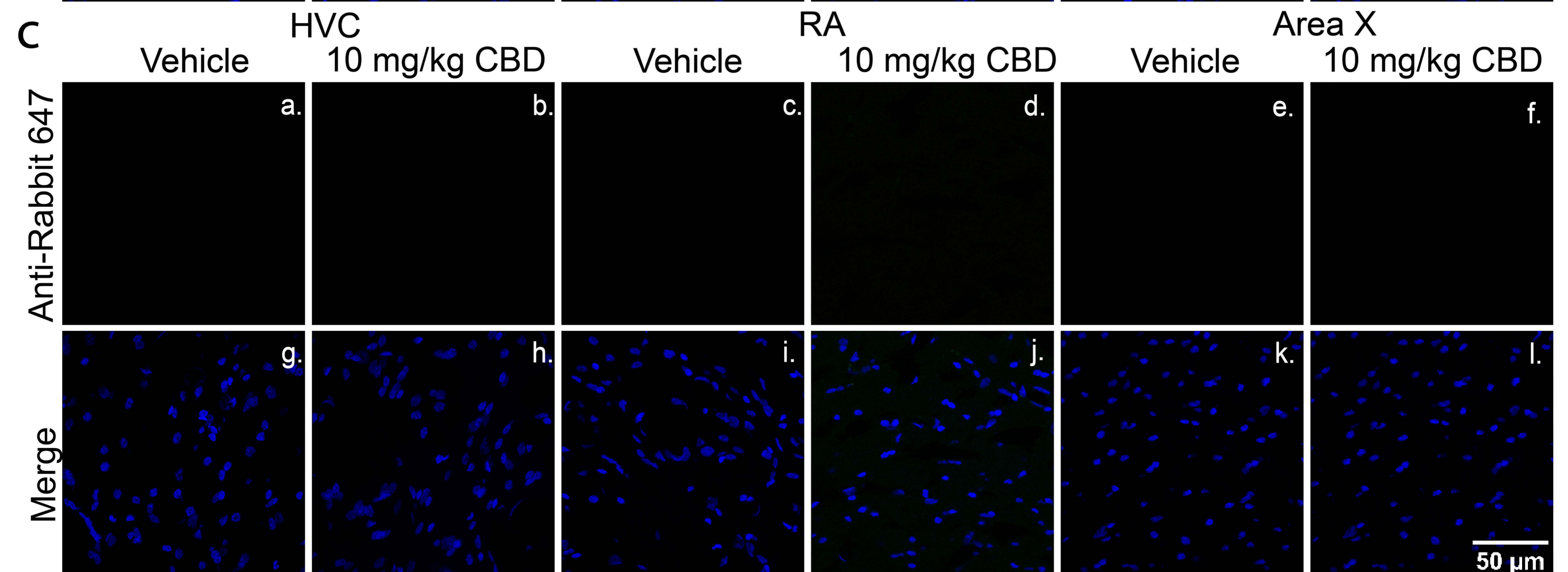
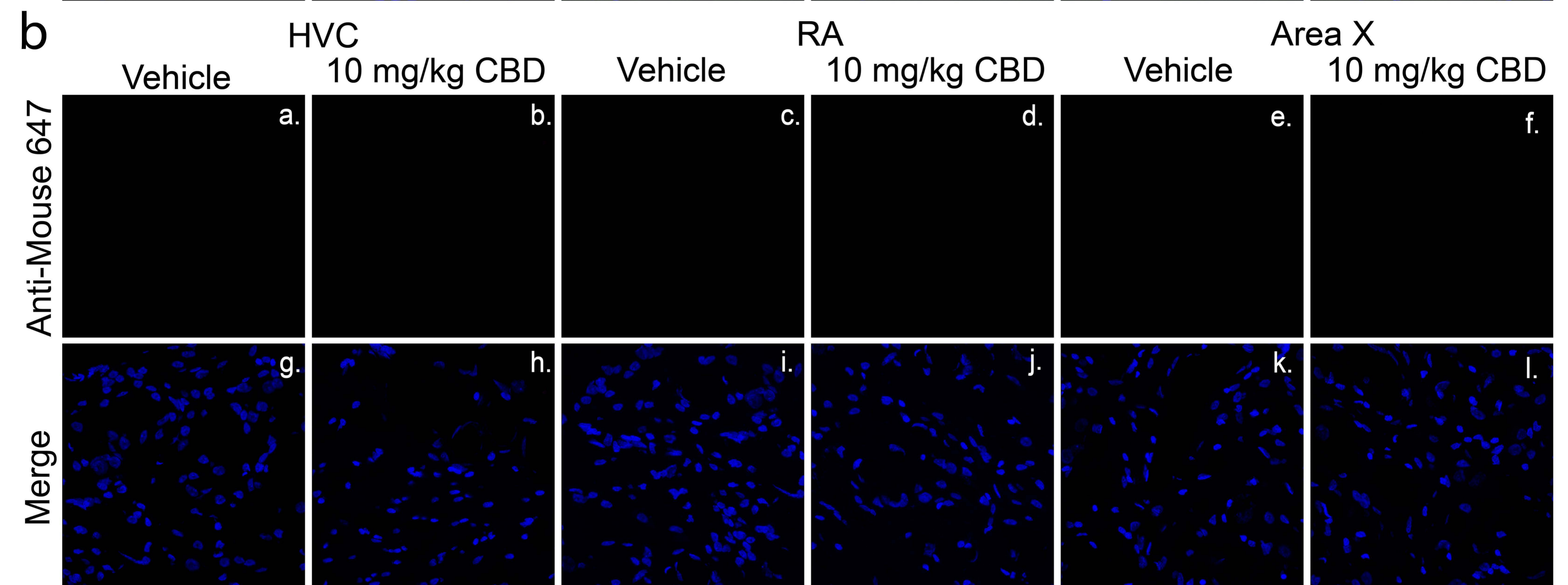
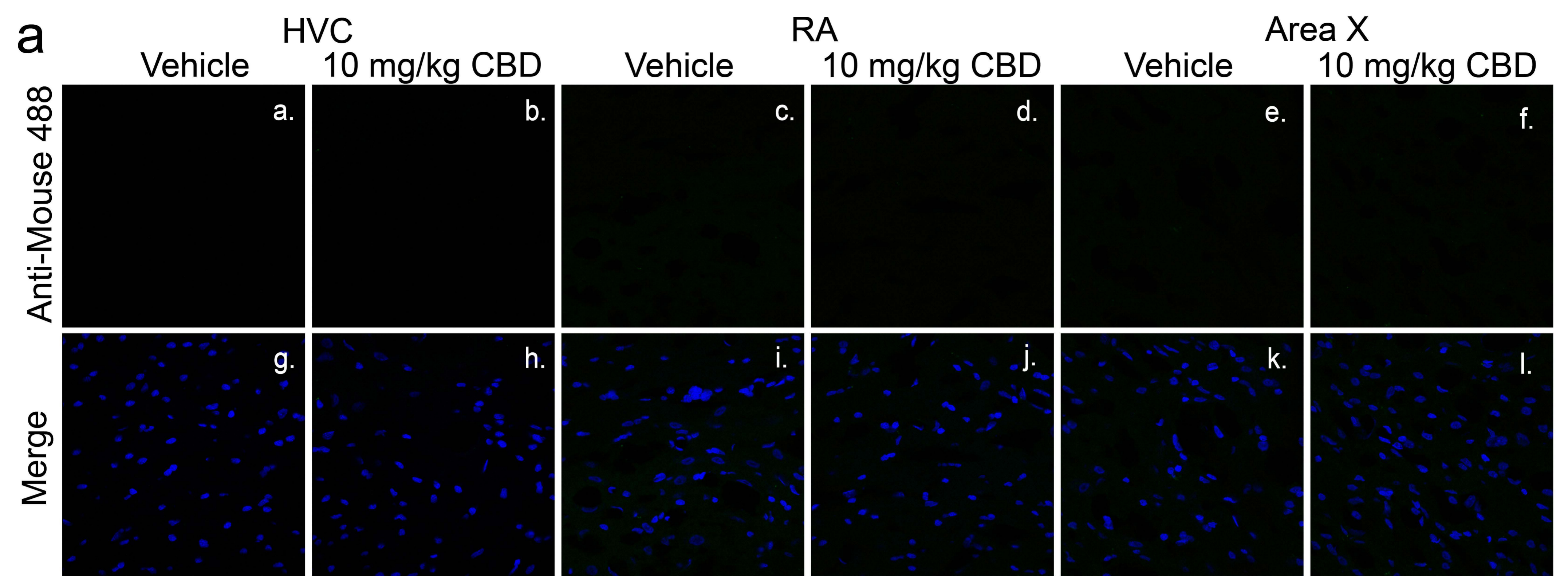
Supplemental Figure 5. Quantification of relative mean grey value of pro- and anti-inflammatory cytokine protein expression. A-C, Relative fluorescence of IL-6 by song region and drug treatment. D-F, Relative fluorescence of IL-1B by song region and drug treatment. G-I, Relative fluorescence of IL-10 by song region and drug treatment.



Supplemental Figure 6. Western blotting supports antibody selectivity with zebra finch protein. Each gel was run with ladder and protein. Each antibody was run on different gels along with its respective ladder.



Supplemental Figure 7. Amino-cupric-silver (A-Cu-Ag) staining to detect neuronal degeneration in zebra finch brain tissue 24 hours after unilateral microlesion (left is anterior, top is dorsal). A, representative image showing microlesion ablation of HVC, significant darkening of projected region RA and slight darkening of Area X. Note, there is axonal degradation from HVC to RA. B, outline of tissue with regions of interest labeled (HVC, RA, and Area X).



Supplemental Figure 8. No primary controls for immunofluorescent staining. The samples followed the same immunofluorescence staining protocol but were incubated with only the diluent (no primary antibody) and then with the respective secondary antibody. Images were taken using the same LSM700 settings as images taken with primary antibodies.

Gene Symbol	Accession Number	Forward Primer (5'-3')	Reverse Primer
GAPDH	NM_001198610.1	TGCTGCTCAGAACATTATCCC	TTTCCCACAGCCTAGCAGCT
IL-1 β	XM_002195564.2	TTCCGGTGCATCAGAGGCAGTTAT	GCACGAAGCACTTGTGGTCAATGT
IL-6	XM_002191284.2	CGTCTGCCAGAACAGCATGGAAAT	TATCCTCATTAAAGCCGGCGAGCA
IL-10	XM_002194605.4	CAAGCTCAAAGAGCTGAGGC	GCCCTTGATTCCTCCAGCA
TNF α	XM_002197321.2	TGTCCCATCTGC ACCACCTTCTTA	ATTCCCTCCATCTGGCTTCTGT
SOD2	NM_001245469.2	ACAGGGGCGCCTACAGATAG	CATGTTCCCATACGTCAATGCC
BDNF	XM_012573739.4	TCCAGCATCTGTTGGAGAGAC	CGAAGACCTGGGTAAGCCAA
RPS6KA5 (MSK1)	XM_030274847.3	CGGAGTGCCCCTGGAAAATA	GGCCGTTACTTTCCTCACT
Arc/Arg3.1	XM_002186825.6	TGGAAGAAGTCCATCAAGGC	TTGCGCCAGAGGAACTGGTC

Supplemental Table 1. Table listing gene symbol, accession number, and forward and reverse primers used for quantitative RT-PCR.

References

1. Alalawi, A. *et al.* Cannabidiol improves vocal learning-dependent recovery from, and reduces magnitude of deficits following, damage to a cortical-like brain region in a songbird pre-clinical animal model. *Neuropharmacology* **158**, 107716 (2019).
2. Soderstrom, K. & Alalawi, A. Software for objective comparison of vocal acoustic features over weeks of audio recording: KLFromRecordingDays. *SoftwareX* **6**, 271–277 (2017).