

# Journal of Visualized Experiments

## Localization of the locus coeruleus in the mouse brain

--Manuscript Draft--

<b>Article Type:</b>	Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE58652R2
<b>Full Title:</b>	Localization of the locus coeruleus in the mouse brain
<b>Keywords:</b>	Brain, locus coeruleus, metals, immunohistochemistry, X-ray fluorescence microscopy, norepinephrine, copper, DBH, TH, ATP7A, ATP7B
<b>Corresponding Author:</b>	Katharina Henny Schmidt, Ph.D. Johns Hopkins Medicine Baltimore, Maryland UNITED STATES
<b>Corresponding Author's Institution:</b>	Johns Hopkins Medicine
<b>Corresponding Author E-Mail:</b>	kschmi25@jhmi.edu
<b>Order of Authors:</b>	Katharina Henny Schmidt, Ph.D. Bilal Bari Martina Ralle Evan Maxey Svetlana Lutsenko
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Baltimore, MD, USA

**TITLE:**

Localization of the Locus Coeruleus in the Mouse Brain

**AUTHORS AND AFFILIATIONS:**

Katharina Schmidt<sup>1</sup>, Bilal Bari<sup>2</sup>, Martina Ralle<sup>3</sup>, Abigael Muchenditsi<sup>1</sup>, Evan Maxey<sup>4</sup>, Svetlana Lutsenko<sup>1</sup>

<sup>1</sup>Department of Physiology, Johns Hopkins University, School of Medicine, Baltimore, MD

<sup>2</sup>Department of Neuroscience, Brain Science Institute, Kavli Neuroscience Discovery Institute, Johns Hopkins University, Baltimore, MD

<sup>3</sup>Department of Molecular and Medical Genetics, OHSU, Portland, OR

<sup>4</sup>X-ray science division, Advanced Photon Source, Argonne National Laboratory

**Corresponding Author:**

Katharina Schmidt (kschmi25@jhmi.edu)

**Email Addresses of Co-authors:**

Bilal Bari (bbari1@jhmi.edu)

Martina Ralle (rallem@ohsu.edu)

Abigael Muchenditsi (amuchen1@jhmi.edu)

Evan Maxey (emaxey@aps.anl.gov)

Svetlana Lutsenko (lutsenko@jhmi.edu)

**KEYWORDS:**

Brain, locus coeruleus, metals, immunohistochemistry, X-ray fluorescence microscopy, norepinephrine, copper, DBH, TH, ATP7A, ATP7B

**SUMMARY:**

The locus coeruleus is a small cluster of neurons involved in a variety of physiological processes. Here, we describe a protocol to prepare mouse brain sections for studies of proteins and metals in this nucleus.

**ABSTRACT:**

The locus coeruleus (LC) is a major hub of norepinephrine producing neurons that modulate a number of physiological functions. Structural or functional abnormalities of LC impact several brain regions including cortex, hippocampus, and cerebellum and may contribute to depression, bipolar disorder, anxiety, as well as Parkinson disease and Alzheimer disease. These disorders are often associated with metal misbalance, but the role of metals in LC is only partially understood. Morphologic and functional studies of LC are needed to better understand the human pathologies and contribution of metals. Mice are a widely used experimental model, but the mouse LC is small (~0.3 mm diameter) and hard to identify for a non-expert. Here, we describe a step-by-step immunohistochemistry-based protocol to localize the LC in the mouse brain. Dopamine- $\beta$ -hydroxylase (DBH), and alternatively, tyrosine hydroxylase (TH), both enzymes highly expressed in the LC, are used as immunohistochemical markers in brain slices. Sections

adjacent to LC-containing sections can be used for further analysis, including histology for morphological studies, metabolic testing, as well as metal imaging by X-ray fluorescence microscopy (XFM).

## **INTRODUCTION:**

The locus coeruleus (LC) is an important region in the brainstem and a major site of norepinephrine (NE) production<sup>1</sup>. The LC sends projections throughout the brain<sup>2</sup> to the cortex, the hippocampus and the cerebellum<sup>3</sup> and regulates major physiological processes, including circadian rhythm<sup>4,5</sup>, attention and memory<sup>6</sup>, stress<sup>7</sup>, cognitive processes<sup>8</sup>, and emotion<sup>9,10</sup>. Dysfunction of LC has been implicated in neurological and neuropsychiatric disorders<sup>11</sup>, including Parkinson disease<sup>12-14</sup>, Alzheimer disease<sup>14</sup>, depression<sup>15-17</sup>, bipolar disorder<sup>18,19</sup>, and anxiety<sup>20-24</sup>. Given these roles, analysis of LC is crucial to studying its function and dysfunction.

Mice are widely used for studies of physiologic and pathophysiologic processes. Due to their small size, the mouse LC has an average diameter of ~300  $\mu$ m, leading to difficulty locating the structure. During brain sectioning, the LC can easily be missed in either coronal or sagittal sections. Available studies describing identification of LC in animals do not offer a step-by-step protocol that a non-expert can follow<sup>1,25</sup>. Thus, to offer guidance for the localization of LC, we describe a protocol that we developed to locate this region in the mouse brain for several applications (**Figure 1-3**). The protocol applies carefully controlled brain sectioning and immunohistochemical detection of DBH<sup>26,27</sup>, or alternatively TH<sup>24</sup>, both enzymes highly enriched in the LC<sup>28</sup>. Once LC is located by immunohistochemistry, adjacent brain slices can be used for further studies, including morphological and metabolic analyses, as well as metal imaging studies via X-ray fluorescence microscopy (XFM)<sup>29</sup>. We describe XFM as an example in this protocol (**Figure 3**).

## **PROTOCOL:**

Studies of animals was approved by Johns Hopkins University Animal Care and use (ACUC) protocol number M017M385.

### **1 Brain Slicing**

1.1 To immobilize, anesthetize mice by the application of 3% isoflurane.

1.1.1 Soak a cotton ball with drops of isoflurane and place it in a 15 mL microcentrifuge tube. Place the animal's nose into the tube and allow it to inhale the isoflurane. Check for the depth of anesthesia by the lack of response to toe-pinch.

1.2 Place the animal on its back and immobilize it by pinning its extremities down with a T pin while having access to its abdomen.

1.3 Cut the animal with surgical scissors by making a snip from the abdominal skin and cut through the skin in the thorax region. Pin the skin down using T pins. Then break the peritoneal

membrane up to the thorax. Expose the heart by cracking the thoracic cavity and cutting the diaphragm.

1.4 Cut the right atrium to allow the blood to flow out of the animal. Insert a 10 mL syringe with a 25-gauge needle into the left ventricle and perfuse with 10 mL of phosphate-buffered saline (PBS).

NOTE: This allows the solution to flow through systemic circulation and exit through the right atrium.

1.5 Remove the 10 mL syringe and insert a 25-gauge needle attached to the 60 mL syringe. Perfuse through the left ventricle with 50 mL of ice cold 4% paraformaldehyde (PFA).

1.5.1 Prepare ice cold 4% PFA solution by diluting 10% PFA solution in H<sub>2</sub>O and chilling the final 4% PFA solution at 4 °C.

1.6 Isolate the head of the mouse and remove the brain from the skull.

1.6.1 Cut the skin from the neck, and then cut towards the eyes to expose the skull. Crack the skull from the neck to the nose, and then from one eyeball to the other. Peel the skull out and excise the whole brain.

1.7 Incubate the brain in 4% PFA for 24 h at 4 °C.

1.8 Transfer the brain with forceps into a 50 mL conical tube filled with 25 mL of 30% sucrose solution. Keep it at 4 °C for 48-72 h until the brain sinks to the bottom of tube.

1.9 Cut the brain with an adult mouse brain slicer matrix coronal through the midbrain (~3 mm posterior of bregma). Keep the brain section containing the brainstem.

NOTE: This will result in two brain sections – one containing most of cortex (anterior of the cut) and one containing the brainstem/cerebellum (posterior of the cut). Use the brainstem section for the following steps.

1.10 Embed the brainstem section with the cut surface placed on the bottom of an embedding mold, surrounded by optimal cutting temperature compound (OCT); move the embedded brain to a -80 °C freezer and freeze for at least 12 h – until further use.

1.11 Cutting at the cryostat: place the embedding mold containing the brain in OCT into the cryostat; incubate it in the cryostat for several hours to adjust the temperature of the brain block to that of the cryostat.

1.12 Peel away the embedding mold to expose the OCT block containing the brain.

1.13 Use razor blades to remove excess of OCT from the surface of the block without touching the brain.

1.14 Mount the OCT block on the chuck of the cryostat, exposing the cut surface of the brain toward the front.

1.15 Adjust the cut surface of the brain so that it is oriented in parallel to the razorblades of the cryostat.

1.16 Trim the brain beginning at the medulla, cutting 100  $\mu$ m sections rostrally.

1.17 Trim rostrally until the cerebellum and brainstem cut as one continuous slice. Begin collecting slices at 50  $\mu$ m thickness.

NOTE: As one trims rostrally from the medulla, the brainstem and cerebellum will cut as two separate sections. In rostral sections, the brainstem and cerebellum will eventually merge at the level of the 4<sup>th</sup> ventricle. Once the lateral edges of the 4<sup>th</sup> ventricle are well-formed, then the cerebellum and brainstem will come out as one continuous slice.

1.17.1 Collect OCT-surrounded brain slice with forceps and place it in a well of a 24-well plate filled with PBS (**Figure 2a**). The LC will be most prominent when the cerebellum and inferior colliculus meet one another at  $\sim$ -5.52 mm posterior of bregma (**Figure 1b**).

NOTE: The most anterior part of LC will disappear once the cerebellum has been fully sectioned and no longer surrounds the inferior colliculus at  $\sim$ -5.34 mm posterior of bregma (**Figure 1c**).

## 2 Immunohistochemistry for Dopamine $\beta$ -Hydroxylase or Tyrosine Hydroxylase (Figure 2)

### 2.1 Day 1

2.1.1 Wash the selected slices three times for 5 min in PBS.

2.1.2 Permeabilize for 24 h in 0.5% phosphate buffered saline with detergent (PBSD) at 4  $^{\circ}$ C.

2.1.2.1 Dilute 125  $\mu$ L of detergent in 25 mL of PBS.

### 2.2 Day 2

2.2.1 Wash slices three times for 5 min in 0.5% PBSD.

2.2.2 Add the primary antibody, anti-DBH or anti-TH for 18 h at a dilution of 1:500 in 0.5% PBSD at 4  $^{\circ}$ C.

### 2.3 Day 3

177  
178 2.3.1 Wash slices three times for 10 min in 0.5% PBSD.

179  
180 2.3.2 Add the desired secondary antibody (488 donkey anti-rabbit for green fluorescence) at a  
181 dilution of 1:1000 in 0.5% PBSD for 16 h.

182  
183 2.3.3 Wrap the 24 well plate in aluminum and place at 4 °C.

184  
185 2.3.4 Wash slices three times for 5 min in 0.5% PBSD.

186  
187 2.3.5 Wash for 5 min in PBS.

188  
189 2.3.6 Transfer slices with a pencil brush into a water container.

190  
191 2.3.7 Mount slices floating in water on charged slides.

192  
193 2.3.8 Coverslip sections with hard-set mounting media (without DAPI).

194  
195 2.3.9 Dry the mounted brain sections for 30 min at room temperature.

196  
197 2.3.10 Image brain slices at a confocal or fluorescent microscope with settings to detect signal  
198 from appropriate secondary antibody fluorophore wavelength.

199  
200 2.3.11 Adjust the microscope to the focal plane of the brain slice and take a single image at 10X  
201 magnification.

202  
203 2.3.12 To locate a possible LC region in the brain slice, use the 4<sup>th</sup> ventricle for orientation; the  
204 cerebellum is located above the ventricle, pons and brainstem are found below<sup>30</sup>.

205  
206 2.3.13 To locate the LC, focus on the lateral edges of the 4<sup>th</sup> ventricle; the LC originates from the  
207 edges of the 4<sup>th</sup> ventricle and points toward the pons/ brainstem region (**Figure 2b, 2c**).

208  
209 2.3.14 Following imaging and localization of the LC in certain brain slices, store slides containing  
210 brain slices at 4 °C.

### 211 212 **3 Detection of the LC in Brain Slices**

213  
214 3.1 To locate the brain section containing the LC, slice the mouse brain as described above  
215 and collect sections in PBS filled 24 well dishes, as shown in **Figure 2a**.

216  
217 3.1.1 Place one brain section per well to allow for the proper localization of the LC.

218  
219 3.2 Collect a total of 48 brain slices that will be immunostained per brain.

220

NOTE: All the wells of the two dishes shown in **Figure 2a** contain brain slices from one animal.

3.3 Immunostain every third to fifth slice for DBH, or TH. Preferably, perform immunohistochemistry of those brain slices that are adjacent to those that are assayed further (via X-ray fluorescence microscopy, XFM).

NOTE: This procedure allows for exact localization of the LC in the final assay slices (**Figure 2a**; labeled with numbers between the wells).

3.4 Adjust the number of brain slices that are immunostained depending on the application, *e.g.*, whether they require exact location of the center and edge of the LC, or just a rough approximation.

3.5 Following immunohistochemistry, detect brain slices containing LC via a characteristic pattern of expression at both sides of the 4<sup>th</sup> ventricle (**Figure 2b, 2c**). Magnification of the DBH signal in the LC of consecutive brain slices is shown in **Figure 2d, 2e**; the magnified image of the LC that is stained for TH is shown in **Figure 2f**.

3.6 Use the sections adjacent to those containing LC for further studies - in this case for XFM to quantify metal levels (**Figure 3**).

3.7 To perform XFM, collect 10-30  $\mu\text{m}$  (depending on the setup) thin coronal brain slices on thin polymeric film with which they can be mounted on sample holders and imaged at the synchrotron.

3.8 Store brain slices which are prepared on thin polymeric film for XFM at room temperature and perform XFM.

#### **4 Metal Imaging in the LC via XFM**

4.1 Slice example mouse brain as described above, determine LC, and measure metal levels via XFM from these brain slices containing the LC.

4.2 Image elemental distributions on beamline 2-ID-E at the Advanced Photon Source (Argonne National Laboratory, Argonne IL).

4.3 Record data 'on the fly' as described previously<sup>31</sup>.

4.4 Determine elemental concentrations in brain slices containing the LC using the program MAPS<sup>32,33</sup>.

#### **REPRESENTATIVE RESULTS:**

Changes in metal homeostasis (such as Cu, Fe, Zn, and Mn) are often observed in neurologic disorders, including changes in the LC<sup>34,35</sup>. Thus, determining metal levels in the brain is necessary

for understanding of disease mechanisms. The brain sections generated using the described protocol can be used to quantify the levels of Cu and other metals in the LC and compare them to the levels in regions outside of the LC. (**Figure 3**). In our example, the brain slice that was cut through the LC, phosphate, potassium, chloride and copper was measured. Only copper was specifically elevated in the LC. Higher resolution XFM imaging (not shown here) can also be performed for detection of subcellular distribution of metal levels. Further possible applications of this protocol include the detection of abundance and intracellular distribution of DBH (**Figure 2b, 2d, 2e**), TH (**Figure 2c, 2f**), and other proteins expressed in the LC individually or in the co-staining assays, studies of LC morphology and neuronal density.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Localization of LC in the mouse brainstem.** (a) Schematic demonstrating the region of the brainstem that will be sectioned to localize the LC. (b) Based on the Paxinos and Franklin brain atlas<sup>30</sup>, LC will be most prominent when the cerebellum and inferior colliculus meet one another (-5.52 mm posterior of bregma). The left image shows a coronal section cut through the LC, while the right image demonstrates localization of LC on the lateral edges of the 4<sup>th</sup> ventricle via Nissl staining. (c) The most anterior part of LC will disappear once the cerebellum has been fully sectioned and no longer surrounds the inferior colliculus (-5.34 mm posterior of bregma). Nissl staining on the right image shows that LC is only marginally present in this coronal slice.

**Figure 2: Detection of LC by immunostaining brain slices for DBH or TH.** (a) One mouse brain was sectioned into 50  $\mu$ m slices around the brain stem and collected in two 24 well dishes. Every 5<sup>th</sup> to 8<sup>th</sup> collected brain slice was mounted on film covered coverslip for imaging via XFM. These slices are labeled with blue numbers between the wells. Adjacent slices floating in PBS were immunostained for DBH to detect LC (labeled with red cross on wells). Green circles denote slices positive for DBH signal, and thus contain LC. (b) A coronal brain slice containing LC was immunostained with DBH and imaged on a confocal microscope. The image shows strong signal in the LC (in green). (c) A coronal slice containing LC was immunostained for tyrosine hydroxylase (TH) and imaged on a fluorescent microscope. Image shows strong signal for both left and right LC. (d) Slices were immunostained for DBH, and slices 7 (on the left) and 9 (on the right) contained LC. Adjacent sections were selected for XFM analysis. (e) Slice 10 also showed LC. In this section, the left LC was cut through its center while the right LC was captured at its anterior-most edge. (f) A section containing LC was immunostained for TH and imaged on a confocal microscope. Image shows TH expressing neurons in the LC labeled in green.

**Figure 3: Imaging of metal levels in the LC.** The brain of a male 12-week-old mouse with a knockout of Cu-transporter ATP7B was isolated and prepared as described in this protocol. Non-stained slices adjacent to LC-containing sections were taken and metal levels were measured via XFM. Cu levels were specifically increased in the LC (labeled with yellow arrows) as compared to the surrounding brain region, while other elements (K, P, and Cl) were unchanged.

#### DISCUSSION:

Properly orienting the specimen is a crucial step in this protocol. Because we are using anatomical



features of the dorsal surface of the brain to locate LC (boundary between cerebellum and inferior colliculus), it is important that the sections be aligned properly. This requires care in properly setting the brain into the mouse brain slicer matrix. We recommend cutting ~500  $\mu\text{m}$  more tissue anterior and posterior to LC to avoid missing the nucleus. The most common mistake is to cut too few sections that results in missing the LC entirely. Thus, for one's first time following this protocol, we recommend cutting more sections than necessary. Careful study of the brain atlas images prior to staining is very helpful. The appearance of the brainstem changes appreciably every few hundred microns and, with some experience, it is possible to know what sections are worth staining simply by the macroscopic appearance.

During the process of localizing the LC, there might be variations in the signal depending on how well the brain was oriented during sectioning. When cutting through the center of the LC, the signal is bright and covers a larger area as compared to the edge of the LC, which will show up as a signal over a much smaller area. In the case that coronal slices are slightly tilted, the LC of one side of the 4<sup>th</sup> ventricle might be apparent and the one on the other side might only be visible in an adjacent slide. Thus, one cannot always expect the appearance of both LC regions at maximum intensity within one brain slice. This artifact can be avoided by cutting the brain exactly coronal in the mouse brain slicer matrix and carefully embedding the brain into the cubical embedding mold with OCT.

Immunostaining, at least with the anti-tyrosine hydroxylase antibody, is extremely forgiving and, in our experience, works on sections up to 100  $\mu\text{m}$  in thickness. We have found that blocking solution is not necessary for high signal-to-noise staining of LC, reducing cost and reducing the amount of time needed to locate LC. In our experience, the staining protocol can be sped up – albeit with reduced quality and penetrance of staining – by reducing permeabilization to 2 h, primary antibody for 8 h, and secondary antibody for 2 h. Additionally, if one is simply interested in locating LC (*e.g.*, for validating injection of a virus/tracer), sections from a fixed brain can be cut on a vibratome at 100  $\mu\text{m}$  thickness.

One limitation of this protocol is it, by design, requires euthanizing the animal and removing the brain. Therefore, it is not useful for *in vivo* localization (*e.g.*, electrophysiologic recordings). Another limitation is that this protocol requires PFA fixation which might alter the native state of the tissue. These alterations include the elemental content such as copper, calcium, iron and zinc<sup>36</sup>. The actual alteration of metal distribution caused by PFA fixation may be tested in one sample which can be run in parallel to a non-fixed sample. A comparison of the metal distribution between these two samples will provide evidence on the effect of PFA fixation on the distribution of the metal which is of interest in a certain study. If PFA fixation must be avoided, the general principle of this protocol (locating LC by immunostaining and using adjacent sections for follow-up experiments) can be extended to frozen sections without fixation.

We note that this protocol is largely a refinement of existing methods to solve this problem. The novelty exists in tailoring previous approaches to locate a very small, easily missed nucleus. We expect that this protocol can be easily modified and extended based on need (*e.g.*, using transgenic animals expressing fluorophores in LC to avoid immunostaining).

**ACKNOWLEDGMENTS:**

We thank Abigael Muchenditsi for the maintenance of the mouse colony. Use of the Advanced Photon Source at Argonne National Laboratory was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract number: DE-AC02-06CH11357. We thank Olga Antipova and Dr. Stefan Vogt for user support and assistance at the Advanced Photon Source. This work was funded by the National Institute of Health grant 2R01GM101502 to SL.

**DISCLOSURES:**

None.

**REFERENCES:**

- 1 Robertson, S. D., Plummer, N. W., de Marchena, J. & Jensen, P. Developmental origins of central norepinephrine neuron diversity. *Nature neuroscience* **16**, 1016-1023, doi:10.1038/nn.3458 (2013).
- 2 Kobayashi, R. M., Palkovits, M., Jacobowitz, D. M. & Kopin, I. J. Biochemical mapping of the noradrenergic projection from the locus coeruleus. A model for studies of brain neuronal pathways. *Neurology* **25**, 223-233 (1975).
- 3 Olson, L. & Fuxe, K. On the projections from the locus coeruleus noradrenaline neurons: the cerebellar innervation. *Brain research* **28**, 165-171 (1971).
- 4 Costa, A., Castro-Zaballa, S., Lagos, P., Chase, M. H. & Torterolo, P. Distribution of MCH-containing fibers in the feline brainstem: Relevance for REM sleep regulation. *Peptides* **104**, 50-61, doi:10.1016/j.peptides.2018.04.009 (2018).
- 5 Semba, J., Toru, M. & Mataga, N. Twenty-four hour rhythms of norepinephrine and serotonin in nucleus suprachiasmaticus, raphe nuclei, and locus coeruleus in the rat. *Sleep* **7**, 211-218 (1984).
- 6 Takeuchi, T. *et al.* Locus coeruleus and dopaminergic consolidation of everyday memory. *Nature* **537**, 357-362, doi:10.1038/nature19325 (2016).
- 7 Korf, J., Aghajanian, G. K. & Roth, R. H. Increased turnover of norepinephrine in the rat cerebral cortex during stress: role of the locus coeruleus. *Neuropharmacology* **12**, 933-938 (1973).
- 8 Sara, S. J. & Segal, M. Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: implications for cognition. *Progress in brain research* **88**, 571-585 (1991).
- 9 Markevich, V. A. & Voronin, L. L. [Synaptic reactions of sensomotor cortex neurons to stimulation of emotionally significant brain structures]. *Zhurnal vysshei nervnoi deiatelnosti imeni I P Pavlova* **29**, 1248-1257 (1979).
- 10 File, S. E., Deakin, J. F., Longden, A. & Crow, T. J. An investigation of the role of the locus coeruleus in anxiety and agonistic behaviour. *Brain research* **169**, 411-420 (1979).
- 11 Pamphlett, R. Uptake of environmental toxicants by the locus ceruleus: a potential trigger for neurodegenerative, demyelinating and psychiatric disorders. *Medical hypotheses* **82**, 97-104, doi:10.1016/j.mehy.2013.11.016 (2014).
- 12 Wang, J. *et al.* Neuromelanin-sensitive magnetic resonance imaging features of the substantia nigra and locus coeruleus in de novo Parkinson's disease and its phenotypes. *European*

397 *journal of neurology* **25**, 949-e973, doi:10.1111/ene.13628 (2018).

398 13 Oliveira, L. M., Tuppy, M., Moreira, T. S. & Takakura, A. C. Role of the locus coeruleus  
399 catecholaminergic neurons in the chemosensory control of breathing in a Parkinson's disease  
400 model. *Experimental neurology* **293**, 172-180, doi:10.1016/j.expneurol.2017.04.006 (2017).

401 14 Zarow, C., Lyness, S. A., Mortimer, J. A. & Chui, H. C. Neuronal loss is greater in the locus  
402 coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Archives*  
403 *of neurology* **60**, 337-341 (2003).

404 15 Chandley, M. J. *et al.* Gene expression deficits in pontine locus coeruleus astrocytes in  
405 men with major depressive disorder. *Journal of psychiatry & neuroscience : JPN* **38**, 276-284,  
406 doi:10.1503/jpn.120110 (2013).

407 16 Bernard, R. *et al.* Altered expression of glutamate signaling, growth factor, and glia genes  
408 in the locus coeruleus of patients with major depression. *Molecular psychiatry* **16**, 634-646,  
409 doi:10.1038/mp.2010.44 (2011).

410 17 Gos, T. *et al.* Tyrosine hydroxylase immunoreactivity in the locus coeruleus is elevated in  
411 violent suicidal depressive patients. *European archives of psychiatry and clinical neuroscience*  
412 **258**, 513-520, doi:10.1007/s00406-008-0825-8 (2008).

413 18 Biela, H. *et al.* Immunohistochemical evidence for impaired nitric oxide signaling of the  
414 locus coeruleus in bipolar disorder. *Brain research* **1459**, 91-99,  
415 doi:10.1016/j.brainres.2012.04.022 (2012).

416 19 Wiste, A. K., Arango, V., Ellis, S. P., Mann, J. J. & Underwood, M. D. Norepinephrine and  
417 serotonin imbalance in the locus coeruleus in bipolar disorder. *Bipolar disorders* **10**, 349-359,  
418 doi:10.1111/j.1399-5618.2007.00528.x (2008).

419 20 Borodovitsyna, O., Flamini, M. D. & Chandler, D. J. Acute Stress Persistently Alters Locus  
420 Coeruleus Function and Anxiety-like Behavior in Adolescent Rats. *Neuroscience* **373**, 7-19,  
421 doi:10.1016/j.neuroscience.2018.01.020 (2018).

422 21 Hirschberg, S., Li, Y., Randall, A., Kremer, E. J. & Pickering, A. E. Functional dichotomy in  
423 spinal- vs prefrontal-projecting locus coeruleus modules splits descending noradrenergic  
424 analgesia from ascending aversion and anxiety in rats. *eLife* **6**, doi:10.7554/eLife.29808 (2017).

425 22 McCall, J. G. *et al.* CRH Engagement of the Locus Coeruleus Noradrenergic System  
426 Mediates Stress-Induced Anxiety. *Neuron* **87**, 605-620, doi:10.1016/j.neuron.2015.07.002  
427 (2015).

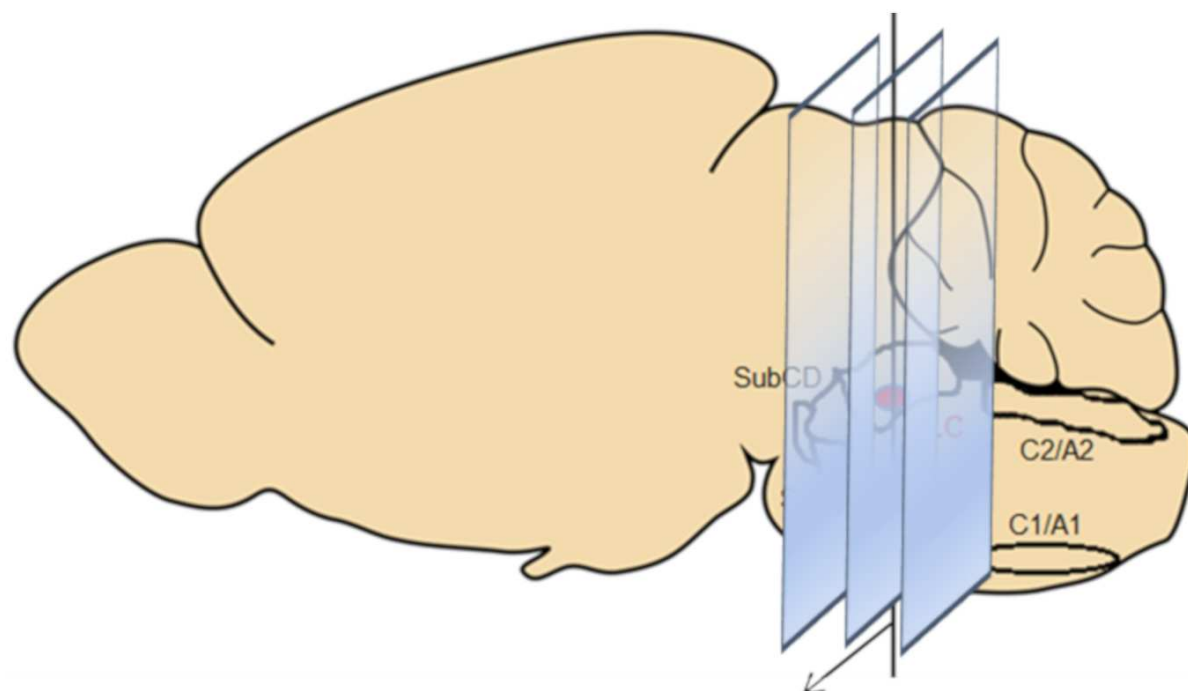
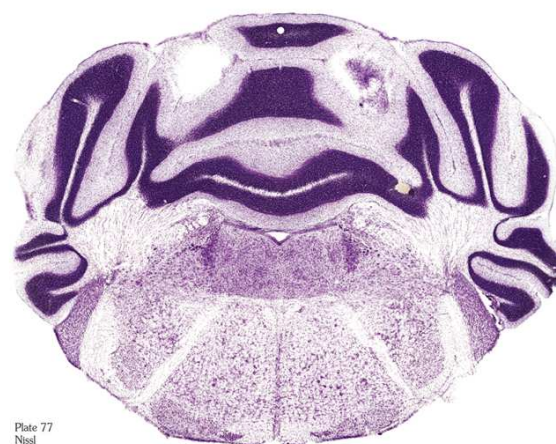
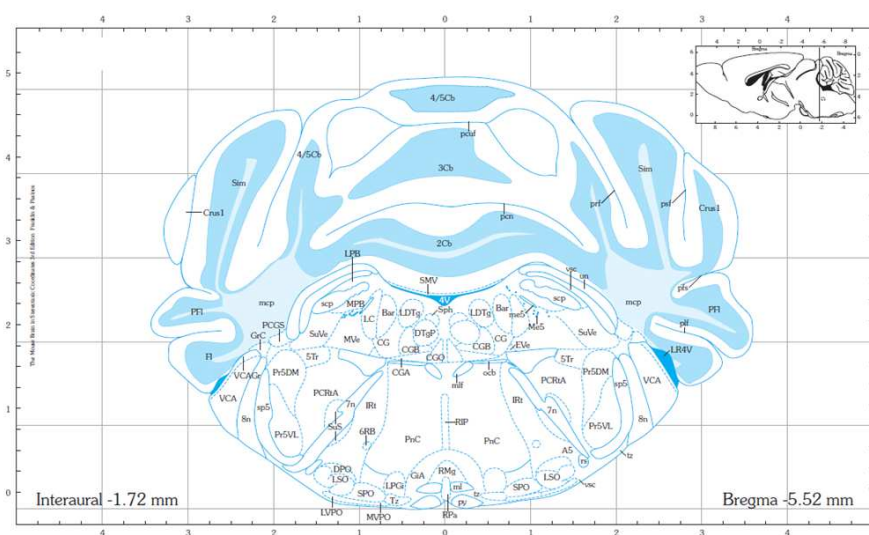
428 23 Borges, G. P., Mico, J. A., Neto, F. L. & Berrocoso, E. Corticotropin-Releasing Factor  
429 Mediates Pain-Induced Anxiety through the ERK1/2 Signaling Cascade in Locus Coeruleus  
430 Neurons. *The international journal of neuropsychopharmacology* **18**, doi:10.1093/ijnp/pyv019  
431 (2015).

432 24 Simone, J. *et al.* Ethinyl estradiol and levonorgestrel alter cognition and anxiety in rats  
433 concurrent with a decrease in tyrosine hydroxylase expression in the locus coeruleus and brain-  
434 derived neurotrophic factor expression in the hippocampus. *Psychoneuroendocrinology* **62**, 265-  
435 278, doi:10.1016/j.psyneuen.2015.08.015 (2015).

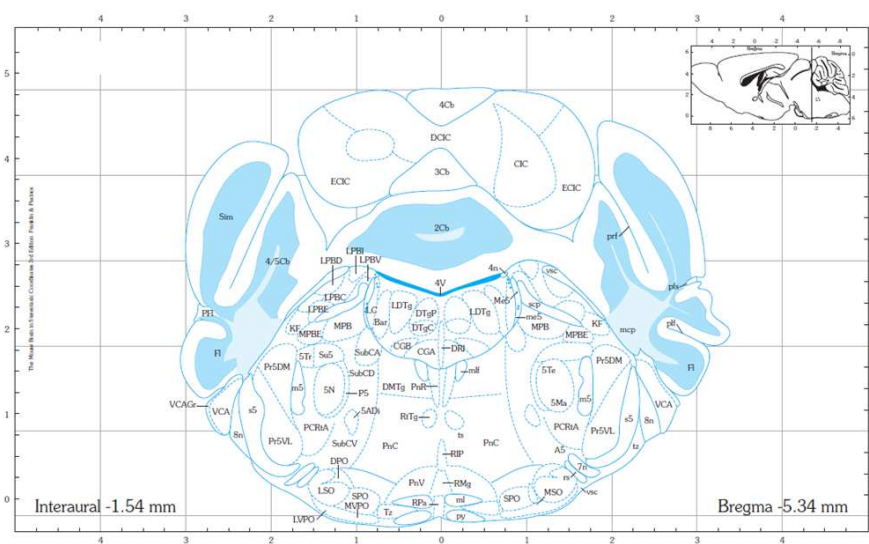
436 25 Carter, M. E. *et al.* Tuning arousal with optogenetic modulation of locus coeruleus  
437 neurons. *Nature neuroscience* **13**, 1526-1533, doi:10.1038/nn.2682 (2010).

438 26 Fan, Y. *et al.* Corticosterone administration up-regulated expression of norepinephrine  
439 transporter and dopamine beta-hydroxylase in rat locus coeruleus and its terminal regions.  
440 *Journal of neurochemistry* **128**, 445-458, doi:10.1111/jnc.12459 (2014).

- 27 Xiao, T. *et al.* Copper regulates rest-activity cycles through the locus coeruleus-norepinephrine system. *Nature chemical biology* **14**, 655-663, doi:10.1038/s41589-018-0062-z (2018).
- 28 Amaral, D. G. & Sinnamon, H. M. The locus coeruleus: neurobiology of a central noradrenergic nucleus. *Progress in neurobiology* **9**, 147-196 (1977).
- 29 Ralle, M. *et al.* Wilson Disease at a Single Cell Level: intracellular copper trafficking activates compartment-specific responses in hepatocytes. *The Journal of Biological Chemistry* **285**, 30875-30883, doi:10.1074/jbc.M110.114447 (2010).
- 30 Paxinos, G. (ed Keith B. J. Franklin) (Boston :, Amsterdam :, 2013).
- 31 Bonnemaïson, M. L. *et al.* Copper, zinc and calcium: imaging and quantification in anterior pituitary secretory granules. *Metallomics : integrated biometal science* **8**, 1012-1022, doi:10.1039/c6mt00079g (2016).
- 32 Nietzold, T. *et al.* Quantifying X-Ray Fluorescence Data Using MAPS. *Journal of visualized experiments : JoVE*, doi:10.3791/56042 (2018).
- 33 Vogt, S. MAPS : A set of software tools for analysis and visualization of 3D X-ray fluorescence data sets. *J. Phys. IV France* **104**, 635-638 (2003).
- 34 Davies, K. M. *et al.* Copper pathology in vulnerable brain regions in Parkinson's disease. *Neurobiology of aging* **35**, 858-866, doi:10.1016/j.neurobiolaging.2013.09.034 (2014).
- 35 Davies, K. M., Mercer, J. F., Chen, N. & Double, K. L. Copper dyshomeostasis in Parkinson's disease: implications for pathogenesis and indications for novel therapeutics. *Clinical science (London, England : 1979)* **130**, 565-574, doi:10.1042/cs20150153 (2016).
- 36 James, S. A. *et al.* Quantitative comparison of preparation methodologies for X-ray fluorescence microscopy of brain tissue. *Analytical and bioanalytical chemistry* **401**, 853-864, doi:10.1007/s00216-011-4978-3 (2011).

**A****B**

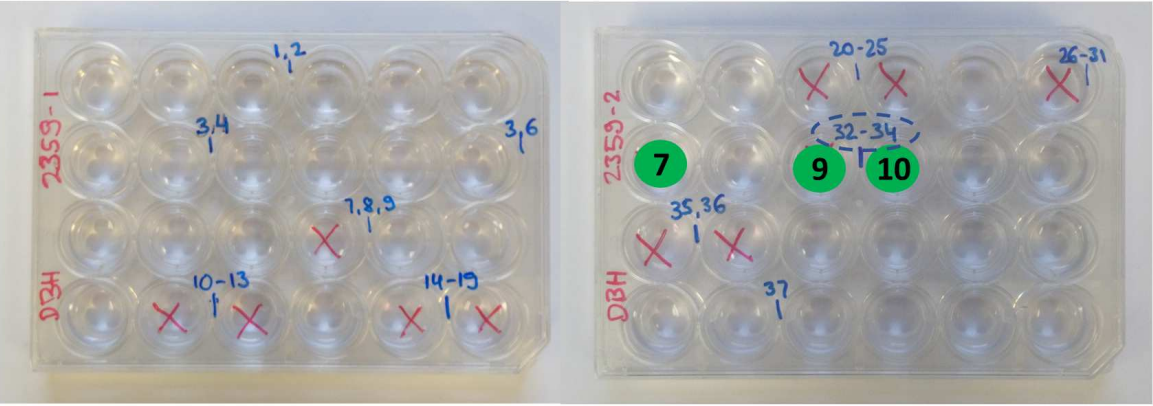
[Figure](#) | [Full Screen](#) | [Open](#) | [Home](#) | [<](#) | [>](#)

**C**

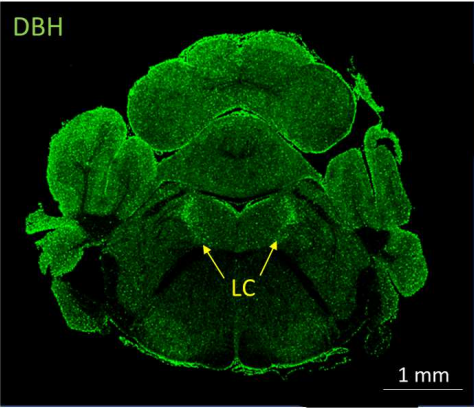
[Figure](#) | [Full Screen](#) | [Open](#) | [Home](#) | [<](#) | [>](#)



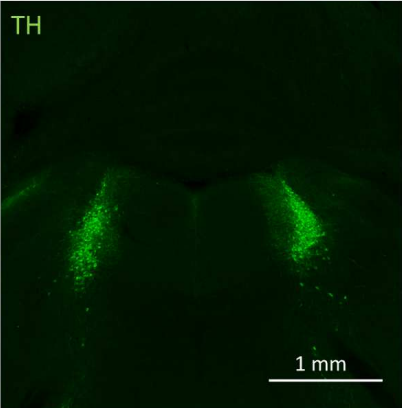
**A**



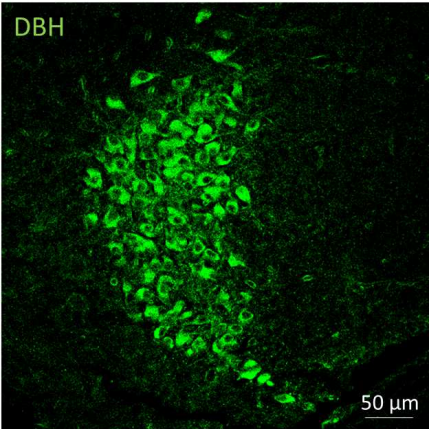
**B**



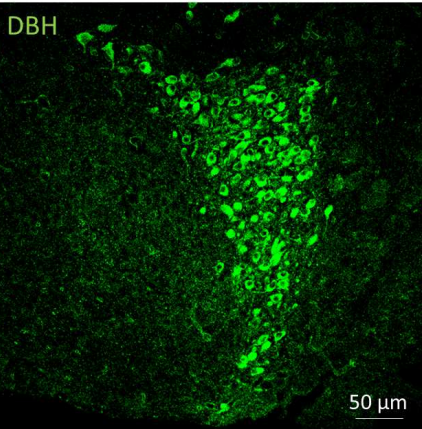
**C**



**D**

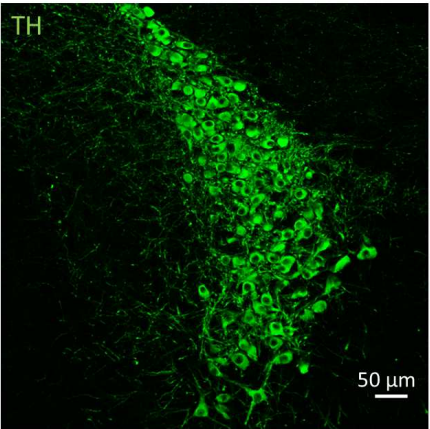


2359-2-7 left LC

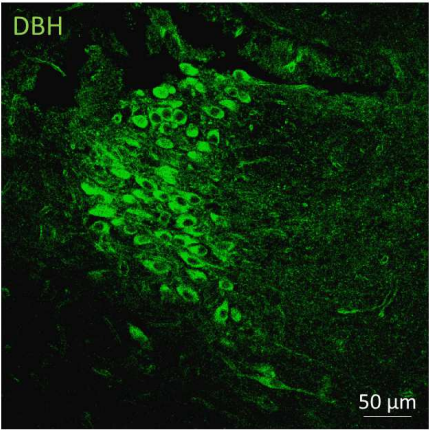


2359-2-9 right LC

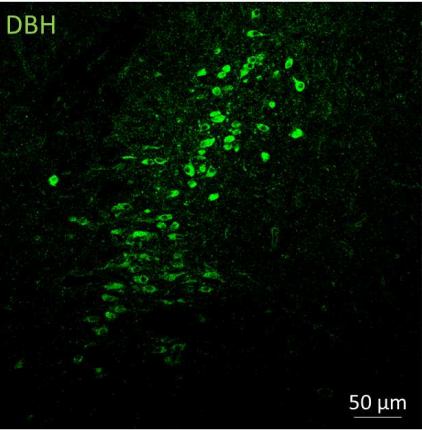
**F**



**E**



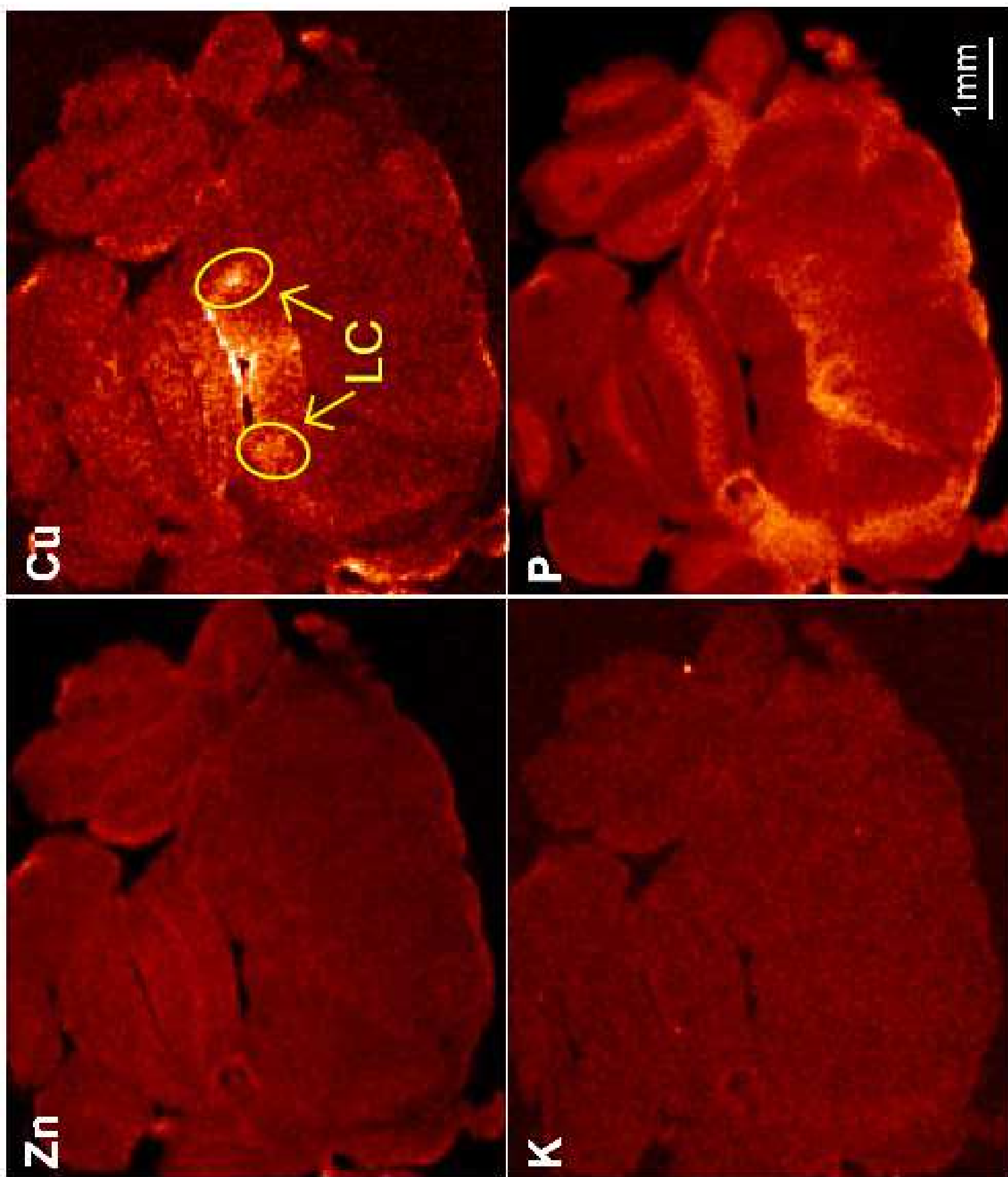
2359-2-10 left LC



2359-2-10 edge of right LC

Figure 3

[Click here to access/download;Figure;Fig3 R2.eps](#)



Name of Material/ Equipment	Company	Catalog Number
Adult mouse brain slicer matrix	Zivic Instruments	BSMAS001-1
Anti-rabbit secondary antibody, Alexa Fluor 488 (source - donkey)	Thermo Fisher Scientific	A-21206
Charged glass slides	Genesee	29-107
Confocal microscope	Zeiss	LSM 800
Cryostat	Microm GmbH	HM 505E
	Thermo Fisher Scientific	
Cryostat cutting blades	Scientific	MX35
Scissors Mini, 9.5cm	Antech Diagnostcs	503241
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich	D9542-10MG
Dopamine $\beta$ -hydroxylase (DBH) antibody - inhouse production (source - rabbit)	B. Eipper	-
Dopamine $\beta$ -hydroxylase (DBH) antibody - commercially availabe (source - rabbit)	Cell Signaling	8586
Falcon tubes, 50ml	USA Scientific	339652
Forane (isofluorane)	Baxter	NDC 1019-360-60
Forceps Micro Adson	Antech Diagnostcs	501245
Hardset mounting media	EM sciences	17984-24
Microscope	Pascal	LSM 5
	Thermo Fisher Scientific	
Multi-well plates, 24 wells	Scientific	930186
Optimal cutting temperature compound (OCT)	VWR/ tissue tech	102094-106
Paraformaldehyde (PFA)/ formalin 10%	Fisher Scientific	SF98-4
Peel-A-Way disposable embedding molds	Polysciences Inc.	18646A
Pencil brush		
Phosphate buffered saline (PBS)	Life Tech	14190250
Razor blades	Amazon	ASIN: B000CMFJZ2
Spatulas	Antech Diagnostcs	14374
T pins	Office Depot	344615
		ISBN: 978-
The Mouse Brain in Stereotaxic Coordinates, Paxinos and Franklin, 3rd Edition	Amazon	0123694607
Triton-X 100 (to prepare PBS)	Sigma-Aldrich	T8787



Tween 20

Tyrosine hydroxylase (TH) antibody (source - rabbit)

Ultralene thin film for XRF

Wide-field fluorescent microscope

Sigma-Aldrich

EMD Millipore

SPEX Sample Prep

Zeiss

P7949-500ml

AB152

3525

Axio Zoom.V16



1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Localization of the locus coeruleus in the mouse brain

Author(s):

Katharina Schmidt, Bilal Bari, Martina Ralle, Evan Maxey, Svetlana Lutsenko

Item 1 (check one box): The Author elects to have the Materials be made available (as described at

<http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

## ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

**11. Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

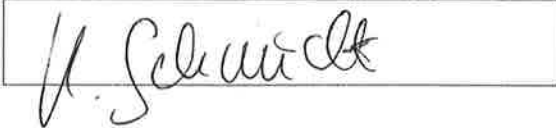
expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

**12. Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

**13. Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name: Katharina Henny Schmidt  
Department: Physiology  
Institution: Johns Hopkins School of Medicine  
Article Title: Localization of the locus coeruleus in the mouse brain  
Signature:  Date: 06/21/18

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

August 30<sup>th</sup>, 2018

Dear Dr. Bajaj,

We sincerely appreciate the editorial comments in the second revision phase of our manuscript JoVE58652. Thank you very much for formatting our manuscript according to the JoVE guidelines. My co-authors and I read and addressed your edits carefully, and we hope that we followed all instructions with satisfaction.

In the introduction part, we added the requested references to provide a comprehensive background for the protocol.

In the protocol part itself, we addressed all comments and added details on the instructions for the dissection part as well as the brain slicing, immunostaining and LC identification. As requested, we also provided citations in this part. Concerning the video production, we labeled 2.75 pages which are essential for the process of identifying the LC in yellow.

Concerning the preparation of Figure 3, we selected an image with higher resolution and provided the essential scale bar. To address the editor's comment 4, we have not published any of the figures previously.

We are looking forward to your feedback. Thank you very much in advance.

Sincerely,

Katharina Schmidt