Investigating the Neuronal Circuit of the Lateral Hypothalamus Related to Anxiety

Gina Christoffersen
gina.christoffersen@gmail.com

under the direction of
Prof. Konstantinos Meletis
Ph.D. Daniela Calvigioni
Ph.D. János Fuzik
Department of Neuroscience
Karolinska Institutet

Research Academy for Young Scientists
July 11, 2018

Abstract

Mental illness is a global problem which lacks the cell specific answers. To heal a damaged brain, researchers firstly need to comprehend the neuronal circuit in a functional brain. The aim of this study was to map the spatial location in the lateral hypothalamus of mouse brains, a region known to be related to anxiety. Retrobeads were injected in the lateral habenula and the periaqueductal gray, retrogradely tracing neurons in the lateral hypothalamus. Immunohistochemistry was performed on mouse brain sections to reveal cell specific protein expressions. In addition to this, individual neurons were filled with biocytocin and with a laser scanning microscope, gathered dendritic morphological features. The results of the differences in dendritic structures, indicated that there are two different types of cells in the lateral hypothalamus projecting to the lateral habenula. Neurons expressing estrogen and melanin-concentrating hormone, were found in the lateral hypothalamus, suggesting their influence on anxiety. The two observed regions' projection from the lateral hypothalamus, shows a complex network which requires further cell specific knowledge.

Acknowledgements

Firsly, I want to thank my mentor Konstantinos Meletis, who gave me the opportunity to stay at Karolinska Institutet and work at the Department of Neuroscience. My lab partners Elin Hermann, Hedieh Mohammadi and Naixuan Pei, were fun to work with and spread a lot of joy. I also want to give my best appreciation to Daniela Calvigioni and János Fuzik, who helped me and taught me a lot a long the way. Lastly, I want to thank Rays for Excellence and their collaborators AstraZeneca and Kjell och Märta Beijers Stiftelse, which all made this study possible including the nicest of counselors; Jonna Karlberg, Hampus Gummesson Svensson, Jakob Broman and Jenny Angelin.

Contents

List of Abbreviations **1**

1 Introduction **2**

1.1 Previous Research 2

1.1.1 Brain Regions Related to Anxiety 2

1.1.2 Protein Expressions 3

1.2 Background 4

1.2.1 Tissue Clearing for Better Imaging 4

1.2.2 Targeting Neurons using Immunohistochemistry 4

1.2.3 Mapping the Axonal Projection 5

1.2.4 Nuclear Staining 5

1.2.5 Electrophysiology and Singel-cell Recording 5

1.3 Aim of The Study 6

2 Method and Materials **6**

2.1 Immunohistochemistry 6

2.2 Tissue Clearing 8

2.3 Imaging and Analysis 8

2.4 Anatomical Connections 8

2.5 Author Contribution 9

3 Results **10**

3.1 Molecular Features 10

3.2 Cellular Features 11

3.3 Morphological Features 12

4 Discussion **15**

4.1 Different Cells Within the LHA 15

4.2 Complex Projection to the LHB and PAG 15
4.3 Interneurons of the LHA 16
4.4 Animal Model for Anxiety 17
4.5 Conclusion 17

References **19**

A Confocal images of neuronal tracing with retrobeads **21**

List of Abbreviations

DAPI Diamidino-2-phenylindole.

ER Estrogen.

LHA Lateral Hypothalamus.

LHB Lateral Habenula.

MCH Melanin-Concentrating Hormone.

PAG Periaqueductal Gray.

PB Phosphate Buffer.

PV Parvalbumin.

1 Introduction

The central goal in neuroscience is to understand the communication of regions in the central nervous system and their constituting circuitry elements, the neurons. The complexity cannot only be defined by the number of neurons in the brain, but all the connections that link them together. A complex network like the brain can be studied on different levels; regions, cell populations, synapses and within the neuron. There is an enormous diversity of neurons, with different structures and functions [1]. Fortunately, there are multiple approaches to understand the fundamental properties of individual neurons and the complete neuronal circuit. This study will use immunohistochemistry and retrobeads and focus mainly on the lateral hypothalamus (LHA).

1.1 Previous Research

1.1.1 Brain Regions Related to Anxiety

The hypothalamus is one of the most conserved regions in the vertebrate brain, which proves its fundamental purpose [2]. It consists of a diversity of cell populations that control vital autonomic functions such as feeding, energy balance and reproduction [3]. It forms the side walls and the floor of the third brain chamber. Furthermore, it is responsible for the neuroendocrine and metabolic responses to stress. A study made on patients with generalized anxiety disorder (GAD), show decreased hypothalamic volumes. This indicates that specific cells found in the hypothalamus affect the development of anxiety [4].

Two other conserved regions in vertebrate brains, are the lateral habenula (LHB) and periaqueductal gray (PAG). They both receive inputs from the LHA and are related to anxiety [5]. The habenula is small like a pea in the human brain and works as a critical neuroanatomical hub, connecting different brain regions. The habenula consists of two subregions with very distinct gene expressions; the medial and lateral habenula.Their anatomical connectivity also differ, which suggest different functions. The LHB is thought

to be involved in motivated and social behavior. Neurons found in this region have shown to inhibit dopaminergic neurons which indicate that the LHB play an important role in reward systems. An increased activity in the LHB, has been found in rodent models of depression. Furthermore, many of the afferent inputs to the LHB, co-release glutamate (excitatory) and GABA (inhibitory). This indicates its complexity, thus complicate the path towards understanding the basis of behaviour [6].

PAG is a region located around the cerebral aqueduct within the tegmentum of the midbrain. Its major function is related to pain, fear and anxiety, and receives inputs from neurons in the spinal cord. The circuit within the PAG, consists mainly of GABAergic neurons. This means that local inhibition of this network, regulates the activity of outputs from the PAG [7].

Investigating different cell types within the regions, will contribute to more detailed information about the function. Different cell types based on protein expression can differ depending on neuropeptides, receptors and neurotransmission.

1.1.2 Protein Expressions

Melanin-concentrating hormone (MCH) is a neuropeptide which is known to regulate food intake. Studies show that it is related to stress, which have lead to further research about the MCH receptors and antidepressants [8]. Furthermore, methods like optogenetics suggest that MCH neurons are functionally GABAergic [9, 10].

Parvalbumin (PV) is a calcium-binding protein, found in many GABAergic neurons. Many of these local inhibitory interneurons, are found in the cortex which possess the cognitive functions. PV works as a slow Ca2+ buffer that can affect the action potential and regulate short-term synaptic plasticity [11] [12].

Estrogen (ER) is another hormone which has shown to affect more than reproductive behaviors, also interfering with anxiety and fear [13]. Studies show that development of anxiety when exposed to stress, differ in males and females. Females are more likely to develop an anxiety disorder, which also has been proven in animal models [14].

1.2 Background

1.2.1 Tissue Clearing for Better Imaging

A new method for clearing tissue called CUBIC (clear unobstructed brain imaging cocktails) was introduced in 2014 [15]. It uses high triton levels to maximize lipid removal and makes the tissue transparent. The transparency removes the problem of light scattering which limits penetration through tissue. This enables imaging of thicker tissues, thus three-dimensional visualizations of cellular circuits are possible. This is important in many fields when investigating within whole organs, but in particularly neurons that extend in many directions. Studies also show that this method efficiently cleared whole brains of adult mice without signal loss from fluorescence proteins [16, 17].

1.2.2 Targeting Neurons using Immunohistochemistry

Targeting specific cells based on protein expression, is a way to further investigate neurons and their function. Immunohistochemistry is a widely used method for characterization of tissue. It is sensitive and very specific, thus useful for molecular identification. An antigen of interest is injected in for example a rabbit, whose immune system will create antibodies against this antigen. When isolated these antibodies, it is injected in another species who will create secondary antibodies targeting the primary. Secondary antibodies are favourable for easier detection with various fluorescence markers and also used as an error reduction. Non-specific site block is also needed to decrease the risk of low affinity antibodies binding.

Other steps are the antibody incubation on a section tissue, interpretation and quantification of the obtained expression. Using a light microscopy, the various fluorescence probe emit light with different wavelengths and it is possible to distinguish between different types of neurons, specific cellular populations can then be identified [18].

1.2.3 Mapping the Axonal Projection

Retrogradely tracing neurons is a widely used method and have provided much information about the neuronal circuit in the brain. Viruses or Fluorescent latex microspheres (retrobeads) are injected in tissue and enter axons and axon terminals. The beads will then retrogradely travel to the original soma. Labeling neurons based on their pathway in the brain is a way to investigate neurons' connectivity. This method can also help to identify neurons within a mixed cell population [19].

1.2.4 Nuclear Staining

Labeling nucleus with DAPI (4',6-diamidino-2-phenylindole) can visualize nuclear DNA in both living and fixed cells [20]. The method is used for various purposes, for example nuclear quantification or comparison between other stainings on the same tissue. In order for DAPI to enter the cell and bind to DNA, it either has to be fixed or permeabilized [21].

1.2.5 Electrophysiology and Singel-cell Recording

A method used to investigate neurons' firing patterns is patch-clamp recording. It is an electrophysiological technique that allows the study of individual neurons' electrical properties. Furthermore, it is a way to identify different cell types based on electrical signals and find out when neurons are silence or active. Using a micropipette in tight contact with the cell membrane, it is possible to make accurate measurements without ionic current leakage. This method can be used for neurons in brain slices as well as anesthetized or awake animals [22].

Singel-cell recordings have showed that structurally distinct neurons also have different firing patterns and synaptic characteristics [23]. Filling a cell with biocytin, it is possible to record morphological features and for example determine dendritic location, thus the potential inputs that a specific neuron receives.

1.3 Aim of The Study

The aim of the study is to investigate the neuronal circuit of the LHA and its relation to anxiety. Experiments done on mouse brains, will study the communication between different regions based on axonal projection to the LHB and the PAG. Furthermore, identification of differences within cell populations can contribute to a potential anxiety model in mice.

2 Method and Materials

Three main methods were used in this study; protein markers, tracing neurons with retrobeads and filling neurons with biocytin.

2.1 Immunohistochemistry

Three months old female wild type mice were anesthetized with pentobarbital and transcardially perfused with 100 mM PBS followed by 4% paraformaldehyde. The brains were then removed and postfixed for 18 hours at 4°C.

Two of the brains were cut off halfway the cerebellum and sectioned in 50 µm thin layers using a vibratome (Leica VT1200, Leica Microsystems, Nussloch GmbH, Germany). The brains were sliced in coronal planes, anterior and posterior to the LHA. All slices were separated into a multywell, filled with 500 µL phosphate buffer (PB) in each well.

All brain sections were incubated in blocking solution (100µL normal donkey serum, 875µL) carrier and 25µL triton (20%)) for two hours and then washed with PB. Pretreating the slices with donkey serum was necessary to block low affinity antibodies. The primary antibody solutions were added to the brain sections (see Table 1), followed by incubation for two days. The samples were washed with PB and the secondary antibody solutions (see Table 2) were incubated overnight. The next days the nuclear staining (DAPI) was added and washed away with PB after 10 -15 minutes.

Table 1: This table shows the primary antibody solutions. Rabbit (Rb) antibodies against estrogen (ER), rat antibodies against somatostatin (SST), guinea pig (GP) antibodies against parvalbumin (PV), mouse antibodies against paired box protein (Pax-6), mouse antibodies against choline acetyltransferase (CHAT) and Rb against melanin-concentrating hormone (MCH).

Solution	**Content**	**Dilution**	**Amount** (µL)
1	Carrier		971
	Triton 20%		25
	ER Rb	1:500	2
	SST Rat	1:1000	1
	PV GP	1:1000	1
2	Carrier		970
	Triton 20%		25
	ER Rb	1:500	2
	PAX6 Mouse	1:500	2
	SST Rat	1:1000	1
3	Carrier		972
	Triton 20%		25
	PV GP	1:1000	1
	CHAT Mouse	1:1000	1
	MCH Rb	1:1000	1

Table 2: This table shows the secondary antibody (1:500) solutions with fluorescence. Donkey (D) antibody against Rb primary antibodies. Nuclear staining using 4,6-diamidino-2-phenylindole (DAPI).

Solution	**Content**	**Wavelength** (nm)	**Amount** (µL)
1	Carrier		994
	D Rb	633 (Blue)	2
	D Rat	555 (Red)	2
	D GP	488 (Green)	2
	DAPI (1:50000)	405	1
2	Carrier		994
	D Rb	633 (Blue)	2
	D Mouse	555 (Red)	2
	D Rat	488 (Green)	2
	DAPI (1:50000)	405	1
3	Carrier		994
	D GP	633 (Blue)	2
	D Mouse	555 (Red)	2
	D Rb	488 (Green)	2
	DAPI (1:50000)	405	1

2.2 Tissue Clearing

In order to visualize 350 µm thick slices of a brain, they were made transparent. To clear the tissue, CUBIC reagent 1 was used (25 wt% urea, 25 wt% N,N,N,N-tetrakis (2-hydroxypropyl) ethylenediamine and 15 wt% polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100). To further clear the slices, they were repeatedly washed in PB and submerged in CUBIC reagent 2 (50 wt% sucrose, 25 wt% urea, 10 wt% 2,20,20-nitrilotriethanol and 0.1% v/v% Triton X-100).

2.3 Imaging and Analysis

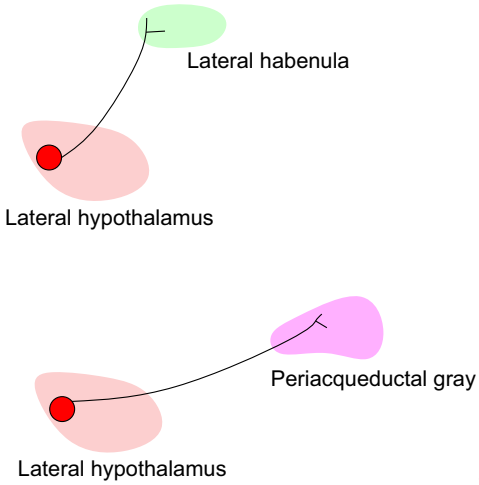
To visualize the results, the brain sections were mounted on glass slides and covered with coverslips. The images were acquired using a confocal laser scanning microscope (Zeiss LSM 880, Germany). Focusing a smaller beam of light in different wavelengths (see Table 2), it is possible to detect the florescence thus identifying target cells and nuclear staining. The cleared tissue was optically imaged through all sections using LSM.

Sholl analysis is a quantitative analysis, calculating the number of intersections correlated with the distance from the soma. This test was performed on the reconstructed neurons to analyze the morphological characteristics of the dendrites.

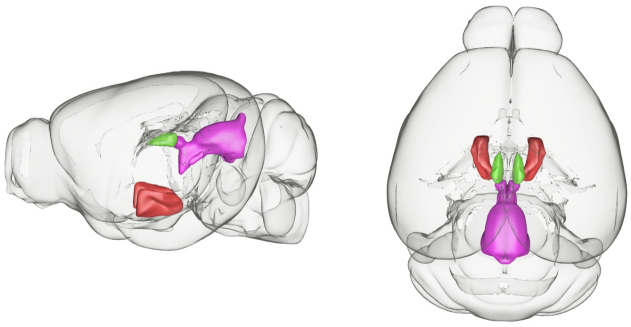
Imaris is an image analysis software which was used to reconstruct the biocytin-filled cells. Fiji is an image processing package of Imagej, that was used for neuronal tracing.

2.4 Anatomical Connections

To investigate different neurons' projection from the LHA, retrobeads were injected in vivo in the brain. 50 nL of beads were injected both in the LHB and the PAG. A stereotaxic injection setup was used to navigate the distance from the bregma. The LHB was injected anterior/posterior (A-P): -1.65mm, lateral/medial (L-M): -0.25mm and depth: 2.3mm. The PAG was injected (A-P): -4.0mm, (L-M): -0.3mm and depth: 1.7mm. Ex vivo sectioning the brains, some of the retrobead-labeled LHA neurons, were patch-clamp



a



b

(a) This figure shows the LHA axonal projection to the LHB and to the PAG.Injection with retrobeads in LHB and PAG, retrogradely labeled (opposite direction an action potential would travel) and coloured the soma, representing the red dots in the LHA.

(b) This figure shows the anatomical positions in the mouse brain. Green represent the LHB, pink represent the PAG and the red represent the LHA

Figure 1: This figure shows the anatomical positions and projections in a mouse brain.

recorded and filled with biocytin. The slices were then post-fixed in 4% paraformaldehyde in PB at 4°C overnight. The tissue was then cleared and it was possible to visualize the biocytin-filled neurons in 3D using LSM. The softer Imaris was used to reconstruct the neuronal morphology of the cells.

2.5 Author Contribution

D.C injected the mice, transcardially perfused and removed the brains. J.F filled neurons with biocytin and imaged them. E.H, H.M, N.N and G.C sectioned the brains, cleared the tissue with CUBIC reagents, did the immunohistochemistry and nuclear staining. Everyone mentioned above imaged the brain sections in the confocal laser scanning microscope.

3 Results

This study focused on three main perspectives to map and target neurons; molecular, cellular and morphological. Different methods were performed on brain tissue on same section coordinates, in order to compare them. To understand the neuronal circuit, both individual cell types and whole regions have been studied.

3.1 Molecular Features

One way to distinguish different cell types is to investigate the differences in their function. The results from the immunohistochemistry show where in the brain different cells express different proteins.

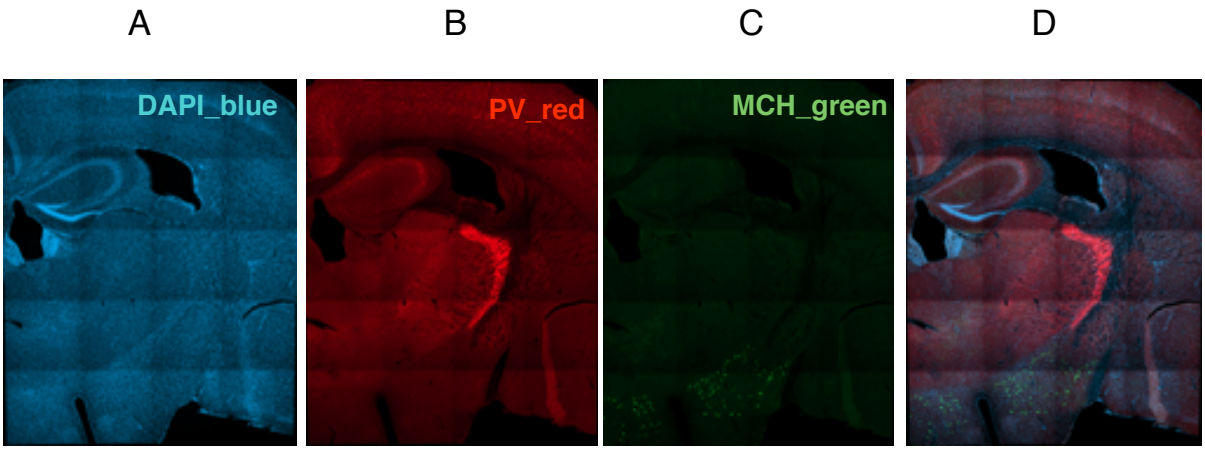


Figure 2: This figure shows results from the immunohistochemistry and the nuclear staining. Every dot in figure (A) represents a nucleus, PV is shown in figure (B), MCH in figure (C) and everything overlapping in figure (D).

The results were successfully obtained. Neurons expressing PV are mainly found in the neocortex, the hippocampus and the reticular nucleus of the thalamus. MCH is only expressed on neurons in the LHA region. The nuclear staining shows more than neurons' nucleus, like blood vessels and glia cells.

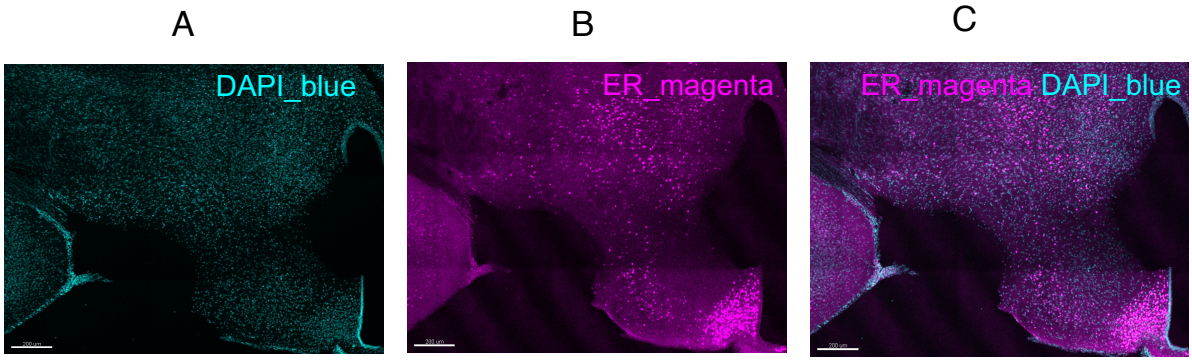


Figure 3: This figure shows upclose the LHA region. Figure (A) is the nuclear staining. In figure (B), neurons which express ER can be found and (C) shows everything overlapping.

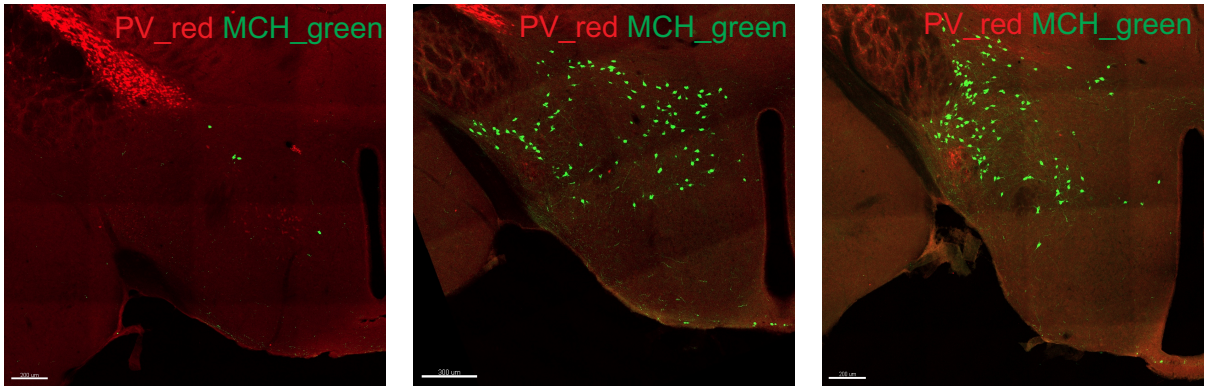


Figure 4: This figure shows results from the immunohistochemistry, comparing sections with different depths in the tissue.

Shown in Figure 2, there are no cells expressing PV in the LHA, but MCH is found. However, there are almost no neurons expressing MCH in the anterior plane in Figure 4.

3.2 Cellular Features

Neuronal tracing with retrobeads shows the communication between different regions of interest. The retrobeads were injected in PAG and in the LHB and retrogradely traced and marked neurons in LHA.

In the anterior plane (A) in Figure 5, there are many neurons in the LHA connected to the LHB. There are fewer and less distributed neurons projecting to PAG. In the medial plane (B), there is a similar amount of neurons projecting to LHB and PAG,

but completely separated. In the posterior plane (C), there are very few neurons but distributed within the whole LHA region.

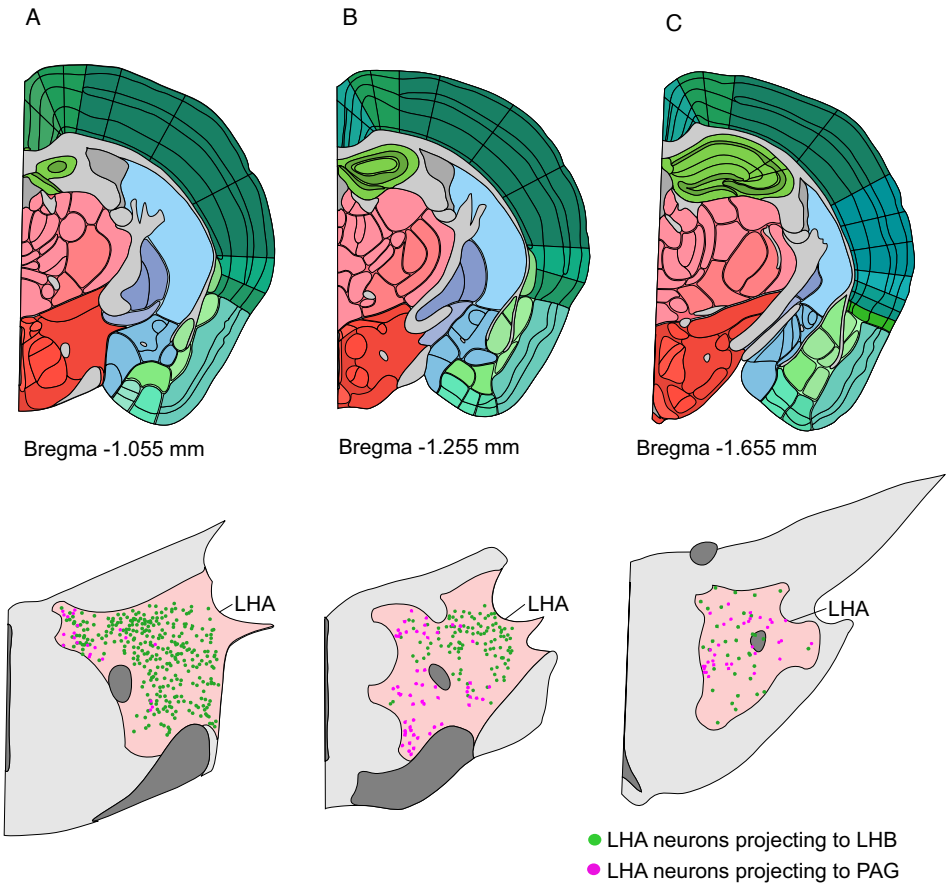


Figure 5: This figure shows the results from the injections of retrobeads. Figure A is the anterior view, B the medial view and C the posterior view of the LHA. The green dots represent projection to the LHB and the pink represent projection to the PAG.

See Appendix for confocal images of the anatomical distribution of retogradely transported retrobeads.

3.3 Morphological Features

Categorizing neurons based on their morphological features is one way to further understand the complex communication. Length, local distribution and number of branches can help classifying different cell types.

The results from the reconstructed biocytin-filled neurons (in Figure 6) show three

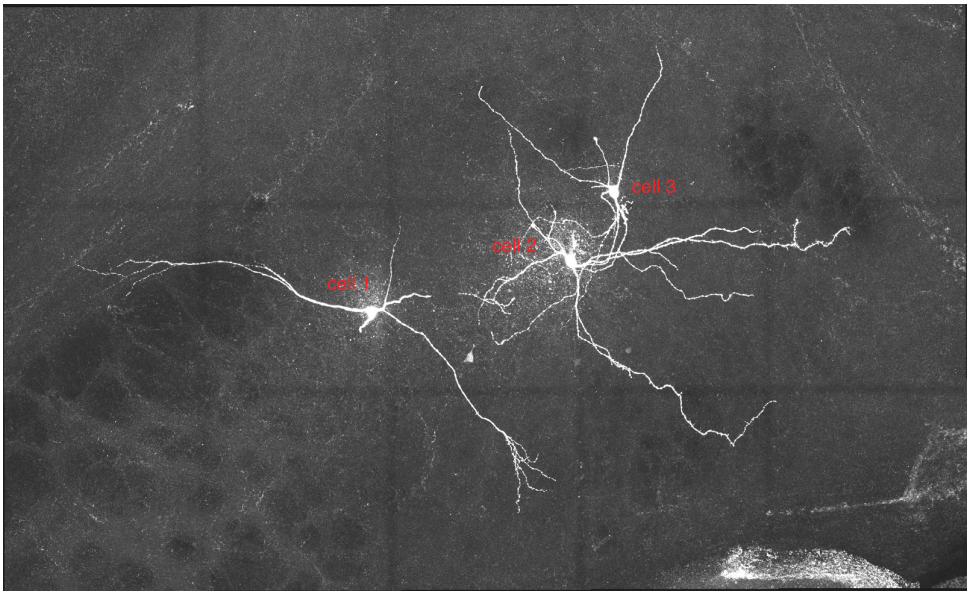


Figure 6: This figure shows biocytin-filled neurons in 3D image from an LSM. Three somas and their dendrites in the LHA, projecting to the LHB are visualized.

populations of cells within the LHA region. All cells in this figure are multipolar, which means that they have multiple long primary branches extending from the soma. Cell 1 and 2 have three primary branches, while cell 3 has four. In total number of branches, cell 1 has 42, cell 2 has 36 and cell 3 has 20. The size of a box in Figure 7, shows that the the total branch number vary much within a cell population, but not so much the total length. The results in Figure 8 also show that there are differences between the three cell population's dendritic structures.

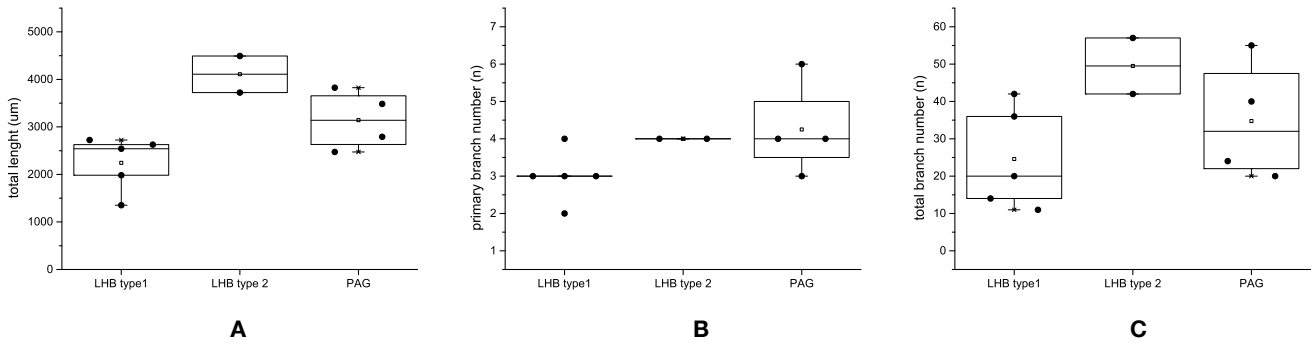


Figure 7: These diagrams show the results from the reconstructed biocytin-filled neurons in the LHA. Every dot within a plotbox, represents information about one neuron. (A) shows the total length, (B) shows the primary branch number and (C) shows the total branch number.

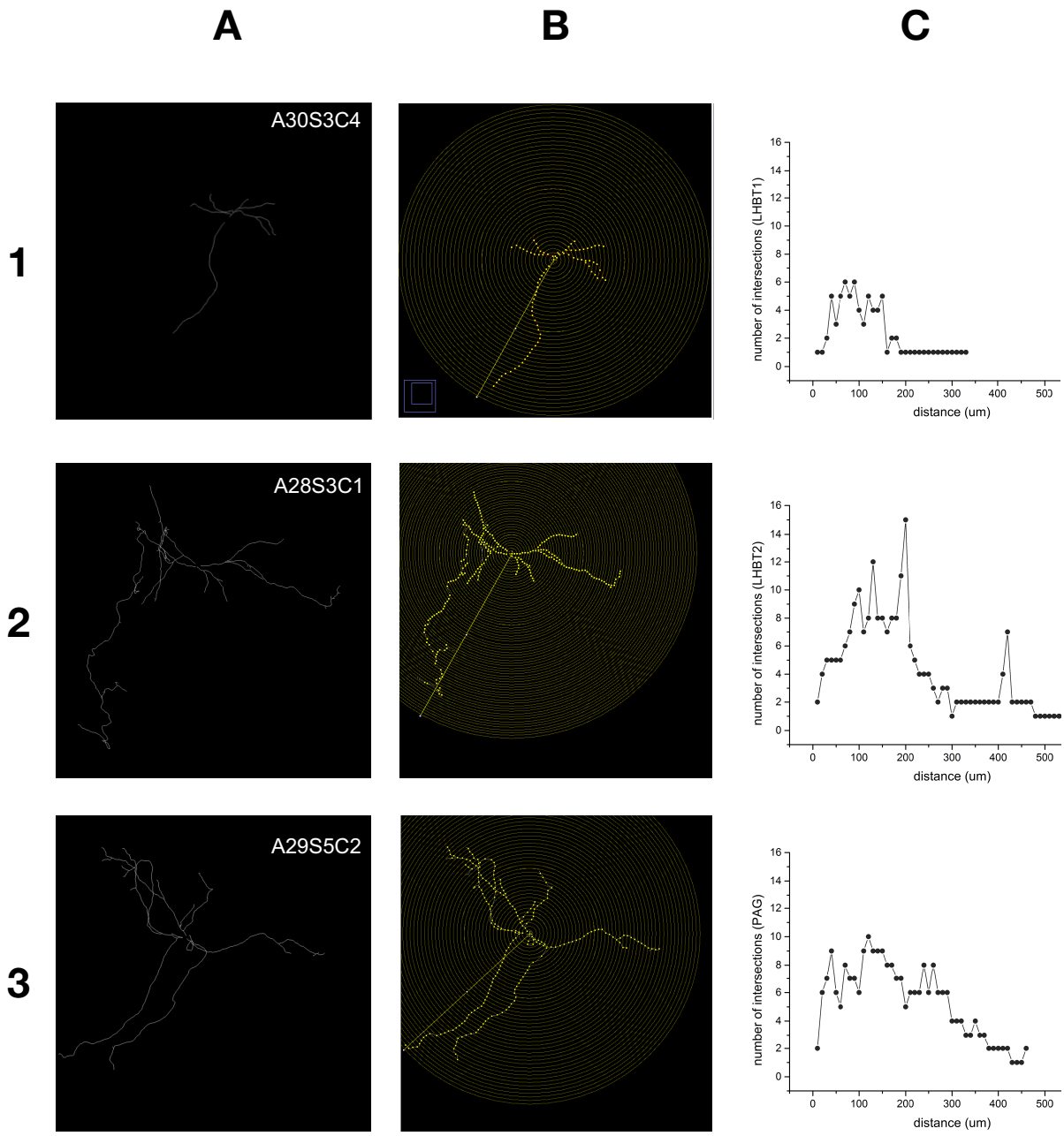


Figure 8: This figure shows the results from the Sholl analysis and give an overview of the differences in dendritic structures of the three cell populations. Column (A) shows three reconstructed biocytin-filled neurons. In column (B), circles from the soma to the end are visible, showing the basis of the Sholl analysis. Column (C) present the data in a diagram with the number of intersection correlated with the distance from the soma.

4 Discussion

4.1 Different Cells Within the LHA

MCH was only expressed in the LHA region. It was not distributed within the whole region, as shown in Figure 4. There were almost no cells expressing MCH in the anterior part, but many more in the medial and posterior parts. This suggests that different areas within a region have different cell types, thus different function. This can also be supported by the fact that cells expressing MCH are found in the same anterior-posterior coordinate as cells expressing ER. In addition to this, they are both expressed in a region related to stress, suggesting a role in anxiety. The fact that females are more likely to suffer from anxiety than males, could be related to the ER level, but the results from this study can not say anything about the function of ER. How anxiety is linked in a more detailed explanation need to be further investigated. If the antibodies were generated in different species, it would be possible to distinguish if some neurons expressed both ER and MCH or if there are two different cell types.

4.2 Complex Projection to the LHB and PAG

Both PV and MCH are known to be expressed on many GABAergic neurons, which means that they both have an inhibitory function. This means that different regions in the brain have inhibitory functions, indicating that regions regulate each other. The anatomical connections between regions are shown in the results from retrogradely tracing neurons. Neurons projecting to PAG are fewer and less distributed. However, in the medial view, there is a similar amount of neurons projecting to LHB and PAG and they are seperated in the middle. This indicates that there are at least two different cell populations in the LHA. Furthermore, neurons in the PAG are known to be GABAergic, indicating that inputs from the LHA can decrease the activity in PAG. This suggests that fear and anxiety, which is controlled by PAG, is partly regulated by the LHA.

Neurons' projection from the LHA to the LHB are many in the anterior plane and

decreases in the more posterior coordinates (see Figure 5). Studies have shown that the activity in the LHB increases when stressed, which can indicate that it is regulated by the many inputs found in the LHA. The LHB is also involved in reward system, which studies show is dysfunctional when suffering from depression [24]. To understand the model of anxiety, the connections between the LHA and LHB are valuable to further investigate.

The reconstructed biocytin-filled neurons in the LHA, show the differences in the cells projecting to LHB and PAG. Three cell populations in total have been found and visible in Figure 7. Categorization of cells based on dendrites total length, primary branch number and total branch number, indicates that there are two types of cells projecting to the LHB. The results from the Sholl analysis support this discovery. In Figure 8 it is visible that cell 1 (projecting to the LHB) has fewer branches and almost non within a distance of 200 µL from the soma. While cell 2 (also projecting to LHB) have many more intersections and is more branched. There is a significant difference and from a morphological perspective, it is suggesting that there are two different cell types in LHA that project to LHB.

Dendritic morphology is important in order to understand the neuronal circuit, mainly because of all connections and inputs, as well as what local areas it covers. Furthermore, both cell 2 and cell 3 (cell 3 projection to PAG) are more branched, indicating that their anatomical connections are more complex. However, in future studies, it should be an increased number of experiments to have more data and better statistics.

4.3 Interneurons of the LHA

PV expressing cells are known to mark one class of interneurons. The immunohistochemistry shows that these are mainly found in the neocortex, hippocampus and the reticular nucleus of the thalamus. This however, is only proved in the chosen sections of the brain, and do not provide information about other regions. There were no cells in the LHA expressing PV, indicating that the PV interneurons are not the primary inhibatory population of the LHA. This does not exclude the major role of other interneuron populations.

Interneurons are local inhibitory cells, controlling the complexity of the brain. Further studies should investigate what type of interneurons are positioned in the LHA.

The neocortex is known to control personality and decision making, hippocampus is involved in memory and the reticular nucleus of the thalamus is important in various states of conscious [25]. This suggest that PV is involved in the more complex systems in the brain. Further studies could be to investigate how the PV level may affect different behaviour and also look into different species from a perspective of complexity.

4.4 Animal Model for Anxiety

This study has provided information about the mouse brain, which match very well with the human brain because the studied regions are old and preserved in all vertebrates brain. The corresponding regions in the human brain have the same basal functions, thus it is hypothesized that there are overlaps in the cellular identity of neurons as well. However, the information received from animals are very likely to be accurate, but in order to apply this knowledge, further studies have to be performed. There is an increased development of animal models for different mental pathologies. They are an important tool in many areas, including disease pathology [26].

4.5 Conclusion

In summary, the results from the immunohistochemistry revealed distribution of protein expressions, indicating that different regions have different functions. PV was expressed in regions important for cognitive functions, suggesting its complex role in the neuronal curcuit. MCH and ER were found in the LHA region which indicate a potential relation to stress and anxiety. However, further investigation of a potential overlap of these expressions is essential, to either merge or separate the function of MCH and ER positive neuronal populations of the LHA.

The results show that the LHA is communicating with LHB and PAG based on

the retro-bead experiments. Both projecting populations are located in the ER positive subregions of the LHA, suggesting their prominent role in anxiety. The difference in their circuit contribution, is to be tested in vivo optogenetic experiments together with the use of animal models of behavior.

Differences in dendritic structures potentially indicate differences in cell types, thus morphology revealed two different types of cells in the LHA projecting to the LHB.

This study contributes to the central goal in neuroscience, to better understand anatomy, morphology and molecular identity of neurons. This has to be done in order to comprehend the major circuitry imbalances and cellular disfunctions. The further understanding of projection-specific localisation of LHA cell types, together with a detailed investigation of the morphological and neurochemical level, provides essential information to an ongoing anxiety research.

References

[1] Giorgio A, Lidia Alonso-Nanclares A, Steward A, German B, Ruth B, et al. Petilla terminology: nomenclature of features of GABAergic interneurons of the cerbral cortex. Nature. 2008; volym 9 : 557 - 67

[2] Xie Y1, Dorsky RI2. Development of the hypothalamus: conservation, modification and innovation. Development. 2017 May 1;144(9):1588-1599. doi: 10.1242/dev.139055.

[3] Patricia Bonnavion, Laura E. Mickelsen, Akie Fujita, Luis de Lecea and Alexander C. Jackson. Hubs and spokes of the lateral hypothalamus: cell types, circuits and behaviour. J Physiol 594.22 (2016) pp 6443–6462

[4] Robert Terlevic, Miriam Isola, Maria Ragogna, Martina Meduri, Francesca Canalaz, Laura Perini. Decreased hypothalamus volumes in generalized anxiety disorder but not in panic disorder. Journal of Affective Disorders 146 (2013) 390–394.

[5] Clas Linnman, Eric A. Moulton, Gabi Barmettler, Lino Becerra, and David Borsook. Neuroimaging of the Periaqueductal Gray: State of the Field. Neuroimage. 2012 Mar; 60(1): 505–522.

[6] Vijay Mohan K. Namboodiri, Jose Rodriguez-Romaguera, and Garret D. Stuber. The habenula. Current Biology 26, R865–R881, October 10, 2016 R877

[7] Behbehani MM. Functional characteristics of the midbrain periaqueductal gray. Prog Neurobiol. 1995 Aug;46(6):575-605.

[8] Hervieu G. Melanin-concentrating hormone functions in the nervous system: food intake and stress. Expert Opin Ther Targets. 2003 Aug;7(4):495-511.

[9] Shinjae Chung, Gregory S. Parks, Cheol Lee, and Olivier Civelli. Recent Updates on the Melanin-Concentrating Hormone (MCH) and Its Receptor System: Lessons from MCH1R Antagonists. J Mol Neurosci. 2011 Jan; 43(1): 115–121.

[10] Jego S1, Glasgow SD, Herrera CG, Ekstrand M, Reed SJ, Boyce R, Friedman J, Burdakov D, Adamantidis AR. Optogenetic identification of a rapid eye movement sleep modulatory circuit in the hypothalamus. Nat Neurosci. 2013 Nov;16(11):1637-43. doi: 10.1038/nn.3522. Epub 2013 Sep 22.

[11] Olivier Caillard, Herman Moreno,‡ Beat Schwaller, Isabel Llano, Marco R. Celio, and Alain Marty. Role of the calcium-binding protein parvalbumin in short-term synaptic plasticity. Proc Natl Acad Sci U S A. 2000 Nov 21; 97(24): 13372–13377. (PV).

[12] Jill R. Glausier, Rosalinda C. Roberts, and David A. Lewis. Ultrastructural Analysis of Parvalbumin Synapses in Human Dorsolateral Prefrontal Cortex. J Comp Neurol. 2017 Jun 15; 525(9): 2075–2089.

[13] Horm Behav. 2001 Dec;40(4):472-82. Effects of estrogen on activity and fear-related behaviors in mice. Morgan MA1, Pfaff DW.

[14] Nina C. Donner and Christopher A. Lowry Sex differences in anxiety and emotional behavior. Pflugers Arch. 2013 May; 465(5): 601–626

[15] Susaki, E.A., Tainaka, K., Perrin, D., Kishino, F., Tawara, T., Watanabe, T.M., Yokoyama, C., Onoe, H., Eguchi, M., Yamaguchi, S., et al. (2014). Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157, 726–739.

[16] Richardson, DSR. Lichtman WJ. Clarifying Tissue Clearing, National Center for Biotechnology Information, U.S. National Library of Medicine, Cell. 2015 Jul 16; 162(2): 246–257.

[17] Kazuki Tainaka, Shimpei I. Kubota, Takeru Q. Suyama, Etsuo A. Susaki, Dimitri Perrin, Maki Ukai-Tadenuma. Whole-Body Imaging with Single-Cell Resolution by Tissue Decolorization. Cell 159, 911–924, November 6, 2014

[18] Leandro Luongo de Matos, Damila Cristina Trufelli, Maria Graciela Luongo de Matos, and Maria Aparecida da Silva PinhaImmunohistochemistry as an Important Tool in Biomarkers Detection and Clinical Practice, lBiomark Insights. 2010; 5: 9–20.

[19] Katz, L.C., Burkhalter, A., and Dreyer, W.J. (1984). Fluorescent latex microspheres as a retrograde neuronal marker for in vivo and in vitro studies of visual cortex. Nature 310, 498–500.

[20] Tarnowski BI1, Spinale FG, Nicholson JH. DAPI as a useful stain for nuclear quantitation.Biotech Histochem. 1991;66(6):297-302.

[21] Chazotte B. Labeling nuclear DNA using DAP, Cold Spring Harb Protoc. 2011 Jan 1;2011(1)

[22] Segev A, Garcia-Oscos F, Kourrich S. Whole-cell Patch-clamp Recordings in Brain Slices. J Vis Exp. 2016 Jun 15;(112).

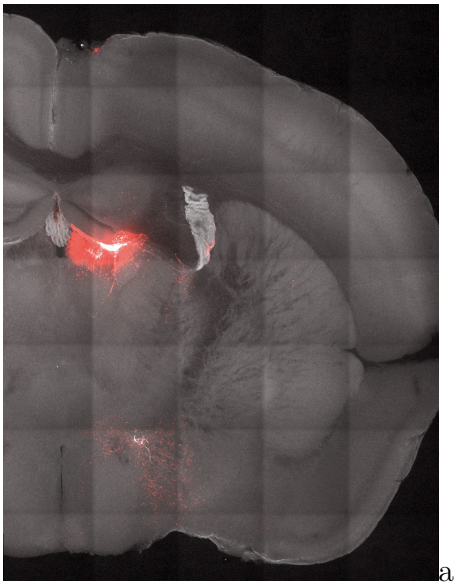
[23] Bogumila Swietek, Akshay Gupta, Archana Proddutur, and Vijayalakshmi Santhakumar. Immunostaining of Biocytin-filled and Processed Sections for Neurochemical Markers. J Vis Exp. 2016; (118): 54880.

[24] Naranjo CA1, Tremblay LK, Busto UE. Prog Neuropsychopharmacol Biol Psychiatry. The role of the brain reward system in depression. 2001 May;25(4):781-823.

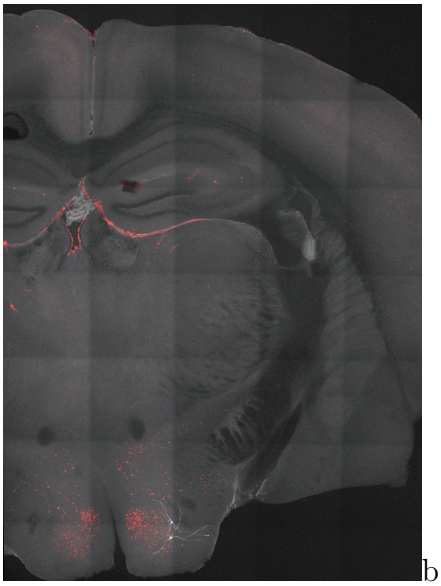
[25] Pinault D. The thalamic reticular nucleus: structure, function and concept. Brain Res Brain Res Rev. 2004 Aug;46(1):1-31.

[26] Eric J Nestler Steven E Hyman. Animal models of neuropsychiatric disorders. nature neuroscience volume 13 number 10 october 2010.

A Confocal images of neuronal tracing with retrobeads


a

(a) This figure shows a brain section from a mouse. The injecting site is in the LHB, visible in the red cluster. The red dots in the LHA indicate neurons projecting to the the LHA, thus have been retrogradely marked.


b

(b) This figure shows a brain section from a mouse.The injecting site is in the PAG, visible as red dots in the lower part of the hippocampus. The red dots in the LHA indicate neurons projecting to the the LHA, thus have been retrogradely marked.

Figure 9: This figure show the confocal images of neuronal tracing with retrobeads.