Final project 76554

COMPUTATIONAL METHODS IN MOLECULAR BIOLOGY LAB

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The Effect of Norepinephrine on Dendrites of Cortical Neurons

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Submitted by

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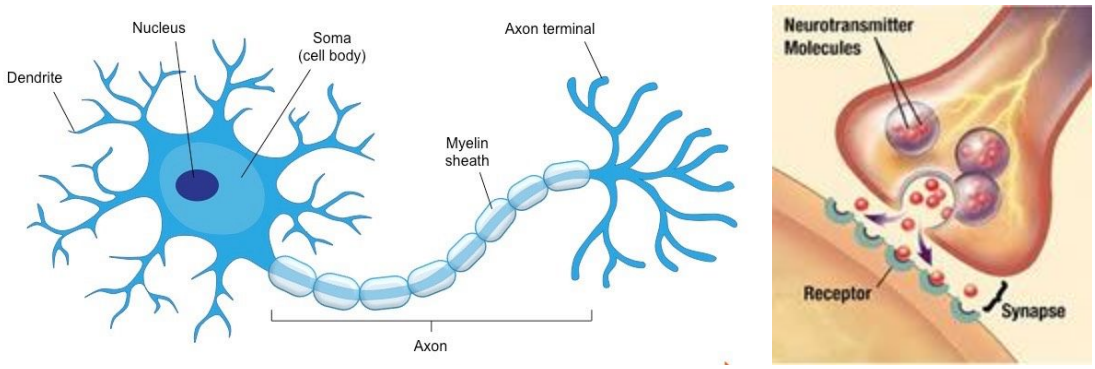
Abstract

Neuromodulation is an important neuronal process by which one or more chemicals regulate the activity of various populations of neurons. One particular neuromodulator is norepinephrine (NE). NE has been demonstrated to have an important role for modulating attention (Berridge and Waterhouse., 2003), arousal (Carter et al., 2010) and working memory (Wang et al., 2007). In the brain, NE release occurs mainly through the activation of a region in the brainstem called the Locus Coeruleus (LC). This small nucleus is the origin of most NE pathways in the brain. Nowadays, guanfacine which is an agonist of NE-receptors, is being used as a component in medication for treating ADHD and has been shown to be effective. Currently, there is no satisfying explanation as to why guanfacine effects ADHD symptoms, and much is yet to be studied about its operation in the brain. Our hypothesis is that NE modulates the activity in the dendrites of pyramidal neurons in the cortex, and my proposed research will test some aspects of this hypothesis.

1 Introduction

1.1 Background Concepts

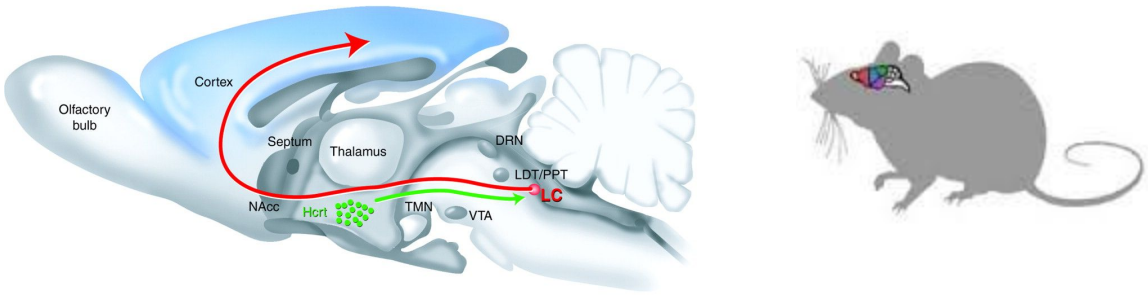
A neuron, also known as nerve cell, is part of our peripheral and central nervous system. A neuron is an electrically excitable cell that processes and transmits information through electrical and chemical signals. A neuron can be divided into 3 segments (see figure 1.1.a), dendrites, which are the input unit, a cell body called the soma, and axons, which is the output unit. The signal transduction between neurons occur via specialized connections called synapses (see figure 1.1.b). A synapse is a small gap between one neuron dendrite to a neighboring neuron axon. A signal transduction occurs when a chemical signal is transferred via a neurotransmitter, which is an endogenous chemical that is stored in a synapse in synaptic vesicles, clustered beneath the membrane in the axon terminal. The NE binds to the dendrite receptors at the postsynaptic neuron. The binding of neurotransmitters may influence the postsynaptic neuron in either an inhibitory or excitatory way. This neuron may be connected to many more neurons, and if the total of excitatory influences are greater than those of inhibitory influences, the neuron will fire a current called spike. The spike is an electrical signal starting at the axon hillock and flows through the entire axon. At the axon terminal, the electrical signal will cause a release of neurotransmitter to the synaptic cleft and pass the signal to the neighboring neuron.



(a) **(b)**

Figure 1.1: (a). A schematic illustration of a neuron showing the dendrites, soma and axon . **(b).** A schematic illustration of a synapse showing the presynaptic side at the axon terminal and the postsynaptic site on the dendrite.

Locus Coeruleus (LC) is the principal site for brain synthesis of the neuromodulator norepinephrine. Neuromodulation is the physiological process by which a given neuron uses one or more chemicals to regulate diverse populations of neurons. This is in contrast to classical synaptic transmission, in which one presynaptic neuron directly influences a single postsynaptic partner. One of the areas which LC projects to is the cortex (see figure 1.2.a), the largest region of the mammalian brain which plays a key role in memory, attention, perception, cognition, awareness, thought, language, and consciousness.



(a) **(b)**

Figure 1.2: (a). A sagittal view of the mouse brain showing the location of Locus Coeruleus located at the brainstem and how it sends axons to the cortex (red arrow). **(b).** An illustration of how panel A is located in the mouse head.

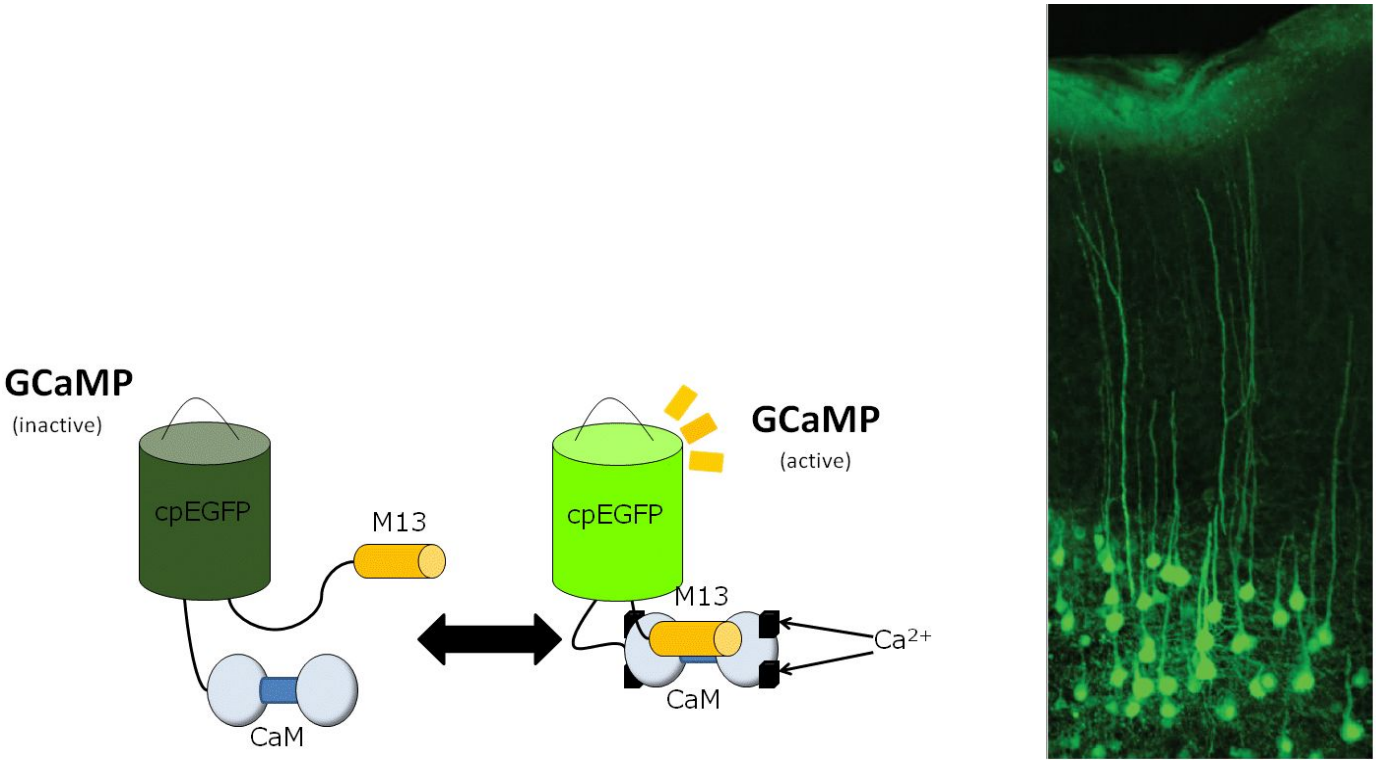
Our hypothesis states that NE modulates the activity in the dendrites of pyramidal neurons in the cortex, but how can we indicate if there is activity of dendrites in the brain?

GCaMP is a genetically encoded calcium indicator, when calcium binding occurs Gcamp is activated and as a response to light emits green fluorescence (figure 1.3.a, Tsai-Wen Chen et al., 2013). Viral vectors encoding the GCaMp protein are injected to the brain, taken up by neurons and after about two weeks it is possible to see its expression.

And now how can we actually visualize this green fluorescence?

Two-photon excitation microscopy is a fluorescence imaging technique which allows imaging of living tissue up to about one millimeter in depth. Two photons of infrared light are fired towards the tissue, hit the GCaMP, and cause it to emit a green fluorescence (assuming it is in active state, figure 1.3.a). The green fluorescence is recorded by the two photon camera (see figure

1.3.b), and the result is a sequence of images which depict the fluorescence levels in the recorded tissue.



(a) (b)

Figure 1.3: (a) GCaMp switches its state from inactive to active when binding calcium.**(b)**. A side view of calcium activity in the cortex using GCaMp, the upper part are dendrites and the lower part somas.

1.2 Motivation

Yair Deitcher is a PhD student in M. London's lab.As part of his research, we would like to answer what is the effect of norepinephrine on the brain? Norepinephrine action mechanism is still currently unknown. In the part I'm involved at also called pharmacological stage, we use an agonist of norepinephrine called guanfacine. Guanfacine binds to adrenoceptors and activates them. The goal of this step is to artificially simulate the release of norepinephrine from the LC by direct assignment of guanfacine on the cortex dendrites (which receive their input from the LC). This leads to my research question:What is the effect of norepinephrine on cortical dendrites?

In order to answer my question, we use recordings from the two photon microscopy. The problem is we receive a lot of data, which up until this point was analyzed in a manual way. We thought an automatic pipeline can help us handle the data more efficiently, and therefore, I developed a pipeline especially for this purpose.

2 Results

2.1 The Experiment

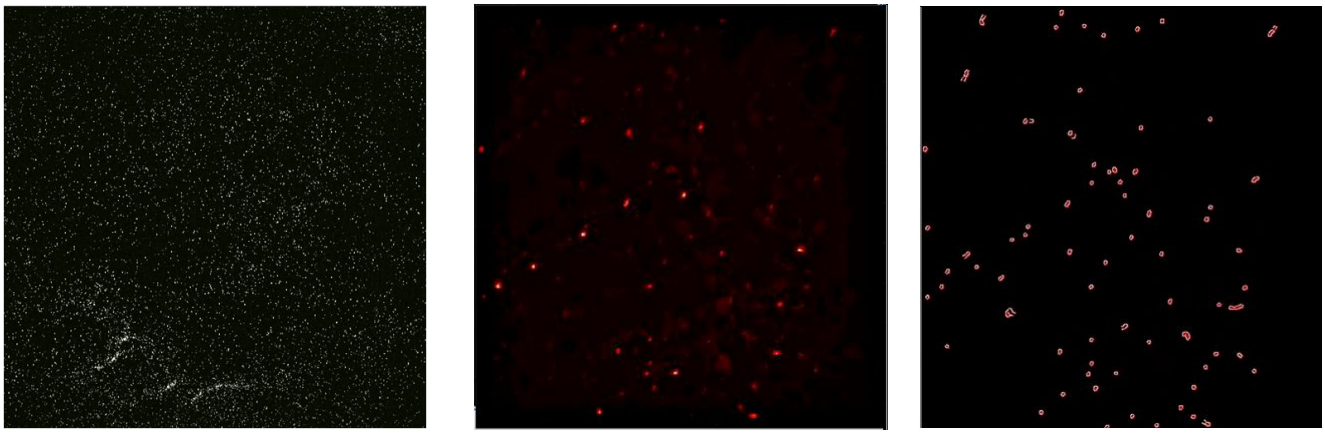
First the mouse are injected with AAV virus which contain two genes, the *loxed* gene for GCaMP and a sparse expression of the *cre* gene. In order to get an expression of the GCaMP protein, two weeks are needed after this time period, a small hole above the desired cortical area in the mouse skull was opened. The final stage is recording by two photon microscopy from above that hole (figure 1.3.b a side view, figure 2.3.1.a taken from above)

2.2 The Data

Sequences of tiff images at 6 different experimental conditions were taken using two photon microscopy. One experimental condition is “Baseline” , before applying guanfacine, and then 5 more experimental conditions: 10 minutes after applying guanfacine, 20, 30, 40 and 50.
The images were taken at an acquisition rate of 40 frames/s for 3 minutes/ experimental condition producing each 7200 images weight about 3.5 GB. Therefore for each experiment with 6 different experimental conditions the total data weight is about 21 GB.

2.3 The Algorithm Flow

From this point and toward in this paper I will refer to dendrite as region of interest (ROI).**First step**, find the regions of interest (ROIs) in the image. In order to analyze the activity in each experimental condition, we need to find ROIs (dendrites) coordinates in each image. Since each single image looks blurry, (see for example figure 2.3.1.a) we are not able to detect the ROIs by analysis of the image itself. Therefore, we decided at lab that the first process would be to turn the image into a standard deviation (std) image (see figure 2.3.1.b) .



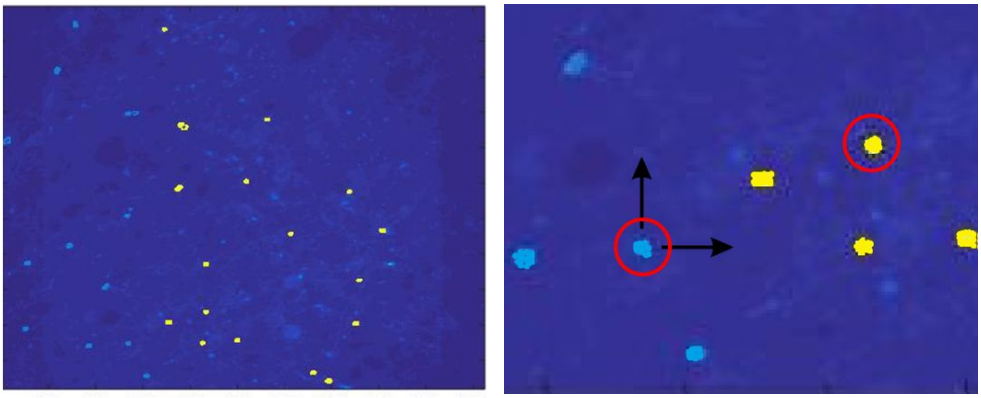
(a) **(b)** **(c)**

Figure 2.3.1: (a). An example of one single image **(b)**. An std image. **(c)**. Std image with applied edge detection.

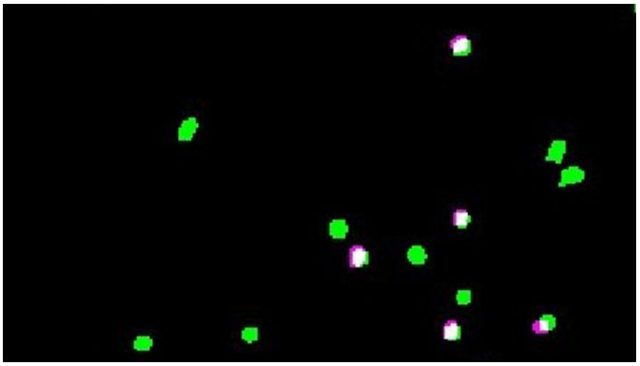
An std image would be an image that each of its pixels is the result of std of the same pixel over all 7200 images per experimental condition, meaning that instead of having 7200 frames per experimental condition, we will end up with one std image. We believe that an std image is a good idea since it would be brighter in areas which change the most during a specific experimental condition, therefore brighter areas will indicate greater activity. Next, the algorithm will apply edge detection combined (see for example figure 2.3.1.c) with connected components on those images in order to find the active ROIs coordinates in the image.

Second step, translation of the images. Watching the image sequences we have noticed that there were small movements of the images between experimental conditions. Therefore, if we will try to contrast the same coordinates between two different experimental conditions (figure 2.3.2.a), we might not compare the same ROI. In order to answer what is the difference between two experimental conditions, namely, how guanfacine effects those dendrites, we need to compare the same ROI in both of the compared experimental conditions.The solution we came up for this problem is computing cross correlation on two marked std images which enables us to find the

wanted translation offset (see example in figure 2.3.2.b) and apply it on the tiff images of the wanted experimental condition (figure 2.3.2.c).



(a) **(b)**

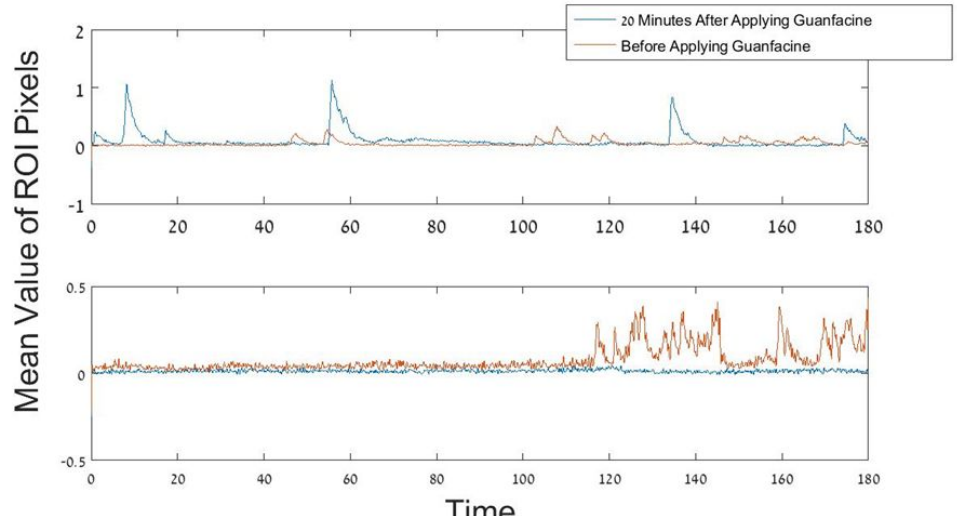


(c)

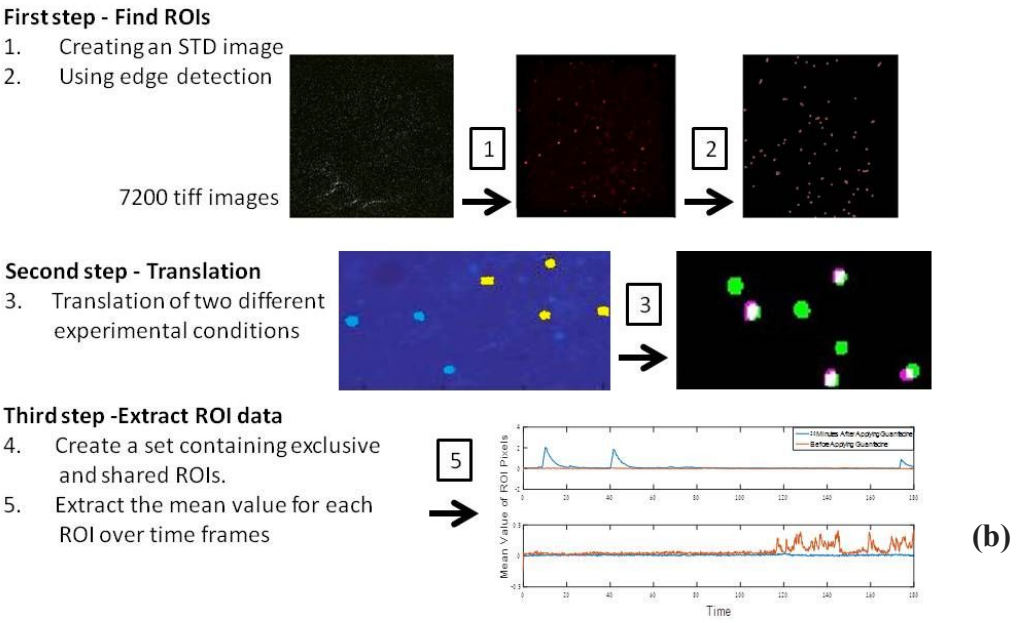
Figure 2.3.2: (a). An image containing ROIs from two different experimental conditions marked by light blue and yellow. **(b)**. Zoom in on one part of image to illustrate the wanted translation**(c)**. Zoom in on part of the translation result, two experimental conditions one - purple dots, second - green dots and the white dots are the two experimental conditions overlapping dots.

Third step, extract ROIs data. Creating a set of ROIs which appear in both of the compared experimental conditions. Each ROI which was included in the set had 3 options for detection (at the pipeline first step). The ROI was detected only in the first experimental condition, only in the second experimental condition or in both of the experimental conditions. Each ROI coordinates values are extracted over the total of 7200 images. After this step we have the normalized mean

values (over 7200 images, figure 2.3.3.a) for each ROI in the set for both of the compared experimental conditions.



(a)



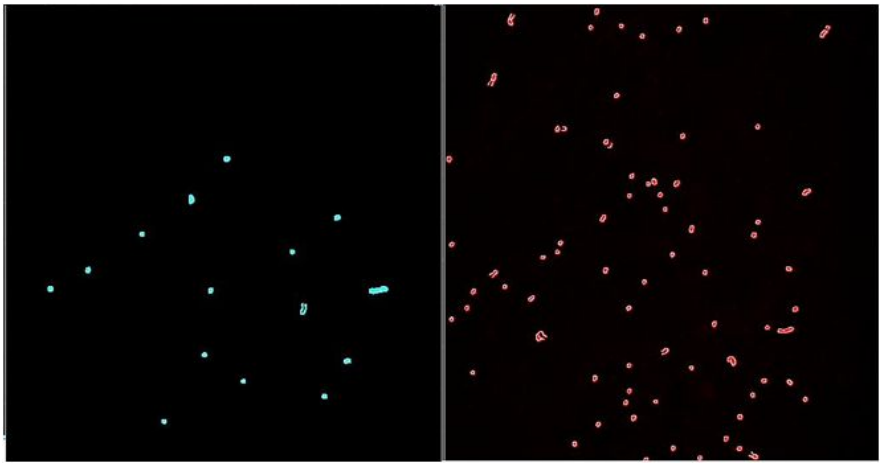
(b)

Figure 2.3.3: (a). Two examples of the raw extracted data, each image describe the fluorescence values of a single ROI over 3 minutes. **(b).** A flow-chart of the pipeline.

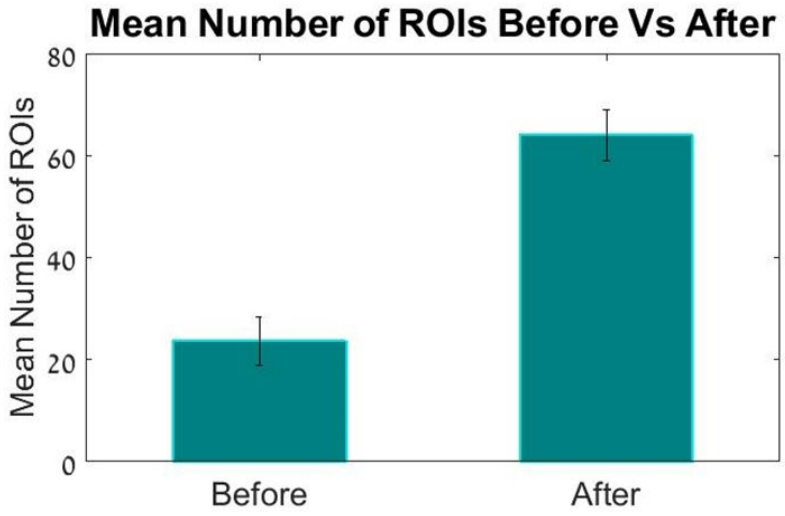
2.4 Analysis Results

2.4.1 The mean number of ROIs after the exposure to guanfacine is bigger compared to their number before the exposure

By using edge detection on the std images it is obvious (figure 2.4.1.a) that the amount of detected ROIs increase after the exposure to guanfacine. The calculated mean number over 3 different experiments is 24 ± 4.7 ROIs before and 64 ± 5.04 after.



(a)



(b)

Figure 2.4.1: (a). Two std images, left - before the exposure to guanfacine, right - 20 minutes after the exposure. **(b).** Mean number of ROIs before the exposure to Guanfacine Vs after (over all the experimental conditions 10 - 50) calculated over 3 different experiments.

2.4.2 The experimental conditions with the highest fluorescence values are 10 and 20 minutes after the exposure to guanfacine

In order to check what is the time with the highest fluorescence values over all the experimental conditions (10,20,30,40,50 minutes after application and before), for each ROI we summed up its fluorescence values over 3 minutes per experimental condition, the final value for each ROI is one scalar representing the fluorescence per experimental condition.

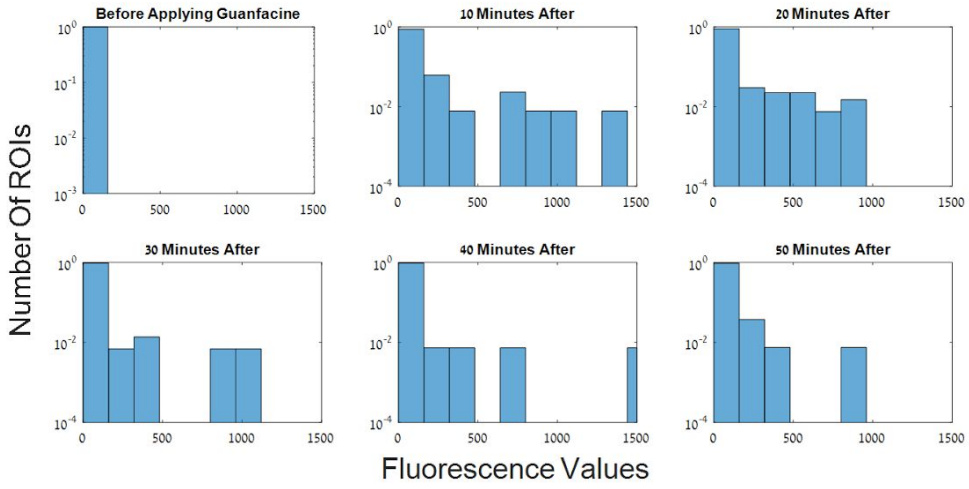


Figure 2.4.2: Histogram for each experimental condition, showing the number of ROIs in a range of fluorescence values.

For each experimental condition, we created a histogram showing how many values there are in each of the fluorescence values. The results show more ROIs at the low fluorescence values for 0, 30, 40, and 50 minutes after the exposure and more high values at 10, 20 minutes after the exposure.

2.4.3 The majority of ROIs have higher fluorescence values 20 minutes after the exposure compared to before the exposure

Next, we took one of the most active experimental conditions, 20 minutes after the exposure to guanfacine and compared it with before the exposure. The comparison was done by summing up their fluorescence values (described in previous point). The result shows that most of the ROIs

(165) have higher fluorescence values after the exposure vs 62 which show the same or lower fluorescence values after the exposure.

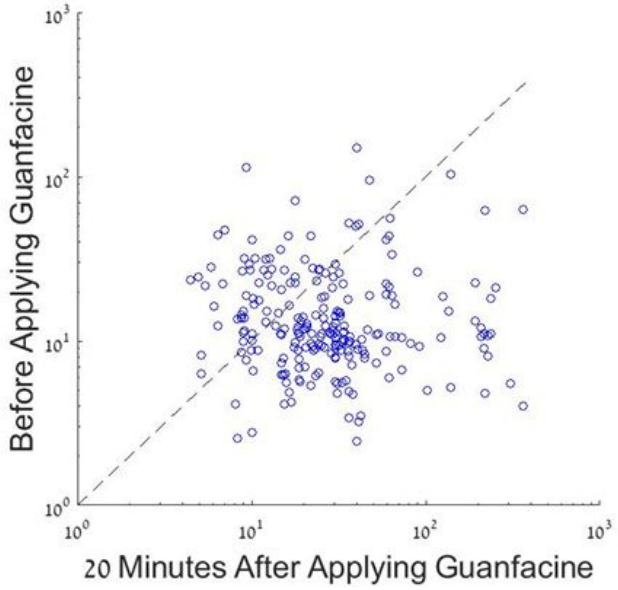


Figure 2.4.3: Fluorescence values per ROI, each dot is an ROI with x coordinate values representing the mean fluorescence value 20 minutes after the exposure, y coordinate represents the mean fluorescence value before the exposure.

2.4.4 A different partition show different perspective on the results

After looking more carefully on the groups embedded in the plotted set, I discovered that by making some different partition we get a surprising result. In figure 2.4.4 we can see that most of the ROIs (43 out of 62) which showed the same or higher fluorescence values before the exposure vs 20 minutes after the exposure are originated from the common ROIs, meaning they were detected independently in each of the compared experimental conditions.

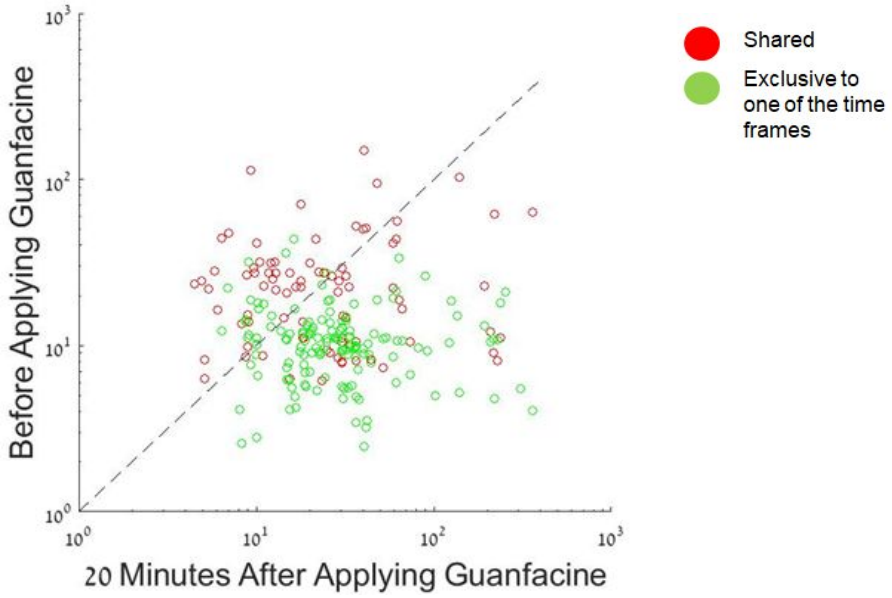


Figure 2.4.4: Same as figure 2.4.3 but with a different partition, red - shared ROIs, green - exclusive ROIs.

3 Discussion

In our research we wanted to answer what is the effect of norepinephrine on cortical dendrites? Using two photon microscopy from mice injected with Gcamp protein we could detect activity in the mouse cortex. In order to analyze the data efficiently, I built an automatic algorithm described in the results, via the algorithm we asked questions which provided us information to answer the research question.

The first basic question we asked was is there any difference in the amount of active dendrites (ROIs) before the exposure in contrast to after the exposure (result 2.4.1) The results show that the mean amount of ROIs before the exposure to guanfacine are lower than the amount detected after the exposure to guanfacine. This basic analysis can indicate that guanfacine effects those dendrites and turn them to be more active after the exposure.

The second question we asked arises from the first. We saw that there is more activity after the exposure, but we wanted to check which experimental condition is the most significant, meaning which experimental condition has more ROIs with high fluorescence values (result 2.3.2). The most active experimental conditions are 10 and 20 minutes after the exposure, we saw that the histogram values get more ROIs with high values compared to before and 30 or above minutes after the exposure where we see that most of the ROIs tend to be at the lower fluorescence values.We assume that the reason is the time it takes guanfacine to diffuse into the cortex (L5) neurons.Therefore, another conclusion was that dendrites are more active 10 or 20 minutes after the exposure to guanfacine.

The third question was comparing between two experimental conditions before and 20 minutes after the exposure (chosen as a result from the previous conclusion).We plotted a scatter plot (figure 2.3.3) presenting those two experimental conditions on the axis . We can see from figure 2.3.3 that there are more ROIs which are more active 20 minutes after the exposure than before the exposure. But we couldn't ignore that there were 62 of the ROIs which showed the same activity or even higher activity before vs 20 minutes after., This result surprised us since we expected to see a rise in the activity after applying guanfacine in all the ROIs (Barth AM.,2008). After looking more carefully on the groups embedded in the plotted set, I discovered that by making some different partition we get another surprising result. In Figure 2.4.4 we can see that most of the ROIs (43 out of 62) which showed the same or higher activity before the exposure vs 20 minutes after the exposure are originated from the common ROIs.

But how can we explain this phenomena?

Every neuron has its own threshold for dendritic Ca^{2+} spike generating. We believe that Guanfacine lowers this threshold and therefore the neuron becomes more sensitive to signals and generates spikes more easily - we see more activity. But what if the initial threshold is already low? Then guanfacine can't lower it more, this is called the floor effect. Our hypothesis is that these group of neurons had a low threshold therefore Guanfacine had no influence on them and that is why they won't show more activity after the exposure.

Another interesting aspect involves a method called transcranial magnetic stimulation (TMS), non-invasive treatment to brain disorders such as schizophrenia, depression and pain (Murphy et al., 2016). TMS is used both to enhance and impair cognitive abilities, simulation is produced by generating a brief, high-intensity magnetic field. According to Murphy et al. the method causes a suppressed dendritic Ca^{2+} activity. Can inhibition of dendritic Ca^{2+} spikes might be the cause for higher cognitive abilities? we showed guanfacine causes less dendritic Ca^{2+} activity for part of the neurons, guanfacine is also given to people with ADHD, so can this inhibition be the key for the effect causes concentration?

3.1 Future plans

At the pharmacological stage, we demonstrated that indeed NE is affecting dendritic excitability. However, it is not clear what is the effect when physiological NE is released. Here, we will take the next step and observe dendritic activity while using a more physiological manipulation of NE. To establish causality, we will use optogenetics and light stimulation in order to manipulate NE activity. This step will also include recording by two photon in different experimental conditions, and with the pipeline I developed, those pictures could be analyzed as well. Moreover we would like to check the phenomena we found, NE has no effect on some of the dendrites because of the floor effect. Validating this assumption is complicated, even if we find a specific neuron which has that property finding its threshold is not a task. Furthermore if we knew how to measure its threshold the analysis needs to happen online in order to enable us to find that specific dendrite. Therefore, checking the actual dendrite we see in my analysis is too far fetched. Our plan is to check how significant is this phenomena, if we will have enough data from pharmacological and physiological experiments we can formulate a statistical hypothesis testing and set it a statistical significant.

As for the algorithm, in order to improve the pipeline experimental conditions analysis we need to handle shifts - the experiments are done in awake mouses which mean they might move

during the procedure. In order to handle this problem and get a more end to end algorithm we will try to develop a part that can handle shifts.

4 Methods and Materials

4.1 Virus injection (GCaMP)

Calcium (Ca^{2+}) is a ubiquitous signaling molecule and acts in several physiological processes. To monitor the calcium fluctuations in the cortex, we used genetically encoded Ca2+ indicator (GCaMP6). GCaMP6 was injected to deep layers in the cortex, and Ca2+ activity was imaged through a chronic imaging window. For sparse labeling of the cortical neurons (specifically in L5) with GCaMP6, AAV carrying floxed GCaMP6 was co-injected with a diluted AAV carrying Cre recombinase (~1:100). This approach reduces the cell density of GCaMP6 expressing neurons without suppressing the expression level.

4.2 Two-photon Imaging In Vivo

Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging of living tissue up to about one millimeter in depth. It differs from traditional fluorescence microscopy, in which the excitation wavelength is shorter than the emission wavelength, as the wavelengths of the two exciting photons are longer than the wavelength of the resulting emitted light. For each excitation, two photons of infrared light are absorbed. Using infrared light minimizes scattering in the tissue. Due to the multiphoton absorption, the background signal is strongly suppressed. Both effects lead to an increased penetration depth for these microscopes.

4.3 Mouses

We used C57BL/6 male mice (8–13 weeks old) for all of the experiments.

4.4 Built in matlab features

Edge detection using canny, with sensitivity of 0.1 for after the exposure experimental conditions (10-50 minutes) and 0.25 before the exposure.

Bwconncomp in order to find the connected components in the std image.

Cross correlation part was done by normxcorr2.

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