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Analysis of the Heterogeneity of the Hippocampus through Single Unit Recordings and Confocal Microscopy

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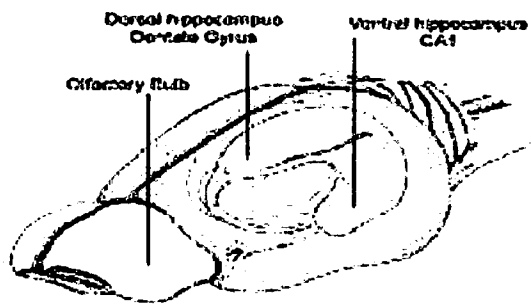
Introduction

The hippocampus is a curved structure located in the medial temporal lobe of the brain. Since the historic case of H.M. the hippocampus has been implicated in the processing of episodic memory, helping us process and store the what, where, when and how of events. Besides memory, this structure also plays important roles in emotional and in spatial processing. Through anatomical studies of neural circuitry, the hippocampus has been proven to be a nonhomogeneous structure (Swanson & Cowan, 1977). The hippocampus is comprised of different cell layers called the CA1, CA2, CA3 and dentate gyrus. These cell layers perform different tasks and all project to different regions of the brain. However, besides these cellular layers, the hippocampus has been proven to possess a functional gradient along the dorsal/ventral pole as well. Current literature implies that the dorsal region of the hippocampus serves as the spatial processing center (Moser et al., 1995) whereas the ventral region is the emotional processing center (Henke, 1990). When as little as 25% of the dorsal hippocampus in rats is lesioned, rats have a significantly more difficult time completing a spatial task such as the morris water maze, during which a rat is required to find a platform in murky water using cues outside the maze (Moser et al., 1995). On the other hand, when the ventral hippocampus is lesioned, there was no deficit in spatial memory (Ferbinteanu & McDonald, 2001). However, these functions are not absolute, in some recent studies, the ventral hippocampus has been implicated in spatial processing as well as emotional processing.



In this paper, I will explore two different methods of hippocampal analysis; single unit recording of individual cells and analysis of gene activation through in vitro hybridization and confocal microscopy, both of which are applied to rats. By using the rat as an animal model, invasive techniques can be used while still maintaining a high level of correlation to the human hippocampus. The dorsal portion of the rat hippocampus is analogous to the posterior portion of the primate hippocampus and the ventral analogous to the anterior (Fanselow & Dong, 2009). Since it is difficult to quantify episodic memories, the rat's capacity for spatial processing serves as the template for research into the hippocampus. In the rat's hippocampus, there are special cells called place cells or pyramidal cells or complex spike cells that specifically encode spatial information. These cells fire when the rat's head is in a specific location and will fire in the same location every time the rat encounters that aspect of space in an environment (O'Keefe & Nadel, 1978). The physical area in which this cell fires is called the place field, the rate at which the cell fires in this area is also specific to location. The formation of these place fields indicates acquisition of spatial information. When the environment, task or trajectory of the animal change, the place fields change. Place fields are quantified through single-unit analysis. This is when a microdrive is surgically implanted into the rat's brain with tetrode wires connecting to a complex spike analysis system. The second way to detect the firing of a place cell is to use immediate early genes and in vitro hybridization to allow the genes to be imaged via confocal microscopy. Immediate early genes are genes that are expressed right after the cell fires. Behavioral tasks are then constructed around this unique feature. Paired with these two different types of analyses are two different behavioral tasks, one centered on remapping and the other focused on emotion.

Behavioral Tasks

The task paired with the single unit recording is a forced-trajectory task based upon a radial arm maze. In this task, there are only two arms of the maze, each of the arms being mobile. The nine food-

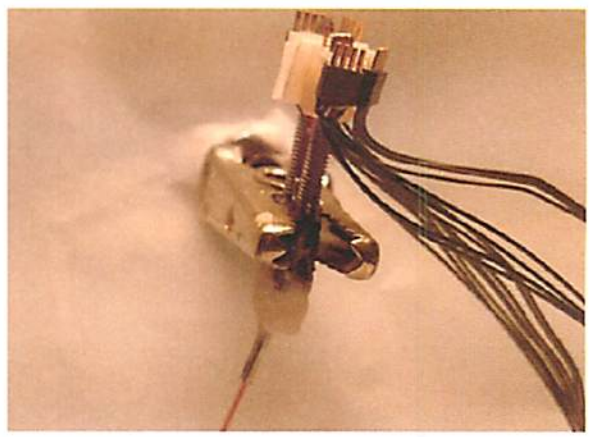
deprived rats are trained in 15 minute intervals with two sessions a day and a five minute break in between sessions. There are food rewards at the end of each arm of the maze. As the rat approaches the end of one arm, a sugar pellet is released into a feeder at the end of the arm, the rat then turns around and repeats this with the second arm. This training ensures that stable place fields are formed and will not fluctuate on recording day with the head stage attached. Once established place fields have been recorded and analyzed, the arms of the maze will slowly change configuration. Then rat will then be run on these different configurations and the changes in the place fields will be recorded. This task aims to shed light upon how different parts of the hippocampus react to gradual spatial changes. It is to determine how salient the difference in spatial orientation must to incite remapping or encoding.

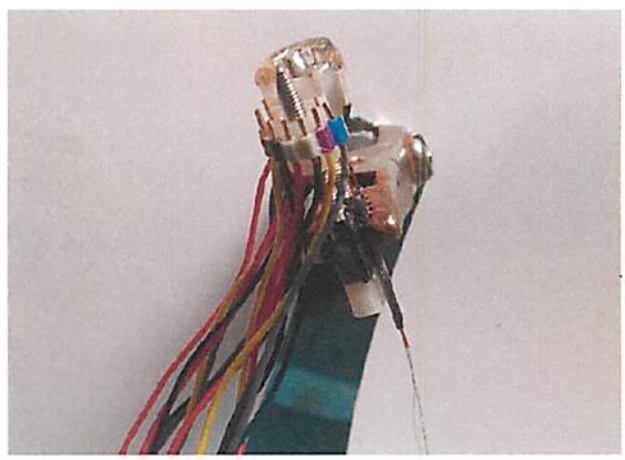
The task paired with the immediate early gene analysis is focused on how different parts of the hippocampus react to emotional situations. This task used a total of 19 rats. The configuration of the maze is a simple horseshoe. There are chambers with feeders at the ends of the horseshoe and an electrified area at the apex to provide a shock. As the rat runs back and forth around the horseshoe, the shock paired with a tone is gradually introduced. The tone starts when the rat is feeding at the ends of the horseshoe so the rat is aware of when the shocks will come. Like the radial arm maze, the rats run two sessions per day, separated by 25 minutes. Each sessions consists of three cycles around the maze, one cycle is completed when the rat returns to the original feeder. During training, the cycles are randomly set to contain shock or to benign. During training, there could be two cycles with shock and one cycle without and other such combinations. Once the rats have learned to associate the tone with the shock, indicated by a significant increase in running speed or by increased time spent in the chambers with the feeders, they are exposed to sessions in which all cycles are accompanied by tone and shock or no cycles are accompanied by tone and shock and finally sacked. At this stage, it is critical that all conditions are the same; if the rats change direction in the middle of their run, extraction cannot occur on that day. This is due to the fact that place cells will fire differently based upon trajectory as well

as spatial location. This task ultimately aims to shed some light onto which regions of the hippocampus are most interested in changes in the emotional climate of a space.

Methods

Two different microdrives were made for this project; one for the dorsal hippocampus and one for the ventral hippocampus. Each microdrive has a base made of plastic and dental cement, two supporting structures with threading on the outside to allow nuts to move freely. This allows the tetrodes inserted through the cannula affixed to the plastic body of the microdrive to be moved up and down into the target cell layer of the hippocampus. The cannula guiding the tetrode wires are straight in a dorsal microdrive. Since the ventral portion of the hippocampus is much lower, the cannula in a ventral microdrive are curved to target the desired portion of the hippocampus. In order to hit different cell layers within the hippocampus, namely CA1 and CA3, the tetrodes are cut to be two millimeters apart. On surgery day, the rats are sedated with gaseous tetra fluorine and treated with 0.1 mg/kg Metacam meloxicam to combat pain after surgery. The microdrives are then implanted and secured onto the scull via scull screws and dental cement. The rats are allowed to recover from surgery, then before any contact with the maze, the tetrodes are turned down until individual place cells are located. The signal from the tetrodes is amplified 2000–5000 times (Neuralynx, Tucson, AZ), then stored for offline analysis.



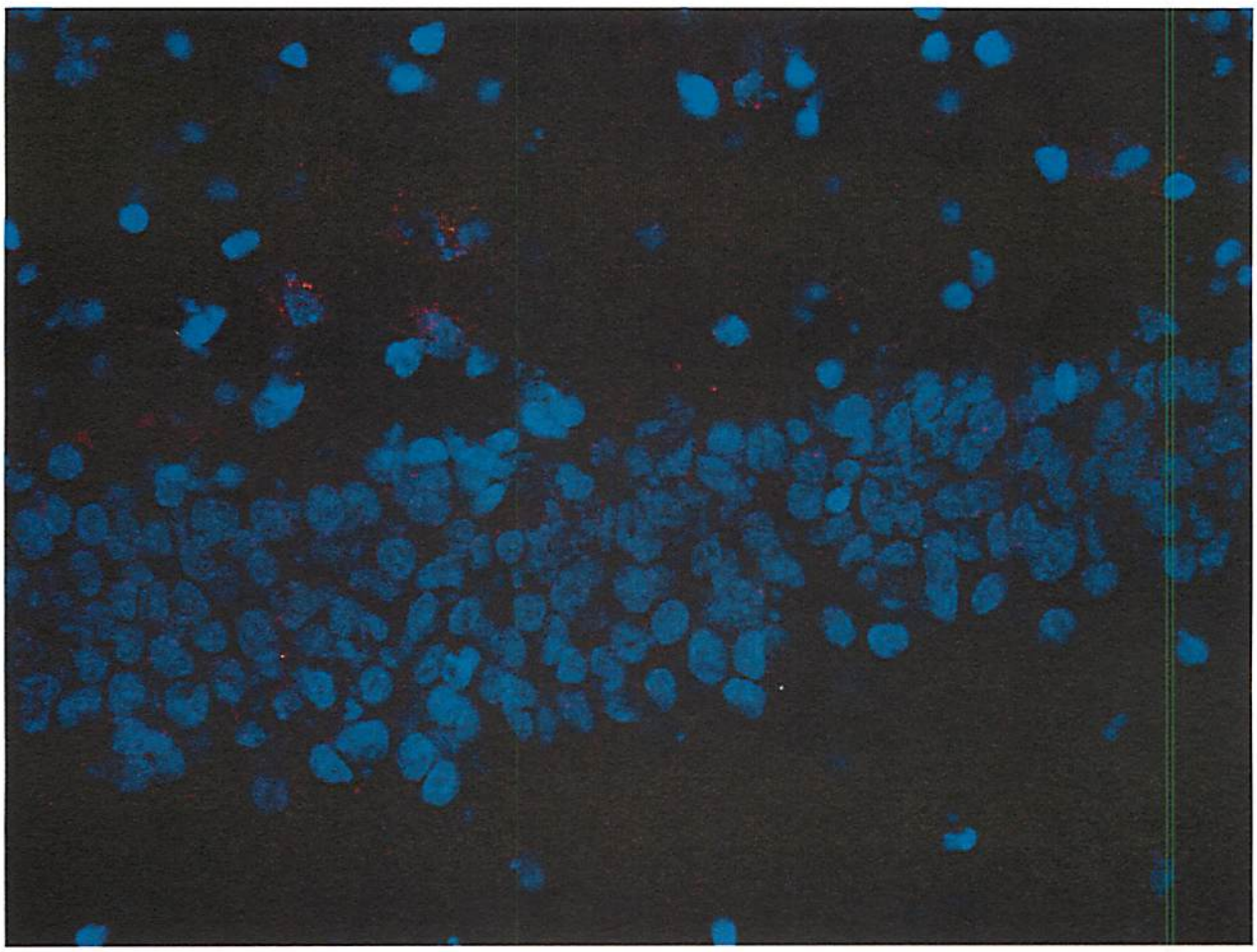


The in vitro hybridization process allows a more comprehensive analysis of the hippocampus. While single unit recording is advantageous because it provides data in real-time, it can only provide data from a couple of cells at a time. Analysis of immediate early genes can occur throughout an entire slice of the brain. After the rats are sacked, their brains are removed within 3 minutes of death and flash frozen and stored at -70 degrees Celsius for analysis. After cutting off the unnecessary cerebellum and prefrontal cortex, hemisections of 6 rats per block are glued into a block of OCT. Using the cryostat, the blocks are sectioned into 20 micrometer slices. The slices are then thaw-mounted onto pre-treated slides and re-frozen to -70 degrees Celsius. Next, the slides are treated to fluorescence through in situ hybridization.

In the dentate gyrus layer of the hippocampus, the most prominent immediate early gene is called zif268. This gene is expressed immediately after a cell fires. After 30 minutes, the mRNA migrates out of the nucleus and into the cytoplasm. This is where the 25 minute break in the behavioral task comes in; if there are signs of zif 268 inside the nucleus, then it means that cell was active for the second session, if there are signs of zif268 in the cytoplasm of the cell, then the cell was active in the first session. Zif268 riboprobes, complementary to the gene, are synthesized using a transcription kit and digoxigenin-labeled UTP, purified in RNA spin columns and then run through gel electrophoresis for confirmation. Once thawed, slides are fixed in 4% paraformaldehyde, treated with 0.5% acetic anhydride, incubated in methanol and acetone (1:1), equilibrated in saline-sodium citrate (SSC), and incubated with prehybridization buffer (Sigma-Aldrich) for 30 min at room temperature. Slides are then incubated in 100 ng of riboprobe in a humid chamber overnight at 56°C. This is followed by continual SSC and RNase A (10 mg/ml) washes at 37°C for 30 min. Then the endogenous peroxidases are quenched with two percent hydrogen peroxide. The tissue is blocked with TSA blocking buffer, containing normal sheep serum, and incubated with anti-digoxigenin-HRP antibody for 2 hours at room temperature. Slides are then washed in Tris-buffered saline with 0.05% Tween 20 and HRP-antibody

conjugates are detected using a CY3 TSA kit. Tissue is then counterstained with DAPI (Sigma), coverslipped, and sealed with Vectashield (Vector Labs). The CY3 and DAPI stains show the gene expression and the cell nucleases respectively.

The finished slides are then stored and refrigerated until ready to image. Images are done at 40X using an Olympus FV1000 confocal microscope. A confocal microscope is a light microscope that uses lasers to excite certain dyes. In the picture below, the blue DAPI dye indicates the cells while the red CY3 dye indicates activation via mRNA. A statistical analysis of the images is done by counting the cells that are active and then comparing the activity of different layers of the hippocampus.



Discussion

Both methods used to investigate the heterogeneity of the brain have faults as well as advantages. Single unit recording is extremely powerful in that the amount of data that can be collected is, in theory, unlimited. If the rat is able to survive surgery and recover well from it, then many manipulations can be done while it is hooked up to the machine. However, its main flaw is that it is limited to recording the number of cells surrounding the tetrode. This means conclusions about an entire neural network must be drawn from a couple of cells. That being said, cross comparisons of the way different cells fire across different regions of the hippocampus can still show contrasts in function. For example, the place cells found in the ventral hippocampus have lower resolution place fields than place cells in the dorsal hippocampus (Jung et al., 1994). Imaging with the confocal microscope, on the other hand, allows the researcher to analyze entire populations of cells at once. This provides a very visual display of the differences between cells of different parts of the hippocampus. One major disadvantage is that it has a limited amount of data; there can only be one rat brain. These are only two methods of data collection, and along with behavioral analysis, genetic manipulations, pharmaceutical studies, EEG recordings and pure anatomical analysis, they are slowly adding to the growing pool of data on the brain.

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Holster Journal

Xiao Li

May 18, 2012

I don't understand how such a small piece of equipment can cause so much grief! After three straight days of work on it, I managed to crush one of the glass tubes in a clamp. Now I have to start all over... The wire tetrode used to actually record the cells in the hippocampus is actually made of four wires twisted together. They are about half the thickness of a strand of hair. Throughout the entire process, the tetrodes need to be perfectly straight. In other words, a wire only as thick as a strand of hair and half as strong, needs to stay pin straight for at least 4 days until the microdrive is finished. On the bright side, the rats are performing well. They have all reached criteria and the automatic system is working smoothly.

June 1, 2012

These last two weeks, I have been working on a different project, setting the microdrives aside for work later. This task is a study of how fear and emotion effect the hippocampus in rats. The best part of this task is that it is short! As long as the rats run back and forth 3 times, they are finished for the day. The rats are already well trained and know the task well. Me getting used to the maze, however, is a different story. The first time that I heard the tone meant signal the shock, I jumped out of my chair in fright before I realized that the maze was supposed to sound like a blaring car horn. I can't believe the rats can stand the sound. I am so excited to be on this project, because it means I get to travel to Canada to help our partner lab up at Wilfred Laurier University to process tissue!

June 15, 2012

Today I experienced my first extraction. Since the brain needs to be alive when it is flash frozen, it needs to be removed within 3 minutes of killing the rat. The blood and debris wasn't what shocked me the most, I was most startled by the amount of noise accompanying the entire process! When the guillotine lands on the rat's neck, there is an accompanying crunch that oddly reminded me of a blunt cleaver cutting through raw celery, I'm not sure how I feel about the vegetable now... After the head is removed, the skull has to be split open and the brain removed. In order to do that, you have to jam a pair of scissors into the rat's forehead, then when the scissors are forced open, the skull cracks like a walnut. All of this within three minutes. At least the process is painless.

June 29, 2012

Well, we've been in Canada for a week now and everything is going smoothly. My three lab mates and I moved into the apartment that they provided us on the 23rd and we started work the following Monday. The lab here is very different from ours, since they do immunohistology, they have fume hoods and chemicals, it is more like a chemistry lab than a psychology lab. When we arrived, the brains hadn't come yet, so we shadowed other members of the lab as they went about finishing other projects. The brains that we extracted back in Connecticut have arrived safely two days ago and I just spent 6 hours slicing them into paper thin slices. The machine we used to cut the brains needs to be freezing so that the brains to not thaw, after 30 minutes I had already lost feeling in my fingers. The process of mounting the slices on its fascinating; all you have to do is hold the slide on top of the slice and because of the heat gradient, the slice just jumps right onto the slide. I sliced about 300 slices, but only about 80 of them were actually good enough to mount on a slide. It's really tedious, but at least we only have to do a couple of days of this.

July 13, 2012

When we finished slicing, the PI taught us how to perform invitro hybridization. Since the process works with DNA and mRNA, the entire area must be clear of the RNA cleaving enzyme RNAase. We have to wear gloves and spray RNAase away on the gloves to get rid of any contamination. Unfortunately, RNAase is present in every single cell, so even if we lean over the counter, it needs to be sanitized before use. I'm so afraid of contamination that I spray it on every few minutes...I might be forming a habit. Depending on how much time is put in, it can take two to four days to prepare ten slides. In some ways, this process is even more annoying than making a microdrive; if a part of the microdrive breaks, I can fix it right away, there is no way of knowing if the hybridization worked or not, so the only way to tell is to run it through gel electrophoresis when the process is complete.

After a couple of batches had been finished, we started to confocal. This amazing microscope has a special feature: it can look through layers of tissue and take pictures of fractions of the slices to make a composite image that looks 3D. The procedure itself is very mundane, all we have to do is find the top and the bottom of the slice and then let the microscope do its work.

July 27, 2012

We said goodbye to Canada on Tuesday and headed home. Despite the fact that we stayed for a month, we still have more tissue to process, the only part of the brain we were able to finish was the dentate gyrus. Now we have the wonderful task of counting cells...it's literally counting the little blue dots and distinguishing them from the little blue dots with a little red in them. We are using a program called imageJ, at least it does the counting for us, but we have to categorized the cells, since the image is a three dimensional stack, we have to run through the entire stack to check a single cell. The program is user made, so there are no nice company trained IT guys to help when there's a problem. It's still full of

inconveniences and bugs. In the end, that's what research is, you make do with what you have and slog through hours of menial work to finally arrive at an amazing breakthrough.