Targeted read-out, analysis, and control to elucidate dynamic-emotional processing

by

Brian D. Allen

B.A. in Cognitive Science and Physics, Northwestern University, 2005

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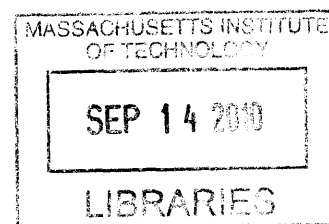in partial fulfillment of the requirements for the degree of

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Abstract

Many psychological disorders, such as panic disorder, are episodic in nature, with unpredictable onsets and similarly unpredictable durations. That the severe symptoms of these disorders come in waves rather than remain at a level of stasis poses a daunting challenge for pharmacological approaches that lack temporal precision. Here, a set of technologies and approaches for examining and treating these conditions are developed, using techniques to monitor brain activity and shut down specific brain sub-regions at times critical to the recall of emotional memory.

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Contents

Chapter 1

Dynamical anxiety disorders persist; drugs lack specificity

The suddenness with which emotions can overcome us is astounding. Even in the absence of proximal external causes, our affective states often wax and wane, most prominently in pathological conditions: patients suffering from depression or anxiety may be suddenly struck by despair without apparent provocation [1], [2]. The collective anxiety disorders, including panic disorder, post-traumatic stress disorder (PTSD), and debilitating phobias, are extremely prevalent in our society, affecting over 28% of us at one point or another [3]. Though the fields of pharmacology and cognitive behavioral therapy have made considerable progress in helping people cope with these maladies, there is still considerable dispute in the literature over the efficacy and widespread applicability of the treatments they offer [4], [5]. Furthermore, drug treatments often come with debilitating side-effects [6], [7]. This likely stems from the fact that the drugs modulate more than their intended targets: the specific circuits that are the sources of pathology in the brain. For an example, selective serotonin reuptake inhibitors (SSRIs) likely modulate activity across the entire serotonin system, a complex web distributed throughout the brain [8] and in the gut [9] that plays a hand in a large amount of disparate neural activity. Serotonin has many proposed functions: a Google Scholar search (http://scholar.google.com) for "The role of serotonin" yields hundreds of results such as "The role of serotonin in premenstrual syndrome", "The role of serotonin in eating disorders", etc, and serotonin has been prominently implicated in depression [7] and irritable bowel syndrome [10].

Furthermore, because we largely lack knowledge of the principles of neural activity underlying the disorders involving serotonin and other neuromodulators, and to a great extent we lack knowledge of the precise method-of-action of the drugs, we don't have a strong grounding on which to improve existing drugs to create more efficacious treatments.

1.1 – Genetic models of anxiety

Anxiety can be thought of as a state of anticipatory fear — a pervasive feeling that something bad is happening or about to happen [11]. While pathological anxiety can be studied at a purely behavioral or pharmacological level, such approaches have yielded unsatisfactory results [12] for many of the millions of Americans suffering from it every day [13]. The application of genetic engineering to the genome of the mouse in 1989 [14], work which garnered the investigators the Nobel Prize in Medicine in 2007, opened up promising new avenues for the scientific exploration of anxiety. Soon after, mouse models of anxiety were created using genetic manipulation to mimic characteristics of anxiety that are thought to be evolutionarily conserved between mice and humans. The behavior of these mice was often assayed with tests of Pavlovian fear conditioning, where an animal is trained to learn that a neutral stimulus such as a tone (termed the conditioned stimulus or CS) predicts an aversive stimulus such as a shock (known as the unconditioned stimulus or US). These fear-conditioning assays yielded data thought to be qualitatively similar across the human and mouse species — where a tone predictive of a foot shock led to a state of immobility in a rodent known as freezing[1] [15], a similar paradigm led to significant responses in several autonomic indices of conditioning, including increased electrodermal activity and heart rate in human subjects [16]. Humans and mice seemed to

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[1] Freezing is defined as a period of immobility in a rodent in which the only movement observed is related to breathing. This expression will be used interchangeably with statements like "behavioral concomitants of fear memory" in this thesis, as it is the gold standard indication of fear memory.

share a common disposition, making it apparent that insight could be gained from scientific manipulation of the latter.

1.2 – Successes in genetic engineering

With the ability to manipulate the genome in mice, researchers were able to start probing the putative mechanisms behind the anxiolytic (anti-anxiety) drugs that were offering relief to some patients. Specifically, in one line of experiments, a receptor class for the neuromodulator serotonin was genetically "knocked out" in a line of mice; the gene encoding for it was turned off. Researchers were led to serotonin because of the success of the aforementioned selective serotonin reuptake inhibitors (SSRIs) in treating some patients suffering from pathological depression or anxiety [17]. For this particular series of studies, the serotonin receptor 5-HT1A knockout mice exhibited many characteristics thought to be related to anxiety — e.g. a combination of decreased exploratory behavior and increased behavioral concomitants of fear when placed in open spaces — and subsequent research suggested that dysfunction of the receptor in early development could predispose one to anxiety disorders [18]. Additionally, a newly-invented "pharmacogenetic" technique — a combination of pharmacological and genetic approaches — for inhibition of some cells of a particular region in the hippocampus (HPC) was shown to reverse many pathological aspects of the phenotype [19]. This represented enormous progress for the following reason. There is good basis to suspect that, in the fully-developed human brain, a widely-projecting neuromodulator such as serotonin may affect separate brain regions, or areas within brain regions, in different ways. Because serotonin's proposed role in many seemingly disparate mental functions suggests that its global modulation would lead to unintended local effects, demonstrating a benefit from its very spatially-limited and precise modulation paves the way for more targeted therapies that are less likely to be

accompanied by side effects. In a similar vein, the next chapter will introduce an approach that is spatially and genetically precise, and additionally allows us to communicate with the brain on a timescale closer to that of the neural activity that underpins dynamic-emotional behavior.

Chapter 2

Attacking emotional dynamics

Recent developments in the field of optogenetics — in which light-sensitive proteins (opsins) are targeted to specific (genetic) classes of neurons, thereby making their neural activity susceptible to modulation by pulses of light — allow for the spatially, genetically, and highly temporally-precise control of neural circuits [20]. Whereas techniques such as the aforementioned pharmacogenetics allow for spatial and genetic specificity, optogenetics allows for even finer spatial resolution [21], and importantly makes possible neural circuit manipulation on the order of milliseconds [20] — the timescale that is characteristic of neuronal firing.

Many groups are now rushing to apply these technologies to develop targeted treatments for a number of neurological disorders, from Parkinson's Disease [22] to epilepsy [23] . Indeed optogenetic research into the nature of emotional disorders, particularly traumatic memories, has become a hot research topic, with the optical manipulation of emotionally salient memories being demonstrated in rodents [24].

Exciting recent developments in the applications of electrophysiological recording techniques to emotional circuitry have simultaneously shed light on some of the pathways critical in emotional processes, suggesting the importance of the dynamical nature of the communication between and among brain regions at various stages relating to the acquisition, retrieval, extinction, and re-instatement of emotionally-salient memories [25], [26]. In one particular set of experiments,

synchronous rhythmic activity from two brain regions important in emotional processing was shown to be concomitant with the expression of acute fear in a mouse model of anxiety [27].

Bearing in mind that recent electrophysiological results suggest the importance of dynamical brain signaling in emotional processing, and that we now have a tool to probe these dynamics in a targeted way at our disposal in optogenetics, let's take a closer look at some paradigms that would be ripe for exploration.

2.1 – Fine-grained time dynamics in behavior and neurons

Figure 2.1 illustrates predictable temporal dynamics in the regime of milliseconds to seconds in mouse behavior and mouse neural activity. Rather than focusing on the details of these graphs just yet, it's important now only to note that both behavioral and neural activity of mice can serve as indicators, of varying degrees of precision, that mice are anticipating a particular event to take place at a particular time.
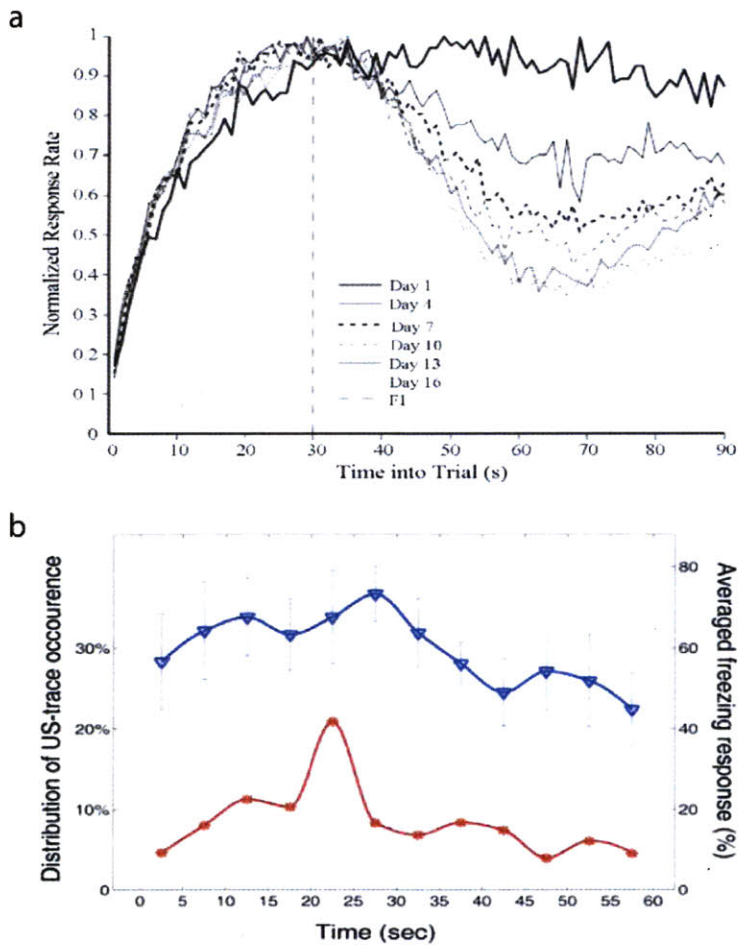
Figure 2.1. Time dynamics in rodent models, as expressed in behavior and neuronal activity.

(a) A mouse is trained over several days to expect a reward upon pressing a lever at a given time after its presentation (in this case 30 seconds, marked by "FI" for "fixed interval"). Robust learning of the time interval, i.e. anticipation of the correct time to respond, is reflected in a peak rate of lever pressing centered around 30s after training [Balci et al, 2009]. **(b)** Large populations of neurons are recorded in the hippocampus during recall testing one day after aversive trace conditioning in 6 mice. The mice were trained the previous day to expect a shock at the 22s timepoint in the graph, which is 20s after the offset of a tone. A distinct peak in the pattern of activity of a population of neurons in the CA1 region of the hippocampus is seen at the point of expected shock in red [Chen, Wang, and Tsien, 2009]. In blue, a behavioral concomitant of shock memory is also plotted, showing a significant correlation to the plotted neural activity ($r = 0.5851$, p,0.05), but not displaying as distinct of a peak.

In part (a) of Figure 2.1, mice are trained to anticipate a reward if they press a lever arm 30s after it has been extracted; in part (b), mice are trained to expect a shock 20s after the offset of a tone. The lever-pressing frequency of the mice peaks at 30s in the experiment of (a), and certain patterns of neural activity associated with the memory of a shock become particularly active at 20s in that of (b). Interestingly, drug treatments can alter the response curve of the former example [28], and it seems likely that neural activity in the latter could be pharmacologically perturbed. Timing can definitely be affected with drugs, almost certainly bringing unintended consequences as well. The constant, pathological fear of an aversive event could similarly be soothed by completely numbing the brain, but cognitive deficits would likely occur. Additionally, fear/anxiety can actually be quite useful when they come about at the appropriate time (imagine how well you'd fare if you weren't scared of a lion that's a few feet in front of you)! Because of the inherent lack of precision of pharmacological or purely genetic methods, the principles underlying these neural and behavioral activities seem out of reach. To better understand why that's the case, let's look more closely at the paradigm referenced in part (b) of Figure 2.1, of "trace" conditioning.

2.2 – Aversive Trace conditioning: an intriguing model of anticipatory anxiety

Trace conditioning has attracted considerable interest in recent years as a paradigm for anxiety and hippocampus-dependent memory [29], [30]. For concreteness, observe the following protocol in Figure 2.2, which was followed in a pilot study for this thesis, and which was adapted from [31].
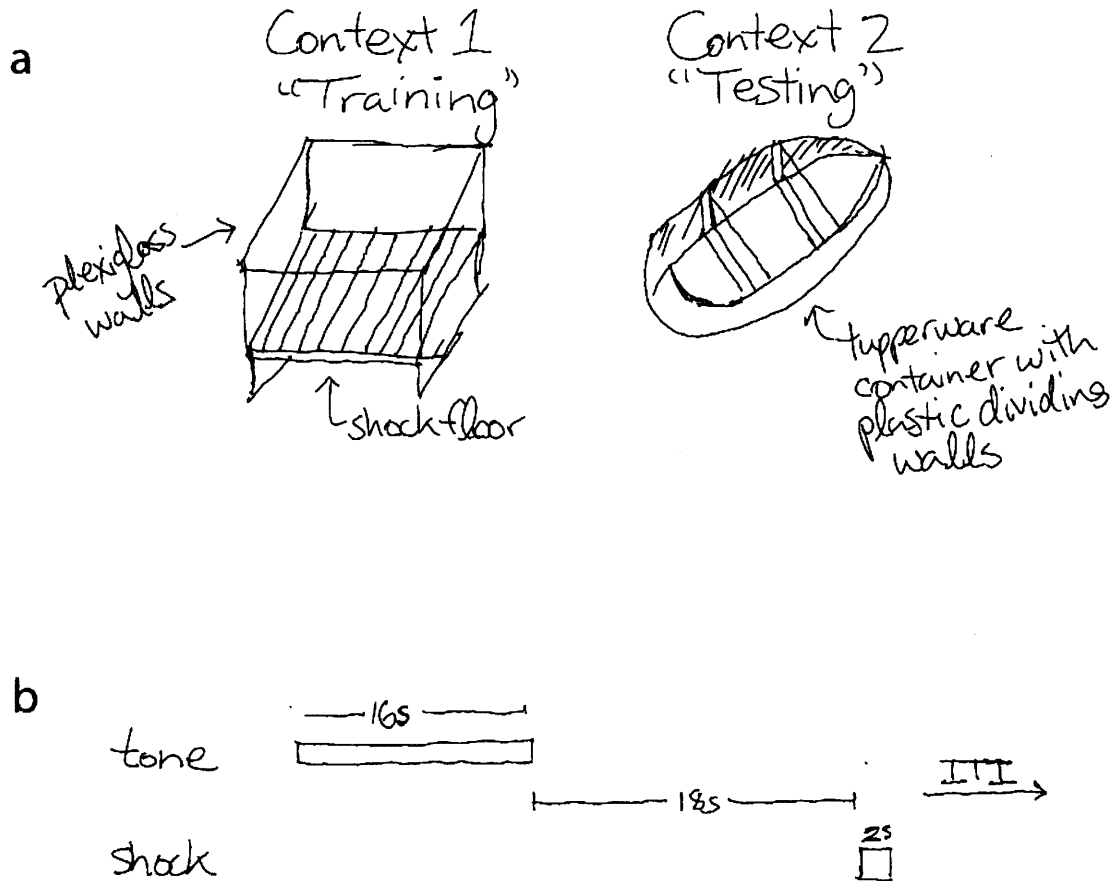
Figure 2.2. Trace conditioning paradigm. a) Subjects are trained and tested in different contexts, to ensure they do not have access to external cues reminding them of the shock during testing. b) A single training trial consists of a 16s tone, followed by an 18s "trace period" (no stimulus), followed by a 2s foot shock, and ending with a pseudo-randomized 4-minute inter-trial interval (ITI) [3:30, 3:45, 4:00, 4:15, or 4:30]. The behavioral paradigm proceeds as follows:

Day 0: Habituation. 20 minute baseline period in Context 1.

Day 1: Training. 20 minute baseline period, followed by 6 trace trials (see (b)), followed by a 3-minute pause before removal, all in Context 1.

Day 2: Testing. 5 minute baseline period, followed by 3 trials identical to the trials of day 1, but without the footshock.

Mice essentially learn that they should expect a shock (US) soon after the offset of a previously-innocuous sounding tone (CS) after some training. Mice can actually learn this quite well, as evidenced by data from the pilot study shown in Figure 2.3.
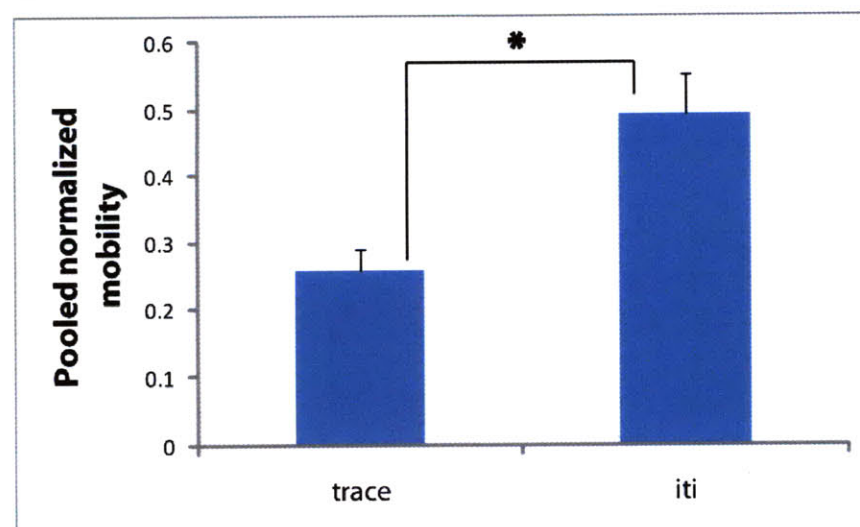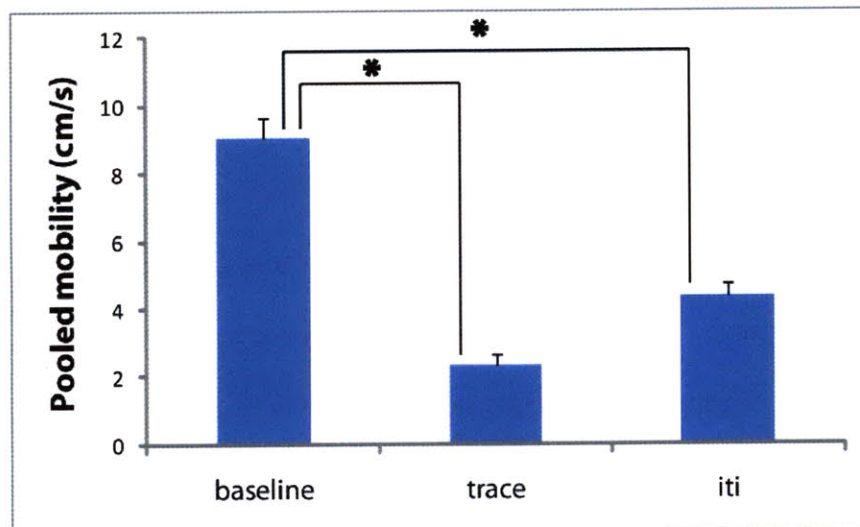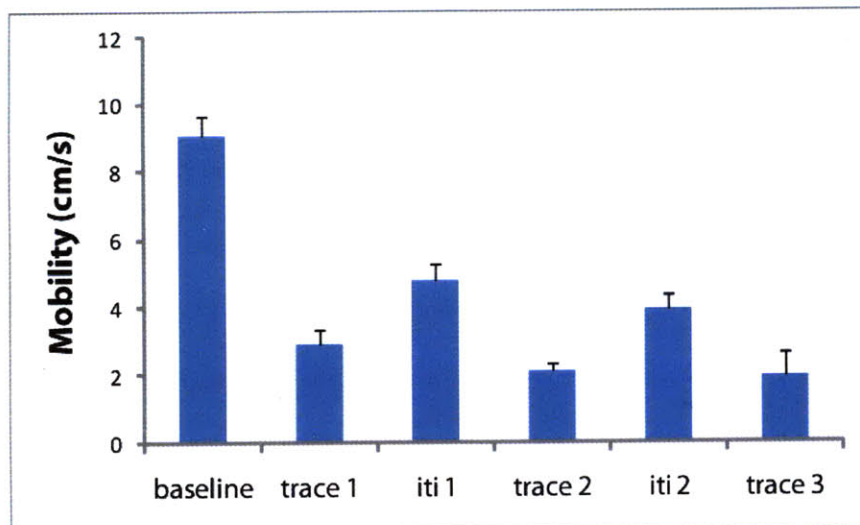
Figure 2.3. Trace conditioning pilot results on the testing (retrieval) day.

a) Mobility during the 5 minute baseline period, 3 trace periods, and 2 inter-trial intervals (iti), as measured by the Noldus Ethovision 7.1 software package. b) Mobility pooled across 3 trace trials and 2 iti's. Baseline mobility is significantly different from trace ($p=3.2 \times 10^{-4}$) and iti mobility ($p=.0032$), even with Bonferroni correction, as measured by paired t-tests. c) Pooled mobility during the trace, normalized by baseline mobility is significantly different from iti mobility ($p=.011$).

Mice display bouts of immobility during the period of time after the tone when they have been conditioned to anticipate a shock soon, while they are significantly more active during the other time points in the experiment. Through lesion studies, we now know that certain parts of the brain are necessary for the learning of this association. In particular, one region, the hippocampus (HPC), is necessary for this type of conditioning to successfully take place, though it isn't necessary for the general display of fearful behavior. For example, the HPC does not need to be intact for an animal to learn to associate a neutral-sounding tone with a shock if the two stimuli overlap in time sufficiently during the animal's training (such as in a popular paradigm known as delay conditioning) [32]. What makes trace conditioning particularly interesting is that it requires the engagement of brain structures that are important for attention and episodic memory, concepts that we might associate with conscious awareness [31]. In the interest of not delving into nebulous concepts like consciousness, it should suffice to say that because of the demands of the task and the brain structures consequently recruited, trace conditioning seems to approximate the type of anticipatory fear that occurs when we're actively expecting something bad to happen, as can be the case with anxiety bouts such as panic attacks.

Chapter 3

Sci-fi & sci

Let's make a brief digression into the world of science fiction, to illustrate what we one day may be

able to do for a patient suffering from severe, unpredictable bouts of anxiety. Let's say that, given

what we know about the HPC's role in trace conditioning, we believe that reverberating neural activity

within the structure reflects anticipatory dread of an aversive event (if you're not convinced that this

hypothesis is worthy of a thought experiment, please flip to Chapter 4 for details and scientific

grounding). Perhaps it would be possible to design a device that would non-invasively send a brief

pulse to the HPC to act as a sort of "reset".
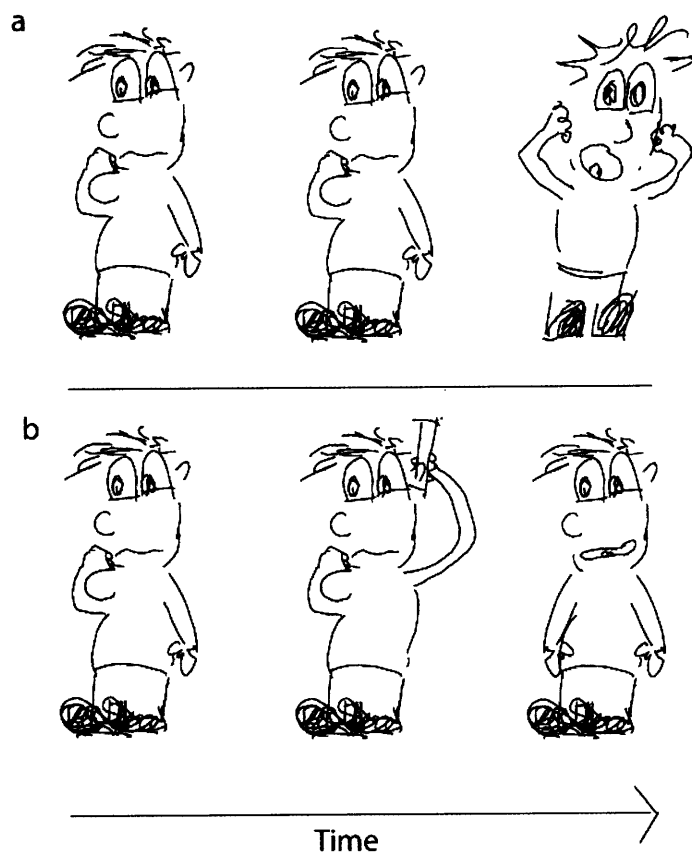
Figure 3.1. A hypothetical device to stop an impending attack of acute anxiety.

a) Fezziwig feels a bout of severe anxiety coming on, and then is overtaken with despair. b) Fezziwig anticipates the bout, uses his hippocampal pulser, and avoids despair.

This likely seems pretty silly. But great strides in the development of non-invasive neural technologies are being made, allowing for more spatially focal stimulation and silencing than previously thought possible [33]. So let's say that the only remaining piece of the puzzle to enable a "hippocampal pulser" device would be a demonstration that silencing activity in the HPC, even briefly, would be enough to prevent the onset of an acute bout of anxiety. Such an experiment would have to be invasive, if we really wanted to nail down the principles underlying the anxiety episode. Therefore it would be entirely unethical to start exploration of the idea with human subjects. So let's go back to our anxiety model of trace conditioning in mice. Recall the conditioning paradigm of Figure 2.2. Now imagine that, during testing of trace conditioning, we briefly shut down the hippocampus while the animal is anticipating a shock. Would the animal "snap out" of its anticipatory fear and go about its activities as if nothing was wrong? Such an experiment would look like the following:

a

training

tone

—— 16s ——

|—— 18s ——|

ITI →

shock

2s

---

b

testing — control group

tone

—— 16s ——

|—— 18s ——|

ITI →

shock anticipation?

---

c

testing — experimental group

tone

—— 16s ——

* hippocampal silencing pulse

|—— 18s ——|

ITI →

shock anticipation?
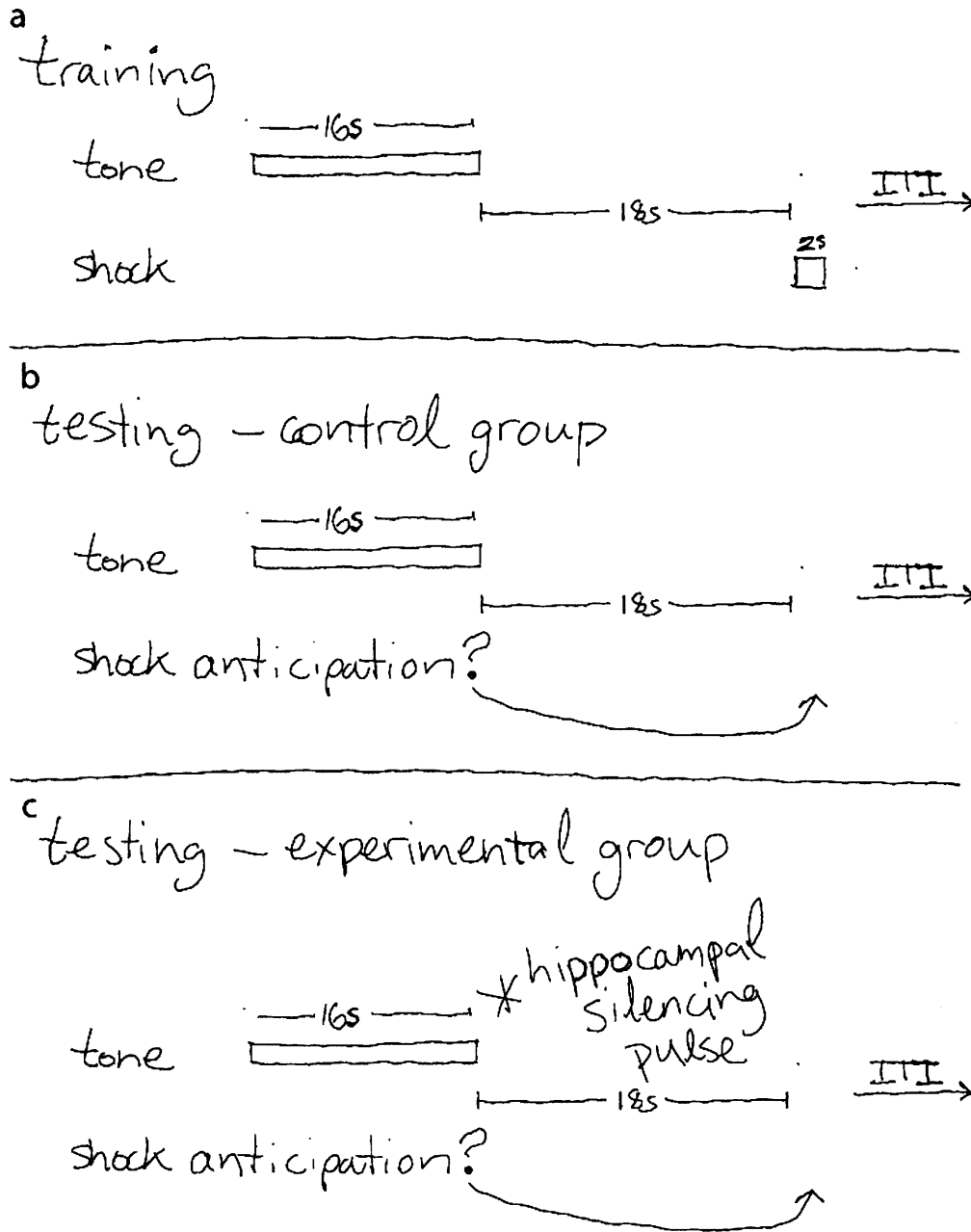
Figure 3.2. Hippocampal silencing experiment.

(a) Animals in both the control and experimental groups are given identical training. Animals in the control group (b) are tested as usual, while animals in the experimental group (c) are given a hippocampal silencing pulse after the offset of the tone. In (b) and (c), if the animals had a somewhat accurate sense of timing, they would anticipate the shock at 18s after the offset of the tone, and perhaps continue to anticipate it for some period of time into the ITI.

In order to do this experiment, we would need a system to selectively target and shut down the hippocampus on demand, reversibly, and for a brief period of time. Happily, thanks largely to the work of individuals in the Synthetic Neurobiology Group at MIT, we now have the requisite technology at hand. A brief schematic of the technologies, which will be elaborated upon in the following chapters, follows:

a

b

c
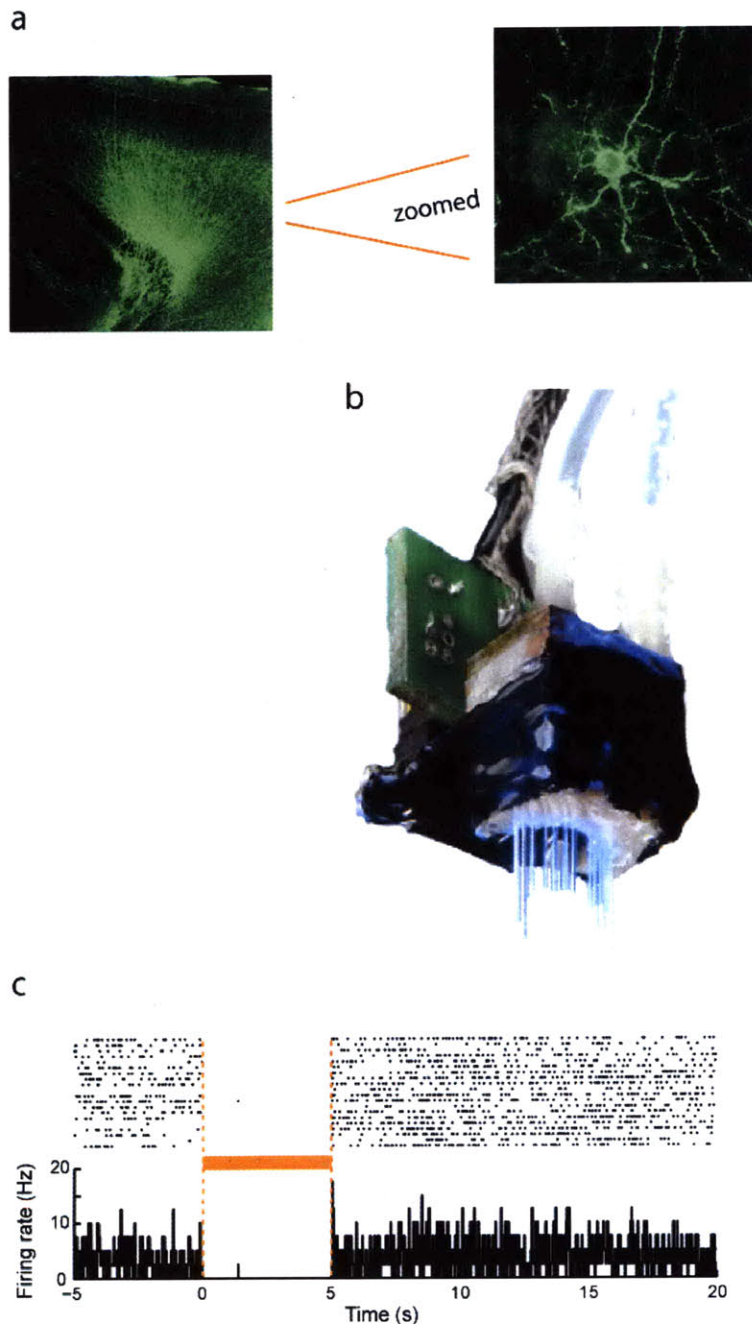
Figure 3.3. Tools from the Synthetic Neurobiology Group allowing for the probing of dynamical neural activity.

a) Neurons in a specific brain region are infected with a virus, which carries a payload that makes them susceptible to silencing with light [adapted from Chow & Han et al. 2010]. b) A fiber-coupled LED array, designed by Jake Bernstein and Ed Boyden allows for the precise-light targeting of the hippocampus in a mouse [Bernstein, et al. 2010]. c) An example of infected neurons being silenced by a pulse of light [adapted from Chow & Han et al. 2010].

While these technologies opened up the possibility of doing experiments like the aforementioned trace conditioning paradigm (Figure 3.2), challenges abounded that would require some innovation for their practical use. Specifically, later chapters will focus on the development of techniques for coupling recording electrodes to the hippocampal LED array (Chapter 5), writing of software for

synchronous control, analysis, and read-out (Chapter 6), techniques for successful surgical implantation (Chapter 7), techniques for widespread virus expression specific to the hippocampus (Chapter 8), and a design for putting it all together (Chapter 9)[2]. Before that, however, it would be useful to take a brief look at recent research into the HPC's role in trace conditioning, to further build the case for investigating its time dynamics.

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[2] All of the work in the following chapters, as well as the previously-mentioned behavioral pilot study, was performed by the author, unless explicitly stated otherwise.

Chapter 4

The hippocampus and trace conditioning

What evidence is there to suggest that disrupting the HPC during the trace period – in which the animal is presumably anticipating an aversive event — would lead to a return to behavioral normalcy? In the following discussion, when the word "trace" is used, it will refer to the hypothetical neural activity that must be ongoing during that interval of anticipation. To see why there must be such ongoing activity, notice that there is no external cue available to the animal during the time after the offset of the tone to indicate that anything is going to happen (such as the occurrence of a shock), yet the animal still reacts as if there is; therefore the trace signal must persist in the animal's brain. It might be pointed out that the animal could possibly be reacting to its own bodily signals [34]— say freezing that occurred during the tone presentation persists with some particular half-life, causing the animal to continue to freeze. This however wouldn't account for how the animal was able to learn the tone-shock relationship during training, and would also incorrectly predict that animals would show no dependence of trace interval on behavioral concomitants of fear such as freezing. To continue playing devil's advocate, let's also say that a signal must persist in the animal's brain during this period; that's a given. However, it is possible that the signal consists of a process with a very long half-life rather than reverberating neuronal firing. This however would be counter to much of what we know about neural computation — which seems to rely to a large extent on temporal sequences of spiking activity [35] — and would not be consistent with results such as those reported in Figure 2.1b, and what we'll soon see in Figure 4.1. By now I hope you are convinced that the "trace" is reflected in ongoing neural activity. The neuronal underpinnings of the trace, i.e. where it's located and how it's communicated, is the subject of the next topic.

4.1 – What is the HPC doing during the trace?

Because the HPC is necessary for trace conditioning and subsequent memory retrieval of the trace, there are several possibilities regarding its actual role in the process. The following are three possibilities:

1. *"HPC as passageway of the trace":*

The HPC is a necessary conduit for the trace, but plays no role in encoding it. Another region or regions is generating the signal, and sending it through the HPC.

2. *"HPC as generator of the trace":*

The trace is encoded in reverberating activity within the HPC, or between the HPC and another region or regions.

3. *"HPC as helper of the trace":*

The HPC is providing a supporting role for the trace. Another region or regions is performing the computation, and the HPC is merely providing some sort of sustenance to allow the region (s) to continue with its normal functioning.

Possibility 3 however would be inconsistent with electrophysiological recordings of the HPC during the trace in studies like those reported in Figure 2.1, so it will be ruled out. In case that argument isn't

satisfying, observe below results from a trace conditioning experiment in which rabbits were

conditioned to a 20s interval and tested the next day. A significant number of neurons in the CA1

region of the HPC displayed their peak firing rate at the interval +/- 2s of the end of the trace period

(when the shock was expected) [36]. This strongly indicates that activity in the HPC is "anticipating" an

event with a fixed time of occurrence.
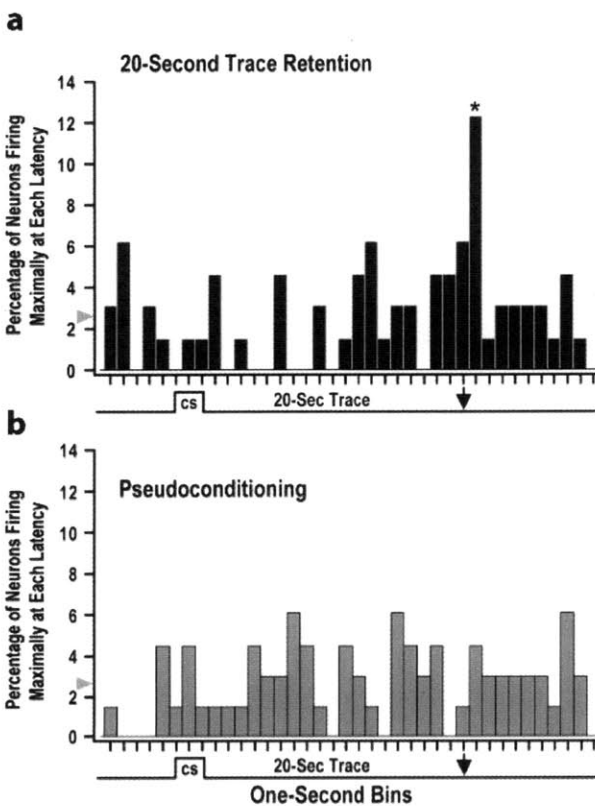
Figure 4.1. Maximal neural firing rate locked to trace period (Adapted from McEchron et al. 2003). a) During trace recall, a statistically-significant percentage of neurons recorded in CA1 exhibited their maximal firing rate at +/- 2s of the end of the trace period (when shock would be expected). b) A control group in which conditioning took place, but tone and shock were not exhibited with a consistent temporal relationship with respect to each other.

The trace experiment outlined in Figure 3.2 would distinguish between possibilities 1 and 2: if a brief

silencing pulse to the HPC leads to a return to behavioral normalcy, with no return of freezing

behavior several seconds after the pulse, possibility 2 is ruled out. Alternatively, if the HPC is merely a

conduit for the trace, and if the trace is continually feeding though the HPC, shutting down the HPC briefly should lead to only a momentary lapse in freezing.

Now assuming we're convinced that the trace either courses through the HPC or reverberates within it, it would be interesting and scientifically useful to determine whether the entire structure takes part, or only subsections.

4.2 – Factors in trace conditioning: hippocampal sub-region and timing

The hippocampus is divided into many sub-regions, with different hypothesized roles. For the purposes of this discussion, we will consider the HPC as consisting of two parts, a dorsal and ventral region, and a special cell layer termed CA1 that runs across the entire dorsal/ventral length. The dorsal/ventral distinction is motivated by considerations of connectivity and function. For an example, the CA1 region of the ventral, but not dorsal, HPC projects directly to the amygdala, a brain region critical for emotional memory [37] . The dorsal HPC is additionally required for spatial learning, with rats able to learn to navigate a water maze even if 75% of the HPC is ablated, as long as the remaining structure is within the dorsal region [38]. Additionally, a targeted knockout mouse[3] study suggests that long-term potentiation or depression[4] in the CA1 region of the dorsal HPC is crucially involved in the formation of spatial memory [39].

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[3] Remember from Chapter 1.2 that a "knockout mouse" is essentially a mouse who has had one of his genes "turned off"
[4] Long-term potentiation and depression result in increased and decreased synaptic strength, respectively.
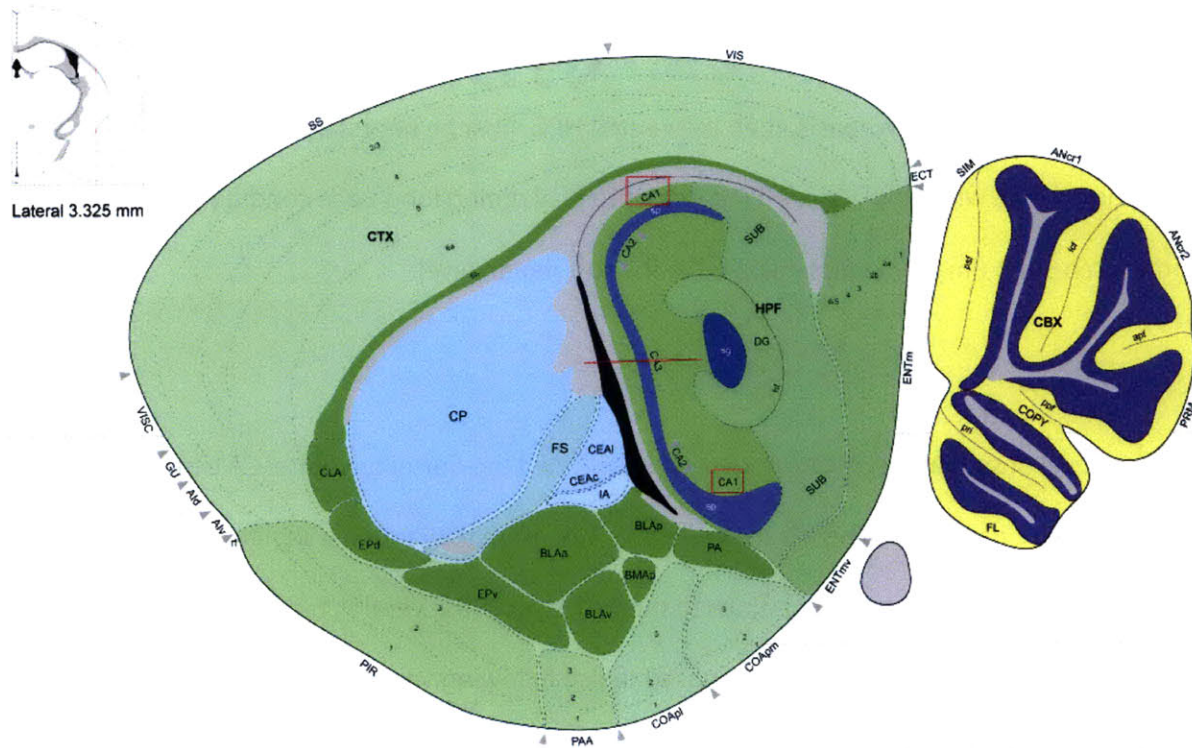
Figure 4.2. Sagittal view of the hippocampus. The red horizontal line near the center roughly separates the dorsal (top) from the ventral (bottom) hippocampus. The CA1 region is pointed out in both the dorsal and ventral regions with red boxes (image from the Allen Reference Atlas [51]).

Though there are somewhat conflicting results in the literature over which sub-regions of the HPC are necessary for trace training and subsequent recall, it now appears that some of these discrepancies may be explained by a dependence of the length of the trace period on the regions recruited. Chowdhury, et al, found that post-training lesions to the dorsal HPC resulted in trace recall deficits only when the trace period was 20s, instead of shorter intervals tested of 1s or 3s [40]. Similarly, Misane, et al, found that post-training lesions of the same region attenuated recall only in groups with traces of at least 15s [41]. In a more recent study, rats trained with a 30s trace period showed a severe deficit in recall when either their dorsal or ventral HPC was lesioned [42]. While the time-dependence of the ventral HPC is unknown in this particular paradigm, it seems likely that it is necessary at short as well as long trace periods. This stems from the fact that in a related but not identical trace paradigm,

in which eye-blink trace conditioning is employed in rabbits, the hippocampus is necessary for learning of trace intervals as short as 500 milliseconds [43]. If the dorsal HPC is not necessary at this time scale, but the HPC as a whole is, then the ventral HPC must be necessary by deduction[5]. These results suggest that, while the whole HPC comes into play during long trace conditioning, there may be some interesting time dynamics involved at the sub-region level.

At a higher level of spatial specificity, Rogers, et al. lesioned the dorsal or ventral CA1 cell layers in the HPC, respectively, in a study using a trace interval of 12s. Here they found a significant reduction in trace recall in rats with post-training lesions to the CA1 layer in the ventral, but not dorsal layer [44]. Additionally, they determined that the proposed deficit following ventral lesioning was not the result of hyperactivity. Specifically, hyperactivity has been reported by some authors who employed blunt lesioning of the entire ventral HPC [45], but Rogers et al. concluded that the fine-grained lesioning of ventral (or dorsal) CA1 did not yield this. Normal baseline activity as well as normal levels of freezing during acquisition (not retrieval—the authors ran several studies with either pre-training or post-training lesioning) were presented as supporting evidence. Interestingly, the rats with post-training ventral lesioning did not freeze any less than controls during the period of tone presentation; only during the trace period did they exhibit significantly less freezing. This indicates that their deficit reflects specifically the time period during which there is no external cue to remind them of the shock.

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[5] That is unless compensatory mechanisms come into play and other brain regions pick up the slack. This is a concern for lesion studies in which a large amount of time passes between the lesion surgery and testing, but will likely not be an issue for the real-time silencing study proposed in this thesis due to the extremely short time periods of neural perturbation inherent in our approach.

4.3 – The hippocampal LED-coupled fiber array

Clearly interesting time and spatial dynamics within the HPC abound during trace recall, but the spatial and temporal specificity of hippocampal sub-region recruitment would be difficult to probe at a fine level of detail with traditional technologies. However, they are ripe for exploration with the technologies presented in Figure 3.3. The hippocampal array in Figure 3.3b has the power to independently address various regions of the hippocampus at arbitrary time points. It was designed to focus its maximum light intensity on the CA1 region, allowing for the replication of experiments such as those of Rogers et al, assuming adequate virus expression to add light sensitivity (see Figure 3.3a), and equipped with the additional features of exquisite temporal and spatial control. The process of endowing the hippocampal array with the ability to sense in addition to perturb neural activity is the focus of the next chapter.

Chapter 5

Coupling recording electrodes to the array

With the advent of the hippocampal fiber-coupled LED array detailed Figure 3.3b, new avenues were opened up for real-time hippocampal modulation. However, the addition of a capacity for electrophysiological read-out was desired for a number of reasons[6]. Most prominently, if electrodes could be coupled to the optical fibers in such a way as to give reliable measurement of spikes from neighboring neurons in the modulated region, real-time validation of neural silencing could be verified. Ideally the spikes from many neurons would be picked up by the electrode. But in choosing a larger electrode to yield more neural signals, one sacrifices specificity of the neural signal — i.e. though many spikes can be seen, it becomes difficult to verify whether a particular spike was produced by a specific neuron. However, this wasn't a primary concern here for the following reason. In validating neural silencing, being able to isolate particular neurons takes a back seat to the ability to show that a larger population of (somewhat indistinguishable) neurons have decreased or ceased their spiking activity. Therefore, in the trade-off between specificity and bulk information, the latter wins out for this application. Ideally we wouldn't have to make such a trade-off, particularly as single-unit recording (as recording isolated neurons is known) can yield extremely interesting information about neural processing at a given time (refer back to Figure 4.1). However, designing a system to record and isolate a vast number of densely-packed neurons in conjunction with LED-coupled fiber array control would be quite formidable, and not absolutely necessary for addressing the scientific questions at hand.

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[6] It should be noted that, in the Master's thesis introducing the hippocampal array [47], preliminary results demonstrating the coupling of an electrode to an optical fiber that yielded neural recordings were presented. However, similar recordings in freely-moving animals or in the dense 3D geometry of the optical fibers in the full-scale array had not been demonstrated.

Methods

Printed circuit boards (here referred to as "electrode interface boards", or EIBs) were designed by Jake Bernstein, obtained, and soldered to a Samtec connector (Samtec Inc). Polyimide-insulated 50 μm diameter Tungsten wire was chosen for the electrodes (California Fine Wire), as its relatively large diameter afforded the best shot at seeing a lot of neural activity. An electrode for analog reference, made of the same material, was coupled to one of the optical fibers and cut to terminate in the corpus callosum of the mouse. This reference location was chosen for its proximity to the HPC, and presumed lack of detectable neural spiking activity within the region [46]. All other electrodes were coupled to the optical fibers, one-per-fiber, and designed to terminate 250μm beyond the tip of the fibers. This distance was chosen because the fibers were designed to terminate 250μm from their target, the pyramidal layer of the CA1 region of the HPC. The design choice of the fiber length was made to minimize tissue damage in the region of interest, and to be well-positioned to deliver light to the entire-desired area. An electrical ground wire of silver-chloride was brazed to a screw to be inserted in the mouse's skull during implantation, and pinned into the EIB, along with the other electrodes.

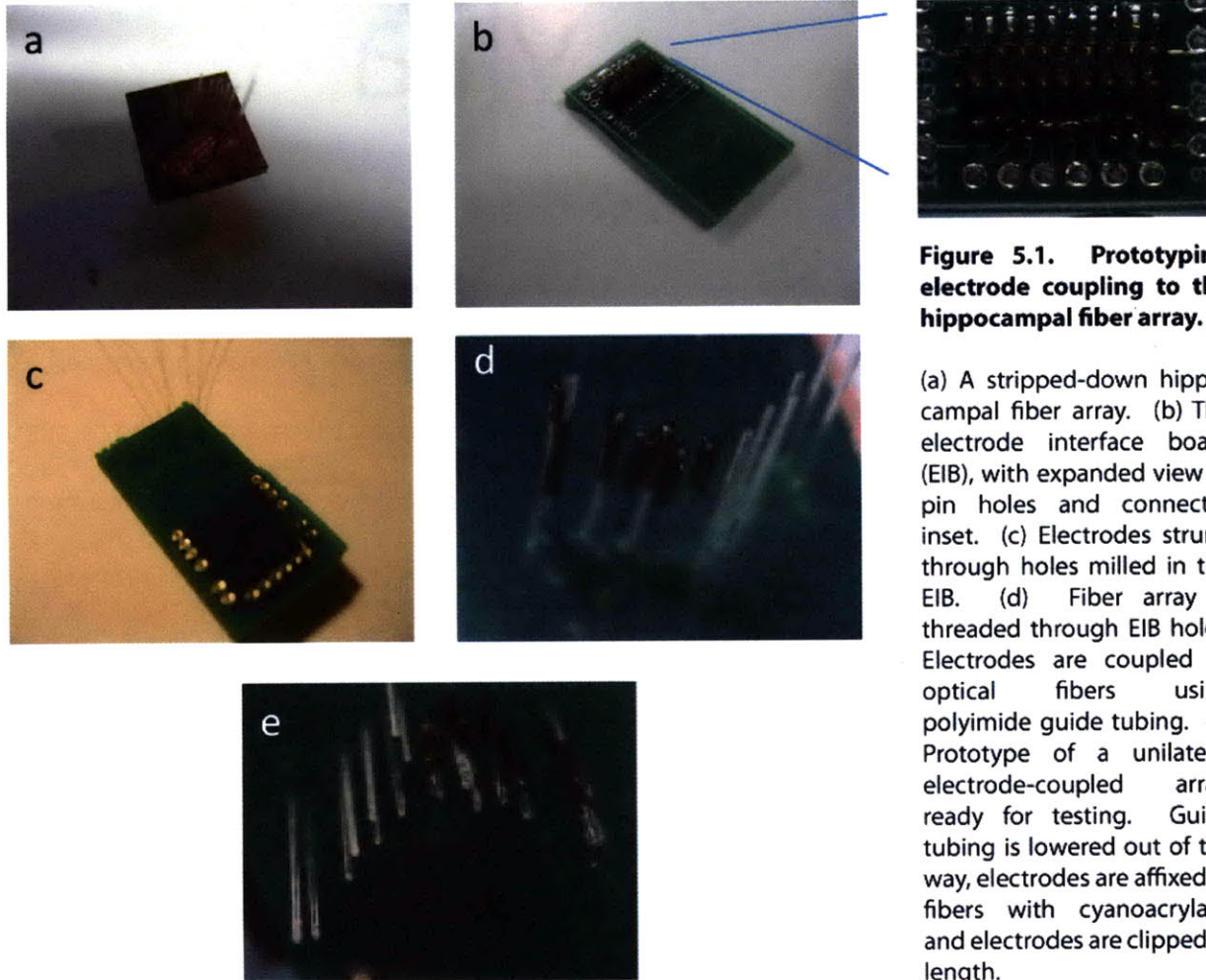Assembly steps for the device are illustrated in the following diagram:

Figure 5.1.   Prototyping electrode coupling to the hippocampal fiber array.

(a) A stripped-down hippo-campal fiber array. (b) The electrode interface board (EIB), with expanded view of pin holes and connector inset. (c) Electrodes strung through holes milled in the EIB.   (d)   Fiber array is threaded through EIB holes. Electrodes are coupled to optical   fibers   using polyimide guide tubing. (e) Prototype of a unilateral electrode-coupled   array, ready for testing.   Guide tubing is lowered out of the way, electrodes are affixed to fibers with cyanoacrylate, and electrodes are clipped to length.

Results

An implanted array was able to pick up neural spiking and local field potential activity, as

demonstrated in Figure 5.2. Real-time validation of silencing using the devices is beyond the scope of

this thesis, and will depend on the level of virus expression in the brain (Chapter 8).
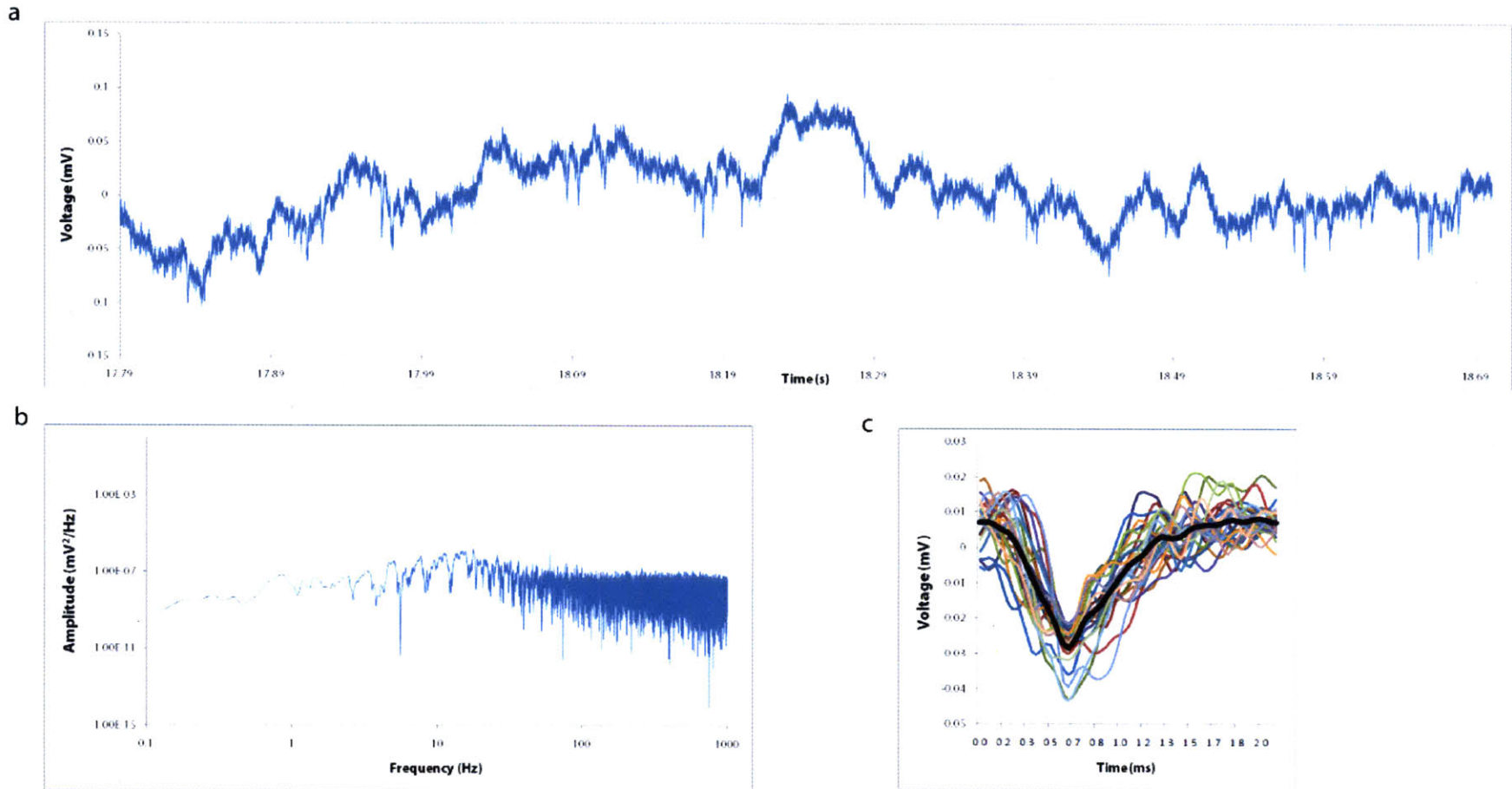
Figure 5.2. Recording with the hippocampal array. a) A representative electrophysiological recording from one channel of an electrode coupled to an optical fiber of the hippocampal array. b) A power spectrum of the recording. c) Spikes from a candidate single neuron, overlaid on the average waveform of these spikes. Spike detection and clustering were performed using Rodrigo Quiroga's wave_clus package [Quiroga, 2004]. As noted in the text, single-unit discrimination is not an immediate focus of the technology. The putative single unit displayed here is provided for the purpose of illustration.

Chapter 6

Software / hardware for acquisition, analysis, and control

While systems for electrophysiological recording certainly exist, and a system for simply controlling LED arrays has been developed in the Synthetic Neurobiology Group at MIT (Bernstein, et al. 2010 – in preparation), there was a desire to create a single platform for neural recording, LED control, video and instrument control. This was motivated by the need for exquisite synchrony among the systems to yield meaningful results, and the realization that the incorporation of a mixture of proprietary and open-source systems would yield a system that likely wouldn't be easy to debug or particularly robust. Additionally, real-time analysis and visualization, though not an absolute necessity for this particular project, would surely prove useful for planned, future experiments.

Methods

The particular specifications for robust neural recording, notably a digitization rate of 30kHz, low electrical noise levels, 14-to-16-bit data resolution, and the ability to easily integrate the system with other components via software, led us to a series of digitizers produced by National Instruments. The NI USB-6259 (Nidaq) was chosen, as it came equipped with these specifications, and supported the 16 channels of analog digitization that were desired for the project. The Nidaq clocked the camera, the behavioral equipment, and data acquisition (for schematic of the whole set-up, see Figure 9.1), and served as the primary input / output device for all of these except the camera (while the camera

acquisition was triggered by the Nidaq, video was pulled into the computer using a USB port and the FlyCapture software by Point Grey). LabView, the graphical programming development environment from National Instruments, proved to be appropriate for our purposes, though a number of other programming languages were initially used for development.

Results

The software developed worked as a flexible platform for acquisition, real-time analysis — such as filtering — and control. Below is a screenshot of the visualization interface.
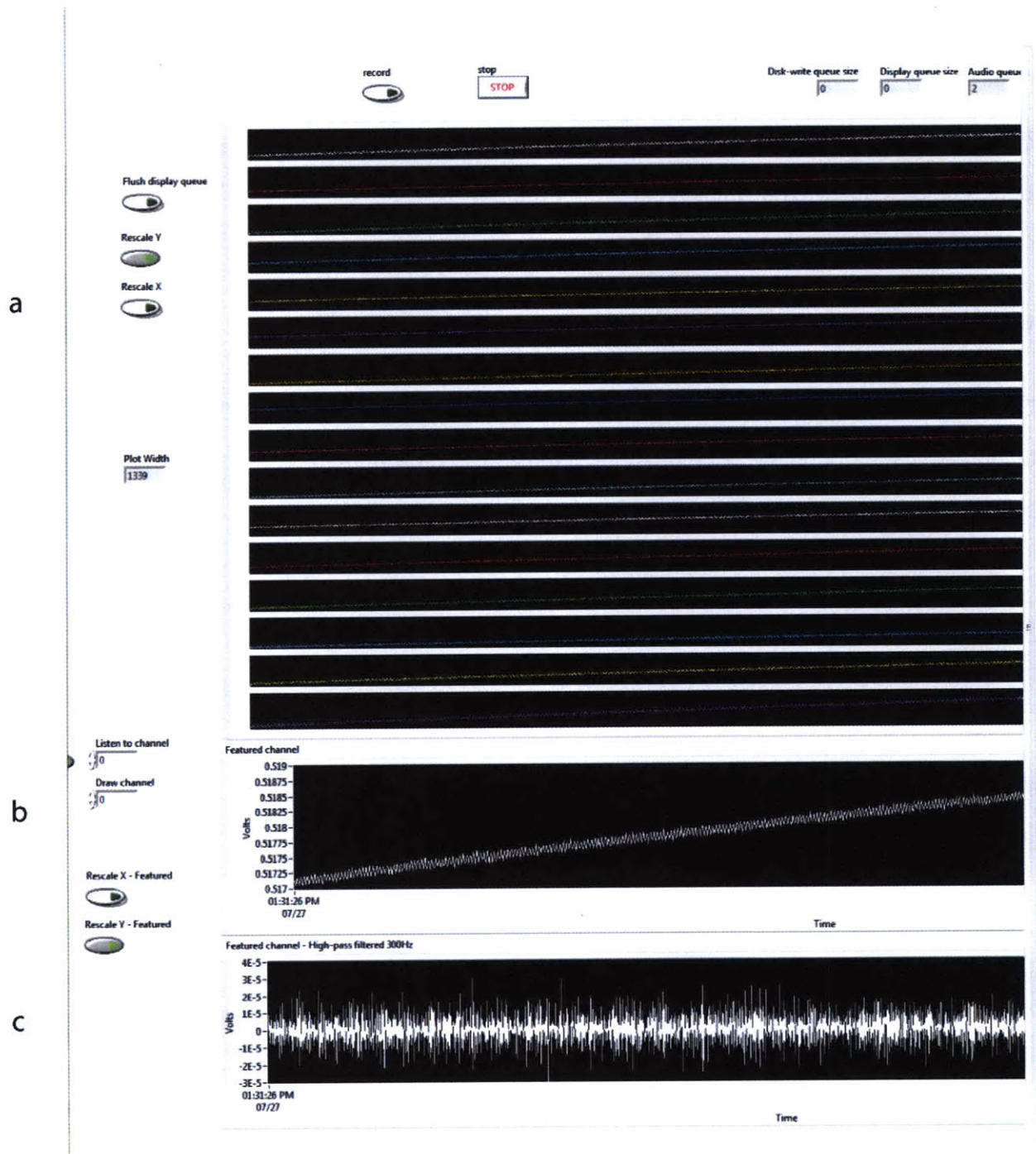
Figure 6.1. Acquisition and control software.

(a) Live visualization of neural signals. (b) Expanded view of a featured signal, with an option to listen to the signal through digital audio. (c) High-pass filtered view of featured signal, for easier spike visualization.

Chapter 7

Surgical techniques

Implantation of the HPC array proved to be a formidable task, though ultimately a system was devised

to make it feasible. At the time of its introduction in [47], it had never been implanted in a mouse. The

dense packing of the fibers made drilling individual holes in the skull for each of them prohibitively

difficult (optical fibers for the array are spaced roughly 0.7mm apart).

Methods

Consequently a large, bilateral craniotomy technique was developed and employed (Figure 7.1), in

which the outline of the region of interest is traced and thinned down with a dental drill, the skull is

peeled off, and silicon elastomer (Kwik-Sil from World Precision Instruments) is applied on top of the

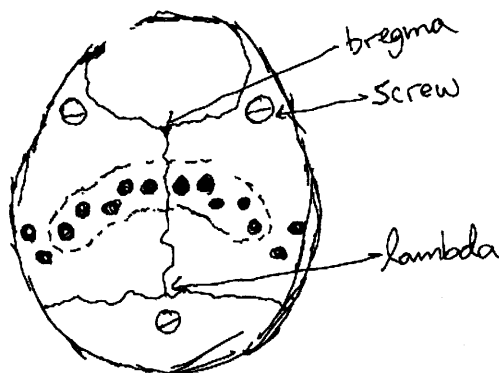brain to prevent drying out and trauma.

Figure 7.1. Large craniotomy strategy.

Drilling a large, bilateral craniotomy (area encompassed in dotted lines) made the surgery feasible. The remaining posterior-lateral targets were drilled individually and used to guide the implantation of the array. The lambda and bregma points on the skull, fiduciary marks used during the surgery, are labeled for reference.

Results and discussion

Implantation accuracy was assessed, and fibers were found to exhibit a tip-to-target accuracy of about

250μm on average (Bernstein, et al, 2010 in preparation). Given the large number of fibers (14) and

their diameter (200μm), a reasonable concern would be that the fibers ablate such a large volume of

brain upon implantation, that the mouse would not exhibit normal behavior and learning thereafter.

Below is a diagram of the fibers overlaid on a three-dimensional representation of a mouse brain, to

scale.
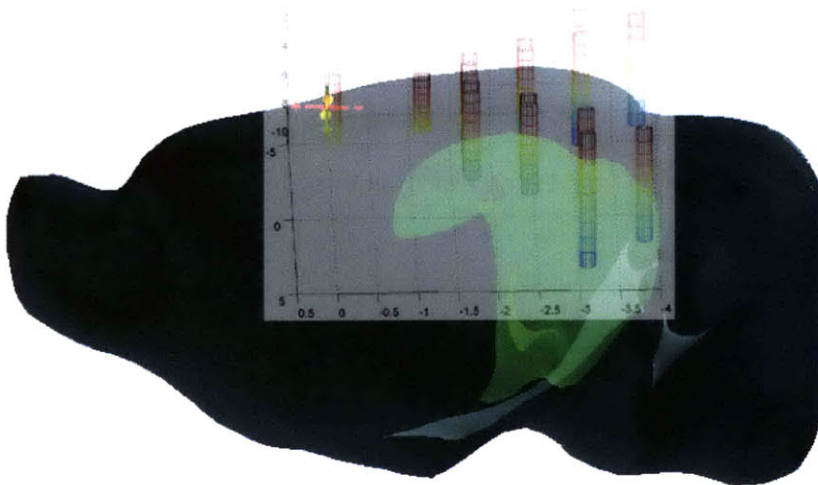
Figure 7.2. Optical fiber size, relative to one hemisphere of a mouse brain. 200 micron diameter optical fibers are used. The two most anterior fibers are used as fiducial markers during the surgery, and do not enter the brain. 3D brain representation was taken from the Allen Brain Explorer [51].

Though it can be seen that a non-trivial amount of brain will be displaced and likely ablated by the fibers, such tissue damage shouldn't pose an insurmountable obstacle to the scientific question at hand. Specifically, it has been shown that rats, close relatives of mice, with complete cortex ablation dorsal to the hippocampus (the area that may be somewhat ablated by the fibers), are still able to condition to the trace protocol robustly, and without deficit with respect to healthy controls [13].

Chapter 8

Virus expression

Light sensitivity of neurons to allow neural silencing was desired in the dorsal and ventral areas of the CA1 region of the HPC, as suggested in Chapter 4.3.

Methods

Lentivirus carrying a payload of the light-activated proton pump termed Arch for silencing, together with green fluorescent protein (GFP) for labeling, and with the Fck promoter for expression exclusively in excitatory HPC neurons was chosen. The Fck promoter ensured viral expression in cells containing α-CamKII [48], specifically the primary cells[7] in the CA1 layer.

Arch has recently been discovered and shown to yield extremely robust neural silencing when pulsed with light near the 550nm wavelength [49]. In order to express Arch throughout the entire dorsal / ventral length of the HPC, a large injection technique (a "flood" injection) was employed near the geometric middle of the hippocampus. Below are coronal diagrams demonstrating the large area that the virus needed to cover.

---

[7] Pyramidal neurons
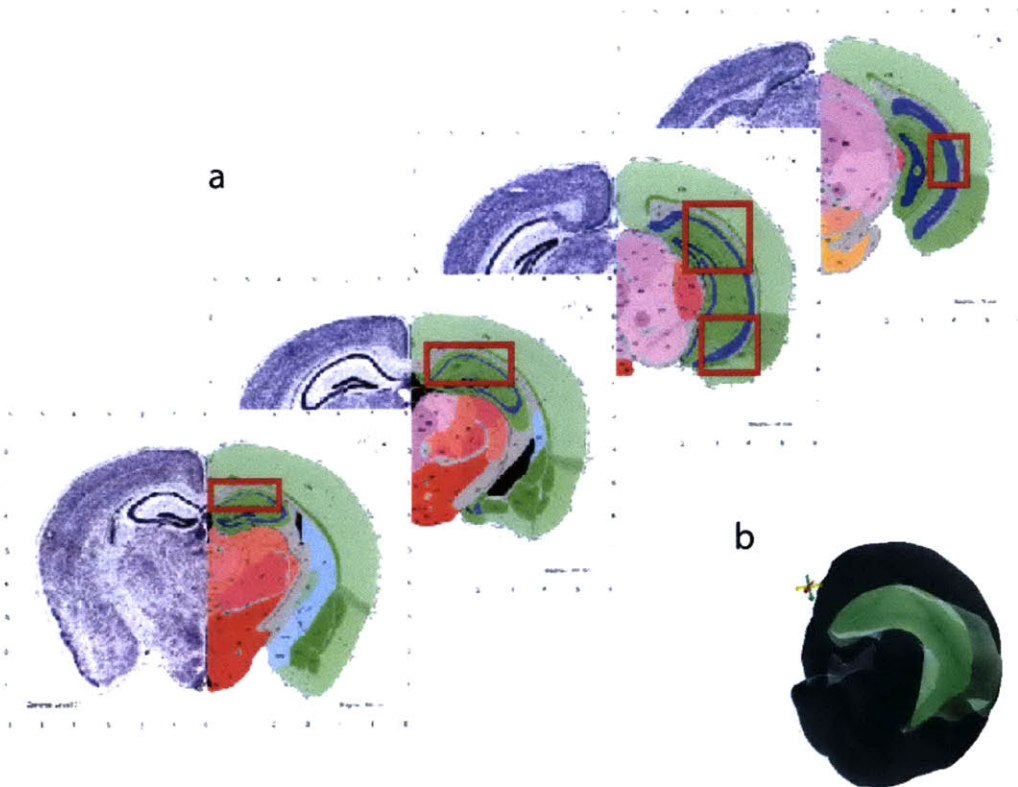
Figure 8.1. The CA1 layer of the hippocampus extends throughout much of the mouse brain. (a) Coronal slices showing the four layers that the CA1 array targets (images from the Allen Reference Atlas [51]). (b) Three-dimensional view of the CA1 region (in lime green), in the context of a half-mouse brain [51].

Results and Discussion

Interestingly, the virus spread throughout the extent of the hippocampus, but little to no viral labeling was observed outside of the structure. The hippocampus in this respect seems to be somewhat self-contained, with surrounding white matter creating a barrier for liquid diffusion (though the precise reason for this virus localization was not investigated). Below are pictures of one 7uL injection, expressed in the mouse brain 10 days post-injection.
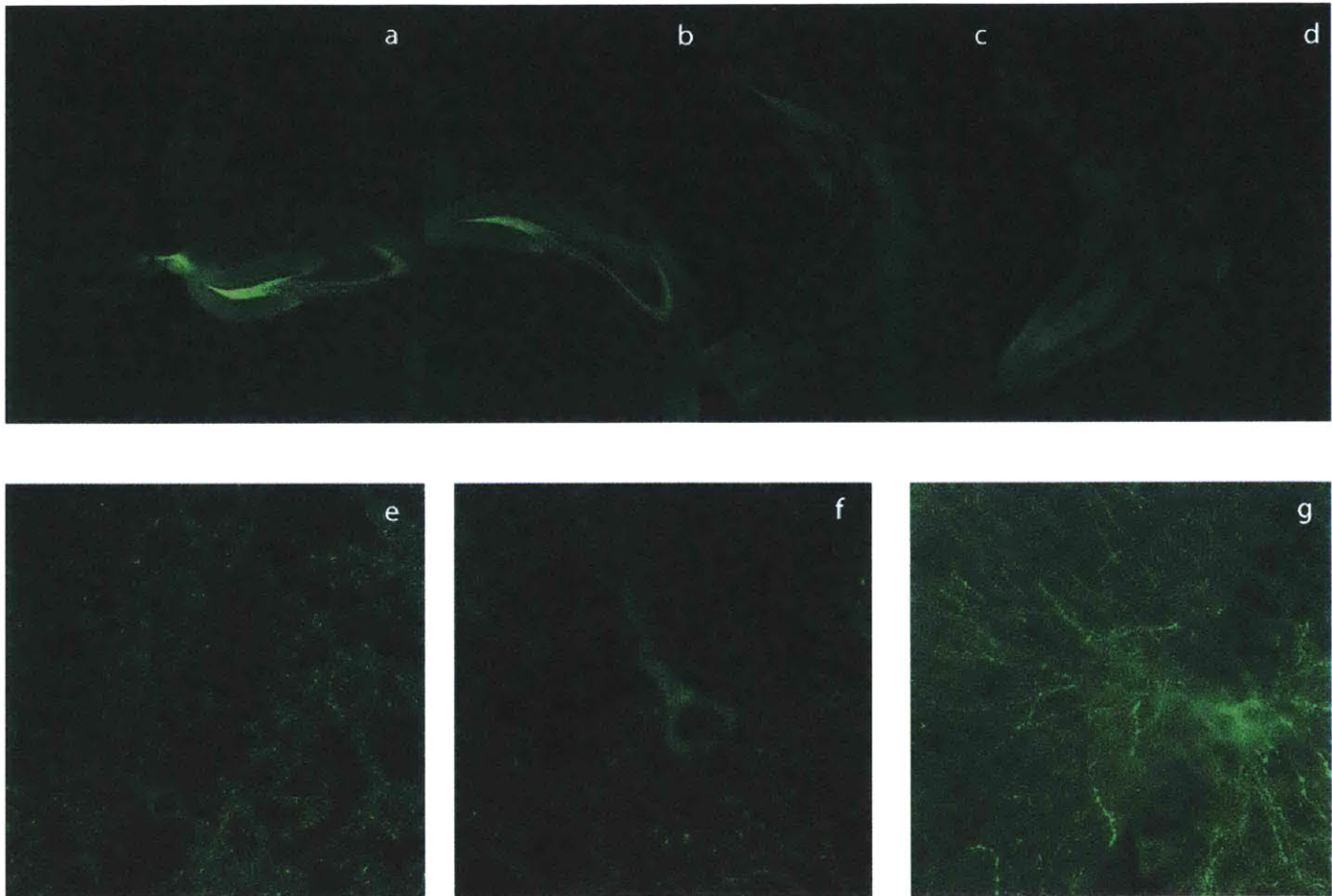
Figure 8.2. Viral expression of Fck-Arch-GFP across the entire length of the hippo-campus.

Data shown is from a 7uL injection at the coordinates [-2.88, -3.25, -3.50], at 2.5x objective magnification for a-d. a) -1.7mm anterior/posterior relative to the bregma landmark. b) -2.4mm. c) -3.1mm. d) -3.8mm. e) Fluorescence in pyramidal neurons in the CA1 region at coronal level -3.1mm, at 63x magnification. f) At 150x magnification. e) A 3-dimensional slice projection of the same layer at 63x.

Note that viral labeling was not restricted to the CA1 region. In particular, extremely dense labeling is seen within the dentate gyrus of the hippocampal formation, where large numbers of α-CamKII-positive neurons are also located. In the proposed experimental design, light delivery will be restricted to roughly 1 mm^2 of the tip of each optical fiber (Bernstein et al, 2010, in preparation), so the CA1 region should be preferentially silenced, despite the labeling in other substructures of the hippocampal formation. It should be noted that there may be a desire to make absolutely sure that only the CA1 region is manipulated, for reasons of scientific clarity. In this case, a genetically-modified mouse created with the Cre-loxP recombination system for exclusive viral targeting in CA1, as developed by Tsien et al, [50] may be used.

Chapter 9

Putting it all together

With the components to perform optical neuromodulation in a trace experiment all in place, including

behavioral validation, neural recording, software/hardware, and virus expression, the goal of this

thesis is fulfilled. Below is a diagram of the components of the entire system, as they are set up to

allow for the first exquisitely targeted system of experiments to investigate the time dynamics of

emotion, to be further elaborated on in the final chapter.

a

power supply
Amplifier

cords for
camera clocking,
buzzer control

Faraday-
shielded
behavior box

shock grid
power supply

LED control
box

breakout
board

digitizer/controller
"Nidaq"

computer

b

camera

tone
buzzer

red
light
bulb

headstage
amplifier

Faraday
cage

electrode-
coupled
optical fiber
LED array

plexiglass
walls

mouse
(not to scale)

shock floor
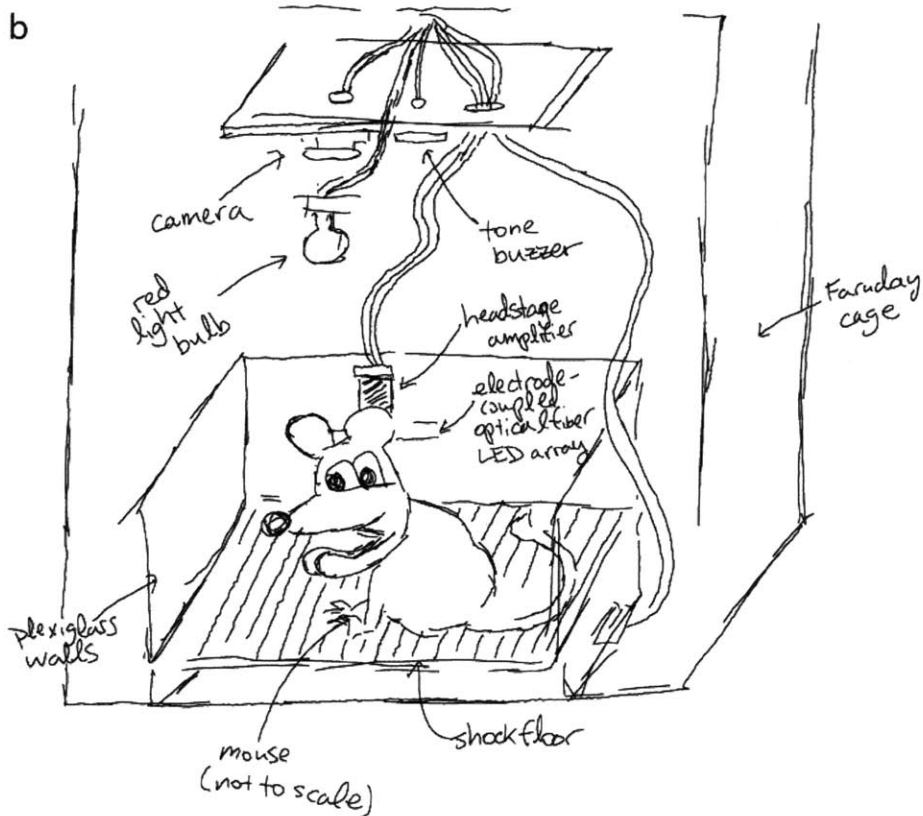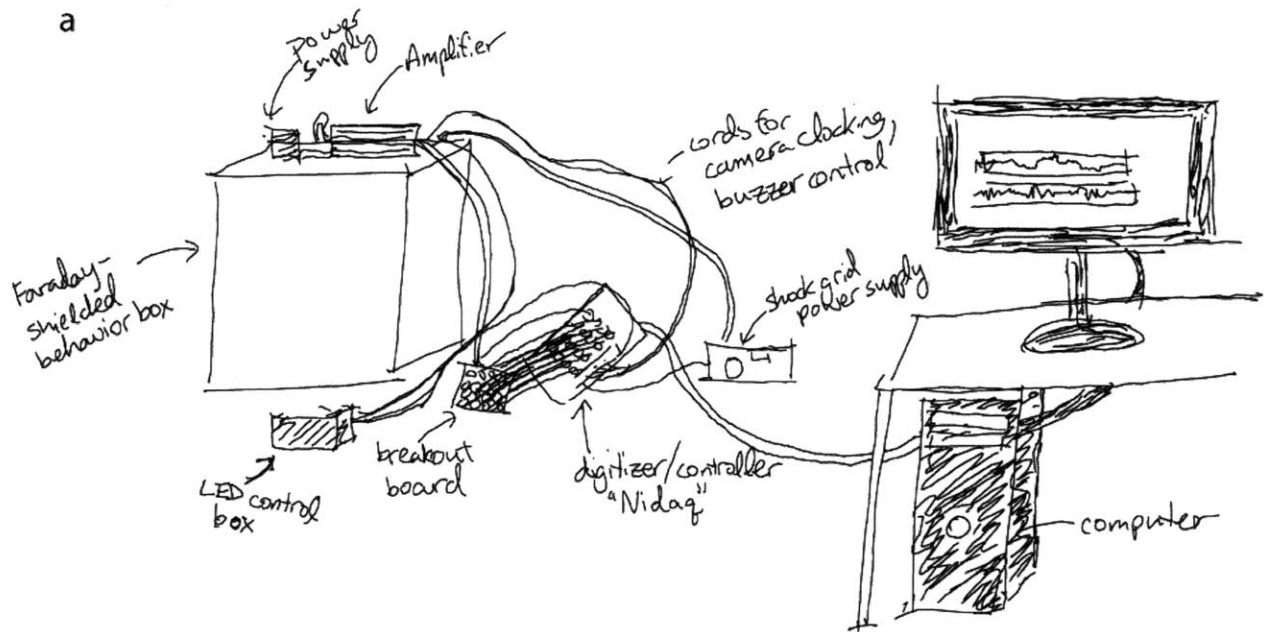
9.1. Hardware overview. (a) Outside the behavior box. (b) Inside the box. See appendix for list of parts.

Chapter 10

Bring on the experiments

Hopefully it is now readily apparent that the experiment of Figure 3.2 is possible, given the developments outlined in this thesis. While preparation for that experiment is underway, it would be useful to think of the other experiments and controls that could be run with the system developed here.

10.1 – Controls

First off, the control experiment from Figure 3.2 may not seem wholly satisfying. Comparing a mouse with a large array on his head to an unequipped mouse hardly seems fair. After all, there is a small but non-negligible chance that unintended effects of the array would alter the mouse's performance. For an example, a trace conditioning experiment was performed in which light was flashed during the trace period of the training day. The flashing of the light led to a deficit in conditioning, leading the authors to believe that attention was disrupted, and leading them to hypothesize that attention is a crucial element in trace conditioning [31]. Therefore, there's a possibility that the large arrays will distract the mice (despite the fact that the mice will have had several weeks to acclimatize to them), yielding less overall conditioning, and making interpretation of the experimental results difficult. So it will be important for the control mice to be implanted with the array and either be a) injected with the same virus, but not have light pulsed during the trace, or be b) injected with a neutral substance such as saline, and have light pulsed during the trace, at the same time that its pulsed for the experimental group.

Furthermore, if it is shown that a brief hippocampal silencing pulse causes an animal to "snap out" of

its state of fear, there may be a desire to demonstrate that this hippocampal perturbation would not

result in the same behavioral outcome in a task that isn't hippocampal-dependent. For an example, as

mentioned in 2.2, delay conditioning is a paradigm in which the tone and shock overlap in time.

Specifically, the tone begins; late into the tone (say 16s after the onset), a shock is delivered that co-

terminates with the tone. On the testing day, a silencing pulse could be administered shortly after the

offset of the tone (at the same relative time after the tone as it was for the trace experiment group). If

the animal "snaps out" of freezing, interpretation of the trace experiment results would be difficult.

For an example, silencing the HPC could lead to bizarre network effects that reduce the expression of

fear, regardless of its potential role in actually encoding the trace.

10.2 – Experiments

Experimentally, given the differences in anatomy and function of the dorsal and ventral sub-regions

highlighted in Chapter 4.2, these regions could be differentially silenced during the experiment. This

is made possible by the fact that the LED's in the hippocampal fiber array are individually addressable.

Another interesting avenue to explore would be the possible role of compensatory mechanisms

within the HPC. If the dorsal HPC is shut down during trace conditioning, does the ventral HPC pick up

some of the slack, as might be reflected in increased neural activity? Could this be an explanation for

why the dorsal HPC is only necessary at long trace intervals? Specifically, does compensatory activity

in the ventral HPC become "overloaded" when it needs to hold a trace for a sufficiently extended

period of time?

The ability to address basic scientific questions such as those just mentioned will likely increase our understanding of the time dynamics of the HPC and emotional memory. Additionally, it might just pave the ground for future technologies such as the hypothetical "hippocampal silencer" of Figure 3.1.

Appendix

1 - Coordinates of the hippocampal array

Locations for fiber tip placement were derived from the Allen Mouse Brain Reference Atlas [51], and

are as follows, listed in the format of [Anterior/Posterior, Medial/Lateral, Dorsal/Ventral]:

[-1.7, +/-0.6, 1.25]
[-1.7, +/-1.3, 1.0]
[-2.4, +/-1.5, 0.9]
[-2.4, +/-2.2, 1.1]
[-3.1, +/-4.1, 4.25]
[-3.1, +/-2.5, 1.2]
[-3.8, +/-3.85, 2.75]

Appendix

2 - A system for rapidly validating array coordinates

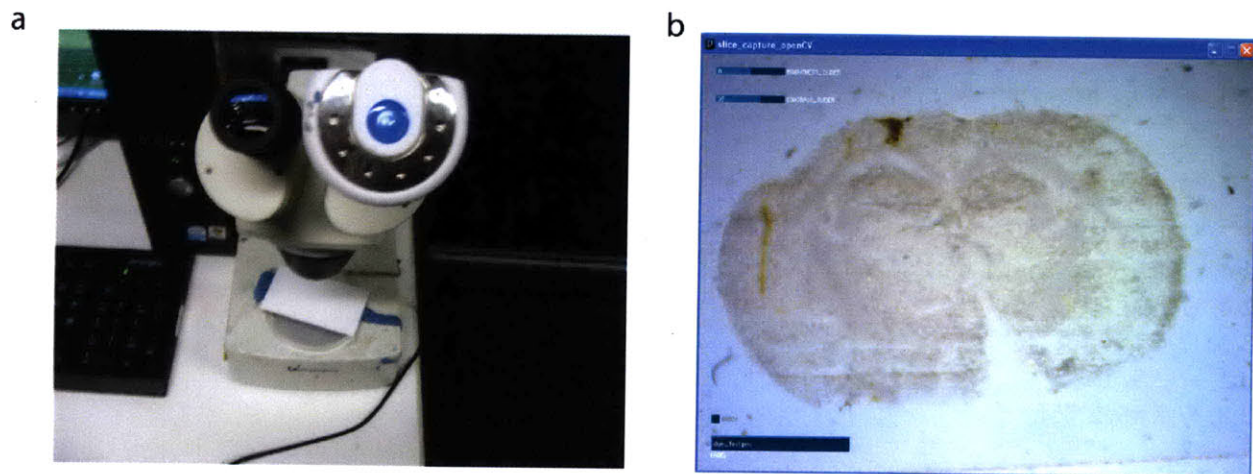**Figure A.2. System for rapidly evaluating fiber and dye placement.**

(a) A simple shop scope is equipped with an inexpensive, USB eyepiece camera. **(b)** Software written in Processing with OpenCV allows for easy image capture and contrast control. Additionally, a semi-transparent "ghost image" of the previous slice captured can be displayed by clicking on a checkbox, allowing for accurate alignment from slice to slice.

Appendix

3 - Parts list

Item	Company	Catalog number
Amplifier	Plexon	PBX2/16wb-G50-.7Hz-8kHz
Amplifier power supply	Plexon	POW/PBX-110
Breakout board	Plexon	BNC/16-B
Camera	Point Grey	Firefly MV
Faraday-shielded box	80/20	Made from a number of parts
Headstage amplifier	Plexon	HST/16V-G20
LED control box	Arduino + custom equipment	Duemilanove + other
Nidaq	National Instruments	NI USB 6259
Shock floor	Med Associates	ENV-005A
Shock grid power supply	Med Associates	ENV-414S
Tone buzzer	Digikey	102-1285-ND

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