Progress Report

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Summary

I have been working on the following:

I. MEA recordings from dissociated cortical cultures during chronic activity blockade, followed by whole-cell patch recordings to assess synaptic strength
II. monitoring expression of ChR2-transfected cortical cultures using confocal microscopy and MEA recordings during light stimulation
III. culturing P0 cortical cells
IV. scientific writing
V. mentoring undergraduates

I. Chronic CNQX or TTX Treatment of Cortical Cultures

A. Motivation

In my previous progress report, I described a several experiments where I had applied the AMPA receptor antagonist cyano-7-nitroquinoxaline-2,3-dione (CNQX) to neuronal cultures grown *in vitro* on multielectrode arrays (MEAs) and recorded network spiking throughout the blockade. These experiments showed that spiking activity was reduced, but not abolished, and that "normal" activity patterns seemed to recover after a few days. My initial whole-cell recordings of mEPSCs from CNQX-treated cultures showed that upward shifts in synaptic strength (as compared to a vehicle-treated sister control cultures) still occurred. I have continued these chronic CNQX treatment experiments to observe whether the increase I observed in synaptic strength is robust across cultures from different platings or at different developmental ages. I have also begun conducting identical experiments using the voltage-gated sodium channel antagonist, tetrodotoxin (TTX), to verify that upward synaptic scaling can also be triggered by blocking network firing.

B. Experimental Design

Model System. Currently I am conducting all experiments using our lab's standard dissociated cortical cultures derived from E18 rat embryos. I have recently one experiment using cultures derived from P0 rat embryos (Section III), however this data is not yet analyzed.

Timeline. After 1-2 weeks *in vitro*, I begin electrophysiological recordings and pharmacological perturbations according to the following schedule:

- Phase 1 (Pre-Drug Network Activity Assessment):
  12 hour MEA recording of spontaneous extracellular spiking in Jimbo's medium

- Phase 2 (Disrupted Network Activity Assessment):
  48 hour MEA recording in the presence of CNQX, TTX, or vehicle dissolved in Jimbo's medium; drug replenished approximately every 24 hours
- Phase 3 (Synaptic Strength Assessment):
  ~5 hour whole-cell voltage clamp recordings in the presence of TTX to isolate miniature postsynaptic current (mPSC or mini), or TTX+bicuculline to isolate glutamatergic minis, in oxygenated artificial cerebrospinal fluid (aCSF); in cases where CNQX is used in Phase 2, these recordings follow a 4-stage wash with Jimbo's medium; in cases where TTX or vehicle is used in Phase 2, no wash is conducted before changing to aCSF
- Phase 4 (Post-Drug Network Activity Assessment):
  12 hour MEA recording of spontaneous extracellular spiking in Jimbo's medium following a 4-stage wash with Jimbo's medium
- note: in some cases, Phase 3 is skipped in order to see the immediate results of a washout rather than doing an intermediate patch experiment

Pharmacology. For each drug mentioned above, I use the following concentrations, taken from Turrigiano et al., 1998:

- 20μM CNQX
- 1μM TTX
- 20μM bicuculline

MEA Data Analysis. I am recording spike times, channels, and waveform snippets from 59 electrodes using the Neurorighter system. For analysis, I had been using SqueakySpk to sort units and validate spike waveforms offline for 30-minute epochs. However, I have become increasingly interested in tracking units throughout the duration of the recording, and have found that clustering algorithm employed be SqueakySpk (WaveClus) to be quite time-consuming for my 72+ hour recordings (e.g. sorting a single channel with very active units can take up to a week). Jon Newman has begun working on an unsupervised offline spike sorter called RapidSort to help me (and others) conduct fast sorting for long-term experiments. I am in the process of testing this new sorter and assessing its efficacy in comparison to the clustering algorithm built into SqueakySpk. By sorting units across the entire recording (instead of in 30-minute increments as I had done before), I can better assess the variability in response to drug application. My undergraduate assistant, Marc Powell, has been generating plots to qualitatively illustrate changes in firing rate throughout the experiment.

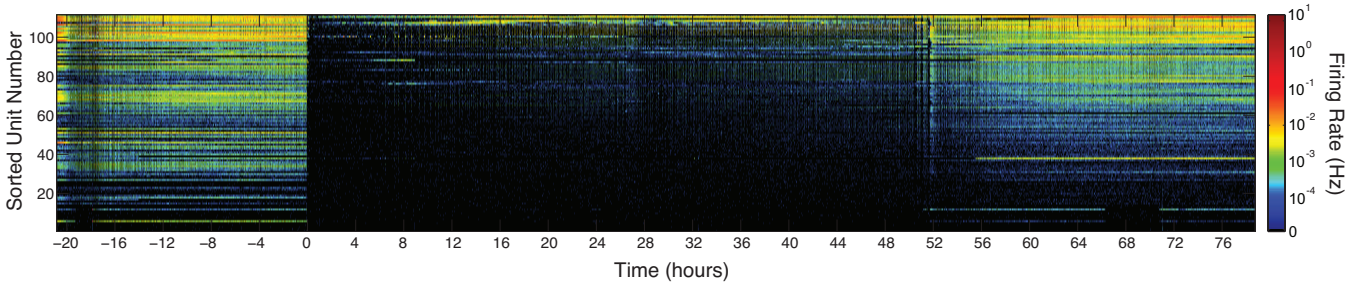

Figure 1: Unit-specific firing rate reveal individual units that have variable responses to CNQX application. The plot was generated from a 97-hour MEA recording. CNQX was added at 0 hours and replenished at 26.5 hours. The drug was washed in four stages between 50.5 and 51.5 hours. Units are sorted according to the number of spikes that occurred during CNQX treatment (most active units have higher unit numbers). The data show that spiking is completely eliminated by CNQX in some units, and that the recovery of activity during the treatment is mediated by only a subset of active units.

Patch Data Analysis. I use the HEKA EPC8 voltage clamp amplifier for my patch recordings with a holding potential of -70mV. In recent experiments, I have been recording minis with TTX in the bath, and then perfusing in bicuculline in order to discriminate between glutamatergic and GABAergic minis. I use MiniAnalysis to analyze mEPSCs.

C. Results & Discussion

In my previous progress report, I showed that:

- CNQX reduces, but does not eliminate spiking activity
- bursting activity appears to slowly recover by the end of the 48 hour drug application
- after washout, activity is increased above that of pre-CNQX levels or sister control cultures

In this report, I show unit-specific firing rates across the entire recording for these CNQX data (Fig. 1). This analysis reveals that this recovery is mediated by reinstatement of spiking activity in only a portion of the units (as opposed to all units globally recovering activity). Further, after the washout, the increased spiking activity appears to be mediated by increased firing rate in almost all units; still, it appears that some units that were active prior to the activity disruption never return.

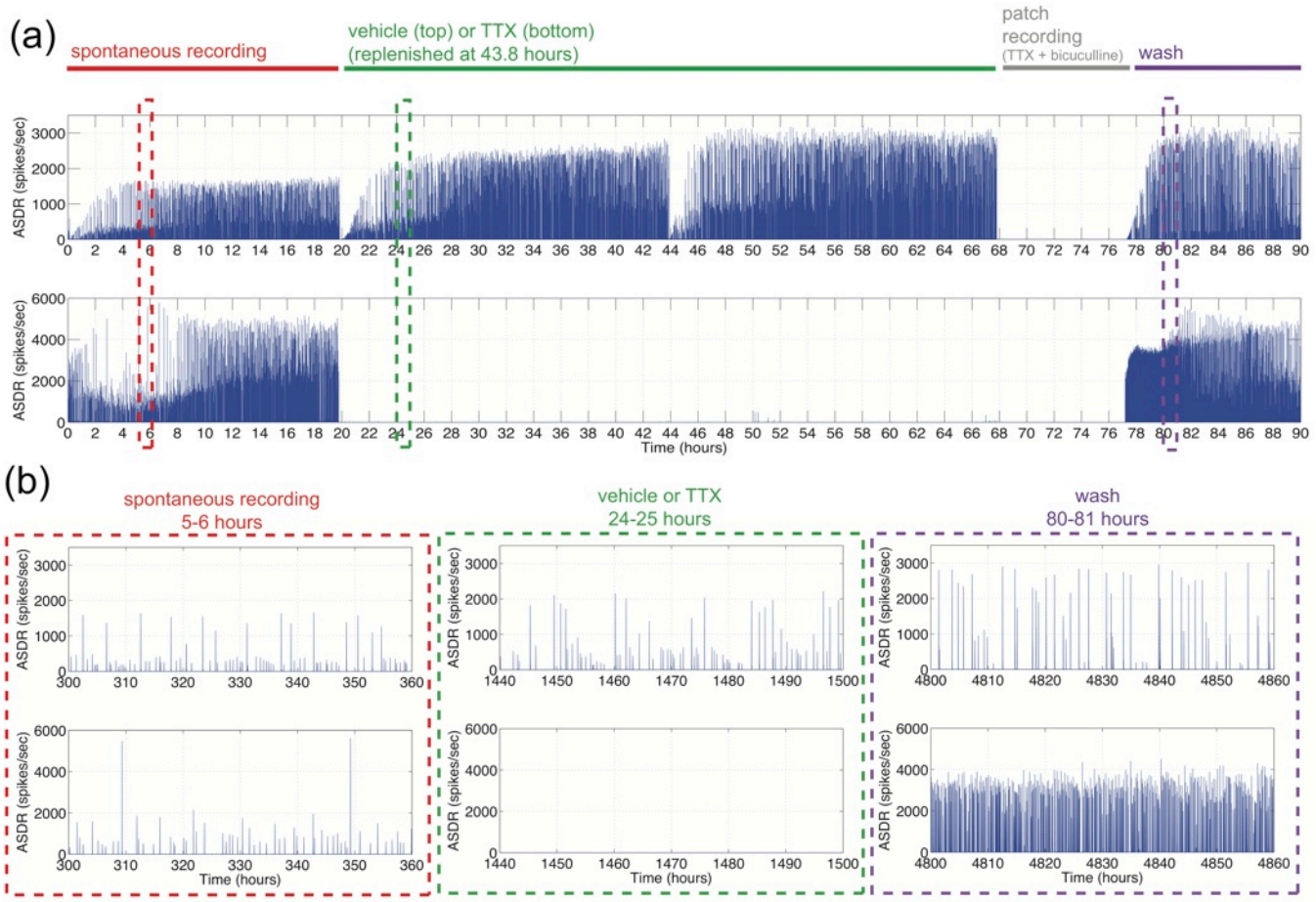

Figure 2: Array-wide spike detection rate reveals that chronic TTX application mediates a homeostatic increase in network activity. These data were collected from sister cultures being recorded from on two different Neurorighter rigs simultaneously, and treated with vehicle (top subplots) or TTX (bottom subplots). (a) Array-wide spike detection rate (ASDR, 1 second bins) for entire recording. Note that there is a ~10 hour gap during which patch recordings (5 hours each) are conducted on the two cultures. (b) One-hour snippets of ASDR during pre-drug spontaneous recording, drug treatment, and post-patch washout. Dotted lines correspond to boxes in (a). The TTX-treated culture shows no spiking activity during drug treatment and significantly elevated activity after the washout compared to pre-drug recordings and its

Fig. 2 shows array-wide firing rates for a TTX-treated culture and vehicle-treated sister control culture. As expected, TTX completely eliminates spiking activity, and the culture is significantly more active after the washout as compared to pre-TTX activity or the sister control culture.

My patch data shows the spread of mPSC amplitudes recorded for all CNQX-treated, TTX-treated, and vehicle-treated cells. As expected, chronic CNQX and TTX treatment elicit compensatory increases in excitatory synaptic strength. Interestingly, the amplitude of GABAergic minis appears to be larger than glutamatergic minis in control cultures; however, in activity-disrupted cultures, this difference is less apparent. This suggests the ratio between glutamatergic and GABAergic mPSC amplitude may be shifted in activity-disrupted cultures in a direction that allows better propagation of excitatory signaling. I do not yet have polished figures showing this data, but plan to have this ready for my upcoming lab meeting presentation.

D. Future Directions

The next step will be to correlate changes in MEA activity with changes in synaptic strength on a by-culture basis. Qualitatively, I have not seen much of a difference between the change in synaptic strength as compared to how effectively activity was blocked during the 48 hour drug treatment (e.g. the degree of synaptic scaling in CNQX-treated cultures does not seem to depend on how well spiking activity recovered during the treatment window). I suspect that the change in synaptic strength may be related to another aspect of activity such as burst rate or nominal firing rate (spiking outside of bursts). Another direction for this work is to begin re-introducing activity during a CNQX blockade. My plan is to use closed-loop optogenetic stimulation for this purpose; I have been working with Jon toward this eventual goal, and some of our work is discussed below in Section II.

II. Monitoring ChR2 Expression Through Microscopy and Electrophysiology

A. Motivation

Jon has been developing a closed-loop optogenetic stimulation system for dissociated cortical cultures grown on MEAs. Over the summer we outlined a set of experiments that we would like to conduct and publish in the near future. In the last progress report I discussed hardware that we had put together for this system and a preliminary open-loop experiment that I conducted. In this progress report, I discuss the progress we have made toward collecting publication-quality data.

B. Methods

I plated three E18 cortical cultures and transfected them with channelrhodopsin-2 (ChR2) using an AAV2-CamKIIa-ChR2-mCherry construct. Beginning at 5DIV, I took confocal micrographs of these cultures every three days in the same place on the array using the same settings. I chose these settings based on the maximum expression levels that I had previously observed in ChR2-expressing cultures after 1 month in vitro. The purpose of this was to track the expression of ChR2 in our cultures through development during the time window we

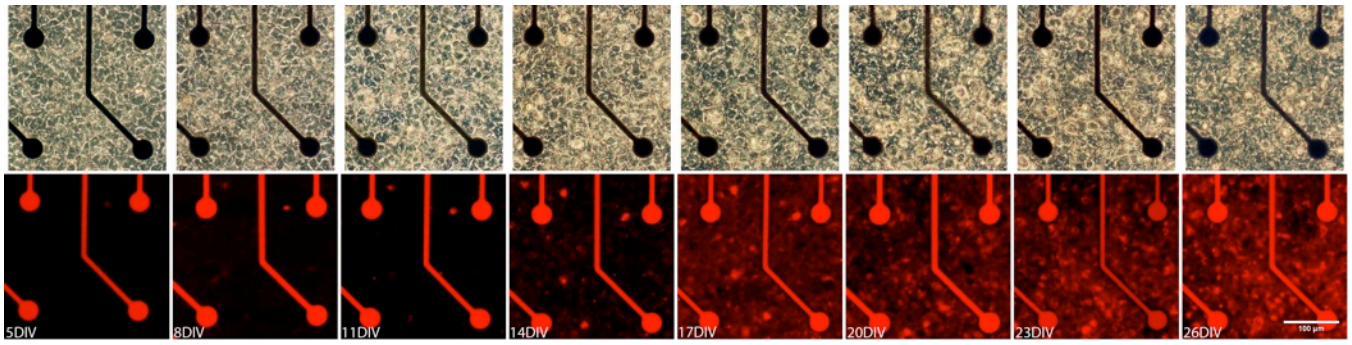

Figure 3: Development of ChR2 expression over 1 month in vitro. Phase-contrast and confocal micrographs of an E18 cortical culture plated on a micro-electrode array and transfected at 1DIV using AAV2-CamKIIa-ChR2-mCherry. Spacing between electrodes is 200um.

expect to perform closed-loop experiments. I continued these imaging sessions through 4 weeks in vitro. During the same 4 week period, Jon and I also collected open-loop data from the three cultures almost every day to characterize the response to stimuli of various pulse-widths, frequencies, and light intensities.

C. Results

Fig. 3 shows phase contrast images and confocal micrographs of the same spot on a culture at three days increments during the first month in vitro. ChR2 expression begins to appear as early as 5DIV in isolated cell bodies, and begins migrating to more distal compartments around 14DIV. As expected, overall protein expression increases throughout development,

Fig. 4 shows the entire array for the period when ChR2 begins to be expressed in processes (14DIV), and progresses to the end of the first month in vitro. At 17 and 20DIV, ChR2-expressing cells seem uniformly distributed across the array. By 23 and 26DIV, we begin to see the emergence of clusters; ChR2-expressing cells and processes are denser in some portions of the dish than others. This same pattern was reflected in the electrophysiological recordings. This suggests that the best time for us to do experiments on these cultures is probably between 2 and 3 weeks in vitro. For this reason, the experiments I described in the grants that our lab recently submitted (Section IV) use this time window.

Jon is currently analyzing our open-loop data from this time period so we can correlate it to expression levels that I have qualitatively shown here.

D. Future Directions

Based on the results of these open-loop experiments, we will be embarking on a similar set of experiments using a closed-loop controller. This will help us to track the ideal developmental time and number of days post-transfection for conducting our close-loop control experiments. In addition, I will be conducting another 2-day CNQX experiment (as described in Section I) but restoring normal levels of activity using closed-loop ChR2 stimulation. I think this data will be very useful in information the direction of my thesis, and will provide excellent data for upcoming papers and grants showing that we can control firing rate during a chronic pharmacological perturbation.

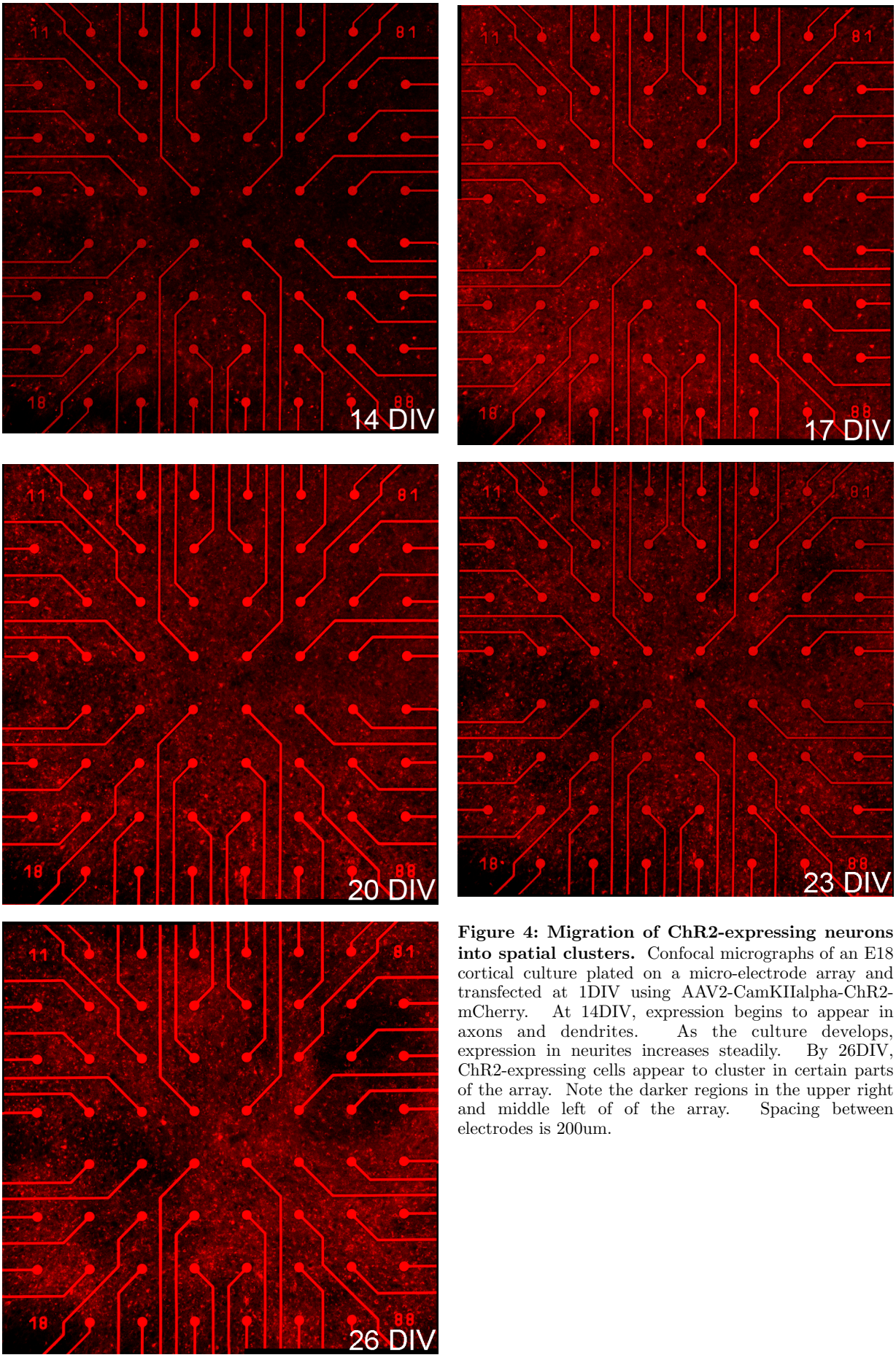

Figure 4: Migration of ChR2-expressing neurons into spatial clusters. Confocal micrographs of an E18 cortical culture plated on a micro-electrode array and transfected at 1DIV using AAV2-CamKIIalpha-ChR2-mCherry. At 14DIV, expression begins to appear in axons and dendrites. As the culture develops, expression in neurites increases steadily. By 26DIV, ChR2-expressing cells appear to cluster in certain parts of the array. Note the darker regions in the upper right and middle left of of the array. Spacing between electrodes is 200um.

III. P0 Cortical Cell Platings

A. Motivation

JT Shoemaker has kindly been providing our lab with E18 rat cortical cells; however, there are many weeks where he only harvests P0 cells. Given the Potter lab's limited funds, I am attempting to develop a protocol for plating P0 cells so that we can take advantage for this available tissue.

B. Approach

I used the current Potter lab E18 cell culturing protocol as a template, and made some modifications based on discussions with others who have worked with P0 tissue, Dr. Potter, and the Banker & Goslin text. This was the general procedure:

1. Coat dishes as usual (PEI in borate buffer at room temperature and ambient CO2; laminin in Jimbo's at 35°C and 5% CO2).
2. Mince cortical tissue into 2mm^2 pieces. Incubate in papain and DNAse for 45min at 35°C with gentle shaking.
3. Replace papain solution with Jimbo's. Triturate 3x. Strain suspension, but set aside undissociated chunks for further enzymatic dissociation.
4. Move strained suspension into 15mL centrifuge tube and bring volume to 3.5mL with Jimbo's. Layer 0.5mL bovine serum albumin at bottom of tube.
5. Centrifuge for 6min at 2 r.c.f. Remove supernatant. Add 1mL Jimbo's and re-suspend.
6. Count cells and dilute to 3000 cells/uL. Plate 15uL of cell suspension on a 10uL laminin footprint.
7. Flood with B27-supplemented neurobasal medium.
8. After 12 hours, do a full media exchange with fresh B27-supplemented neurobasal medium.
9. At 3DIV, do a full media exchange with serum-containing Jimbo's medium; from here on out, do a half media exchange every three days with fresh Jimbo's.

I took phase-constrast photographs of the cells after each media change to assess cell health. Some of these photos are shown in Fig. 5. I have also recorded extracellular action potentials via MEAs and mEPSCs via patch electrodes from these cells indicating healthy development of activity and synaptic connections, as with our E18 platings.

III. Scientific Communication

A. Motivation

Writing is an important skill for any scientist. I have exercised this skills in a number of ways during the past month.

B. NSF Biophotonics Grant and NIH R01

I worked with Dr. Potter and Jon Newman on a grant focused on designing a closed-loop optogenetic stimulation system, and using this system to influence homeostatic synaptic plasticity. My primary contribution was in motivating and formulating experiments for influencing synaptic strength, with an end goal of attenuating pathological network-wide bursting in cortical cultures. Other contributions included generating figures, writing portions

of the preliminary data section, re-organizing preliminary data and experimental sections, writing the first draft of the project summary, and general editing. In the process of writing this grant, I learned a great deal about collaboration--although I can be a bit of a 'control freak' sometimes, I did my best to show restraint and be useful in whatever capacity possible rather than trying to take the primary leadership role. I look forward to getting reviews back so that I can learn and improve... and I hope the reviews will be favorable enough to warrant funding! Subsequently, Dr. Potter modified this grant to be submitted as an NIH R01; besides co-authored the original draft, my role in this grant was in editing and checking for scientific accuracy.

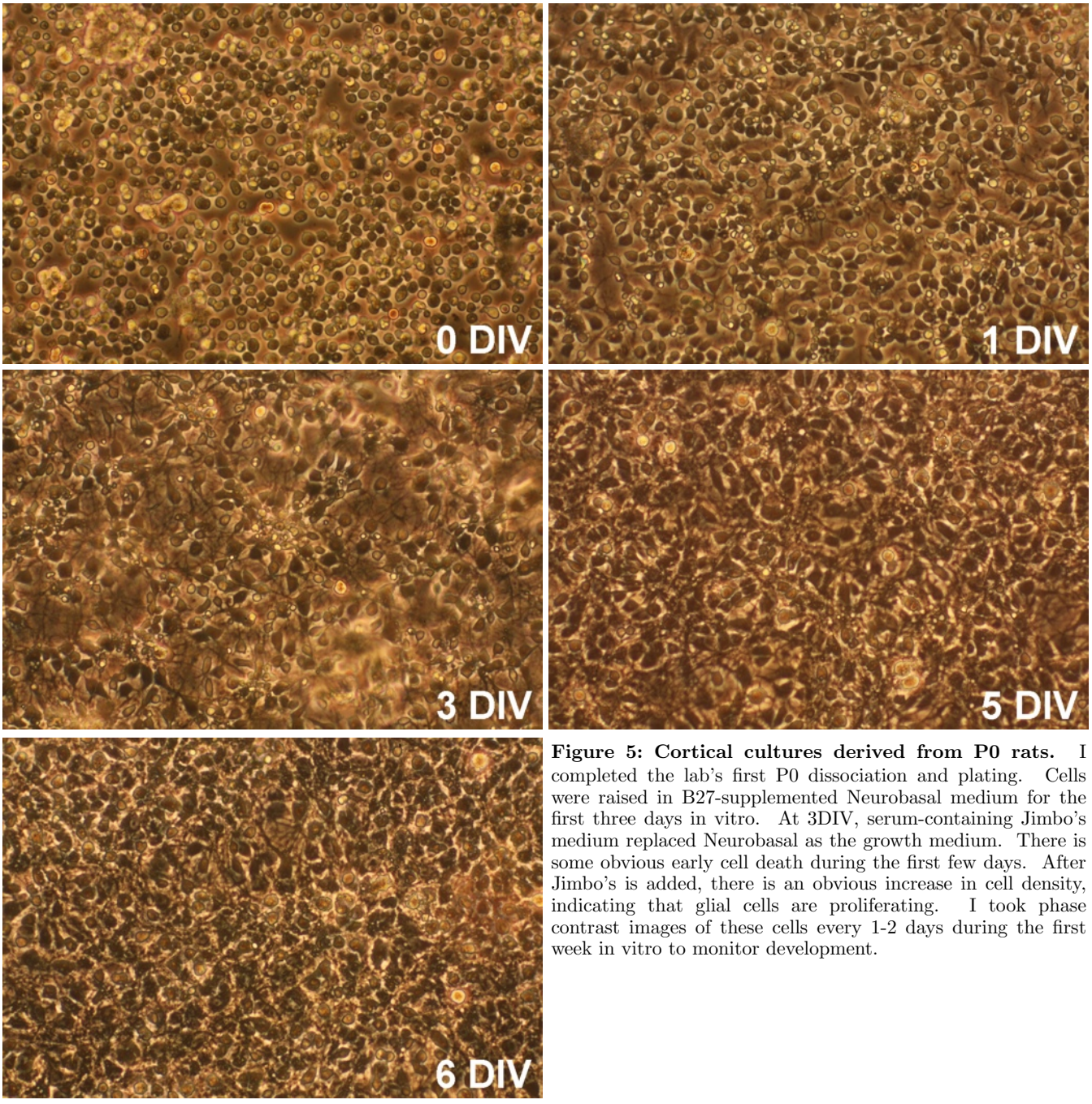

Figure 5: Cortical cultures derived from P0 rats. I completed the lab's first P0 dissociation and plating. Cells were raised in B27-supplemented Neurobasal medium for the first three days in vitro. At 3DIV, serum-containing Jimbo's medium replaced Neurobasal as the growth medium. There is some obvious early cell death during the first few days. After Jimbo's is added, there is an obvious increase in cell density, indicating that glial cells are proliferating. I took phase contrast images of these cells every 1-2 days during the first week in vitro to monitor development.

C. Journal of Neurophysiology Review

I worked with Dr. Potter on a review of a manuscript submitted to the Journal of Neurophysiology (I'll refrain from saying the authors or topic for confidentiality purposes). I wrote a critical review of the manuscript and discussed this review with Dr. Potter. Things that I learned in the process were: do not summarize the experiments or results, be more concise with criticism (simply point out the problem without elaborating), be sure to maintain confidentiality, and point out both large and small mistakes. Also, a reviewer should consider that his or her major goals are: [1] to serve as a filter for poor work from being published, [2] to strengthen work before it is published, [3] to protect one's reputation as a reviewer. This process was educational for me and I was happy to have the opportunity.

D. Abstracts

I am preparing for some poster presentations. At the upcoming Society for Neuroscience Annual Meeting, I have one first-author and two second-author abstracts:

- **Fong M**, Newman JP, Potter SM, Wenner P. 2011. Microelectrode array recordings of cultured cortical networks help identify activity perturbations that trigger homeostatic synaptic plasticity. Annual Meeting Society for Neuroscience. Washington, D.C.
- Newman JP, **Fong M**, Ghosh U, French T, Potter SM. 2011. Long-term, continuous tuning of network excitability in dissociated cortical networks by spatially-distributed electrical stimulation. Annual Meeting Society for Neuroscience. Washington, D.C.
- Heidemann M, **Fong M**, Streit J. 2011. Development of an in vitro model for studying functional recovery after spinal cord lesions. Annual Meeting Society for Neuroscience. Washington, D.C.

I have also submitted an abstract for the upcoming "Chemistry Woven through our Lives: Celebrating the International Year of Chemistry" event run by the Georgia Tech Libraries. The tentative title is "Chemistry woven through our brains: homeostatic regulation of chemical synapses".

E. Dissertation Committee Meeting

I have set up my 2nd committee meeting for the end of this month and am currently working to prepare a brief presentation to give regarding the work I completed during the past year. My plan is to review the first aim of my thesis and discuss progress (and changes) in this aim during the past 12 months since my previous meeting.

F. Cell Care Log Website

I care for cultures used in pharmacology experiments (conducted by Marc and me), optogenetics experiments (conducted by Jon and me), and immunocytochemistry work (conducted by Candace and me). I have two very responsible undergraduate assistants who share the burden of feeding these cultures and monitoring cell health with me. In order to keep track of our cell care duties, I have created a website (https://sites.google.com/site/cellcarelog/) where we organize culture information and responsibilities, and log our platings and media changes in a central location. This website has been very useful for us and I have been pleased with how this communication tool has worked out so far. My advisors (Pete

Wenner and Steve Potter) and my primary collaborator (Jon Newman) also have view-access to the site in case they would like to check on the status of any cultures we use for experiments.

V. Mentoring

A. Motivation

Teaching is an important skill for any scientist. I look for mentoring opportunities in my everyday activities, and try to integrate these teaching moments into my research.

B. Undergraduate Mentoring

I have mentored three undergraduate students during the past month.

Marc Powell is a sophomore biomedical engineering major at Georgia Tech who I began mentoring in May 2011. Over the summer he worked on instrumentation for the patch clamp rig, assisted with patch experiments, and became an expert with preparation and maintenance of 2D cortical cultures. Given the time constraints imposed by a more rigorous class schedule, Marc's project has shifted toward assistance with longterm MEA experiments and analysis of MEA data. He has become proficient at setting up an MEA experiment in Neurorighter and applying various pharmacological agents during these experiments. He has also been writing MATLAB scripts to analyze MEA data, with a focus on understanding how different extracellularly-recorded units respond to TTX or CNQX. We are currently in the process of applying for a Petit Fellowship to fund his work during the upcoming calendar year.

Nisha Bhat is a sophomore chemistry major at Princeton University who I mentored for 1 month at the beginning of the fall semester. She was interested getting some basic lab experience, so I taught her to culture and care for cortical cells. Despite some early missteps, she became quite proficient with these tasks by the end of her month here. I hope that she had a rewarding experience in our lab and can perhaps apply these skills to another research position in her future.

Candace Law is a sophomore biomedical engineering major at Georgia Tech who I began mentoring at the beginning of the fall semester. So far she has learned to plate and care for cells, to image fluorescence in fixed tissue, and to perform immunocytochemistry for cytosolic and nuclear proteins. I am very happy with the progress that she has been making, although I am somewhat concerned that we do not have antibodies (or funds to obtain antibodies) to continue her study. Since Brad Cooke is using several of the same primary antibodies for his study, and because I gifted him several cultures for testing his immunocytochemistry protocols, I have asked him about sharing these with us; however, but have not heard back. In the mean time, Candace is doing a great job caring for cells and learning general laboratory skills.

I am quite pleased with the work that Marc and Candace have done so far this semester. They are both dependable and hardworking, and bring unique perspectives to my research. They consistently attend lab meetings (for the time duration they are able to stay given their class schedules), and complete weekly reading assignments to broaden their knowledge of basic neuroscience. I welcome advice on how to improve in mentoring them.