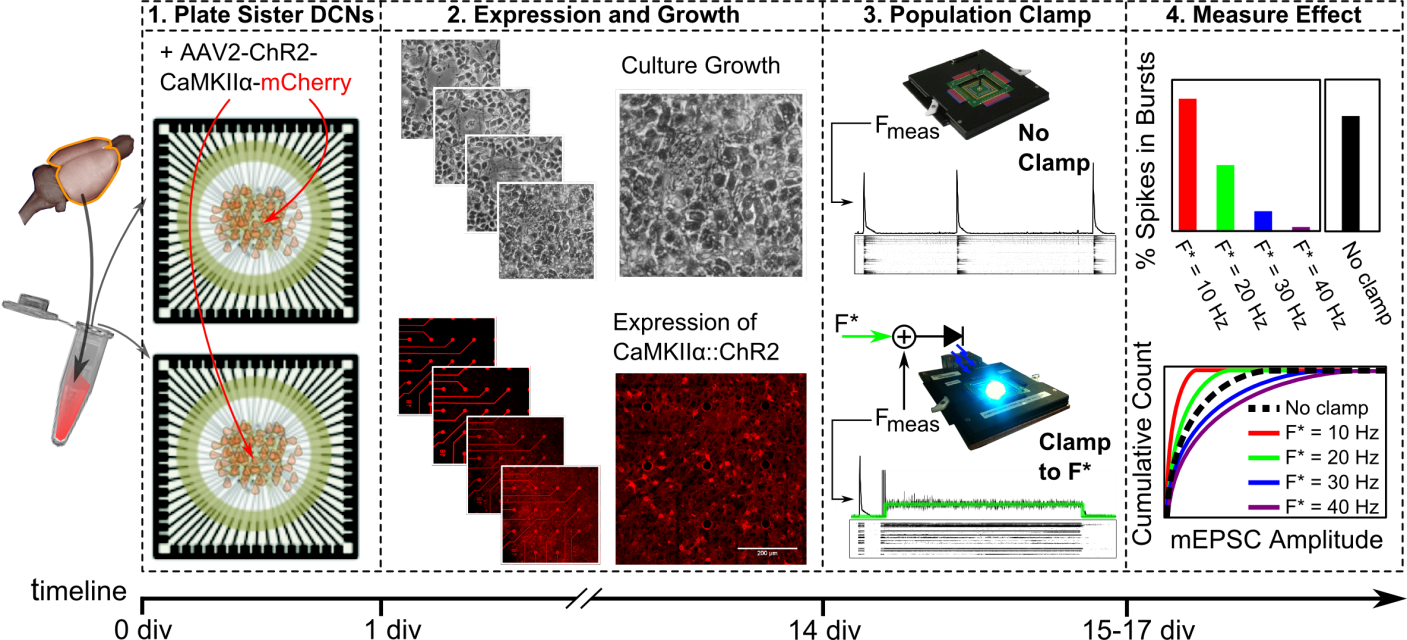
## Research Design and Methods

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Table XX:** Timeline for completion of proposed research. | | | **Year 2** | **Year 2** | **Year 3** |
| **Specific Aim 1.** Design and implement optogenetic population clamp | | |  |  |  |
|  | 1. | Virally transduce optogenetic activators and silencers in pyramidal and GABAergic subpopulations within DCNs. |  |  |  |
|  | 2. | Control design: optimize population clamp performance. |  |  |  |
| **Specific Aim 2.** Manipulate the homeostatic state of DNCs | | |  |  |  |
|  | 1. | Trigger homeostatic increases synaptic strength and cellular excitability using optogenetic population clamp. |  |  |  |
|  | 2. | Trigger homeostatic decreases synaptic strength and cellular excitability using optogenetic population clamp. |  |  |  |
|  | 3. | Induce bidirectional homeostatic changes within DCNs by clamping population activity over a range of firing rates. |  |  |  |
|  | 4. | Induce long-term plasticity in the homeostatic state of DCNs that eliminates the propensity for pathological bursting. |  |  |  |

**Rationale:** Our preliminary data has demonstrated that using a feedback controller and optogenetic excitation, the population firing rate of a heterogeneous cortical networks can be clamped to specific set-points. This technique opens the door to new types of studies because the property of a cortical network that is controlled by homeostatic regulation - the mean activity state of the network - can be externally forced to different values. Voltage and current clamping of individual neurons analogously has provided many insights into the physiology of excitable membranes. Dynamic clamping (artificial current injection) revealed new insights about the role of a neuron in circuit function. A population clamp would allow experimenters to reverse engineer the mechanics of homeostatic plasticity by decoupling experimental perturbations (e.g. addition of a synaptic blocker) from their would-be effects on population activity levels. Additionally, it will a handle to shape the homeostatic state of the network, potentially righting instabilities induced by a lack of external drive due to deafferentation.

*Our goal is to use the optogenetic population clamp to induce a known effect on the balance of synaptic input to pyramidal cells within DCNs in order to stabilize network activity states*. [Table XX] summarizes the project. **In Specific Aim 1**, we will prepare 4 optogenetic constructs to in order to control the activity level of DCNs in 4 physiologically different manners. We will tailor a feedback control architecture around each construct to ensure fast dynamic response and stability in tracking set-points for long time periods [Astrom]. **In Specific Aim 2**, we will use the population clamp to manipulate the homeostatic state of DCNs. We will perform experiments in which the activation of excitatory and inhibitory neuronal sub-populations are used to clamp network firing rate. The each effect of each population clamp will be compared to intracellular measures of homeostatic plasticity to determine the most effective means of modulating homeostatic plasticity within the DCN. Finally, using the neuronal subpopulation capable of inducing the largest homeostatic effect, we will stabilize DCNs by adjusting their homeostatic state. The experimental flow is outlined in [Fig XX].

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**Fig XX.** General experimental flow. Sister DCNs are plated on MEAs then transfected with various optogenetic constructs and given time to grow and express. The networks are subsequently clamped to a specific population firing rate or left uncontrolled. The effect on synaptic excitability, network stability and firing rate statistics is then quantified.

**Aim 1: Implement and tune 4 forms of the optogenetic population clamp**

**A. CONTROLLING NETWORK ACTIVITY USING DIFFERENT NEURONAL SUBPOPULATIONS**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | **Construct** | **Excitation Wavelength** | **Transfected**  **Cell Activity** | **Network**  **Activity** | **Bidirectional**  **Combos** |
| **Cell Type** | **Pyramidal Cells (Glutamatergic)** | **A** | CamKIIα-ChR2-mCherry | 480 nm |  |  | 1  2  3  4 |
| **B** | CamKIIα-ArchT-eGFP | 570 nm | ⬇ | ⬇ |  |
| **Parvalbumin+**  **Interneurons (GABAergic)** | **C** | fPV-C1V1-eGFP | 570 nm |  | ⬇ |  |
| **D** | fPV-Mac-eGFP | 620 nm | ⬇ |  |  |

Rationale:A high performance population clamp should allow for control of network dynamics over a wide range of activity set-points, both above and below spontaneous network firing rates. However, the physiological means by which network activity is modulated will have consequences on the degree of homeostatic change induced [22]. Additionally, certain neuronal subpopulations may be more effective than others at controlling network-wide firing rate over long time periods. We will investigate four optogenetic constructs as handles for network activity that employ distinct neural circuitry:

**Table XX:** Four optogenetic strategies for bi-directional population control using genetically specified neuronal subpopulations. The separated action spectrum of construct pairs allows co-excitation without interference.

Methods: With the Gross lab at Emory University (see attached letter of support) we have employed an AAV2-CaMKIIα -ChR2-mCherry to drive ChR2 expression in neocortical PY cells, and verified its expression through imaging, MEA recordings, and whole-cell patch clamp recordings (Preliminary Data). Using constructs obtained from Karl Diesseroth, Ed Boyden, and Edward Callaway, we will create custom plasmids for the remaining three constructs shown in Table XX. In order to generate pAAV2-CamKIIα-ArchT-eGFP, cDNAs encoding the CaMKIIα promoter will be subcloned by PCR into the SmaI and Xbal restriction sites of the vector pAAV2-ArchT-GFP. For pAAV2-fPV-C1V1-eYFP, we will subclone the fPV promoter by PCR into pAAV-C1V1-eYFP at \_?\_ and \_?\_ (insert restriction sites). For pAAV2-fPV-Mac-GFP, Mac cDNA will be subcloned by PCR into pAAV-fPV-GFP at \_?\_ and \_?\_ (insert restriction sites). All of these plasmids will be validated in HEK 293 cells. Viral constructs will then be generated and amplified at the UNC Viral Vector Core (<http://genetherapy.unc.edu/services.htm>) to a titer of 10^2 viral genomes/mL.

Transfection and construct efficacy will then be tested in neocortical cells will be dissociated from the brains of embryonic day 18 rats and plated on glass-bottom petri dishes (MatTek, P50G-1.5) or 60 channel MEAs (Multi Channel Systems, Reutlingen, Germany) as described in our publications [xxx]. Briefly, cortical tissue will be ezymatically dissociated for 30 minutes in 2.5 U/mL papain solution. Dissociated cells will be plated onto culturing surfaces precoated with polyethyleneimine and laminin [potter and demarse]. Viral transfections will be performed at 1 DIV by adding 2 uL of 10^12 c.f.u mL-1 viral solution to each cultures 1 mL of cell culture medium. Virus will be allowed to incubate with the culture for 3 days before removal. No antibotics or glial growth inhibitors will be added to culturing media since these practices may have confounding effects on neurotransmission [x], cell health [Potter Demarse], and homeostatic mechanisms [x].

Starting at 4 DIV, DCNs will be imaged on a Zeiss LSM 510-META confocal microscope every three days to monitor the expression of marker proteins. At 14-17 DIV (13-16 days post transfection, DPT), the photocurrents induced by light-excitation of each construct will be verified in comparison with published levels [Chr2 - 2a][C1V1 - 13][ArchT/Mac - 7] using whole-cell voltage-clamp recordings. These will be conducted on an upright Nikon Eclipse e600fn microscope using an Axopatch 1D voltage-clamp amplifier, Digidata A/D converter and PClamp recording software (Molecular Devices, Sunnyvale, CA). Photocurrents will be induced by driving the appropriate LED on our optical stimulator (preliminary results) coupled to the trans-illumination port of the microscope using digital signals produced by PClamp. Light intensities will be adjusted to appropriate levels before experimentation by manually adjusting power on our LED driver and measuring optical power using a Newport 1830c meter (Newport Corp., Irvine, CA). All recordings will be performed in the presence of 0.5 uM tetrodotoxin (TTX) to prevent post-synaptic currents from interfering with the measurement of light-induced currents.

After the efficacy of constructs has been verified DCNs on 4 MEAs will be transfected with the 4 constructs listed in [table XX]. Starting at 4 DPT, MEA recordings will be performed daily using a commercial 60 channel amplifier (Multi Channel Systems, Reutlingen, Germany) and our NR acquisition platform. Interleaved 30 second epochs of stimulation trains each train applying a random combination of three parameters - stimulation frequency, pulse-width, and light power - will be applied. All transfected MEA's will be stored in light-tight dishes.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **Construct** | |
|  |  | ChR2, V1C1 | ArchT, Mac |
| **Stimulation**  **Parameter** |  | {1,5, 10, 20, 40} | {0.5, 1, 5, 10} |
|  | {0.1, 0.5, 1.0, 5} | {10, 100, 500,1000} |
|  | {0.5, 1, 1.5, 2, 4} @ 480 nm | {1, 2, 5, 10} @ 590 nm |

**Table XX:** Stimulation parameters to explore for each construct type. Values are chosen to cover a wide range of parameters relevant to the on/off kinetics and light-power requirements of each construct type [2a] [13] [7].

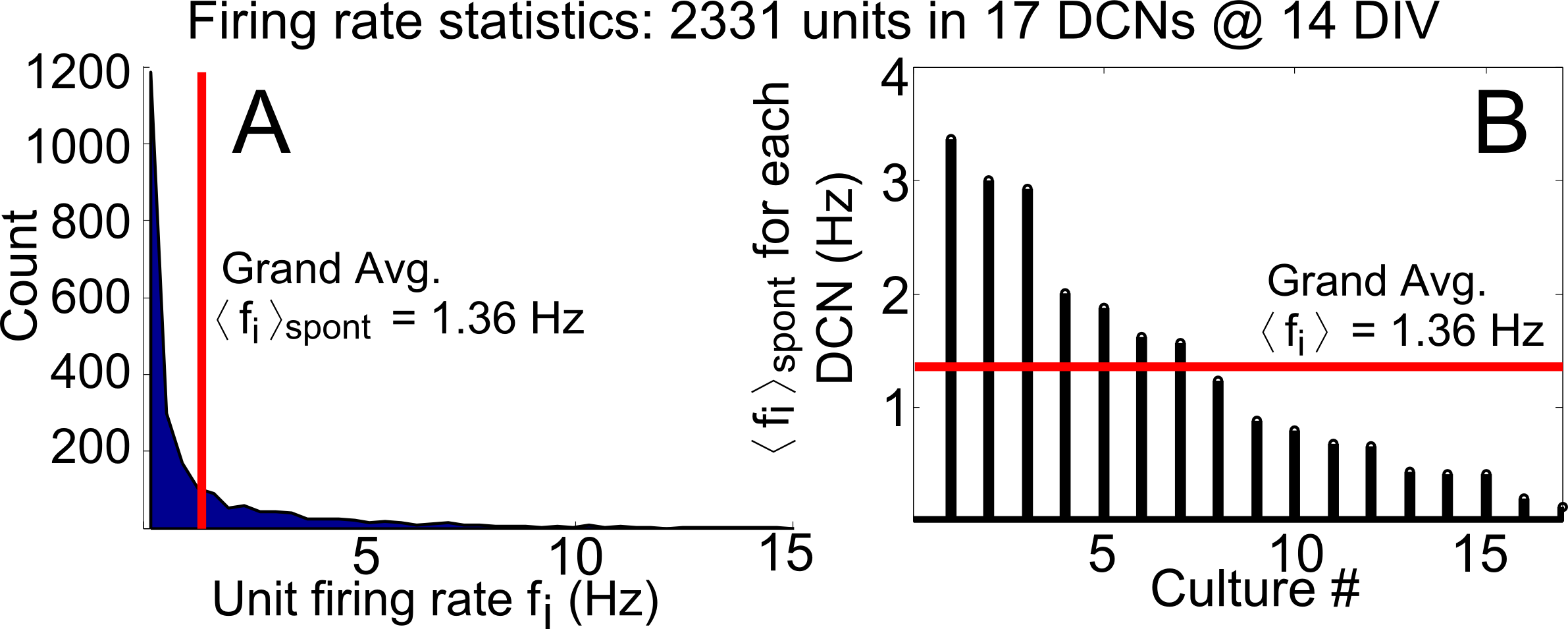
Spike detection and sorting will be conducted during recordings with NR. Sorting will be performed first using principal component analysis for dimensional reduction of spike waveforms and then unsupervised Gaussian Mixture modeling for classification (real-time C# port of Charles Bowman's 'Cluster', written in C [Bowman]). Data generated will consist of three binary file types: spike files containing spike objects (time, channel, 3-ms voltage waveform, unit #); digital files containing digital objects denoting the 32 bit port state and encoding stimulation timing, frequency, pulse-width and amplitude; and 'analog' files containing raw traces of LED current measurements. For each DIV and each construct combination, the normalized steady state firing rate of unit , , will be calculated. Here, is the steady-state firing rate for a particular a particular combination of control values , or . is stimulation frequency at a given pulse-width and irradiance, is duty-cycle at a given irradiance and is average light power over time. The ensemble average of the set of normalized per unit firing rates, , will be compared with each prospective control parameter, . This method examines the effect of a control signal on an 'average unit' within each DNC, and therefore accounts for variability in the number of neurons that happen to be picked up by a given MEA. The monotonicity, signal to noise ratio, and dynamic range of the relationship between and will be considered for given construct and the most highest performing control signal will be designated as the control signal for a that construct.

**B. OPTIMIZE CONTROL PERFORMANCE OF POPULATION CLAMP**

Rationale: Control performance for tracking static set-points is measured in step response time and stability (no oscillations around the target value). Additionally, since the changes in cellular physiology associated with homeostatic plasticity take place over relatively long time scales (many hours or days) our population clamp must be capable of holding firing rates at a desired set-point over these time periods.

Methods: We will aim for a step-response time-constant of 10 seconds and a steady-state tracking error of 0.5 RMS Hz in the average unit firing rate, , about the target rate . We will attempt to achieve these targets our existing PI design (preliminary data, equations) since there is a large body of literature dedicated to PI/PID tuning [Astrom]. However, if the PID architecture fails to meet design requirements we turn to more sophisticated methods capable of handling strong non-linearities, namely model-predictive control and/or fuzzy control.

**Fig X:** The meaning of high and low population firing rate is dependent on the DCN being controlled. (A) Histogram of spontaneous firing rates for 2331 units across 17 DCNs (B) The average firing rate of units varies across DCNs.



We will use our previously written population clamp DLL (preliminary data) to calculate online, every 100 ms. A feedback control signal will be sent to the LED of appropriate wavelength according to eq. 2, with the new, per-unit error term . In [figure xx] the spontaneous firing rate statistics of spontaneous activity from 2331 detected units across 17 DCNs at 14 DIV is presented (data from [p3]). The ensemble average firing rate per unit, , is calculated across half-hour recordings of spontaneous activity for each DCN. Because there is large variability in the value of for different cultures, the value of will be dependent on an estimate of the spontaneous average unit firing rate for a given culture,. For each rate, we will tune the PID algorithm using the Zeiger-Nichols method presented in the preliminary data section and our NR hardware (fig. XX).To test the efficacy of each construct and corresponding feedback control parameter set, we will first perform 30 minute sessions at the following set-points:

1. for the AAV2-CaMKIIα-ArchT-eGFP and AAV2-fPV-C1V1-eGFP constructs
2. for AAV2-CaMKIIα-ChR2-mCherry and AAV2-fPV-Mac-CFP constructs

If these set-points are met we will push setpoints to more extreme values until we no longer see changes in population activity with increasing control inputs. When control bounds have been determined, we will attempt to hold DCNs for 24 hour periods at extreme and intermediate set-points. Directly following these control sessions, we will perform a live-dead assay using ad propidium idodide

**EXPECTED OUTCOMES FOR SPECIFIC AIM 1** We expect that each construct will have strengths and weaknesses in controlling population activity, and this variability will be accounted for in control design. Additionally, we expect that there will be a biological relevant range of population activity levels that we can hold DCNs at for extended (>24 hour) periods without excitotoxicity or control failure.

**ANTICIPATED DIFFICULTIES AND ALTERNATIVE APPROACHES FOR SPECIFIC AIM 1** We must find the range of set-points that is safe to drive the network to and those that are unobtainable or detrimental to culture. health ArchT and Mac are more sensitive than older silencing constructs, such as Halorhodopsin. However it is still possible that we will need a brighter light source than our LED system for effective activation of these constructs. We have included funds for a 200 mW, 532 nm, fiber-coupled laser to fix this problem if it arises (Shanghai Laser, Shanghai, China). Secondly, it is our experience that suppression of network inhibition via the addition of GABAzine (analogous to the action of FPV::Mac) leads to an extreme propensity for bursting which may override our attempts to hold static increases in population firing rate. If this is the case, we will perform FPV::Mac based elevations in firing rate in co-transfected FPV::Mac and CaMKIIα::ArchT cultures so that bursts can be suppressed, via concurrent suppressive control via CaMKIIa::ArchT [013b].

### Aim 2: Optogenetic population clamp to manipulate the homeostatic state of DCNs.

#### A. TRIGGERING HOMEOSTATIC INCREASES IN SYNAPTIC STRENGTH

Rationale: An appropriate balance of excitation and inhibition is necessary for the proper functioning of neural circuits. Reduced levels of network spiking, e.g., due to pharmacological deafferentation, lead to compensatory increases in excitatory synaptic strength and cellular excitability in neocortical pyramidal neurons (Turrigiano et al., 1998; Desai et al., 1999).  In addition, homeostatic plasticity mechanisms decrease inhibitory input to pyramidal cells via compensatory alterations in mIPSC frequency, probability of release, and number of GABAergic neurons (Bartley, 2008; Hartman, 2006; Kilman, 2002; Rutherford,1997). In these studies, activity was suppressed using pharmacological blockade of spiking activity using TTX.  This approach does not tell us whether the homeostatic plasticity mechanisms that lead to an increase in activity are triggered by too little excitation or too much inhibition. Answering this question is crucial in the development of therapeutics aimed at inducing long-term changes in pathologically-behaving networks. Changes that might disrupt the balance of excitation and inhibition could be reduced glutamatergic transmission or increased GABAergic transmission. The former has been tested via pharmacological blockade of non-NMDA type glutamate receptors and shown to effectively decrease synaptic strength (Turrigiano et al., 1998).  Increasing GABA drive in order to reduce network activity has not been explored. We propose to test the cases of reduced glutamatergic transmission via optogenetic suppression of activity in CaMKIIα -expressing pyramidal cells, and elevated GABAergic transmission via optogenetic elevation of FPV+ inhibitory interneurons.

Methods: Sister DCNs will be plated on MEAs as previously described. All DCNs will be transfected with either AAV2-fPV-C1V1-eGFP or AAV2-CaMKIIα-ArchT-eGFP. At 14-17 DIV, network firing rates will be clamped to for 24 hours in half of the DCNs and left unregulated in remaining cultures. MEA data will be collected concurrently from clamped and control cultures stored in a single incubator using two NR rigs. Up to three culture pairs will be controlled sequentially in the 14-17 DIV time window. Following 48 hours of population clamp, whole-cell voltage-clamp recordings will be obtained from pyramidal cells from all DCNs. Recordings will be conducted oxygenated aCSF with 0.5 uM TTX.  mPSC amplitude and frequency distributions will be calculated post-hoc using MiniAnalysis (Synaptosoft, Decatur, GA). Distributions from clamped and non-clamped DCNs will be compared..

Potential Outcomes: Because the two clamp types (B and C in Table XX) reduces population activity levels using a different neuronal subpopulation, the corresponding increases in synaptic strength can be compared for each clamp type. There are three possible results. (1) **The best handle for upward SS is firing rate.** This is the case if we see no difference in SS between reducing activity using either form of population clamp. (2) **The best handle for upward SS is reduction of glutamatergic neurotransmission**. This is the case if increases in synaptic strength are most dramatic in the CaMKIIα::ArchT controlled cultures. (3) **The best handle for upward SS is elevation of GABAergic neurotransmission**. This is the case if increases in SS are more dramatic in the FPV::C1V1 controlled cultures.

#### B. TRIGGERING HOMEOSTATIC DECREASES IN SYNAPTIC STRENGTH

Rationale: Elevated levels of network spiking lead to compensatory decreases in excitatory synaptic strength in neocortical pyramidal neurons (Turrigiano, 1998) and increassed inhibitory input to pyramidal cells via compensatory increases in mEPSC amplitude in inhibitory interneurons (Chang, 2010; Doyle, 2010).  In these studies, a chronic activity elevation was achieved via elevation of glutamatergic transmission (using kainate injection) or blockade of inhibitory transmission (using bicuculline or gabazine). Two recent studies used genetic targeting of cation channels to elevate activity in individual cells and saw cell-autonomous changes (i.e. changes not requiring changes in activity at the network level) in synaptic strength.  In the first study, activation of overexpressed TRFPV1 channels via capsaicin resulted in Increased in mIPSC amplitude in pyramidal cells (Peng et al., 2010).   In the second, activation of ChR2 channels using pulsatile blue light led to reductions in miniature and evoked EPSC amplitude (Goull & Nicoll, 2010).  However, these manipulations have not been performed at the network level, nor have activity states been clamped to set levels. In this aim we take inspiration from studies that alter glutamatergic or GABAergic transmission at the network level, but use the cell-type specific targeting approach to better tease apart circuits that are involved in homeostatic plasticity mechanisms. To increase network activity we test two approaches: elevation of glutamatergic transmission via optogenetic excitation of pyramidal cells, or suppression of GABAergic transmission via optogenetic inhibition of FPV+ inhibitory interneurons.

Methods: Sister DCNs will be plated on MEAs as previously described. Both DCNs will be transfected with either AAV2-FPV-C1V1-eGFP or AAV2-CaMKIIα-ArchT-eGFP. network firing rates will be clamped to for 24 hours in half of the DCNs and left unregulated in remaining cultures for 24 hours. Intracellular recordings will be conducted as in aim 2A.

Potential Outcomes: Because the two clamp types (A and D in Table XX) increases population activity levels using a different neuronal subpopulation, the corresponding increases in synaptic strength can be compared for each clamp type. There are three possible results. (1) **The best handle for downward SS is firing rate.** This is the case if we see no difference in SS between reducing activity using either form of population clamp. (2) **The best handle for downward SS is the elevation of glutamatergic neurotransmission**. This is the case if increases in synaptic strength are most dramatic in the CaMKIIα::ChrR2 controlled cultures. (3) **The best handle for downward SS is the reduction of GABAergic neurotransmission**. This is the case if increases in SS are more dramatic in the FPV::C1V1 controlled cultures.

**C. BIDIRECTION CONTROL OF SYNAPTIC STRENGTH BY CLAMPING POPULATION ACTIVITY OVER A RANGE OF SETPOINT FIRING RATES.**

Rationale: Many diseases and disorders of the cortex may be due to maladaptive plasticity mechanisms (homeostasis gone wrong).  In order curb these problems, a system capable of controlling the homeostatic state of the heterogeneous cortical networks is required.  This includes being able to precisely drive synaptic strength in an upward or downward direction across a spectrum of values. Aim 2A and 2B will reveal what strategies might be best for increases or decreases in synaptic strength, so the purpose of aim 2C is to combine these strategies for smooth bidirectional control of synaptic strength over a wide range.

Methods: We will co-transfect DCNs on MEAs with the two viruses that show the greatest effect on synaptic strength from Aims 2A and 2B. At 14-17 DIV, network firing rates will be clamped one of the set of rates or left unregulated. Directly following the clamping period, intracellular recordings will be conducted as in aim 2A.

Potential Outcomes: We envision two potential outcomes for this aim: (1) There is a smooth monotonic and inverse relationship between clamped firing rate and changes in excitatory synaptic strength. If this is the case, using the bidirectional population clamp, we should be capable of driving the homeostatic state of a DCN to a specific set point over this range. (2) The relationship between clamped firing rate and changes in excitatory synaptic strength is monotonic and inverse, but very steep. It this is the case, we will see scaling occur for dramatic increases or decreases in firing rate, but not for smaller deviations from activity. This would indicate that achieving a functional effect in terms of curbing aberrant bursting activity in a DCN will require fairly drastic changes in the network's firing rate.

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#### D. LONG-TERM PLASTICITY IN THE HOMEOSTATIC STATE OF A DCN THAT REDUCES OR ELIMINATE THE NETWORK’S TENDENCY TOWARD PATHOLOGICAL BURSTING.

Rationale: DCNs are de-afferented cortical networks that develop synchronized population-wide bursting activity after two weeks in vitro (Wagenaar, BMC Neuro).  These bursts are reminiscent of pathological bursting that develops in vivo after cortical  de-afferentation.  It is likely that in both these networks, loss of afferent induces homeostatic mechanisms to overcompensate for the lack of excitatory drive [16] [16a] [16d].  A potential therapeutic approach is to control these homeostatic mechanisms to prevent the pathological overcompensation.  In Aim 2C we will establish a way to precisely control homeostatic mechanisms using our optogenetic population clamp.  Using this new tool, we seek to induce plasticity mechanisms that eliminate these pathological bursts in DCNs for extended time periods after the clamp has been turned off.

Methods: DCNs will be prepared as in Aim 2C. At 13-14DIV, the spontaneous spiking activity of each DCN will be continuously recorded. Starting at 14 DIV, population firing rates will be clamped to one of the following target rates: for two days.  Every 12 hours, the population clamp will be paused to allow a 30 min recording of spontaneous activity.  At 16 DIV, the population clamp will be stopped. Immediately after this, a 1 hour

maybe we can put a poisson light train here and ask the culture to decode it. This way we could measure a defined effect on information transmission.

permanently and a 24-hour spontaneous recording will be performed.

Following these recordings, we will compare levels of bursting, spatial/temporal spike correlations and information processing across all population set points, . Specifically,

- Burstiness index

- autocorrelation width

- ability to properly encode poisson spike train

Potential Outcomes:

* + **12 hours of stimulation is sufficient to prevent bursting**, e.g. we see tons of bursting during the pre-recording, but no bursting after the first 12 hours.  same idea applies to 24 and 36 hour markers.
  + **Clamping at a high firing rate is the most effective way to maintain burst-free activity**, e.g. during the 12 hour post-recording, bursting comes back that latest for the culture clamped at the highest FR.  same idea applies to other clamped firing rates.

**ANTICIPATED DIFFICULTIES AND ALTERNATIVE APPROACHES FOR SPECIFIC AIM 2**

* Our use of as a basis of a DCNs nominal firing rate is based on a large set of spontaneous data at 14 DIV. however, it is possible that the large variability in across cultures is simply due to random variations in the number of fast spiking interneurons that are picked up by the MEA. If this is the case, we would expect to see poor correlations between the relationship between a given value of R and a predictable change in synaptic strength. If this occurs, we will switch to absolute values for F\* = {0.01 0.25, 0.5, 2, 5, 10} Hz.