OMB Number: 4040-0001
Expiration Date: 10/31/2019

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

c. Previous Grants.gov Tracking Number

2. DATE SUBMITTED | **Application Identifier**

5. APPLICANT INFORMATION **Organizational DUNS*:** 0521841160000

Legal Name*: CARNEGIE-MELLON UNIVERSITY
Department:
Division:
Street1*: 5000 Forbes Ave
Street2:
City*: PITTSBURGH
County: Allegheny
State*: PA: Pennsylvania
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 15213-3815

Person to be contacted on matters involving this application
Prefix: Ms. First Name*: Jessica Middle Name: J Last Name*: Higby Suffix:
Position/Title: Pre-Award Administrator
Street1*: 4802 Fifth Avenue
Street2: 1015 WQED Building
City*: Pittsburgh
County: Allegheny
State*: PA: Pennsylvania
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 15213-2942
Phone Number*: 412-268-2813 Fax Number: 412-268-6279 Email: jvig@andrew.cmu.edu

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** 1250969449A1

7. TYPE OF APPLICANT* O: Private Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Sources of Cholinergic Modulation of Cortical Microcircuits

12. PROPOSED PROJECT
Start Date* Ending Date*
09/01/2018 08/31/2022

13. CONGRESSIONAL DISTRICTS OF APPLICANT
PA-014

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Stephanie Middle Name: E. Last Name*: Myal Suffix:
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15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$296,176.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$296,176.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Ms. First Name*: Linda Middle Name: Feuster Last Name*: Duffy Suffix:
Position/Title*: Assoc Vice President of Sponsored Programs
Organization Name*: Carnegie Mellon University
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Signature of Authorized Representative*
Jessica Viglione

Date Signed*
04/09/2018

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:Myal_cover.pdf

424 R&R and PHS-398 Specific
Table Of Contents

SF 424 R&R Cover Page....1
Table of Contents....3
Performance Sites....4
Research & Related Other Project Information....5
Project Summary/Abstract(Description)....6
Project Narrative....7
Bibliography & References Cited....8
Facilities & Other Resources....13
Equipment....16
Research & Related Senior/Key Person....18
PHS Fellowship Supplemental....28
Applicant's Background and Goals for Fellowship Training....31
Specific Aims....37
Research Strategy....38
Respective Contributions....44
Selection of Sponsor and Institution....45
Training in the Responsible Conduct of Research....46
Sponsor and Co-Sponsor Statements....47
Letters of Support from Collaborators, Contributors, and Consultants....53
Description of Institutional Environment and Commitment to Training....57
PHS Human Subjects and Clinical Trials Information....59
Vertebrate Animals....60
Select Agent Research....62
Resource Sharing Plan....63
Authentication of Key Biological and/or Chemical Resources....64

OMB Number: 4040-0010
Expiration Date: 10/31/2019

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:	CARNEGIE-MELLON UNIVERSITY
Duns Number:	0521841160000
Street1*:	4400 Fifth Avenue
Street2:	MI159B
City*:	PITTSBURGH
County:	Allegheny
State*:	PA: Pennsylvania
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	15213-2683
Project/Performance Site Congressional District*:	PA-014

Additional Location(s) File Name:

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ● No

IACUC Approval Date: 12-07-2017

Animal Welfare Assurance Number A3352-01

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	Myal_Project_Title_and_Summary.pdf
8. Project Narrative*	Myal_Project_Narrative.pdf
9. Bibliography & References Cited	Myal_References_cited.pdf
10.Facilities & Other Resources	Myal_Facilities_and_Training_Environment.pdf
11.Equipment	Myal_Equipment.pdf

PROJECT TITLE

Sources of Cholinergic Modulation of Cortical Microcircuits

PROJECT SUMMARY

The cognitive symptoms of schizophrenia are subtle, persistent, debilitating, and more strongly linked to treatment non-adherence and poor prognosis than the overt, better-known psychotic symptoms. They are also poorly treated by antipsychotics. A large body of indirect evidence implicates deficient signaling of acetylcholine (ACh) in schizophrenia cognitive symptoms. ACh normally acts as an attention signal, activating a chain of GABA-ergic inhibition in the cerebral cortex that increases the activity of output neurons. In our published work, we see that ACh acts in a precise manner to strengthen specific synapses in the mouse somatosensory cortex (S1). Preliminarily, we see that different cell types respond distinctly to ACh, mediated by nicotinic and muscarinic ACh receptors. We are interested in how ACh from different sources affects cortical circuits, and whether ACh release sites are preferentially associated with specific intra-cortical cell types and thalamocortical synapses. The main source of cortical ACh is the basal forebrain, but there are also intrinsic cortical ACh neurons of unknown function which may augment ACh signaling in a precise manner. In **Aim 1.1**, we propose to anatomically test the relative contribution of extrinsic and intrinsic sources of ACh inputs using immunolabeling of ACh release sites for advanced confocal imaging and 3D reconstructions of ACh release sites. In **Aim 1.2**, we will functionally assess the contribution of these sources in whole-cell recordings, using optogenetics to evoke ACh release in brain slices. **We hypothesize that endogenous ACh has cell type- and layer-specific responses differentially controlled by intrinsic and extrinsic ACh sources.**

We also suspect that the precise localization of ACh release sites contributes to synapse-specific thalamocortical effects of ACh. In our recent paper, we found that endogenous ACh strengthened excitatory synapses onto particular interneuron types via presynaptic mechanisms. However, when we activated cholinergic receptors pharmacologically, both synapses were strengthened, suggesting that ACh receptors and/or release sites are spatially segregated to precisely tune excitation. Prior pharmacological work suggests that thalamic inputs from the ventral posterior medial nucleus (VPM, a lower-order sensory thalamic nucleus) express nicotinic receptors, but it is unknown whether these inputs would be strengthened by endogenous ACh, or whether inputs from a higher order sensory thalamic nucleus (the posterior medial nucleus, POm) are also influenced by ACh. In **Aim 2.1**, we will anatomically compare whether ACh release sites are more common at VPM or POm synapses. In **Aim 2.2** we will functionally assess whether endogenous ACh strengthens POm and VPM inputs using whole-cell recordings with dual-color optogenetics in brain slices. **We hypothesize that ACh release sites are associated with POm inputs, and that timed endogenous ACh enhances thalamocortical inputs onto specific cell types,** potentially shifting cortical circuits to favor different thalamic information streams during states of attention or disease-related cholinergic dysfunction.

PROJECT NARRATIVE

Schizophrenia is characterized by overt psychosis, but the subtler cognitive symptoms have a greater impact on quality of life and prognosis and are poorly treated by antipsychotics. Acetylcholine, a neurotransmitter important for cognition and attention, fine-tunes cortical circuits to achieve a complex balance of excitation and inhibition that is disrupted in schizophrenia. The proposed work will test the effects of acetylcholine from diverse sources on neural circuits in the mouse somatosensory cortex, and will inform the ongoing development of cholinergic therapies for cognitive impairment in schizophrenia and other psychiatric diseases.

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Facilities & Other Resources

Laboratory

My sponsor, Alison Barth, occupies approx. 1800 sq. ft. of laboratory space on the first floor of the Mellon Institute (*MI*, Rm. 157) at Carnegie Mellon University (*CMU*). The Barth lab space contains four rigs for brain slice recordings, one *in vivo* two-photon microscopy unit, a confocal microscope, and equipment for perfusions, genotyping, brain slice preparation, and immunohistochemistry. I have my own experimental space (MI 159D) that contains my dedicated equipment for whole-cell patch clamp recordings as well as a data analysis computer, -20°C refrigerator, and prep area for chemicals and tissue samples. The Barth lab is located in the *CMU "Great Hall of Brain Science*:" a collaborative, open environment that also houses the following independent neuroscience investigators:

-Aryn Gittis, PhD (thesis committee chair): physiology of the basal ganglia in models of Parkinson's disease
-Sandra Kuhlman, PhD: *in vivo* experience-dependent plasticity in the visual cortex
-Eric Yttri, PhD: *in vivo* and computational studies of striatal activity during motor decision making
-Andreas Pfenning, PhD: genetic and transcriptomic classification of cortical interneuron types

This collaborative space also contains a lunchroom, two nearby conference rooms, a lounge area for quiet study, and ample space to work, read, and discuss research with fellow lab-mates. Occasionally, short, non-credit "boot camps" are offered by Great Hall of Brain Science faculty to teach useful experimental techniques. Previous boot camps have taught electrical circuit design, cranial window construction for two-photon imaging, and other topics. I plan to attend these "boot camps" when relevant to my work and interests to continue building my technical skillset.

The Barth lab also has full access to, and daily contact with, on-premises facilities at the Molecular Biosensor and Imaging Center (MBIC), including additional imaging and electron microscopy resources (see "Equipment" section). The Barth lab has extensive on-premises animal care support from the Mellon Institute Core Veterinarians (MICV), a comprehensive and AAALAC-accredited housing and breeding facility for transgenic mice on the 2nd floor of MI. In addition to breeding space in the MICV, the Barth lab has one dedicated surgical suite and two temperature- and light-controlled rooms for behavioral testing. The Center for the Neural Basis of Cognition (*CNBC*) main office is also housed in MI, down the hall from my laboratory. The CNBC program combines the resources of CMU and the Center for Neuroscience graduate program at Pitt in a unique environment with >20 basic and translational neuroscience faculty offices as well as diverse education and training facilities and a dedicated library space.

Office and Computer

I have dedicated access to two offices: 1) a workspace in the sponsor's laboratory, situated directly across from the thesis mentor's office, and 2) a designated desk in a secluded office space reserved for graduate students. I have full access to all hardware and software needed for data entry and analysis pertinent to this proposal, including:

- Sole use of a Dell Intel Xeon computer linked to the CMU mainframe
- Access to all lab-shared computers (Windows desktop computer, Dell laptop and iPad for portable use, two workstations for 3D neuronal reconstructions)
- Software for electrophysiological data collection (MultiClamp 700B Commander, IgorPro 6.23)
- Software for image processing (QCapture, ImageJ, Adobe Illustrator, Inkscape)
- Software for data analysis (Origin 2017, GraphPad Prism, Matlab 2017)

The CMU mainframe includes a VPM with free access to a large library of statistical software including SPSS, Matlab and Simulink, as well as tutorials and software for video editing, reference management, programming and data sharing. The CMU Biological Sciences department has a dedicated library staff (including Melanie Gainey, a neuroscience PhD and expertise in electrophysiology) to assist students with project design, data sharing and archiving, record keeping, and manuscript preparation. As a member of the CNBC, I can also receive, upon request, a one-time reimbursement of $1000 to purchase a personal or office computer, should additional hardware become necessary.

The Barth lab also has access to technical support and IT services in the Department of Biological Sciences. Al Scheuring, our Systems Administrator, is available to assist with computer hardware issues, software upgrades, mainframe support, and data storage.

Statistical Consulting

I will perform all statistical analyses myself, with statistical advice from CNBC faculty who specialize in neural data analysis. The lab has regular contact and collaboration with eminent researchers in neurostatistics (Rob Kass, PhD; Brent Doiron, PhD) and electrophysiological data interpretation (Steve Chase, PhD, Carl Olson, PhD). In addition, the Pitt Health Sciences Library System (affiliated with the School of Medicine) and graduate Department of Statistics at Pitt provide statistical and IT-oriented consulting in design, execution, analysis, and reporting of general research studies. I will also benefit from intellectual expertise in clinical and translational research design, analysis, and reporting through the Pitt. Institute for Clinical Research Education and the Clinical and Translational Science Institute. These resources will hone the skills in comprehensive research planning and analysis that I will need for success in translational neuroscience.

Clinical

This is Dr. Barth's first time mentoring an MSTP student. However, my committee benefits from the strong clinical perspective of Susanne Ahmari, MD, PhD (committee member, collaborator), an accomplished young physician-scientist affiliated with the Western Psychiatric institute and Clinic and the Department of Psychiatry at the Pitt Medical Center (UPMC). Dr. Ahmari is both a dedicated clinician and researcher with lab space in the well-equipped and nearby BST and Bridgeside Point research facilities at UPMC.

My thesis committee also includes experts in translational research. Aryn Gittis, PhD (committee chair, referee) is a young investigator focused on developing new therapies for movement disorders. My MSTP career advisor, Teresa Hastings, PhD (referee), is a senior researcher with expertise in defects of cell metabolism in in neurodegeneration, and will participate on my thesis committee as a non-voting member.

During my PhD training, I will complete two longitudinal clinical clerkships in psychiatry and neurology. These outpatient clerkships (consisting of one half-day per week for 20 weeks) combine hands-on patient contact with one-on-one mentoring by a practicing physician-scientist. Clinical facilities relevant to this project, where I will perform my LCCs, include:

--the Western Psychiatric Institute and Clinic, one of the nation's top-ranked psychiatry training programs and largest freestanding psychiatric hospitals, with dedicated wings for schizophrenia and acute psychosis treatment as well as mood disorders, eating disorders, pediatric services and dual-diagnosis (mental illness and substance abuse). As the largest, most comprehensive psychiatric facility in western Pennsylvania, these facilities offer access to a uniquely large and varied clinical population and a superb faculty base.

--the UPMC Departments of Neurology and Neurosurgery, specializing in surgical and outpatient maintenance of epilepsy, stroke, and movement disorders. The neurosurgery department is one of the nation's top five training programs and has the highest patient volume of any neurosurgical program in the country. Both departments have a renowned research base in functional anatomy, and their combined facilities include a high-definition fiber tractography core, a large postmortem brain bank, and non-human primate facilities focused on studying cortical anatomy and plasticity. The neurology department has extensive facilities at the UPMC main hospital as well as the large and comprehensive nearby care centers at the Pittsburgh Veteran's Administration, the Children's Hospital of Pittsburgh, and the nearby UPMC Shadyside and Mercy hospitals.

These combined facilities provide comprehensive neurological and psychiatric diagnosis, prevention and early detection, nutrition, education, genetic counseling, physical and occupational therapy, palliative care, complementary and alternative approaches, and behavioral counseling in inpatient and outpatient settings. The majority of UPMC's teaching faculty are MD or MD-PhD physician-scientists, and this overlap of roles creates an ideal mentoring environment for MSTP trainees.

Outside of my LCCs, I have maintained contact with these programs by attending research seminars and Grand Rounds, performing clinical research, and participating in mentored activities via the Neuroscience Area of Concentration: a "minor" in neuroscience offered by the School of Medicine. My career development will continue to benefit from the superb, copious and diverse patient care environment of UPMC.

Contribution of the Training Environment to Success

CMU Biological Sciences is a truly cross-disciplinary research department, drawing from CMU's extensive computational and technological expertise as well as Pitt's broad expertise in translational cell biology.

My experimental training will take place in the Mellon Institute, which houses the CMU departments of Biological Sciences, Chemistry and Physics at CMU. The Biological Sciences department has strong expertise in basic cell biology, computational genomics, and developing novel tools for genetic manipulation and visualization of biological systems. CMU Biology has the benefits of a small research department at an

undergraduate institution (faculty availability, small graduate class sizes, ample teaching opportunities and lab assistance from undergraduates) while maintaining strong connections to Pitt and UPMC. Neuroscience training is an area of active expansion within the department, supported by the CMU BrainHub initiative (formerly directed by my mentor, Alison Barth) to increase collaboration between computational and basic research labs as well as support public engagement and outreach.

The "Great Hall of Brain Science" in MI, which includes the Barth lab and several others, is a tight-knit environment with shared space, monthly joint lab meetings, and daily collaboration with an enthusiastic and driven group of faculty and students. The MI is located very close to Pitt (two blocks away) and the UPMC main campus (five blocks away), facilitating my LCCs as well as networking with faculty at these institutions.

The University of Pittsburgh (*Pitt*) is a major academic medical center with a well-established record of high quality, federally funded research. Pitt consistently ranks among the top 10 recipients of NIH funding and has recently risen to the fifth-highest recipient; the University and its affiliates received over $315 million in NIH support in 2017. Pitt's many research centers devoted to neuroscience create an exceptional training environment that is particularly strong in the areas of sensory biology, movement disorders, computational research, and neuroimaging. Pitt's programs for graduate medical education are highly regarded, with the Psychiatry and Neurosurgery departments consistently ranking in the top 10 programs nationally. Pitt is truly a collaborative place, as evidenced by its close ties to CMU. I have found the faculty to be extremely accessible, and will continue to make invaluable connections with leading neuroscientists and physician-scientists throughout my training.

The University of Pittsburgh Medical Center (*UPMC*) is a non-profit health system comprising over 30 hospitals and 2700 physicians. In 2016, UPMC was ranked 12th nationally in *U.S. News and World Report's* Honor Roll of America's Best Hospitals. UPMC provides a large, diverse patient population for translational studies. Further, UPMC provides a dynamic hospital system for medical students to train. No other medical schools have access to UPMC facilities, guaranteeing access to the world-renowned faculty and resources of UPMC for Pitt medical students. Since there are few non-UPMC healthcare facilities in the region for specialized care, Pitt students encounter a diverse patient population from rural and urban areas within a ~150 mile radius of Pittsburgh, encompassing all of western Pennsylvania as well parts of West Virginia, New York, and Ohio. UPMC's generous financial support of biomedical research at the University of Pittsburgh further stabilizes research funding. Overall, UPMC contributes substantially to the financial and translational success of research at the University of Pittsburgh, in addition to providing excellent clinical training to MSTP students (see "Additional Education Information" for MSTP details).

The Pitt-CMU Center for the Neural Basis of Cognition (CNBC) is one of the nation's largest and most well-regarded cross-institutional programs in neuroscience. The program includes graduate students and faculty from Pitt's Center for Neuroscience (CNUP) and CMU Biological Sciences as well as departments of Computational Biology, Computer Science, Biomedical Engineering, Psychology, Statistics, Philosophy, and Machine Learning. The program includes over 200 faculty across numerous departments at Pitt, CMU, the CNBC, and UPMC, and numerous opportunities to interact with these faculty at multiple seminar series on both general and specialized neuroscience topics. The CNUP is supported in part by a large NIH pre-doctoral training grant and was one of eight centers nationwide to participate in the Carnegie Foundation's Initiative on the Doctorate to assess graduate education in neuroscience. The CNBC's unique setting fosters tight collaborations with researchers in UPMC departments of Psychiatry, Neurology, Neurosurgery, Nursing, Physical Therapy and Biomedical Engineering, other departments on campus, and other universities and institutes.

The CNBC offers regular seminars, retreats and opportunities for networking and data presentation. As part of the CNBC, I retain access to all neuroscience training facilities at Pitt, and have frequent interactions with a diverse faculty body at neuroscience seminars and retreats. The staggering amount of CNBC, UPMC, Pitt and CMU-hosted conferenced have allowed me to participate in numerous regional meetings headguartered in Pittsburgh on topics as diverse as deep brain stimulation (Neuroethics of Implantable Brain Stimulation Devices, 2017), statistical analysis of neural data (SAND, 2017), brain development studies to improve early childhood education (Neurons to Neighborhoods, 2016), and applying emerging research technologies to solve large-scale health problems (White House Frontiers Conference, 2016). These incredible opportunities will allow me to form lasting networks of engaged colleagues across diverse disciplines, in neuroscience and all connected fields, and become truly adept at cross-disciplinary collaboration and communication.

Equipment

Electrophysiology: The Barth laboratory contains about 1,800 sq. ft. of space and includes four acute brain-slice electrophysiology rigs designed for fluorescence-visualized, whole-cell patch clamping. In addition to equipment shared with the lab, **I have sole use of a patch clamping station** containing the following:

- A fixed-stage Olympus BX51 WI microscope with fluorescence capabilities including an XL Fluor4x/340 0.28 N/A objective for fluorescence visualization at low magnification, green and blue optical filters, brightfield illumination, a mercury lamp for fluorescent imaging, and a Retiga CCD camera system for visualized whole-cell recordings
- Dual Sutter MP-225 electrode micromanipulators and headstages for paired whole-cell recordings (Molecular Devices)
- 1 Multiclamp 700B two-channel amplifier (Molecular Devices), 1 filter/digitizer (National Instruments)
- 1 Plexon instruments white-light LED, 1 Prizmatix LED power supply, and a Master-8 controller for optogenetic stimulation at multiple wavelengths
- A Dell Xeon desktop PC for data acquisition and analysis
- 1 vertical micropipette puller
- A large workshop of electrical and power tools

The lab also has one in vivo electrophysiology set-up for extracellular single-unit recordings. This set-up consists of a Knopf stereotaxic frame with mouse adaptor, an FHC temperature controller, a Zeiss OPMI 1-FC surgical microscope, a CED Micro 1401 A:D board, a Neurolog amplifier and pulse generator, Hitachi V-252 oscilloscope, and a dedicated Dell desktop PC for data acquisition. The Barth lab also has one dedicated setup for 2-photon-guided targeted patch-clamp and juxtacellular recording in living animals, including a laser (Mai-Tai; Spectraphysics) and integrated microscope system (Femtonics). The Femto2D 2-photon laser scanning microscope is based on an Olympus BX61WI microscope, with laser control software Mai Tai 2.x and data acquisition through MES v.5.2878, and custom adjustments for in vivo imaging and recording. Three recording microelectrodes (Luigs & Neumann SM-7 remote control and SM10) are mounted on a Luigs & Neumann X-Y translator. The system is mounted on a Newport ST series smart table with IQ damping technology. The recording set-up is equipped with a Tektronix DPO 2004B Digital Phosphor Oscilloscope and electrophysiological data are acquired using two, dual-headstage Axon CNS MultiClamp 700B amplifier (Molecular Devices). Imaging and recording data is collected by a system-dedicated Dell desktop PC. The 2-photon microscope has a dedicated surgical preparation area for making craniotomies, including a digital display Kopf stereotax (Model 923-B), with a specialized mouse anesthesia head mount (Scivena Scientific anesthesia innovations M3000), a surgical microscope (Carl Zeiss OPMI 1-FC), a high-intensity fiber-optic illuminator (Edmund Optics MI-150), a hand drill, and heating pad to maintain animal temperature (FHC).

General Equipment: Shared facilities in immediate proximity provide an additional micropipette puller, a dry ice chest, O_2/CO_2 tanks for tissue oxygenation, and a horizontal micropipette puller. Space and fixtures include two fume hoods, a dark room, a confocal imaging suite room, and dedicated rooms in the animal facility with temperature and lighting control for behavioral testing. The lab also possesses a Leica SM 2000R freezing microtome for basic histology, a deli case with electrical supply for immunohistochemistry, and assorted water baths and incubators. Equipment for molecular biology includes heat blocks, shakers, microcentrifuges, incubators, and a -80°C freezer. The lab has an RT-PCR machine and gel electrophoresis equipment dedicated for animal genotyping. Additional light and confocal microscopes are shared between the Barth lab and the Department of Biological Sciences, where Dr. Barth holds a faculty appointment.

Histology and imaging: The Barth lab contains all equipment I will need to complete my anatomic experiments. The lab contains a high-resolution confocal microscope (ZEISS Airyscan LSM 880), a fume hood for mouse perfusions, a tissue prep area, 2 incubator-shakers, and an extensive library of antibodies for postmortem immunostaining. Through the lab's collaboration with the Molecular Biosensors and Imaging Center (MBIC) at CMU, we have access to multiple light microscopes, a temperature- and CO2- controlled time-lapse imaging microscope, Leica, Olympus, and Zeiss confocal and two-photon microscopes, and other equipment necessary for advanced multichannel fluorescent microscopy as well as Clarity, Brainbow and expansion microscopic techniques, should this level of anatomic characterization become necessary.

Office space and software: All Barth lab students and associated personnel have dedicated office space. I will work at my electrophysiology set-up, and use a separate office space with PCs containing analysis software for

off-line data analysis. The Barth lab has two licenses for the Imaris image analysis program (Bitplane), with two high-performance computers and >10 TB storage space dedicated for image analysis and 3D modeling of confocal images. The Barth laboratory has individual PCs associate with each electrophysiology set-up (4 total), plus additional computers for microscopy and lab use (6 total). The lab also has an established relationship with the Pittsburgh Supercomputing Center for high performance computing, adding to the computational power of the lab.

Vivarium: The Barth lab maintains ~200 mouse cages in a dedicated breeding facility in the MI Central Vivarium, located on the second floor of the Mellon Institute. Our breeding facility houses 27 different transgenic lines, with space to accommodate at least 100 more cages. This facility is equipped for BSL-3 level virus work, and this proposal will use BSL-1 pathogens. My proposed work will use 9 transgenic mouse strains, all of which are currently breeding successfully in the facility.

Media and Library Services: At CMU, I have full access to extensive services including 3D printing and poster printing via the CMU Media Services office, as well as numerous licenses for biostatistics and cloud-based data storage. I also have access to the Pitt main campus and health sciences library systems, which provide a suite of office software as well as literature search, data analysis, and scientific writing tutorials and consultation services.

Core and Affiliated Facilities: The Barth lab has an ongoing collaboration with the Pfenning molecular biology lab at CMU, which allows us to do fluorescence-activated cell sorting (FACS) to isolate specific cell types in brain tissue and analyze cell-type-specific differences in cellular features via ATAC-seq, RNA-seq, and high-throughput proteometry. We also have ongoing collaborations with Yongxin (Leon) Zhao, an expert in expansion microscopy. My affiliation with the Pitt system grants me access to over 30 core facilities for mouse genotyping, genomic and proteomic analysis, cell culture, and advanced fluorescent tissue analysis, should these become necessary. Relevant Pitt facilities include a Proteomics and Mass Spec Core with comprehensive DNA sequencing capabilities; a Peptide Synthesis Facility, services for Tissue and Research Pathology, a Biostatistics Core for statistical and computer-related expertise in design, execution, analysis, and reporting of research studies, an extensive Neuropathology research core. Additional clinical research expertise is also available through the Pitt Institute for Clinical Research Education and the Clinical and Translational Science Institute.

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

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POSITION TITLE:	MSTP Trainee

EDUCATION/TRAINING INSTITUTION AND LOCATION	DEGREE *(if applicable)*	START DATE MM/YYYY	END DATE *(or expected)* MM/YYYY	FIELD OF STUDY
Arkansas State University (ASU), Jonesboro, AR	B.S.	08/2007	05/2010	Biology
	B.A.	08/2007	05/2010	Chemistry
University of Pittsburgh (*Pitt*), Pittsburgh, PA	M.D.	06/2014	05/2022	Medicine
Carnegie Mellon University (*CMU*), Pittsburgh, PA	Ph.D.	07/2016	05/2020	Neuroscience

A. Personal Statement

I am a second-year PhD student in the Pitt-CMU MSTP. My career goal is to identify mechanisms of complex cognition as a principal investigator at a major medical center, and share my knowledge with patients and students in a part-time teaching practice. I want to study neural circuits and use my findings to improve the diagnostic criteria for cognitive disorders, refine neuromodulatory therapies, and improve quality of life for patients with severe disease. The rigorous training plan I have designed with my thesis mentor (Alison Barth), the MSTP and many scientific mentors will help me design, execute and communicate this incisive set of experiments into the actions of acetylcholine in cortical networks, a topic with direct applications to my interest in neurologic and psychiatric disease mechanisms.

Dr. Barth is an expert in synapse physiology of the neocortex, and my thesis committee includes strong translational research expertise and substantial input from a successful physician-scientist (Susanne Ahmari, MD, PhD, Associate Professor, Pitt/UPMC Psychiatry). Dr. Barth's lab is dynamic and fast-paced. In my first year, I contributed to a paper in *Neuron*, and saw that acetylcholine (ACh) receptors in the mouse barrel cortex are precisely expressed in particular synaptic compartments. My proposed work expounds on these findings. I will assay postsynaptic responses to ACh across cortical layers, test whether an enigmatic group of cholinergic cortical neurons contributes to these responses, and assess whether endogenous ACh strengthens thalamocortical inputs.

My training plan capitalizes on the extensive facilities and mentorship resources of Pitt, CMU, and their unified neuroscience training program, the Center for the Neural Basis of Cognition. These centers are at the forefront of technology development in brain research and are an ideal place to train. I will also take advantage of Pittsburgh's superb clinical instruction. In years 3-4 of my PhD, I will do two longitudinal clerkships with practicing physician-scientists in neurology and psychiatry to maintain my clinical skills and learn to successfully balance patient care and research.

I have always been interested in neuromodulation. After graduating from Arkansas State University at age 19, I studied reward-related plasticity and drug abuse for 4 years before joining the MSTP. I was a post-baccalaureate IRTA fellow at the National Institute on Drug Abuse, a research assistant at the University of Maryland School of Medicine, and a technician at the Center for Translational Neuroscience at the University of Arkansas. I have received exceptional training so far. I am an author of 7 publications and have presented my work at more than 20 regional and national conferences. I love making scientific discoveries. I am excited to continue training in the vibrant, intersectional environment of CMU and Pitt. The field of neuroscience is rapidly evolving and I look forward to contributing to it as a young investigator.

B. Academic and Professional Honors

2005-10 ASU University Honors, Dean's List, President's List, *Summa Cum Laude (GPA 4.0/4.0)*
2008 ASU Honors Summer Internship in Biotechnology *(competitive 10-week research fellowship)*
2009 ASU Private Scholarships (3 endowed scholarships for academic distinction, totaling ½ tuition)
2010 Chancellor's Scholar (*Most distinguished graduate, ASU College of Science & Mathematics*)
2010 ASU B.S. Biology Pre-Professional Award (*Most distinguished graduate in that major*)
2010 ASU B.A. Chemistry Award (*Most distinguished graduate in that major*)
2010 Phi Kappa Phi and Phi Theta Kappa interdisciplinary honor societies
2011-13 **Post-baccalaureate Intramural Research Training Award (IRTA)**, NIDA, NIH
2012 1st Prize Trainee Poster, NIDA Intramural Research Program Annual Research Symposium
2013 Outstanding Poster, NIH Postbac. Poster Day

2014-16 **Medical Scientist Training Program trainee (T32 NRSA)**, Pitt MSTP
2015 2nd place poster, Autopsy Discovery Program, UPMC Department of Pathology
2016 Finalist, Randall Family Big Idea Competition for Entrepreneurs
2016 Blast Furnace, Pitt Innovation Institute *(9-week business incubator; declined*)
2017 White House Frontiers Conference at CMU (*closed invitation, Personalized Medicine section*)

B. Contributions to Science

1. **Cholinergic modulation of neocortical microcircuits.** For my PhD in the Barth lab (Fall 2016-present), I am comparing the effects of acetylcholine (ACh) from diverse sources in the mouse barrel cortex, and testing whether ACh has targeted effects on thalamocortical synapses. In a recent paper, I used paired whole-cell recordings with optogenetics in acute brain slices to show that endogenous ACh alters cortical networks differently from cholinergic agonist drugs. Excitatory synapses onto PV interneurons are strengthened by an ACh agonist but not endogenous ACh release, whereas excitatory synapses onto SST cells are strengthened by both ACh and agonists via presynaptic nicotinic receptors and G-protein-coupled signaling **(Urban-Ciecko et al. 2018)**. I have found that endogenous ACh release activates excitatory and inhibitory neurons across cortical layers. I will use these cells as an assay to gauge the contribution of extrinsic and intrinsic sources of ACh to their intrinsic properties and responses to thalamic inputs.

   Peer-Reviewed Publications:

   Urban-Ciecko J, Jouhanneau J-S, **Myal SE,** Poulet JFA, Barth AL (2018). *Precisely timed nicotinic activation drives SST inhibition in neocortical circuits. Neuron* 97(3): 611-625. PMID: 29420933

   Myal S, Barth AL (2018). *Rapid, layer-specific responses to endogenous acetylcholine in neocortical interneurons.* In preparation.

   Selected Abstracts:

   Myal S, Urban-Ciecko J, Barth AL (2017). *Cholinergic modulation of PV interneuron activity in sensory neocortex*. Posters: Barrels 30; Society for Neuroscience (SfN).

   Audette N, Matsushita M, **Myal SE**, Grant R, Bernhard S, Barth AL (2017). *Automated sensory association training causes input-specific thalamocortical plasticity in mouse barrel cortex*. Posters: Barrels 30; SfN.

2. **Neural circuitry of reward and drug abuse.** Before joining the MSTP, I worked with Patricio O'Donnell, MD, PhD at the University of Maryland School of Medicine (2013-2014). I used targeted intracranial drug injections during a sucrose drinking task in rats to show that striatal metabotropic glutamate receptors underlie developmental differences in reward seeking behavior (**Myal et al. 2015**). As an IRTA fellow at the National Institute on Drug Abuse (NIDA, 2011-2013), I worked with Roy Wise, PhD to show that cocaine's actions in the peripheral nervous system are an important learned cue during drug taking **(Wang et al. 2013)** and showed that opiates most effectively reduce heroin craving, causing "satiety," when they act in the posterior VTA and rostromedial tegmental nucleus, areas rich in mu-opioid receptors **(Steidl et al. 2015)**. At NIDA, I also trained with Eugene Kiyatkin, MD, PhD, and used *in vivo* amperometry with implanted biosensors to show that striatal glutamate and glucose levels fluctuate distinctly during drinking behavior, likely due to the engagement of inhibition **(Wakabayashi et al., 2015)**.

   Peer-Reviewed Publications:

   Myal S, O'Donnell P, Counotte DS (2015). *Nucleus accumbens injections of the mGluR2/3 agonist LY379268 increase cue-induced sucrose seeking following adult, but not adolescent sucrose self-administration*. Neuroscience 305: 309-15. PMID: 26241341

   Steidl S, **Myal S**, Wise RA (2015). *Supplemental morphine infusion into the posterior ventral tegmentum extends the satiating effects of self-administered intravenous heroin*. Pharmacology, Biochemistry & Behavior 134: 1-5. PMID: 25913296

   Wakabayashi KT, **Myal S,** Kiyatkin EA (2015). *Fluctuations in Nucleus Accumbens Extracellular Glutamate and Glucose during Motivated Glucose-drinking Behavior: Dissecting the Neurochemistry of Reward.* J. Neurochemistry 132(3): 327-341. PMID: 25393775

Wang B, You ZB, Oleson EB, Cheer JF, **Myal S,** Wise RA (2013). *Conditioned contribution of peripheral cocaine actions to cocaine reward and cocaine-seeking*. Neuropsychopharmacology 38(9): 1763-9. PMID: 23535778

3. **Mechanisms and outcomes of neuromodulatory therapies**. During medical school (2014-15), I worked closely with a practicing neurosurgeon-scientist to track neuropsychological outcomes of deep brain stimulation for patients with essential tremor, dystonia and Parkinson's disease (Advisor: R. Mark Richardson, MD, PhD, FAANS, Associate Professor and Director, Adult Epilepsy and Movement Disorders Surgery Program and Brain Modulation Laboratory, UPMC Department of Neurosurgery). After college (2010-11), I worked with Edgar Garcia-Rill, PhD (Director, Center for Translational Neuroscience, University of Arkansas for Medical Sciences) to develop a rat model of transcranial magnetic stimulation therapy for nicotine abuse and investigate potential neuroprotective properties of the stimulant modafinil.

Conference Proceedings:

Saad A, Harty S, Richardson R, **Myal S**, Pardini J, Henry L (2017). *Emotion and Cognition in Movement Disorders: Comparing Pre-operative Deep Brain Stimulation Patients with Parkinson's Disease and Essential Tremor*. Archives of Clinical Neuropsychology 32(6): 667–765. https://doi.org/10.1093/arclin/acx076.68

Selected Abstracts:

Myal S, Hayar AM, Buchanan R, and Garcia-Rill E (2011). *Effect of repetitive transcranial magnetic stimulation (rTMS) on nicotine-induced suppression of P13 auditory evoked potential.* Poster: SfN.

Bisagno V, Smith K, Urbano FJ, Custer CE, **Myal S**, Lozama A, Prisinzano TE, Fantegrossi WE, Randolph M, and Garcia-Rill E (2011). *Automated gait analysis in mice after administration of an acute toxic dose of methamphetamine and treatment with modafinil*. Poster: SfN.

4. **Medical education and entrepreneurship.** In medical school (2016), I collaborated with a chronic pain specialist and a neurosurgeon (Debra Weiner, MD, Associate Professor of Medicine and Psychiatry, UPMC Institute on Aging; Raymond Sekula Jr., MD, MBA, Associate Professor and Director, Cranial Nerve Disorders Program, UPMC Neurosurgery) to create a business model for a scientifically validated chronic pain tracker for mobile devices. Our prototype was selected as a finalist in an entrepreneurship competition sponsored by the University of Pittsburgh Innovation Institute, and we were invited to develop our product in a competitive summer incubator.
As president of a neuroscience-focused medical student group (2014-16), I worked with a resident neurosurgeon (Nitin Agarwal, MD, PGY-4, UPMC Neurosurgery) to organize hands-on training events to increase residency preparedness and documented student outcomes for a paper on medical education.

Clinical Publications:

Kashkoush AI, Feroze R, **Myal S**, Prabhu AV, Sansosti A, Tonetti D, Agarwal N (2017). *Fostering Student Interest in Neurological Surgery: The University of Pittsburgh Experience.* World Neurosurg. PMID: 28866067

D. Research Support

NIH T32GM008208-26/27 06/2014-2016 PI: Richard Steinman, PhD Role: MSTP Student Trainee

E. Professional Society Membership

2010- Society for Neuroscience
2014- American Medical Association
2014- American Medical Student Association
2015- American Association of Neurological Surgeons, Women in Neurosurgery
2015- American Psychiatric Association
2016- American Physician Scientist Association

F. Scholastic performance

Graduate Courses (GPA: 4.03/4.33)

Year	Title	Grade
Carnegie Mellon University Biological Sciences, Center for the Neural Basis of Cognition		
2017	Cognitive Neuroscience	A-
2017	Proposal Preparation and Peer Review	S
2016-	Graduate Seminar and Research Seminar	S
2016	Statistical Models of the Brain	A
2016	MATLAB mini-course	S
2016	Advanced Cellular Neuroscience	A+
University of Pittsburgh School of Medicine		
2016	Family Medicine Clerkship	P
2016	GI/Endocrine/Hematology/Skin and Musculoskeletal/Reproduction & Development	S
2015	Neuroscience/Psychiatry	S
2014-16	Introduction to Patient Care 1-4	S
2014-16	Patient, Physician & Society 1-3: Ethics, Behavioral Medicine, Population Health	S
2014-15	Basic Science Fundamentals 1-3	S
2014	Evidence-Based Medicine (Clinical Statistics)	S
2014	Body Fluid Homeostasis	S
University of Pittsburgh MSTP		
2017	Ethics for Physician-Scientists	S
2016	Professional Development III: Grant Writing	S
2015-	MSTP Seminar	S
2015-16	Laboratory Research Rotations 2 and 3 (Alison Barth, PhD)	S
2015	Professional Development II: Methods and Analysis	S
2015	Research Basis of Medical Knowledge 1-2	S
2014	Scientific Reasoning 1	S
2014	Laboratory Research Rotation 1 (Aryn Gittis, PhD)	S
2014	Professional Development I: Molecular Medicine	S
Elective Coursework		
2016-	CNBC Brain Bag seminars in research ethics	N/A
2016	Benchtop to Bedside: A Scientist's Guide to Commercialization	S
2015-16	Motivational Interviewing I-II	S
2015	Mind-Body Seminar Series, MS1	S
2015	Autopsy Discovery Program, UPMC Pathology	S
2015	Palliative Care Clinical Experiences	S
2014-	Neuroscience Area of Concentration, Univ. of Pittsburgh School of Medicine	N/A

Undergraduate Courses (GPA: 4.0/4.0)

Year	Title	Grade
Arkansas State University, Jonesboro, AR		
2011	Independent Study: Transcranial Magnetic Stimulation	A
2010	Applied Statistics 1	A
2010	Microbiology	A
2010	Quantitative Analysis	A
2010	Physical Chemistry Survey	A
2010	Honors Thesis: Salmonella Serovar Detection	CR
2009	Genetics (+ Lab)	A
2009	Introduction to Pathology	S
2009	Biology Seminar	A
2009	Honors Organic Chemistry 2 (+ Lab)	A
2009	Honors Pathophysiology	A
2009	Physical Diagnosis	A
2009	Descriptive Inorganic Chemistry	A
2009	Honors Biochemistry	A
2009	Accelerated Intermediate Spanish 1	A
2008	Honors Principles of Ecology	A
2008	Honors Science in the Cinema	A
2008-9	Organic Chemistry 1 (+ Lab)	A
2008-9	General Physics 1 and 2 (+ Labs)	A
2007-8	Human Structure and Function 1 and 2 (+ Labs)	A
2007	Honors Biology of the Cell (+ Lab)	A
2007	Honors Biology of Plants (+ Lab)	A
2007	Honors Elementary Spanish II; Elementary Spanish 1	A
2007-8	General Chemistry 1 and 2 (+ Labs)	A
2007	Biology of Animals (+ Lab)	A
Ozarka Technical College, Ash Flat, AR		
2007	English Composition 2	CRE
2007	English Composition 1	A
2007	Developmental Psychology	A
2007	World Literature 1	A
2007	Survey of Calculus	A
2007	General Biology (+ Lab)	A
2006	Intro. to Studio Art	A
2006	Computer Information Systems	A
2006	World Civilization 1	A
2006	American History 2	A
2006	Intro. to Sociology	CRE
2006	Intro. to Geography	A
2006	College Algebra	A
2006	Physical Science (+ Lab)	A
2005	Cultural Anthropology	A
2005-6	Fine Arts Visual, Fine Arts Theater	A
2005	Communications	A
2005	Concepts of Physical Activity	A
2005	General Psychology	A

Grade legend:

"S" = Satisfactory (for courses graded Satisfactory/Unsatisfactory)

"P" = Pass (for clerkships graded Fail/Pass/High Pass/Honors)

"CR" = Credit for fulfillment of honors thesis

"CRE" = Credit by CLEP examination

"N/A" = Not graded or not applicable

OMB No. 0925-0001 and 0925-0002 (Rev. 09/17 Approved Through 03/31/2020)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Alison Louise Barth

eRA COMMONS USER NAME (credential, e.g., agency login): ALBARTH

POSITION TITLE: Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Brown University	A.B.	05/91	Biology
University of California, Berkeley	Ph.D.	05/97	Neurobiology
Cardiff University, Wales UK	postdoctoral	07/98	Neurophysiology
Stanford University	postdoctoral	11/01	Electrophysiology

A. Personal Statement

For over 15 years, I have been using extracellular and whole-cell patch-clamp recording techniques to analyze how neurons in the cortex can be changed over developmental time or with behavioral/sensory perturbations. My long-term interests are in the plasticity of neocortical circuits, where I have investigated the mechanisms underlying experience-dependent synaptic strengthening. I have a long track-record of developing technologies that can be used to address important questions in cellular and systems neuroscience, from the development of the fosGFP transgenic mice more than 10 years ago, to the multiplexing of fluorescent proteins for increasing fluorescence signal, to the application of single-molecule tracking technologies in the study of ion channel trafficking. Recently, we have been developing tools for synapse detection for high-throughput, machine-learning analysis of synapse properties in the mammalian brain, to provide an unbiased platform for data collection to study mechanisms of plasticity in the cerebral cortex. We are also developing tools to precisely label and quantify post-synaptic densities in the cortex from various sources, and the expertise of myself and several postdocs will benefit Stephanie in her immunostaining and imaging assays to quantify acetylcholine release sites from various sources.

Stephanie will have the full benefit of my expertise in her project to assess synapse-specific modulation of cortical circuits by acetylcholine. A major focus of our current work is cell-type specific electrophysiological recordings across the cortical column, to understand how neural circuits are rewired during brain state-dependent neuromodulation and active learning. We have shown that acetylcholine released from endogenous sources has effects that are spatiotemporally quite precise and distinct from cholinergic drugs (Urban-Ciecko…Barth 2018), suggesting that more precise studies are necessary to pinpoint the effects of acetylcholine across time in different cortical areas. We have also characterized cell type-specific projections from higher-order thalamic nuclei (Audette…Barth 2017), and are very interested in whether brain state-dependent neuromodulation can differentially control information flow into the cortex. I am proud to mentor Stephanie, as her work will benefit our understanding of basic circuit motifs as well as their neuromodulation during behavior, and serve as a foundation for our ongoing behavioral studies of cortical and thalamocortical plasticity after sensory learning.

B. Positions and Honors

Positions and Employment

Assistant Professor, Biological Sciences, Carnegie Mellon University 2002-2007
Associate Professor, Biological Sciences, Carnegie Mellon University 2008-2010
Associate Professor (with tenure), Biological Sciences, Carnegie Mellon University 2010-2014
Professor, Biological Sciences, Carnegie Mellon University 2014-present
Interim Director, BrainHub, Carnegie Mellon University 2015-present

Honors and Awards

Burroughs-Wellcome Summer Student Fellowship, June-August 1990
Magna cum laude, Brown University 1991
National Science Foundation Predoctoral Fellowship, 1991-1994
Elizabeth Roboz Einstein Fellowship, 1994-1995
Milton I. and Florence Krenz Mack Award for Developmental Neuroscience, 1996
Hitchings-Elion Postdoctoral Fellowship, Burroughs Wellcome Fund, 1997-2000
NIH Epilepsy training grant, Stanford University 2000-2001
Alfred P. Sloan Research Foundation Fellow, 2003-2005
Research Award for Innovation in Neuroscience, Society for Neuroscience, 2008
Career Development Award, Society for Neuroscience, 2008
Eberly Family Career Development Professorship, 2009
Friedrich Wilhelm Bessel Research Award, Alexander von Humboldt Foundation, 2010
NeuroCure Award, Berlin Germany 2010
Honorable mention, Emerging Female Scientist, Carnegie Science Awards, 2010
McKnight Cognitive and Memory Disorders Award, 2013
Humboldt University Talent Travel Award, 2014
Burroughs Wellcome Collaborative Research Travel Grant, 2016

Selected Professional Associations and Service

Society for Neuroscience, 1993-present
Society for Neuroscience, Program Committee member, 2013-2016
Society for Neuroscience, Government and Public Affairs Committee, 2016-present
American Epilepsy Society, 2009-present
NIH study section ad hoc member, Molecular Neuropharmacology and Signaling
NIH study section member, Learning and Memory 2011-present
reviewer, National Science Foundation
reviewer, Medical Research Council (United Kingdom)
European Union Commission Expert Reviewer
National Science Center, Poland

C. Contributions to Science

Work in my lab centers around one fundamental question: how does experience change neural function? My professional life has been directed to addressing this question at a molecular, cellular, synaptic, and systems level, with a focus on the mouse somatosensory system as a platform to investigate neural plasticity. I consider the following to be my key contributions to the field:

1. **Neuromodulation at precise spatial and temporal scales.** For more than 30 years, synaptic function has been estimated using anatomical or electrophysiological measurements under highly constrained experimental conditions. These measurements provide insight into how synapses can function, but not how they do actually perform in dynamic active neural networks. We are leading the way in understanding how synapses are modulated in complex multicellular neural networks, using precisely-timed, optogenetic release of endogenous neuromodulators to determine synapse-specific effects in the context of network activity. Our studies are revealing remarkable specificity in how neuromodulators influence synapses that was unanticipated by prior pharmacological studies. Importantly, neuromodulators frequently silence or “awaken” synapses, resulting in a functional rewiring of network structure. The fine spatial, temporal, and synapse specificity that we have observed with GABAb and cholinergic modulation suggests that this is the tip of the iceberg, and that many other neuromodulators will have similarly precise effects.

Relevant publications/related work:

a. Urban-Ciecko, J., Fanselow, E.E., and **Barth, A.L.** Neocortical somatostatin neurons reversibly silence excitatory transmission via GABAb receptors (2015) *Current Biology* Mar 16;25(6):722-31.

b. Urban-Ciecko, J., Jouhanneau, J-S., Myal, S.E., Poulet, J.F.A. and **Barth, A.L.** Precisely-timed nicotinic activation drives SST inhibition in neocortical circuits. (2018) *Neuron* Feb 7;97(3):611-625. doi: 10.1016/j.neuron.2018.01.037. PMID: 29420933.

2. ***Experience-dependent synaptic plasticity in the mouse somatosensory system.*** Our knowledge about how synapses can be changed in vitro vastly exceeds our understanding of how and when these pathways are actually engaged during normal experience. We used the fosGFP transgenic mice to identify a tiny region of somatosensory cortex that had undergone experience-dependent changes in evoked firing activity. In 2006, we were the first to discover that native AMPARs were trafficked to activated excitatory synapses – something that had been predicted but never before observed *in vivo* – and our subsequent work in 2008 defined novel changes in NMDAR function that occurred during plasticity induction. Most recently (2013), we have discovered that experience-dependent changes in glutamatergic synapses proceeds through three biochemically distinct stages.

   Relevant publications/related work:
   a. Clem, R.L., Celikel, T. and **Barth, A.L.** Ongoing *in vivo* experience triggers synaptic metaplasticity in the neocortex. (2008) *Science* 319:101-104.

   b. Wen, J.A. and **Barth, A.L.** Initiation, labile, and stabilization phases of experience-dependent plasticity at neocortical synapses. (2013) *Journal of Neuroscience* 33(19):8483-93.

   c. Chandrasekaran, S., Navlakha, S., Audette, N.J., McCreary, D., Souhan, J., Bar-Joseph, Z., and **Barth, A.L.** Unbiased, high-throughput analysis of experience-dependent changes in synaptic density in somatosensory cortex. (2015) *Journal of Neuroscience* Dec 16;35(50):16450-16462.

   d. Audette, N.J., Urban-Ciecko J., Matsushita M., and **Barth, A.L**. POm Thalamocortical Input Drives Layer-Specific Microcircuits in Somatosensory Cortex. (2017) *Cerebral Cortex*28(4): 1312-1328. PMID: 28334225

3. ***Development of the first fluorescence-based reporter for activity-dependent gene expression in rodents***. A critical question in learning and memory studies are which neurons (and which synapses) are altered by experience, a non-trivial question in even rodents that have “only” 80 million neurons. I created the fosGFP transgenic mouse to identify and electrophysiologically characterize the subset of neurons activated by sensory experience and learning. Although there have been a multitude of similar transgenic mice created that followed our initial publication in 2004, I hold the patent for fluorescent-reporters of activity-dependent transcription in mice. These animals have been broadly distributed to over 100 labs worldwide, are available through the MMRRC and the Jackson Laboratory, and have been licensed to more than 5 pharmaceutical companies over the past decade.

   Relevant publications/related work:
   a. **Barth A.L.** Neuronal Activation in a Transgenic Model. (2003) U.S. Patent Application (Patent Application Serial No.: U.S. Patent No. 8,952,213; issue date Feb. 10, 2015).

   b. **Barth A.L.,** Gerkin R.C. and Dean, K.L. Alteration of neuronal firing properties in a fosGFP transgenic mouse. (2004) *Journal of Neuroscience* 24:6466-6475.

   c. Jouhanneau, J.S., Ferrarese, L., Estabanez L., Audette N., Brecht, M., **Barth, A.L.,** and Poulet, J.F.A. Cortical cfos expression reveals broad receptive field excitatory neurons targeted by POm. (2014) *Neuron* Dec 3;84(5):1065-78.

   d. Ye, L., Allen, W.F., Thompson, K.R., Tian, Q., Hsueh, B., Ramakrishnan, C., Wang A.-C., Jennings, J., Adhikari, A., Witten, I.B., **Barth, A.L.,** Luo, L. Halpern C.H., McNab, J., and Deisseroth, K. Long-range wiring and molecular signatures of prefrontal cortex neuronal populations mediating positive or negative valence experience. (2016) *Cell* Jun 16;165(7):1776-88.

4. ***Experience-dependent changes in BK channel activity***. Activity-dependent transcription factors like c-fos regulate gene expression to alter synaptic and cellular output. In 2008, we identified the large-

conductance, Ca^{++} and voltage-gated K^{+} channel BK as an important target for activity-dependent upregulation after chemoconvulsant induced seizures, linked this upregulation to abnormal, high-frequency firing in cortical networks. In 2009, we showed that BK channel antagonists had potent anticonvulsant properties in mice. This functional upregulation is tied not to an increase in BK channel expression, but to altered expression of an accessory subunit that normally sequesters BK channels in the ER (2012). Currently we are developing methods for real-time, single-BK channel imaging in neurons.

Relevant publications/related work:

a. Shruti, S., Clem, R.L., and **Barth A.L.** A seizure-induced gain-of-function in BK channel is associated with elevated firing activity in neocortical pyramidal neurons. (2008) *Neurobiology of Disease* 30(3):323-30.

b. Sheehan, J.S., Benedetti, B.L. and **Barth A.L.** Anticonvulsant effects of the BK channel antagonist paxilline. (2009) *Epilepsia* Apr;50(4):711-20.

c. Shruti, S., Urban-Ciecko, J, Fitzpatrick, J., Brenner, R., Bruchez, M., and **Barth, A.L.** The brain-specific beta 4 subunit downregulates BK channel cell surface expression. (2012) *PlosOne* 2012;7(3):e33429, epub Mar 16.

d. Pratt, C.P., He, J., Wang, Y., **Barth, A.L.,** and Bruchez, M.P. Fluorogenic Green-Inside Red -Outside (GIRO) labeling reveals kinase-dependent control of BKalpha surface expression. (2015) *Bioconjugate Chemistry* Sep 16;26(9):1963-71.

A complete bibliography can be found at:
http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/41139421/?sort=date&direction=ascending.

D. Research Support

NIH R01NS088958 (P.I. Alison L. Barth)
No scientific or budgetary overlap
Dynamic connectivity in neocortical networks
Project goals: To understand the role of SST neurons in GABAb-mediated synaptic silencing
Project period: 4/2016-3/2020

NIH R21 NS104821-01 (P.I. Alison Barth)
Machine learning approaches for electrophysiological cell classification
Project goals: To develop a classifier to differentiate between neural subtypes based upon spike trains
Project period: 10/2017-9/2019

NIH RF1MH114103 (P.I.s Marcel Bruchez and Alison Barth
Potential overlap: additional new tools proposed that might ultimately facilitate the current research plan
High throughput approaches for cell-specific synapse characterization
Project goals: To develop transsynaptic FRET reagents for input-specific labeling
Project period: 9/2017-8/2020

NIH R21NS092019 (P.I.s Marcel Bruchez and Alison Barth)
No scientific or budgetary overlap. This work funded reagent development applied in the current proposal
Brain-scale measurements of cell-specific synaptic contacts
Project period: 4/2015-3/2017, NCE until 3/2018

NIH RF1MH114233 (P.I. Bin He, Co-Investigator Alison Barth)
No scientific or budgetary overlap.
Electrophysiological source imaging guided transcranial focused ultrasound
Project goals: To use ultrasound for spatially-restricted brain stimulation in rodent models
Project period: 2/2018 - 7/2021

PHS Fellowship Supplemental Form

OMB Number: 0925-0001
Expiration Date: 03/31/2020

Introduction

1. Introduction to Application
(for Resubmission applications)

Fellowship Applicant Section

2. Applicant's Background and Goals for Fellowship Training*	Myal_Background_and_goals.pdf

Research Training Plan Section

3. Specific Aims*	Myal_Specific_Aims.pdf
4. Research Strategy*	Myal_Research_Strategy.pdf
5. Respective Contributions*	Myal_Respective_Contributions.pdf
6. Selection of Sponsor and Institution*	Myal_Selection_of_Institution_and_Sponsor.pdf
7. Progress Report Publication List (for Renewal applications)	
8. Training in the Responsible Conduct of Research*	Myal_RCR.pdf

Sponsor(s), Collaborator(s) and Consultant(s) Section

9. Sponsor and Co-Sponsor Statements	Myal_Sponsor_Statement.pdf
10. Letters of Support from Collaborators, Contributors and Consultants	Myal_Letters_of_Support.pdf

Institutional Environment and Commitment to Training Section

11. Description of Institutional Environment and Commitment to Training	Myal_Additional_Educational_Information.pdf

Other Research Training Plan Section

Vertebrate Animals

The following item is taken from the Research & Related Other Project Information form and repeated here for your reference. Any change to this item must be made on the Research & Related Other Project Information form.

Are Vertebrate Animals Used? ☑ Yes ☐ No

12. Are vertebrate animals euthanized?	☑ Yes	☐ No
If "Yes" to euthanasia Is method consistent with American Veterinary Medical Association (AVMA) guidelines?	☑ Yes	☐ No
If "No" to AVMA guidelines, describe method and provide scientific justification		
13. Vertebrate Animals	Myal_Vertebrate_Animals.pdf	

Tracking Number:

Funding Opportunity Number: PA-18-668. Received Date:

PHS Fellowship Supplemental Form

Other Research Training Plan Information

14. Select Agent Research	Myal_Select_Agent_Plan.pdf
15. Resource Sharing Plan	Myal_Resource_Sharing_Plan.pdf
16. Authentication of Key Biological and/or Chemical Resources	Myal_Resource_Authentication.pdf

Additional Information Section

17. Human Embryonic Stem Cells

Does the proposed project involve human embryonic stem cells?* ☐ Yes ☑ No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s), using the registry information provided within the agency instructions. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s):

18. Alternate Phone Number: 870-847-7197

19. Degree Sought During Proposed Award:

Degree:	If "other", indicate degree type:	Expected Completion Date (MM/YYYY):
DOTH: Other Doctorate	M.D./Ph.D.	05/2022

20. Field of Training for Current Proposal*: 160 Neurosciences & Neurobiology

21. Current or Prior Kirschstein-NRSA Support?* ☑ Yes ☐ No

If yes, identify current and prior Kirschstein-NRSA support below:

Level*	Type*	Start Date (if known)	End Date (if known)	Grant Number (if known)
Predoctoral	Institutional	07/01/2014	06/30/2016	5T32GM008208

22. Applications for Concurrent Support?* ☐ Yes ☑ No

If yes, describe in an attached file:

23. Citizenship*

U.S. Citizen — U.S. Citizen or Non-Citizen National? ☑ Yes ☐ No

Non-U.S. Citizen
☐ With a Permanent U.S. Resident Visa
☐ With a Temporary U.S. Visa

If you are a non-U.S. citizen with a temporary visa applying for an award that requires permanent residency status, and expect to be granted a permanent resident visa by the start date of the award, check here: ☐

24. ☐ Change of Sponsoring Institution — Name of Former Institution:*

PHS Fellowship Supplemental Form

Budget Section

All Fellowship Applicants:

25. Tuition and Fees*:

☐ None Requested ☑ Funds Requested

Year 1	$44,500.00
Year 2	$45,500.00
Year 3	$46,500.00
Year 4	$47,500.00
Year 5	$0.00
Year 6 (when applicable)	$0.00
Total Funds Requested:	$184,000.00

Senior Fellowship Applicants Only:

	Amount	Academic Period	Number of Months
26. Present Institutional Base Salary:			

27. Stipends/Salary During First Year of Proposed Fellowship:

	Amount	Number of Months
a. Federal Stipend Requested:		

	Amount	Number of Months
b. Supplementation from Other Sources:		

Type (e.g.,sabbatical leave, salary)

Source

Appendix

28. Appendix

APPLICANT'S BACKGROUND AND GOALS FOR FELLOWSHIP TRAINING

A. Doctoral Dissertation and Research Experience

Undergraduate: As a biology and chemistry dual major at Arkansas State University (2007-2010), I worked for three years in an applied food microbiology research lab at the Arkansas Biosciences Institute, a facility specializing in agricultural and cell biology. During three summers (40h/week, 10 weeks, supported by a $4000 honors research award) and semesters (~20h/wk), I independently developed simple, rapid assays to detect and differentiate six common, pathogenic strains of foodborne *Salmonella enterica*. I worked under the mentorship of Soohyoun Ahn, PhD to create immunomagnetic bead-based and PCR-based detection assays and validation techniques. I mentored three undergraduate students and two Master's degree students in assays I designed, and was an author on a poster at three annual meetings of the Institute for Food Technologies. I became interested in clinical neuroscience based on my own reading, and sought post-baccalaureate research experience in this area.

Post-baccalaureate: After college (2010-2011), I worked as a research technician at the University of Arkansas for Medical Sciences (UAMS) Center for Translational Neuroscience (CTN), studying neural mechanisms of repetitive transcranial magnetic stimulation (rTMS). rTMS is FDA-approved for the treatment of refractory depression, and clinical studies at the CTN were exploring its efficacy as potential therapy for tinnitus, cigarette smoking, and other conditions. Using rats, I tested whether rTMS administered at high frequency (>1 Hz, thought to generally increase cortical activity) could attenuate the acute effects of nicotine in decreasing auditory-evoked EEG potentials. I was fascinated by rTMS and its mechanisms, which are still poorly understood and are now being investigated for their ability to preferentially activate different cortical cell types and fiber tracts based on the angle and strength of the magnetic field. This project involved a high amount of technical independence. I found no protective effect of rTMS treatment on intracortical EEGs modulated by nicotine, and no deleterious effects on other evoked potentials, supporting a large body of work suggesting that rTMS is not harmful to cortical function. I also helped develop a gait analysis program and behavioral assay to test whether the non-addictive stimulant modafinil could counteract the acute behavioral effects of methamphetamine, and this projet resulted in my first poster presentation at the 2010 Society for Neuroscience (*SfN*) meeting in San Diego, CA. My projects were co-managed by four PIs - Drs. Roger Buchanan, Robert Skinner, Abdallah Hayar and **Edgar Garcia-Rill (referee)** – whose enthusiastic mentorship inspired me to become a physician-scientist, to investigate and treat fundamental problems in human health.

I decided to follow my interest in drug abuse research to the NIH, where I did a post-baccalaureate IRTA fellowship at the National Institute on Drug Abuse (NIDA) intramural research campus in Baltimore, MD (2011-2013). For my first 18 months at NIDA, I worked with Roy Wise, PhD on several projects, on which I achieved an increasing level of independence from my advisor and postdoctoral mentors and contributed substantially to experiments and manuscript preparation. In one paper (**Steidl et al. 2015**), I examined the distribution of heroin "satiety sites" in the ventral tegmental area (VTA) of the rat midbrain, comparing the efficacy of morphine injections into anterior, posterior, and far posterior "tail" areas of the VTA in reducing operant responding for intravenous (iv) heroin. When trained rats lever-press to receive a drug injection, they wait a fairly stable period before pressing the lever again. This is the "satiety period," and its length is predictable based on the drug's half-life in the body. Opiates presumably have rewarding effects by inhibiting GABAergic neurons in the VTA to disinhibit nearby dopamine cells. When we infused opiates into the VTA by reverse dialysis to increase "satiety period" between self-administered (iv) opiate injections, we discovered that the duration of satiety increased when morphine was given in the "tail" of VTA, confirming recent studies showing the importance of this area in opiate-related behaviors.

In another paper (**Wang et al 2015**), I studied the actions of cocaine methiodide (MI), a blood-brain-barrier-impermeable form of cocaine (COC). COC is presumably rewarding via its blockade of dopamine (DA) reuptake in the brain. MI cannot penetrate the brain, so it fails to trigger DA release in COC-naïve rats. However, in rats previously exposed to COC there is strong DA release, perhaps due to a conditioned association between the "fast," second-scale actions of COC in the peripheral nervous system and COC's "slow," minute-scale central rewarding actions. Over time, DA release shifts forward to coincide with early reward-predictive cues, so this "conditioned peripheral cue" may explain why human addicts feel a "high" immediately after taking COC, even though the drug takes minutes to affect the brain. I handled the "relapse" portion of this project. Switching COC for MI in rats trained to self-administer COC caused them to gradually stop lever pressing, but made them more resistant to start pressing again (reinstate, or "relapse") after COC

injections than control rats switched with saline. By uncoupling this peripheral cue from the central rewarding actions of COC, MI treatments decreased COC-induced relapse & could be useful in treating COC addiction.

I found this "sensory cue" aspect of cocaine fascinating. The lab of Eugene Kiyatkin, MD, PhD at NIDA was studying a similar process with fixed-potential amperometry, measuring MI's effects on glutamate (GLU) levels in the nucleus accumbens shell (NA) of the forebrain in awake, behaving rats. They found that GLU activity (a proxy measure of excitatory neurotransmission in the accumbens) also increases when MI is given to COC-experienced animals, but not COC-naïve animals. Successive MI injections habituate this increase, while successive COC injections cause the GLU signal to increase further, reiterating the importance of COC in altering excitation. Amperometry offered second-scale resolution, and a chance for me to get closer to watching the brain work in real time. I spent my last 6 months at NIDA on this project, and another (**Wakabayashi et al. 2015**) to examine changes in NA GLU & glucose during motivated drinking behaviors. GLU currents measured in amperometry reflect mostly extracellular activity, and glucose currents provide a general picture of regional energy supply & consumption. NA glucose signals appear to increase during and after sensory stimuli (e.g., tail pinch) or sustained behavior (e.g., drinking). However, GLU signals *decrease* during drinking behaviors, suggesting that the increase in metabolism in the NA during this behavior is driven by inhibitory activity that suppresses GLU release. My projects at NIDA were extremely valuable scientific learning experiences, and I practiced communicating my science at multiple poster sessions at Johns Hopkins Hospital, the NIH, SfN, and the Society of Biological Psychiatry.

Next, I joined the lab of Patricio O'Donnell, MD, PhD at the University of Maryland School of Medicine. Here I deepened my interest in investigating mechanisms of appetitive behavior, taking the lead on a project to examine developmental differences in excitatory neurotransmission during sucrose consumption. The lab was exploring a rather counterintuitive finding: that adult rats self-administer more sucrose (becoming more "hooked" on the appetitive substance) than adolescent rats, contradicting the popular notion that adolescence is a time of highest susceptibility to appetitive stimuli. In the adult rats but not adolescents, the ratio of AMPA to NMDA glutamate receptors decreased in the core region of the NA, suggesting that glutamate receptor signaling might underlie this age difference. I tested this hypothesis using targeted bilateral intracranial injections of a glutamate receptor agonist during a reinstatement task, in which rats trained to drink sucrose either as adolescents or as adults were deprived of sucrose for 3 weeks before having the chance to self-administer it again for one more session. I found that, indeed, during this reinstatement session, the agonist (which targeted group 2/3 metabotropic GLU receptors) increased sucrose seeking behavior, but only in adults **(Myal et al. 2015)**. This suggests that age-dependent changes in metabotropic (G protein-coupled) glutamate receptor activity in the nucleus accumbens core region play an important role in responses to appetitive stimuli – and potentially addiction susceptibility – across adulthood and adolescence.

MSTP Rotation #1 (Gittis lab): When I matriculated to the MSTP in summer 2014, I did my first research rotation with **Aryn Gittis, PhD (referee).** I examined basal ganglia circuit changes in the early stages of Parkinson's Disease (PD) using a partial dopamine depletion model in mice. Parkinson's Disease is a very common and debilitating movement disorder that does not present clinically until 80-90% of all dopamine cells have been lost, despite the accumulation of subtle, subclinical pathology for many years. It is unclear why it takes so long for PD symptoms to manifest, and one hypothesis that Dr. Gittis continues to explore is that the substantia nigra (the site of dopamine cell loss) has numerous redundant connections with the striatum (its major output area). Basal ganglia circuits downstream of the substantia nigra may be able to compensate for a very large amount of dopamine loss, but begin to decompensate at extremes of dopamine loss. We wanted to test whether a basic property of the striatum – the presence of long-term depression of synaptic inputs to D2 receptor-expressing striatal inhibitory neurons – would be changed by a "preclinical" amount of dopamine loss. To test this, I injected small amounts of the toxin 6-OHDA into the substantia nigra, killing ~50% of the dopamine cells, and performed patch clamp electrophysiology in fluorescently labeled D2-expressing neurons. We found that LTD was unchanged with 50% dopamine loss, a finding that supports the hypothesis that striatal circuits are robust to asymptomatic amounts of dopamine depletion. In this experience I first learned patch clamp techniques, and became familiar with the field of movement disorders research and novel treatments such as deep brain stimulation (DBS). I presented my work at the Pitt MSTP Retreat in August 2014.

MS1-2 experiences (Richardson lab): During MS1-2, I performed clinical research on neuropsychological outcomes of DBS with **R. Mark Richardson, MD, PhD**, in the UPMC Department of Neurosurgery, examining neuropsychological effects of DBS on cognitive function. I worked closely with Dr. Richardson, the

department's neuropsychologist (**Jamie Pardini, PhD**) and her trainees to collect, collate, and analyze a large battery of neuropsychological testing data collected before and after DBS surgery on all patients with movement disorders who had undergone DBS in the past 5 years: over 200 patients with PD, essential tremor, and dystonia. We hypothesized that, in addition to the huge motor benefits most patients reports after surgery, there may also be subtle cognitive improvements after stimulation. It is known that DBS is linked to small speech articulation deficits, but these do not seem to be correlated with the brain area targeted by the stimulation and could relate to other aspects of the surgical procedure or general anesthesia. Preliminarily, we found that DBS patients tend to have depressive symptoms and may have positive emotional outcomes after surgery; however, these could be related to improved motor functioning and quality of life. We reported this in a conference paper (**Saad et al. 2017**) and are continuing to collect longitudinal follow-up data to properly control for effects of age, medication changes, and other factors to more precisely compare pre- and post-operative changes. Doing clinical research during my preclinical coursework was an incredibly valuable experience that exposed me to a fascinating patient population. It redoubled my interest in understanding higher-order cognition from a basic neural circuits perspective. Dr. Richardson's practical perspective as a surgeon-scientist and the acknowledgement of the field of just how little we still know about the cellular mechanisms of DBS were a major inspiration for me to pursue a basic science thesis related to cognition and psychiatric disease.

MSTP Rotation #2 (Barth lab): I met Dr. Barth during my first lab rotation, and knew by the end of that summer that I would be returning to the Great Hall of Brain Science to work in her lab. With Dr. Barth, I explored a pilot project related to the lab's ongoing work on neural circuits of thermal sensation, and assessed whether the primary somatosensory cortex (S1) was required for the detection of cold stimuli. The TRPM8 receptor, expressed on nerve endings in the skin, is specifically activated by non-painful, mildly cold stimuli. Activation of TRPM8 receptors causes We used menthol (a highly specific ligand of TRPM8, and the reason "cool mint" flavors actually do taste cool) to activate this receptor while silencing S1 optogenetically in awake mice, using a chronically implanted optic fiber to activate transgenically expressed, cell type-nonspecific, light-gated chloride channel halorhodopsin to silence S1. I performed the viral injections, implanted the optic fibers, and adapted two behavioral assays (tail-flick and conditioned place preference) to specifically test responses to cold stimuli before and after S1 silencing. We found no effect of continuous S1 silencing on cold stimulus detection, measured by latency for the mouse to flick its tail out of a bowl of menthol solution, or time spent on either the "warm" side or the "cool" side of a temperature-controlled behavioral chamber. These data match the literature on human stroke patients, who can still detect warm and cool stimuli after massive lesions to S1, and spurred our interest in other brain regions (such as the posterior insula, see below) which might be "where" information about cold stimuli is processed in the brain. I presented my work at the Pitt MSTP Retreat in August of 2015.

MSTP Rotation #3: During my third MSTP rotation with Dr. Barth, I examined the cortical representation of light, pleasant touch. The mas-related G protein-coupled receptor B4 (MgB4) is a unique genetic marker for a set of neurons, with nerve endings in the hairless skin, that respond to light, stroking touch, and activating these neurons optogenetically appears to be rewarding for mice. We were interested in which brain regions were responsible for this representation of pleasurable touch. However, the sensation of touch has many sub-modalities associated with it, such as temperature, pressure, and vibration. We planned to use optogenetic techniques for "touch-less" activation of only the MgB4 receptor, activating nerve endings in the skin using a similar approach to the one I took in my first rotation. We suspected (based on my and the lab's prior work with cold sensation) that the primary somatosensory cortex would be less involved, and that the posterior insula, a more ventral structure that performs multisensory integration and affective roles, would show the most activation, when we immunostained the tissue for the immediate early gene c-Fos. I synthesized the literature to design all aspects of this project, and worked that summer and in the subsequent year to troubleshoot an alkaline phosphatase assay to locate MgB4 neuron arbors in the skin. Concurrently, I learned to perform whole-cell patch clamp experiments, and am now training undergraduate students to pursue the MgB4 project.

Dissertation research: For my PhD in the Barth lab (Fall 2016-present), I am comparing the effects of acetylcholine (ACh) from different sources across cell types in the mouse barrel cortex, and testing whether ACh has targeted effects on thalamocortical synapses. Once I mastered single-cell recordings in brain slices (which took about a month, since I had patched before in a rotation with Dr. Gittis), I began performing paired recordings in Fall 2016 to isolate excitatory synapses onto PV inhibitory neurons in the mouse S1 cortex. We were curious whether endogenous ACh release, induced optogenetically in ChAT-ChR2 mice, would have similar effects to cholinergic agonist drugs, which enhance excitation onto many types of interneurons. Finding connected pairs of neurons was quite challenging, and required an acute awareness of the spatial relationships between cells in the cortical column, informed by the literature and hands-on experience. I

ultimately found that excitatory synapses onto PV interneurons are strengthened by ACh agonist drugs but not endogenous ACh, whereas excitatory synapses onto SST interneurons are strengthened by both ACh and agonists via presynaptic nicotinic receptors and G-protein-coupled signaling. My experiments and analysis were a key part of the lab's recent paper in *Neuron* **(Urban-Ciecko et al. 2018)**, and the subject of data presentations at SfN and Barrels (2017). In following up on this study, I found (see **Research Strategy**) that endogenous ACh release activates diverse cell types across cortical layers, and that cholinergic VIP cells may be quite common and important for cholinergic signaling. I will use ACh responses in diverse cell types as an assay to gauge the contribution of intrinsic and extrinsic ACh sources to total ACh responsivity in S1. The narrative of my thesis and other first-author publications will be based upon the data I collect from the experiments outlined in the Specific Aims of this proposal. The hypotheses and experiments herein have all been explicitly discussed with and approved by the members of my thesis committee, who will provide me advice and support in achieving these Aims. This project will be the most immersive and exciting yet in my extensive body of research experiences. I have begun to develop a strong, unique skillset as a translational cellular neuroscientist, and will make excellent use of this diverse prior experience and ongoing mentorship in completing the proposed Aims.

B. Training Goals and Objectives

Career goals: My overall career goal is to do translational research on the neural circuitry of complex cognition and behavior as an NIH-funded independent investigator, care for patients with neuropsychiatric disease, and teach students, residents, and fellows at an academic medical center. Upon completing my MD/PhD training, I plan to pursue residency training in psychiatry or neurology followed by a subspecialty fellowship. I will seek out a residency program that offers substantial protected time for research, and use this period to collect data for a mentored K award to bridge my transition to a junior research appointment at an academic medical center. I hope to use the majority of my time to run a research lab (80-90% of schedule), and also see patients, perform outcomes studies, and teach (10-20%). I plan to pursue a "bench to bedside to bench" approach, generating novel research toward diagnostic and therapeutic interventions based on my interactions with patients, and gearing my experiments toward accelerated clinical applications as much as possible. As a "bilingual" clinician-scientist, I hope to form new collaborations that could not take place without my training. The intersectional training environment of the Barth lab, CMU, Pitt, and the MSTP will give me ample skill and knowledge to meet these goals.

Scientific and technical skill development: I will spend ~85% of my remaining PhD time doing experiments, performing all data analysis, and preparing results for publication (see **Tables 1 and 2**). The methods I will learn in the proposed work will develop my technical acumen as well as core scientific reasoning skills I will rely on for the rest of my career, if or when these methods become obsolete. With the aid of my collaborators (Susanne Ahmari and Bryan Hooks), Dr. Barth, and various senior students in the lab, I will develop my skills in **whole-cell patch clamp electrophysiology, pharmacology and optogenetics** to manipulate cholinergic and thalamocortical signaling in different cell types, adding dual-color optogenetic stimulation to my technical repertoire. I will also learn **advanced histologic and imaging techniques** in fixed brain tissue, including 3D modeling of neurons and putative synapses, from Dr. Barth and two postdocs experienced in this technique. Applying the techniques outlined in this proposal will also teach me complementary skills, including immunohistochemistry, stereotactic survival surgery, software-based behavioral analysis, and sophisticated concepts in anatomy, pharmacology, and physics relating to electrophysiology. The pairing of these structural and functional approaches to investigate neural circuits will enforce a multifaceted and mechanistic view of neurophysiology. Learning about these basic circuits, which are conserved from rodents to humans and which are the substrates of all psychoactive drugs, will lay a powerful foundation for my understanding of neural mechanisms of complex behaviors and drug actions that will allow me to approach clinical problems in a thoughtful way.

Apart from my own research, this proposal comprises many activities that will enhance my knowledge of cellular neuroscience topics and their applications to clinical practice (see **Sponsor and Co-Sponsor Information, Contribution of Training Environment to Success**). I have worked closely with Dr. Barth, my thesis committee, the CMU Biology department and the MSTP to assemble a comprehensive plan to gain more experience in the intellectual skills I will need to carry out the scientific method: form hypotheses, design experiments, anticipate statistical concerns ahead of time, analyze data appropriately, and critically interpret results in the context of a rapidly evolving literature, among other skills.

Dr. Barth is a very respected electrophysiologist and synaptic biologist with publications in *Neuron,* top journals in the field. She is energetic, very committed to mentoring students scientifically and professionally. I

currently meet with Alison for 1-2 hours each week outside of our 3-hour lab meetings. Alison is very well-supported by her neuroscientist peers in the "Great Hall of Brain Science" at CMU and the larger environment of the CMU Biology Department, the CNBC, and Pitt. All are well-equipped to provide superb, multidisciplinary training on their own, and I will continue to leverage their combined resources to achieve success in my research and set a strong foundation for the next stages of my clinical and research independence.

C. Activities Planned Under This Award

Coursework and Graduate School Milestones: **I have already completed all required semester courses** from the Department of Biological Sciences at CMU and the Graduate Training Program in the Center for the Neural Basis of Cognition (CNBC). I have completed the teaching assistantship and course grading assignments required by my department, and passed the qualifying exam for my thesis proposal. I am also finished with the graduate portion of my MSTP coursework. ***I expect to spend >85% of my remaining PhD time completing the proposed experiments*** (see **Table 2**) and the remainder of the time attending lab-related seminars and meetings, performing two longitudinal clerkships, and continuing to serve the MSTP on its Curriculum Committee and other student-led initiatives to improve training resources for students.

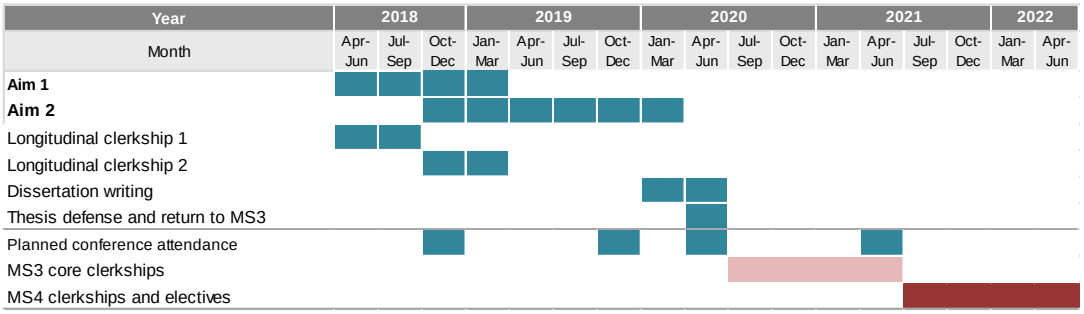

Table 1. Activities and program milestones to be completed during the award period.

Year 1: I will spend 85% of my time in 2018-19 conducting research in the laboratory of Dr. Barth. I plan to perform the experiments proposed in Aim #1 preceding, and concurrently with, Aim #2. I will receive training in the techniques necessary for my proposed studies from senior members of the laboratory and from consultants **(see consultant letters).** An additional 5% of my time will be spent completing Longitudinal Clinical Clerkships (LCC, 5 hours/week for 20 weeks) in neurology and psychiatry. In my LCC I will work as a member of a clinical team delivering care to patients suffering from movement disorders, epilepsy, schizophrenia mood disorders, and other neurologic and psychiatric conditions, a strong parallel to the studies proposed in this application. Finally, the remaining 10% of my time will be spent furthering my professional and scientific development. In 2018, I will present my findings at the Society for Neuroscience and Barrels meetings. I will regularly attend seminars on campus sponsored by the CNUP and Department of Psychiatry. I will also attend the annual MSTP, CMU and CNBC retreats.

Year 2: I will spend 90% of my time in Year 2 completing Aim #2. I will spend an additional 5% of my time completing a second LCC at WPIC, and The remaining 5% of my time will be spent furthering my professional development through attendance at scientific meetings (the American Academy of Neurology, SFN, Biolgoical Psychiatry), departmental seminars, program retreats, and local conferences including WPIC Research Day. Year two will culminate with my thesis defense in March-May 2020.

	G3	G4	MS3	MS4
Activities planned under award	**Year 1**	**Year 2**	**Year 3**	**Year 4**
Research	85	83	12	12
Lab & MSTP Meetings	5	5	2	2
Clerkships	4	4	75	75
Seminars	2	2	5	5
Committees & Outreach	1	1	3	3
Conferences & Presentations	3	5	3	3

% effort

Table 2. Expected percent effort devoted to the proposed research aims, clinical development, and professional development activities during the

Year 3 and 4 (return to MS3 Spring 2020): In the MS3-4 years, I will devote 75-90% of my time to required clinical clerkships. I will spend 5% of my time writing up any unpublished data from my PhD and beginning to develop a research focus that I can bring with me to residency and use to apply for future funding. During this time I will also complete the Step 2CK and Step 2CS portions of the United States Medical Licensure Exams (USMLE).

Progress Meetings: I will collect and analyze my own data, and I will write up all manuscripts on my own and revise them with Dr. Barth's guidance. I will continue my standing weekly meeting with Dr. Barth, and will have

a meeting with my thesis committee (Alison Barth, Aryn Gittis, Anne-Marie Oswald and Susanne Ahmari) every six months to discuss research progress. I will also continue semiannual meetings with my career advisor, Teresa Hastings, to update her on my progress, discuss career goals, and get an impartial second opinion on scientific or personal matters that arise during my training. I will also continue to meet formally on an as-needed basis with the MSTP program director, Richard Steinman, who serves a similar advisory role.

Research Presentations: I will attend and present at both local and national conferences to cultivate my intellectual development as an investigator. I will present my results frequently at local venues, including lab meetings (giving a 3-hour data presentation approx. every 6-8 weeks), Great Hall of Brain Science meetings (one-hour research-in-progress talks, given ~annually), Barth lab journal clubs, CMU journal clubs and data presentation seminars (once per year), semiannual thesis committee meetings, and the CMU, CNBC and MSTP annual retreat events (6 weekend days per year in total). I will also present my work at Pitt's annual Science conference, the Department of Psychiatry's annual Research Day, and other events as time permits.

I will present my work at national conferences with diverse audiences, including the Society for Neuroscience and Barrels meetings, relevant Gordon Research Conferences on synaptic plasticity, and at clinical conferences in my G3-4 years (Society of Biological Psychiatry, American Academy of Neurology). For major society meetings at which I present (i.e., SfN and others), I will apply for predoctoral travel awards. The CNBC also provides up to $1000 in annual travel funds for students, as does the CMU Mellon College of Science (on a competitive basis). The Pitt School of Medicine also offers up to $1000 annually in travel awards, and I will apply for these to attend clinical conferences in the G3,G4, and MS3 years as relevant.

Seminar Participation: My professional development will continue through the MSTP, building off of the foundation I have built from the first 4-course MSTP professional development series and my coursework in the School of Medicine. I will continue to attend monthly MSTP workshops discussing ethics and career development, and will continue to plan one such workshop every year. I will attend workshops at CMU and the CNBC on scientific and professional development topics (alumni speakers, networking, data presentation, communicating science to the public, social media, big data archiving and management, research ethics). Through these experiences I will gain knowledge in diverse topics such as ethical use of animals in research, mentor-mentee relationships, and responsible data presentation and dissemination, among others. I will also pursue substantial training in the responsible conduct of research and principles of grant writing through didactic coursework at the CNBC and elsewhere **(see Responsible Conduct of Research)**.

Clinical: I will complete two Longitudinal Clinical Clerkships (LCCs), each 20 weeks long and consisting of a half-day each week of seeing patients one-on-one with an attending physician-scientist. I am planning LCCs with Dr. Edward Burton (neurology) and a senior attending at UPMC's Western Psychiatric Institute (TBA), taking advantage of opportunities to learn from master clinician-scientists who have successfully balanced their dual roles. As I prepare to return to medical school, I will take the MSTP Clinical Reentry course, which is a case-based review of skills in physical diagnosis, medical decision-making, and treatment planning led by master clinicians.

Extracurricular: I will devote time to community outreach and committee involvement through the medical school, CMU Biology program, and MSTP. My lab participates regularly in community outreach to teach underprivileged youth about science, and has spearheaded a data analysis course for high school students in underserved areas around Pittsburgh. I will continue to hold a leadership position on the MSTP Curriculum Committee, and will stay involved in this and other MSTP support roles as time permits. These experiences will bolster my ability to effectively manage teams to accomplish shared goals.

In my future career as a physician-scientist, I hope to become a skilled, compassionate clinician. During my PhD training, I will complete two Longitudinal Clinical Clerkships and the MSTP's clinical reentry elective to prepare me for the transition to my remaining clinical training years. Much of my clinical training will take place at the Western Psychiatric Institute and Clinic (WPIC), a free-standing psychiatric hospital with over 400 beds that is unique among medical centers in the US for its size and approach to patient care. In my future career, I hope to generate basic science findings that help identify new treatments for neuropsychiatric disorders. To do this, I will need to learn existing limitations of psychiatric therapies and design experiments with maximum potential to address these limitations. My training with Dr. Barth, whose studies have laid the groundwork for understanding cortical function, will be ripe for application to disease states and behavioral investigations of learning and plasticity. My translational knowledge of psychiatric illness will also be enhanced by my frequent interactions with the diverse network of scientists and clinicians in the Department of Psychiatry and WPIC.

SPECIFIC AIMS

The goal of this project is to characterize the effects of acetylcholine (ACh) on cell types and thalamocortical synapses in the cerebral cortex, and explore the role of extrinsic and intrinsic ACh sources in these circuits. ACh signaling is important for attention and stimulus detection[1–3]. Defective ACh signaling is linked to cognitive symptoms of diverse psychiatric disorders, including schizophrenia[4,5], and cholinergic agonist drugs are being explored as novel treatments for these disorders[6–8]. Traditionally, ACh was thought to have generalized effects on the brain through volume transmission[9], but recent evidence points to a much more targeted effect. In the cortex, ACh acts on specific inhibitory interneurons to increase the activity of output neurons[10,11,12,13], but the sequence of these circuit changes is still unclear. In a previous paper[14], we showed that ACh has synapse-selective effects, strengthening excitation of specific inhibitory neurons. We are interested in whether ACh release sites are preferentially located near certain cell types, and whether ACh also strengthens thalamocortical inputs.

There may also be multiple sources of ACh. The cholinergic basal forebrain is the best-known source[15–17], and it densely innervates the cortex[18,19]. However, there are also intrinsic cholinergic neurons in the cortex whose connectivity is unknown[20,21]. No one has ever compared the specific influence of extrinsic and intrinsic ACh sources, or whether they have distinct effects. We propose to use optogenetics in transgenic mice to independently drive the activity of these ACh sources and record their effects in four neuron types (pyramidal, PV, somatostatin and VIP) using patch clamp recordings in brain slices of mouse somatosensory cortex. We will complement these studies with high-resolution confocal imaging to quantify ACh release sites on specific cell types, and on synapses from two distinct thalamic nuclei. We hypothesize that extrinsic and intrinsic ACh sources make distinct contributions to ACh signaling by targeting distinct cell types and cortical layers. The ACh responsivity of higher-order thalamic inputs is unknown, and we will use two-color optogenetics to drive ACh release and thalamic inputs separately in brain slices to test whether ACh modulates thalamic signals.

Aim 1: Identify sources of ACh release and postsynaptic cholinergic activation across cortical layers. Preliminarily, we find that endogenous ACh activates nicotinic or muscarinic receptors in a cell type-specific manner. These effects could result from differences in receptor expression across cell types and/or precise targeting of ACh release sites from different sources. Using transgenic mice for precise cell type labeling, we will electrophysiologically assess responses to optogenetically-evoked ACh release. In parallel, we will use quantitative fluorescence imaging and 3D cellular reconstructions to quantify ACh inputs. We hypothesize that extrinsic and intrinsic ACh sources have distinct cellular and molecular targets.

Aim 2: Test whether endogenous ACh release enhances excitatory inputs from the sensory thalamus. Pharmacologic studies suggest that ACh strengthens thalamic inputs to cortical layer 4 via presynaptic nicotinic receptors[22]. Thus, ACh may reweight excitation to increase sensitivity to incoming sensory stimuli. Here we will test the hypothesis that endogenous ACh strengthens thalamic inputs to distinct cell types and thalamorecipient cortical layers. We will use whole-cell patch clamp recordings with dual-channel optogenetic stimulation of cholinergic and thalamic fibers to test whether ACh strengthens thalamic inputs to different cell types. Anatomically, we will test if ACh release sites are differentially associated with specific thalamic inputs.

Overall Impact: Historically, most studies of ACh effects used *in* vivo or pharmacologic methods, observing network changes over seconds to minutes. Our dual anatomic and functional approach will examine these pathways at the synapse level, with high spatial and temporal resolution. Our investigation of the neuron selectivity of cortical information processing and potential neural substrates of ACh will inform the development of new therapies to precisely treat schizophrenia and other diseases.

Contribution to Training: I hope to become a physician-scientist specializing in the treatment of complex cognitive disorders as a neurologist or psychiatrist. My proposed experiments relate directly to this goal, and will give me insight into the basic physiology of neuropsychiatric diseases. I will hone my skills in synaptic electrophysiology, adding two-color optogenetics to my technical arsenal. I will improve my surgical technique and learn immunohistochemistry and high-throughput imaging techniques. I will refine my experiments to control for potential confounders, such as sparse viral labeling of neurons and spectral crosstalk between optogenetic activators. I will practice parametric and non-parametric statistics (t and U tests, ANOVA and Kruskal-Wallis, multiple comparisons). I will continue to absorb the literature in the rapidly changing field of neural circuitry and neuromodulation, and contribute to the field as a first author. I will present my findings to solicit feedback from colleagues, and put their suggestions to work. I will obtain data and skills in this project that will greatly advance my training as a neuroscientist, physician, and future principal investigator.

A. Significance

Deficits of acetylcholine (ACh) release and signaling are implicated in cognitive symptoms of schizophrenia[5,23,24] as well as bipolar disorder[2526], Alzheimer's disease[27,28,29,30], traumatic brain injury[31,32,33], autism[34,35] and depression[36]. In schizophrenia, these cognitive symptoms include working memory and attention deficits that precede the onset of psychosis[37,38,39], persist or worsen throughout life[38], and are a better predictor of treatment non-adherence and poor outcomes than psychotic symptoms[40]. Cognitive symptoms are poorly treated by antipsychotics[41], but novel ACh agonists have shown recent promise in preclinical studies[42,5,43] and early clinical trials[6,7,8]. However, as it is unclear how ACh signaling contributes to cognition, it remains challenging to develop new drug candidates or predict which ones will be clinically successful[8,7]. We seek to characterize ACh effects on neural circuits, with the goal of informing development of mechanism-based therapies for cognitive disorders.

We and others have found that ACh tunes cortical excitation in a highly precise manner **(Urban-Ciecko et al. 2018)**. However, it is unclear how ACh modulates other types of synapses, and whether this synapse-specificity is due to the close association of ACh *release sites* with particular cell types, in addition to differences in ACh receptor expression. There may also be multiple sources of ACh. Basal forebrain ACh inputs are the best-known source[17,44], but an enigmatic subgroup of cortical ACh neurons has been described[45,20] that could also contribute to local ACh effects. In this project, we will explore the effects of ACh inputs from diverse sources on intra-cortical and thalamocortical synapses. *We hypothesize that ACh has cell type-specific and rapid effects associated with precise localization of ACh release sites and receptors*.

A1. Cholinergic circuits in cognition and schizophrenia.

ACh is rapidly released into the cortex during states of focused attention[2,46,47,48,49,50] and physical activity[51,52], and binds to ionotropic (nicotinic) and metabotropic (muscarinic) receptors[17]. Cortical ACh release improves fine-scale sensory discrimination[1,3,53,54] and is required for sensory stimulus detection in mice[18,30,55] as well as optimal cognition in humans[57]. A large body of evidence implicates cortical ACh signaling defects in schizophrenia[17,58]. Schizophrenics have decreased expression and activity of cortical ACh receptors[59,60,61,62,63,64], and cigarette smoking with nicotine (a behavior 4 times more prevalent in schizophrenia than in the general population[65,66]) transiently improves working memory and other cognitive symptoms[67,68,69,70,71,72,73.74]. New treatments for cognitive symptoms of schizophrenia are sorely needed, and will be informed by understanding how ACh controls neural circuits underlying cognition.

A2. ACh activates select cell types in the cerebral cortex.

ACh release during attention activates a disinhibitory circuit that is conserved across cortical areas (see **Fig. 1**). Here, ACh release activates neurons expressing vasoactive intestinal peptide (**VIP**)[11,10] and somatostatin (**SST**)[12], leading to inhibition of parvalbumin (**PV**) interneurons[13,75,76] and ultimately disinhibition of select groups of stimulus-encoding excitatory (Pyramidal, **Pyr**) neurons[13,77]. These neurons all express different combinations of nicotinic[59,62,80,65,66,] and/or muscarinic[81,82] receptors, though exactly which subcellular compartments contain these receptors across layers is unclear for some cell types. In a notable study[13], the insertion of a nicotinic receptor mutation found in human schizophrenics into VIP interneurons recreated a schizophrenia-like neural phenotype in mice, suggesting that ACh modulation of VIP cells is especially relevant to this circuit, and to symptoms of schizophrenia.

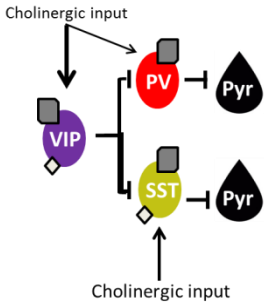

Fig. 1. Simplified micro-circuit in Layer 2/3 of S1. Boxes = nicotinic receptors. Diamonds = muscarinics. Line thickness indicates relative input strength. Adapted from Koukouli et al. 2017.

A3. Endogenous ACh release strengthens excitatory synapses.

Recent transgenic and optogenetic tools have helped reveal cell type- and synapse-specific ACh effects. In a recent paper[14], we showed that endogenous ACh strengthens Pyr-to-SST synapses (but not Pyr-to-Pyr or to PV), via presynaptic nicotinic receptors, acting at precise sites to increase excitation. We hypothesize that other cortical and thalamocortical synapses are strengthened by ACh in a similar manner. The proposed experiments will test this hypothesis anatomically by quantifying ACh release sites and functionally, via *in vitro* patch clamp recordings and optogenetics.

A4. Cholinergic VIP neurons: a secondary source of ACh?

We will also test whether ACh inputs from diverse sources can distinctly influence cortical circuits. In addition to dense, well-studied ACh inputs from basal forebrain nuclei[12,45,46,47] (see **Fig. 2A-B**), there are intrinsic cholinergic neurons in the cortex[23,24,75,76,77] (**Fig. 2C**) whose connectivity is unknown. Single-cell transcriptomics studies show that these are VIP neurons[86] that express enzymes for ACh and GABA synthesis[87]. However, it is unclear whether they actually release ACh or GABA[20,84,85]. These *cholinergic VIP neurons (cVIPs)* constitute ~33% of VIP cells in S1[87], and their intrinsic properties are similar to non-cholinergic VIPs[20]. In general, VIPs

get basal forebrain inputs[88], are clustered in layer 2/3, project downward to layers 4-6[89], and selectively innervate SSTs[90,91]. cVIPs may do this as well. Basal forebrain neurons can co-release ACh and GABA[92], and cVIPs may also[93,94,95]. cVIPs would be well-positioned to provide locally-gated ACh input to particular cell types (possibly SSTs) either in synch with, or independently of, basal forebrain ACh. In Aim 1 we will compare the contribution of intrinsic (cVIPs) and extrinsic (basal forebrain) sources to overall ACh innervation and responses.

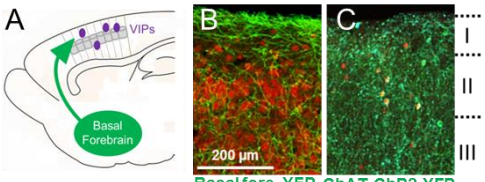

Fig. 2. A) Sources of cortical ACh. Basal forebrain inputs predominate, but cholinergic VIPs may also contribute. **B)** Basal forebrain axons (green) in pre-frontal cortex. Adapted from Bloem et al. 2014. **C)** ACh axons (ChAT-ChR2-YFP, green), VIP neurons (mCherry, red), and cVIPs (overlay, yellow) in Layer 2/3 of S1.

B. Preliminary Studies

B1. ACh responses are common and distinct in S1.

Many landmark studies of cortical ACh effects were done *in vivo*, examining network changes on a timescale of seconds to minutes[12,51,13,96]. Thus, the exact temporal sequence of ACh-mediated network changes remains unclear. To determine which cell types are activated by ACh at millisecond timescales, we performed patch clamp recordings in brain slices of S1 in ChAT-ChR2-YFP transgenic mice (expressing channelrhodopsin in choline acetyltransferase-expressing [ChAT] axons) and evoked ACh release using minimal stimulation (single 10ms blue light pulses, 470 nm).

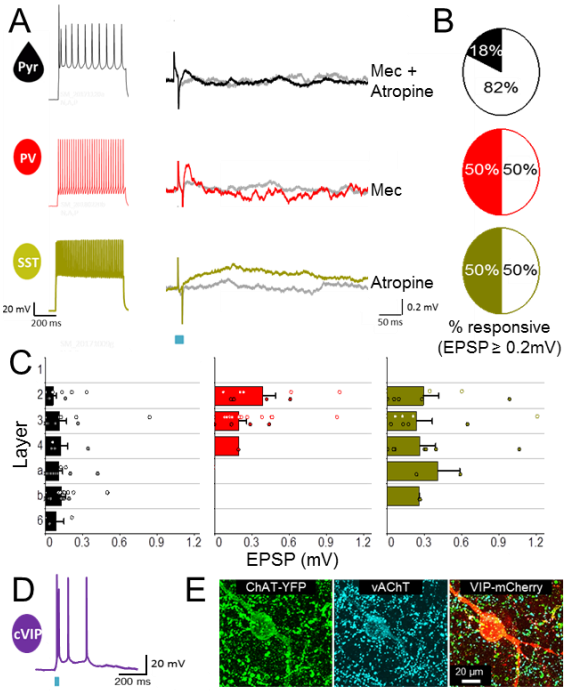

Fig. 3. Distinct responses to endogenous ACh across cell types in ChAT-ChR2 mice (crossed with PV-Cre, SST-Cre or VIP-Cre for flex-AAV fluorescent labeling of interneurons). **A)** Firing phenotype (left) and EPSP (mean of 10 sweeps) to a 10 ms light flash (blue box). Colored trace = baseline. Gray trace = response to ACh antagonists mecamylamine (*Mec*), atropine, or both. **B)** Shaded area of pie charts = percent of cells with EPSPs ≥ 0.2mV. **C)** EPSP size across cortical layers. Open circles = no drugs. Closed circles = recordings in NBQX, AP5 and picrotoxin to block AMPA, NMDA and GABA$_A$ receptors. **D)** ACh-mediated spiking in a putative cVIP cell. **E)** Triple-labeling of a c VIP cell with AAV-flex-mCherry and antibodies for ChR2-tagged YFP (green) and vAChT (blue).

About 50% of SST and PV neurons show rapid excitatory postsynaptic potentials (EPSPs) in response to ACh, compared to 18% of Pyr neurons (**Fig. 3B**). Interestingly, though SSTs are known to express nicotinic and muscarinic receptors[13,80,97], we see mainly slow-rising responses in SSTs that are blocked by atropine, a muscarinic antagonist (**Fig. 3A**, gold trace). Also of interest, we see a fast-peaking response in PV cells that is blocked by the nicotinic antagonist mecamylamine (**Fig. 3A**, red trace). As in prior reports, Layer 2 Pyr neurons have small, infrequent ACh responses[87], and deep-layer Pyrs have larger, more consistent ACh responses[100] (**Fig 3C**) with mixed nicotinic and muscarinic ACh features (**Fig. 3A,** black trace). We will continue this cell type-specific characterization in Aim 1.

In examining ACh effects on VIPs, we encountered cholinergic VIPs. These cells have rapid-onset (<3 ms) ChAT-ChR2 photocurrents and spiking (**Fig. 3D**) that persist in ACh antagonists and the sodium channel blocker tetrodotoxin (not shown), and are immunolabeled with antibodies against the vesicular acetylcholine transporter (vAChT; **Fig. 3E**). *Thus, transgenic ChAT-ChR2 activates both basal forebrain axons and cholinergic VIPs*, and prior studies (including our own) using these mice may have conflated the effects of activating these two populations. If cVIPs release ACh, then both basal forebrain and cVIP activation will each represent a fraction of the "total" ACh responsivity seen with ChAT-ChR2. In Aim 1 we will test this hypothesis. We predict that cVIPs release ACh, and that ACh release from extrinsic sources and cVIPs will target distinct cell types and cortical layers.

C. Approach

C1. Specific Aim 1: Identify sources of ACh release and cholinergic activation across cortical layers.

Hypothesis 1a: ACh release sites from diverse sources preferentially localize near distinct cell types.

Rationale: Cortical circuits are densely innervated by the cholinergic basal forebrain[15,101,102.103]. Most of these inputs are not canonical synapses, but rather "partner-less" axon terminals without adjoining postsynaptic densities[45]. This observation, coupled with the poor spatiotemporal resolution of microdialysis[2,9,104] and pharmacologic[22] studies, led to an old assumption that ACh was released diffusely into the extracellular space, and achieved cell type-specificity solely via ACh receptor expression on target neurons. However, we recently showed[14] that endogenous ACh has very different effects from pharmacologic ACh receptor activation. The

ACh agonist carbachol facilitates Pyr-to-PV excitatory synapses but endogenous ACh does not, even when high-frequency stimulation (12-20Hz) is used to increase synaptic spillover. Bath-applied drugs permeate the tissue, "reaching" receptors located in areas inaccessible to endogenous neurotransmitter release. We hypothesize that **ACh *release sites* may be concentrated near specific cell types and compartments, an input-specificity that contributes to precise ACh effects.**

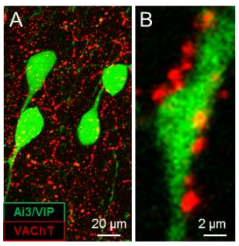

Fig. 4. vAChT inputs to a VIP neuron. **A)** Merged confocal image showing dense vAChT axon staining (red) in S1. **B)** Inset of a single confocal section in (A). vAChT puncta (red) surround the surface of a VIP dendrite (green).

Experiment 1a: Anatomic characterization of ACh release sites from extrinsic and intrinsic sources onto cortical cell types. Basal forebrain ACh inputs to the cortex have been qualitatively but never quantitatively mapped[88], and cVIP neuron connectivity is unknown. *Our goal is to quantify and compare ACh release sites from diverse sources onto specific target cells and subcellular compartments*.

We will label presynaptic (basal forebrain or VIP) neurons and postsynaptic cortical neurons using injections of AAV-encoded fluorophores in transgenic mice (see **Fig. 5**). In fixed tissue of young mice (P21-25), we will immunolabel ACh boutons with antibodies against vAChT in a third color (blue, Alexa 405). Pairing vAChT and presynaptic stains will let us distinguish between the two ACh sources. We will take confocal z-stack images of cells of interest and use Imaris software to create 3D renderings of these neurons and nearby dual-labeled (vAChT + presynaptic) fluorescent puncta. These models let us precisely count ACh release sites and assess their distance from the target cell. Associated vAChT puncta will be counted per compartment (axon, soma, and dendrites) and normalized to cell surface area to yield a puncta density value for comparison across cells and cell types.

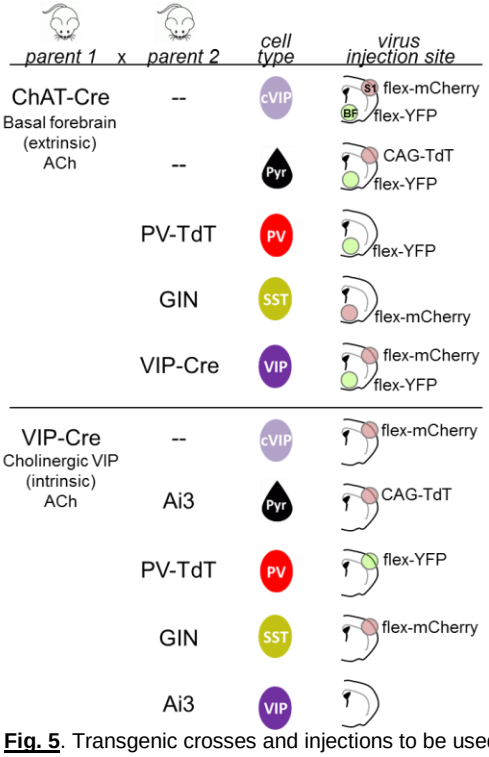

Fig. 5. Transgenic crosses and injections to be used for vAChT immunostaining and imaging, Aim 1.1. Extrinsic (ChAT-Cre) or intrinsic (VIP-Cre) ACh sources in S1will be labeled along with their postsynaptic targets via transgenic or injected AAV-fluorophores. Est. n=8 cells from N=2 mice per group (N=20 total).

Predicted results and interpretation: Based on preliminary confocal images (showing dense vAChT innervation of VIPs; **Fig. 4**), we expect that VIPs will receive dense extrinsic ACh input, possibly more than onto SST or PVs. We also expect that the cVIP subpopulation will receive basal forebrain inputs, and thus be recruited by extrinsic ACh. However, if basal forebrain inputs avoid cVIPs, this could suggest that cortical ACh neurons are active under different brain state conditions, perhaps in response to local inputs. We predict that cVIPs will have the same output distribution as regular VIPs (i.e., sparse lateral axons in Layer 2/3 and dense projections to layers 4-6) and will innervate SSTs. If this is the case, then SSTs would be privileged in their access to *local* ACh inputs, which may influence their activation during attention. If cVIPs instead target PV or Pyr cells, this distinction from non-cholinergic VIPs would suggest a distinct function for these neurons.

Alternative outcomes and potential pitfalls: Our use of viral injections to label presynaptic neurites may underestimate ACh inputs if the labeling is sparse. In VIP-Cre mice, we label 40-60% of VIPs with AAV2. However, this should be sufficient for cellular reconstructions, where sparse labeling is desired to reduce background signal. However, we acknowledge that sparse labeling may deflate our absolute estimate of ACh inputs from a particular source, in relation to the overall vAChT signal. In early studies, we find that viral labeling of cortical cholinergic VIPs in ChAT-Cre mice is sparse (not shown). Since we can identify cVIP neurites using vAChT in VIP-Cre mice, we have opted to use VIP-Cre in most cases to maximize viral labeling. Use of the GIN line (which labels Martinotti-type SST cells[105,106,107]) may limit our study of SSTs to Layers 2 and 5, where Martinotti cells are concentrated[105,106,107]. By using these mice, we exclude X94 and some X98-type SST cells, which we may have sampled in our preliminary data. Generally, we acknowledge that it is hard to predict the functional effect of ACh release from imaging alone. Thus, we will also compare ACh effects from diverse sources electrophysiologically.

Hypothesis 1b: ACh from diverse sources elicits distinct effects across cortical cell types.

Rationale: Our preliminary studies used ChAT-ChR2 to activate both basal forebrain and VIP ACh release, yielding the "total" ACh responsivity of the two sources. We will test whether basal forebrain or VIP-derived ACh causes different postsynaptic responses, favoring specific cell types and layers.

Experiment 1b: Electrophysiological survey of ACh effects from diverse sources. We will continue the approach described in our preliminary data to classify ACh responses in ChAT-ChR2 mice cross-bred with PV-TdT, GIN or VIP-Cre at allow fluorescent targeting of these neurons. Next, we will examine extrinsic and intrinsic ACh sources separately via a similar combination of mice and injections to Aim 1.1, except we will inject an AAV-encoded opsin (ChR2) into the ACh source of interest (basal forebrain or S1; see **Fig. 6**). In young mice (P21-25), we will record from fluorescent PV, SST or VIPs, or non-fluorescent Pyr neurons (identified by morphology and firing phenotype) and measure EPSPs in response to single or multiple light pulses to trigger ACh release. These recordings will be done in NBQX, AP5 and picrotoxin to block AMPA, NMDA and GABA-mediated activity. (Thus, when all VIPs are photo-stimulated, any depolarizing response should be cholinergic). If an EPSP is observed, we will serially add ACh antagonists to test whether the response disappears under nicotinic or muscarinic blockade.

Predicted results and interpretation: We expect our electrophysiological results to parallel the anatomic results in Aim 1.1, our preliminary data, and prior studies[13,80,88]. The basal forebrain likely represents the vast majority of cortical ACh inputs[19,108,109]. Thus, photoactivation of basal forebrain axons may elicit responses of similar amplitude to our preliminary data, and in a similar percentage of cells, and may strongly activate VIP and cVIP cells (high response probability, >1mV EPSP) via nicotinic receptors. We predict that activating VIP cells will produce cholinergic EPSPs in Layer 2/3 SST and PV cells, and frequent, strong EPSPs in Layer 5 SSTs.

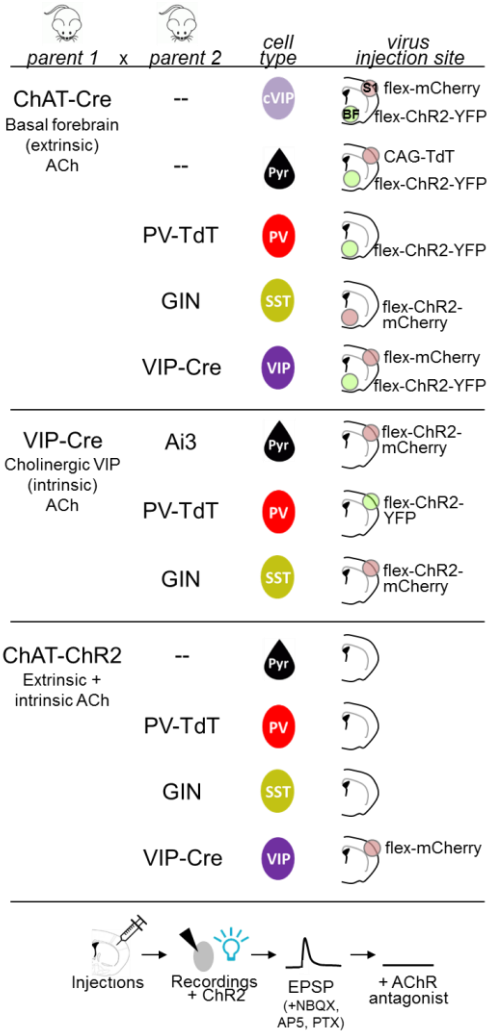

Fig. 6. Transgenic crosses and injections to test effects of ACh release from intrinsic and extrinsic sources in whole-cell recordings, Aim 1.2. Extrinsic (ChAT-Cre) or intrinsic (VIP-Cre) ACh sources will be targeted for injections of AAV-ChR2. Bottom panel: experimental workflow. Est. n=8 cells from N=4 mice needed per group (total N=28).

Alternative outcomes and potential pitfalls: cVIPs may release little to no ACh[20], in which case, we might not see cholinergic EPSPs after VIP photo-activation. The transgene used to create the ChAT-ChR2 line contains a copy of the vAChT gene, leading to vAChT overexpression in this line[110]. Thus, we and others may be overestimating ACh effects due to increased release. In this case, basal forebrain and VIP-evoked responses in the ChAT-Cre and VIP-Cre lines (which do not overexpress vAChT[111]) may yield smaller, more physiologic ACh response patterns.

C2. Specific Aim 2: Quantify cholinergic modulation of thalamocortical excitation.

ACh is capable of tuning intra-cortical excitability[51,12–14,100], and may also directly modulate thalamocortical synapses[22]. The mouse S1 is innervated by two thalamic nuclei. The ventral posterior medial (VPM) nucleus is a "lower-order" structure that relays spinothalamic sensory information to PV, SST, VIP, Pyr[88], and other cells in layers 4 and 6. The posterior medial (POm) nucleus is a "higher-order" structure, which receives cortical layer 5 and brainstem inputs[112,113] and innervates Pyr and VIP cells in Layers 2 and 5[88], and Layer 5 PV cells[114]. ACh release sites or receptors could be specifically associated with either or both inputs, allowing ACh release to gate the flow of overall thalamic information or adjust the strengths of different thalamic streams in a brain state-dependent manner. Pharmacologic studies suggest that VPM thalamocortical synapses express presynaptic nicotinic receptors[22]. However, as we have shown[14], the effects of ACh agonist drugs may not reflect the actions of endogenous ACh, particularly at short timescales. Relatively little is currently known about POm input plasticity[114]. It is unknown whether ACh modulates POm inputs, what the relative strength of ACh input to VPM versus POm synapses might be, and whether the timing of ACh signaling relative to thalamic excitation is important to strengthen these inputs.

Hypothesis 2a: Cholinergic terminals are associated with thalamic inputs to specific cells and layers.
Rationale: VPM axons onto pyramidal neurons appear to express presynaptic nicotinic receptors[22,115] that enhance release[22]. It is known that the VPM innervates Pyr cells and interneurons (SST, PV, and particularly VIP)[88]. ACh interactions with POm inputs are unknown. We seek to compare the relative amount of ACh release sites near POm contacts onto neurons in layers 2 and 5, versus VPM contacts in layer 4.

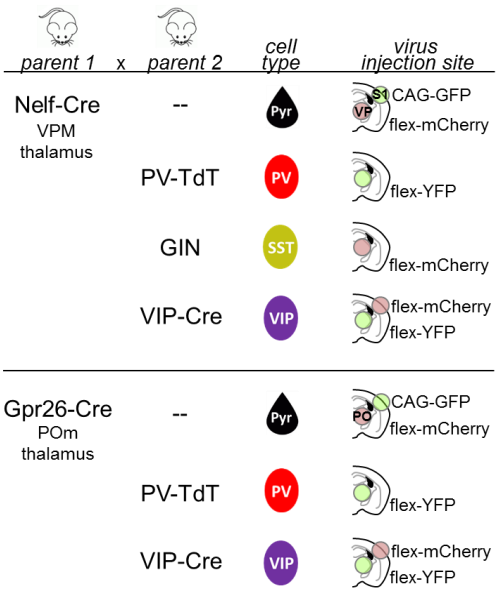

Fig. 7. Transgenic crosses and injections for vAChT immunostaining and imaging of thalamic inputs, Aim 2.1. The ventral posteromedial (VPM, in Nelf-Cre) or posteromedial nucleus (POm, in Gpr26-Cre) will be targeted for AAV-fluorophore injections. Est. n=8 cells from N=2 mice needed per group (total N=20).

Experiment 2a: Anatomy of ACh release at thalamocortical inputs. Our workflow of vAChT immunostaining and confocal image analysis will be similar to Aim 1.1, with a key difference: the targeted injection of presynaptic fluorophores into either POm or VPM thalamus (see **Fig. 7**), which our lab has successfully targeted in prior[114] and ongoing studies. POm inputs occur mainly onto VIP, Pyr and PV neurons but not SST cells[114]; thus, in the POm group we will not examine SSTs. In all others, we will reconstruct cortical neurons of interest from thalamorecipient layers (Layer 4 for VPM, Layer 2 and Layer 5 for POm) and quantify vAChT colocalization with thalamocortical axon-neurite contacts.

Predicted results and interpretation: We predict that vAChT puncta will be closely associated with both VPM and POm putative synapses onto cortical neurons, in a tripartite relationship. Further, we predict that there may be more ACh release sites associated with neurons found to be activated by ACh, namely Layer 5b pyramidal neurons[116], SST cells (for VPM inputs) and VIP cells (for both POm and VPM inputs). The density of extrinsic ACh input differs across cortical layers, with Layer 2/3 receiving less innervation[108]. Thus, we may see fewer ACh inputs onto POm contacts in Layer 2 than in Layer 5.

Alternative outcomes and potential pitfalls: Similar pitfalls apply to these experiments as to Aim 1.1 regarding viral labeling and cell type-specificity of inputs. We acknowledge that it is difficult to speculate synaptic relationships from associated contacts "near" the cell of interest. Here, the electrophysiologic data collected in Aim 2b will be an especially important complement to our assessments.

Hypothesis 2b: Timed endogenous ACh release strengthens POm and VPM inputs

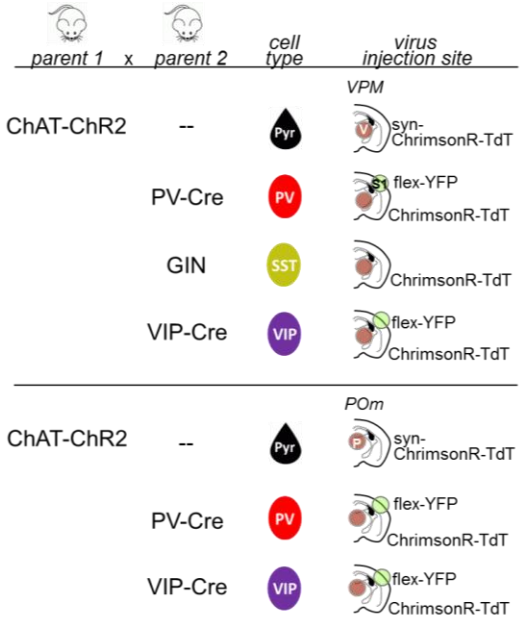

Fig. 8. Transgenic crosses and injections for timed ACh modulation of thalamocortical synapses, Aim 2.1. The ventral posteromedial (VPM, in Nelf-Cre) or posteromedial nucleus (POm, in Gpr26-Cre) will be targeted for AAV-fluorophore injections. Est. n=8 cells from N=4 mice needed per group (total N=28).

Rationale: It is unknown how endogenous ACh differentially gates incoming information from VPM and POm thalamus, potentially regulating the cortical input received during attentional states. Our lab is now studying thalamocortical plasticity in the context of learning, and has preliminary evidence that a sensory learning task involving the whiskers strengthens POm inputs. POm thalamocortical inputs are less well-studied than VPM inputs, but given that they appear plastic with learning, we hypothesize that ACh release coincident with POm stimulation will increase POm-evoked responses in thalamorecipient neurons in Layers 2 and 5.

Experiment 2b: Modulation of thalamocortical synapses by timed, endogenous ACh release. We will compare the degree of facilitation and temporal dynamics of VPM and POm input modulation by ACh using thalamic injections of the red-shifted opsin ChrimsonR in ChAT-ChR2 mice, crossed with reporter lines or Cre drivers to label PV, SST, or VIP. During recordings, we will use ChAT-ChR2 to activate all sources of ACh release with blue light (470nm), and after a delay period (50-500ms), activate AAV-ChrimsonR with red light (600nm). This approach allows us to precisely study the time course of ACh modulation of thalamic inputs. Previously[14], we showed that Pyr-to-SST synapses are not immediately facilitated after ACh release, but require a 200msec delay to activate a calcium-dependent protein kinase A to facilitate

glutamate release, and such presynaptic signaling mechanisms could be important for thalamocortical synapse changes under ACh. Because ACh-induced recurrent activity (i.e. network changes that inhibit or disinhibit thalamorecipient neurons) could also influence thalamocortical postsynaptic effects, we will subtract these out of our recordings by adding synaptic blockers (tetrodotoxin and 4-AP) at the end of the recording. In this case, persistence of the change in EPSP with ACh would suggest a direct effect of ACh on this synapse.

Predicted results and interpretation: We expect that both POm and VPM inputs will be facilitated by ACh, and that the cortical target cell types which show the most facilitation will be the same ones with dense ACh innervation in our anatomic studies (possibly SST and VIPs). We expect that VPM-to-Pyr inputs will be associated with presynaptic nicotinic receptors, as previously described[22], as will POM-to-pyramidal inputs.

Alternative outcomes and potential pitfalls: It is possible that endogenous ACh will not directly affect thalamocortical synapses, and that all effects (if observed) will be due to ACh modulation of recurrent network activity (i.e., EPSPs will be blocked in tetrodotoxin and 4-AP). We may also observe nicotinic or muscarinic activation that *depresses* neurotransmitter release, as this has been reported[117–119] (i.e., ACh antagonists may *increase* the amplitude of thalamic-evoked EPSPs). Due to spectral overlap between Chrimson and ChR2, thalamic inputs will be stimulated during ChAT-ChR2 activation of ACh release (with 470 nm blue light), whereas stimulation of Chrimson (with 600nm red light) will exclusively activate thalamic inputs. We will control for effects of synapse depression or facilitation induced by multiple light pulses; because Chrimson has slower opening kinetics than ChR2[120], we may also be able to reduce crosstalk using short pulses of blue light (2-5 ms) to activate ChR2, in tandem with longer (10ms) pulses to activate Chrimson. I will troubleshoot these parameters with advice from Bryan Hooks (collaborator), who has successfully used dual-channel optogenetics in a similar approach[121], and Susanne Ahmari (collaborator), also an expert in optogenetics.

D. Statistical Methods

For anatomical studies (Aims 1.1 and 2.1), we will use Shapiro-Wilk tests for normal distributions, and then either the mean (if normally distributed) or median vAChT puncta density to compare across compartments and cell types in a 1-Way ANOVA (+ Tukey's post-hoc for multiple comparisons). To compare VPM vs. POm inputs, we will use a 2-way ANOVA (+ Bonferroni post-hoc test). For Aims 1.2 and 2.2, we will determine if ACh-evoked EPSP amplitudes are normally distributed across cells within cell type (Shapiro-Wilk test), and use a two-tailed, paired t or U test to compare mean EPSPs pre vs post cholinergic blockade. 1-way ANOVA or Kruskal-Wallis tests will be used to compare EPSPs across cell types (+ Tukey's post-hoc test). A 2-way ANOVA (+ Bonferroni) will be used to compare effects of extrinsic and intrinsic ACh across cell types.

E. Power Analyses

Due to a lack of preliminary data to estimate effect size and variance across cell types, we are unable to precisely predict sample sizes necessary for statistical power. However, based on ongoing confocal imaging studies of other synapse types in our lab, we estimate needing n=8 cells per cell type for Aim 1.1 (1-way ANOVA by cell type, $1-\beta = 0.8$, $\alpha = 0.05$), multiplied across 5 cell types (Pyr, PV, SST, VIP and cVIP) and 2 input categories [cVIP and basal forebrain] = 80 cells total ÷ 4±2 high-quality cells per mouse = N=20, or 2 mice per group. For Aim 2.1, 8 cells per group x 3-4 cell types (Pyr, PV, VIP, SST) in 2 input categories (VPM and POm) = 112 cells ÷ 4±2 cells per mouse, 4 mice per group: N=28. Based on effect sizes and variance from our preliminary whole-cell recordings, we estimate needing 8 cells per group in Aim 1.2 for within-cell type comparisons (t test, pre-vs-post ACh antagonist). Across 5 cell types and 3 input categories (VIP, basal forebrain, and ChAT-ChR2), we expect to require 144 cells ÷ ~6 Pyr cells or 3-4 interneurons per recording = ~4 mice per group, N=30. For Aim 2.2, 8 cells per group x 3-4 cell types (Pyr, PV, VIP, SST) in 2 input categories (VPM and POm) = 112 cells ÷ 4±2 cells per mouse, 4 mice per group: N=28.

SUMMARY

We will characterize the release patterns and effects of ACh on intra-cortical activity and thalamic sensory inputs. The proposed experiments will fill a critical gap in knowledge of ACh function in the cerebral cortex: the location of ACh release sites and their precisely timed effects on cortical neurons, thalamic inputs and their interactions, which form the basis of sensory stimulus encoding. Our study will be the first to examine thalamocortical modulation by endogenous ACh release, and the first functional study of the input-output connectivity of cortical cholinergic VIPs (a potential novel target for cholinergic therapies). Our study of ACh effects at short timescales is critical to precisely understanding the sequence of cortical circuit changes during cognition, a goal in accordance with many priorities of the NIMH Strategic Plan for Research[122].

Our paired anatomic and functional studies of ACh inputs and effects will provide insight into how cholinergic drugs act in the cortex to modulate cognition, and inform the development of therapies to restore these precise neural circuits in schizophrenia and other diseases of cognitive dysfunction.

Respective Contributions

Preparing proposal:

The Sponsor Information section of this proposal was written by my sponsor, Dr. Alison Barth. The Additional Education Information section was written by the Pittsburgh MSTP program director, Dr. Richard Steinman (referee). I wrote all other sections independently with guidance from my sponsor, Dr. Alison Barth. My meetings with her and with my thesis committee (Anne-Marie Oswald, Susanne Ahmari [collaborator], and Aryn Gittis [referee]) were integral to the construction of this proposal and my preliminary data collection.

I outlined and drafted my experimental background and plans for this proposal in my formal thesis proposal, which I defended on 2/19/2018. There I received thoughtful written feedback and verbal comments that were very helpful in focusing my aims, project design, anticipated results, and backup plans to make this proposal as effective and impactful as possible. I also got helpful feedback from my fellows in the Barth Lab and nearby labs in the CMU "Great Hall of Brain Science." In particular, comments from Dika Kuljis, Nick Audette, Ajit Ray, and Sandra Kuhlman led to useful clarifications and exciting new avenues I had not considered.

Many ideas presented in this proposal follow directly from my recent paper, where we found that acetylcholine has cell type- and synapse-specific effects on excitatory synapses. Joanna Urban-Ciecko, the chief electrophysiologist and first author of the project, provided both technical and scientific inspiration for the experiments proposed. My interest in cholinergic modulation of higher-order thalamic inputs was influenced by ongoing behavioral studies in the lab, where we find that thalamic inputs are strengthened in an attention-based behavioral task. Zhuopin Sun and Dika Kuljis collected the fluorescent images shown in this proposal, and I prepared all preliminary electrophysiological data.

Accomplishing Proposed Project:

I entered Dr. Barth's lab with a strong research background and skillset to pursue most of the tasks necessary to complete the proposed project. I have been well-trained by the MSTP and my prior research to think critically, identify what is feasible and interesting, design tractable experiments, and troubleshoot them. I also have experience in patch clamping, optogenetic manipulations, stereotaxic surgeries to target deep and superficial brain areas, and electrophysiology data analysis. However, there is always greater mastery to be gained in these areas, and I will continue developing my skills under Dr. Barth's guidance. In particular, I will expand my optogenetics repertoire to include dual-color stimulation in brain slices. Bryan "Mac" Hooks at the University of Pittsburgh has successfully used this technique in his prior work and has agreed to provide technical assistance by email or in person as I reconfigure my optogenetic stimulator.

I am currently learning to do confocal microscopy, 3D reconstructions of neurons, and high-throughput analyses to quantify cholinergic inputs to cortical neurons. I am being trained in these techniques by Dika Kuljis, who is co-developing a high-throughput, computerized method for synapse counting along with Dr. Barth. Other members of the lab (Zhuopin Sun, Ajit Ray, and Eunsol Park) have also become proficient in this technique and will provide assistance. I am learning new stereotaxic injections to target two distinct sub-regions of the thalamus. Nick Audette and Ajit Ray have done these injections successfully and are training me in the technique.

In addition to my official advisors, the Center for Neural Basis of Cognition (CNBC) provides me with an exceptional breadth of expertise in neural data analysis, electrophysiology and functional neuroscience. Robert Kass, a renowned expert in neural data analysis, has offered to advise me on statistical methods. My discussions with Peter Strick, Nathan Urban, Sandra Kuhlman, Carl Olson, David Lewis, and Daniel Brasier have also provided new insights that refined this proposal. My interactions with fellow CMU and CNBC students at our lectures and retreats have broadened my view of biology and neuroscience and provided me many opportunities to get feedback from a diverse, friendly cohort of future colleagues.

All manuscripts and presentations arising from the proposed work will be first-drafted by me, and refined by extensive critiques from my sponsor, my committee, and many of the labmates and informal advisors listed above. This diverse input will be critical to making my science communication as broad and effective as possible. The extremely supportive environment of the Barth lab and Great Hall of Brain Science, the excellent collaborations fostered by the CNBC, and the continued translational support of the MSTP have enhanced my early studies in innumerable ways and will continue to contribute to this project. They will provide me with excellent training to pursue a career as a physician-neuroscientist.

Selection of Institution

Out of many options available to me as a University of Pittsburgh (Pitt) – Carnegie Mellon University (CMU) MSTP student, I chose to pursue my PhD in the Department of Biological Sciences at CMU. I am also privileged to be part of the Center for the Neural Basis of Cognition (CNBC), a joint neuroscience training program shared between Pitt and CMU. The CNBC was founded to foster interdisciplinary collaborations between the two universities, and particularly to share technical advances between CMU (which is strong in computation and tool development for basic research) and Pitt (which has exceptional resources for clinical and translational research). CMU Biological Sciences is a small department with a focus on cellular anatomy and physiology. The CNBC brings together world-class faculty from diverse fields at both universities, from mathematics to psychiatry, to do paradigm-shifting, interdisciplinary research on higher-order cognition.

I believe that, as an MD-PhD, it is easy to become a jack-of-all-trades, and that the key to developing mastery is to find a niche that synergizes one's clinical insights with the tools of basic research. I hope to find this niche as a principal investigator, so I sought out a degree program that would give me exceptional training in the cellular mechanisms of brain function, couched in the context of translational discoveries, unconventional collaborations, and powerful new tools. CMU and the CNBC offered this, and were a major reason why I chose to do my MSTP training in Pittsburgh. The CNBC is far ahead of other institutions in its collaborative treatment of fundamental topics in neuroscience. The labs here dig deep into basic biology in an interdisciplinary, creative fashion which I believe will be crucial to redefining diagnostic criteria for cognitive and psychiatric disorders, and ultimately treating them in a precise way that addresses their root causes. The CMU Biology department has given me a superb grounding in broad-based biology research, and its new faculty hires have given me a taste of exciting new transgenic tools currently being up-scaled from cell cultures and zebrafish to rodents. The CNBC has proven to be a truly collaborative place, true to its mission. I have received incredible support from experts in many fields related (and even unrelated) to my research. Whether I seek an expert in somatosensation, optogenetics, expansion microscopy, biomaterials, or rare genetic diseases, I have always found support and resources from CMU and CNBC faculty and students. I look forward to learning from and working alongside these fantastic colleagues.

Selection of Sponsor

I joined Alison Barth's lab after my second MSTP rotation, between the first and second years (MS1 and MS2) of my medical training. Dr. Barth is a highly successful mid-career investigator of synaptic physiology in the neocortex, and an exceptional mentor. She was one of the first to develop transgenic technologies for tracking ensembles of neurons activated by behavioral experience, and is renowned for her expertise in cortical cell type classification and synapse plasticity. Dr. Barth's incisive, challenging and enthusiastic mentoring style made it an easy and smart choice to say yes when she asked if I would come back to her lab after MS2. At the beginning of my studies, she helped train me to perform whole-cell patch clamp recordings and improve my electrophysiological data analysis. I now meet with Dr. Barth weekly (or more often) to discuss new data, and she is always available to talk about technical issues. Dr. Barth gives prompt and insightful feedback when needed while fostering my freedom to choose the direction of my project: a perfect combination for me to grow as an independent researcher. She has also fostered a collaborative, supportive and effective lab environment where I receive generous additional mentorship from a senior graduate student, three postdocs and two technicians. I have also been able to serve as a mentor to talented undergraduate students and PhD rotation students from Pitt and CMU. The lab's journal clubs, data meetings, and informal discussions have dramatically improved my scientific communication and critical thinking skills. Dr. Barth is an energetic mentor who is highly invested in my project and success in the lab. My project topic, cholinergic neuromodulation, is a new area of interest for the lab that fits well with Dr. Barth's current expertise. Dr. Barth is willing to forge new collaborations and explore new techniques to achieve the most effective answers to my proposed experimental questions.

Overall, I sought an institution and sponsor that are deeply passionate and knowledgeable in the cellular neuroscience of cognition. I sought the strongest possible training and resources to pursue my research goals as effectively as possible, in an environment of structured independence. With Dr. Barth as my advisor at CMU, I have found this environment. Her mentorship has allowed me to quickly learn very sophisticated and challenging techniques in electrophysiology, publish a paper quickly on this work, and gain a solid theoretical foundation for my proposed studies. Dr. Barth's mentorship will train me to be the most rigorous and thorough scientist I can be. As a future independent investigator, I could not ask for better training.

Training in Responsible Conduct of Research (RCR)

Scientific integrity is a priority for the Barth lab and the institutions where I am training. It is also a personal priority, as I seek to do science that is maximally effective and unambiguous, as well as publically respected. In addition to the training I received pre-MSTP as an NIH IRTA fellow, I have had extensive recent training in RCR from my lab, the CMU Biological Sciences graduate program, the CNBC, the Pitt MSTP, and the Pitt School of Medicine, all of which have their own independent programs for teaching good scientific practice.

Advisor and Lab: Discussions with my advisor regarding good ethical practice in data presentation and reporting are frequent and ongoing as experiments are planned, designed, analyzed, and prepared for publication. The topic is also frequently discussed in our weekly lab meeting and weekly journal club, where we assess our own work and published work in terms of its understandability, reproducibility, and honest representation of research data.

CMU Biological Sciences: Like all CMU Biology graduate students, I take a weekly *seminar and journal club* course during all years of my PhD that includes RCR topics. Numerous faculty with diverse expertise have participated recently in these 60-minute lectures, including Ann Mathias (Assistant Vice President of Research), John Zimmerman (human subjects researcher at CMU Human-Computer Interaction Institute), Lisa Zilinski (Data Services Librarian); Allison MacFarlan (Senior Information Security Risk Analyst), Lynn Young (Manager of the CMU Office of Sponsored Programs), and Stacey Becker (COI Research Compliance Coordinator). Topic areas have included ethics of authorship and publications, peer review, animal research and IACUC, human subjects research and IRB protocols, conflicts of interest, mentor and mentee responsibilities, data management, data sharing, research misconduct, technology transfer and intellectual property.

RCR was also addressed in my *Proposal Preparation and Peer Review course* required for G2 students. This 8-week course was focused on training students to effectively conduct literature reviews and scientific writing, and also featured a series of video modules on scientific misconduct (via the DHS Office of Research Integrity; https://ori.hhs.gov/THELAB) and a 60-minute discussion of misconduct and authorship ethics, as well as an overview of CMU ethics reporting and ombudsman services.

CNBC: The CNBC Brain Bag lecture series holds an annual series of mandatory, 90-minute ethics roundtable discussions which cover the following topics: 1) ethics of animal research, 2) considerations in human subjects research, and 3) general research ethics including authorship issues and scientific misconduct. I have attended these since joining the CNBC as a G1.

Pitt MSTP: My 10-week *Professional Development II course* in the second summer of the MSTP was devoted to training in data hygiene with the goal of fostering honest reporting and reproducibility. Topics included a module on various rubrics used at high-impact institutions (ARRIVE guidelines, Nature journals' reproducibility checklists), an exercise to identify questionable data from select examples from the literature, and strategies to improve research design and presentation. In the spring of my G1 year, the MSTP held a mandatory, five-week *Ethics for Physician-Scientists course* devoted to RCR in the basic and clinical settings. These two-hour weekly small groups featured discussions of an ethics topic led by the MSTP director, often accompanied by a guest speaker. One speaker was David Wehrle, director of the Conflict of Interest Office at Pitt. As part of this course, we completed online modules offered by the Conflict of Interest Office and were encouraged to discuss how we would adopt "best practices" for RCR in our own labs as young investigators.

Pitt School of Medicine: The School of Medicine addresses research reproducibility and data transparency issues longitudinally. The ethics of human subjects research and data management to ensure confidentiality were discussed in our week-long *Introduction to Being a Physician course* at the beginning of MS1, and also during a module in the *Ethics-Based Medicine* course in MS1, which taught statistical methods and good practice for clinical research. To perform clinical research and have patient contact at UPMC, I was required to take online courses on human research ethics and patient privacy through the *Collaborative Institutional Training Initiative (CITI)*. I intend to renew these courses for the remainder of the program and take additional courses as the need arises.

Section II- Sponsor Information

A. Research support available

NIH/NINDS	R01 NS088958-01	Dynamic connectivity in neocortical networks
Barth PI	Through 04/2020	$313,477 (direct+indirect) FY2017-18
NIH/NINDS	R21 NS104821-01	Machine learning approaches for electrophysiological cell classification
Barth PI	Through 09/19	$237,150 (direct+indirect) FY 2017-18
NIH/NIMH	R01MH114103-01	High throughput approaches for cell-specific synapse characterization
Barth and M. Bruchez PI	Through 08/2020	$309,691(direct+indirect) FY 2017-2018

B. Sponsor's Previous Trainees

My research group currently has 2 PhD students and 3 post-doctoral fellows. Six Ph.D. students and 5 postdocs have trained in my lab to date.

Predoctoral Trainees

- Roger Clem. Completed PhD fall 2007. Roger did a post-doc with Rick Huganir (Johns Hopkins and HHMI) and is now an Associate Professor at Mount Sinai School of Medicine in New York.
- Rick Gerkin. Completed Ph.D. spring 2009. Rick did a post-doc with Nathan Urban (University of Pittsburgh) and is now an Assistant Research Professor at University of Arizona.
- Brett Benedetti. Completed Ph.D. August 2010. Brett did a post-doc with Gord Fishell (NYU; now at Harvard) and is currently an editor at *Nature Medicine*.
- Sonal Shruti. Completed Ph.D. April 2010. Sonal did a post-doc with Eve Marder (Brandeis) and is currently a post-doc with Dr. Vincent Prevot, INSERM Lille, France
- Jing Wen. Completed Ph.D. May 2012. Jing did a post-doc with Nathan Urban (University of Pittsburgh) and is currently working in the finance industry for PNC bank.
- Santosh Chandrasekaran. Completed Ph.D. May 2015. Santosh is currently a post-doc in biomedical engineering with Lee Fisher at the University of Pittsburgh.

Postdoctoral Trainees - selected

- Joanna Urban-Ciecko. Finished postdoc in 2016. Current position: Research scientist, Nencki Institute, Warsaw Poland
- Sarit Goswami. Finished postdoc in 2015. Currently a Data scientist at Kogentix Inc.
- Saket Navlakha. Finished postdoc in 2014. Currently an Assistant professor, Salk Institute, San Diego
- Dr. Guillem Genove. Finished postdoc in 2005. Currently Research faculty at Karolinska Institute in Stockholm, Sweden

C. Training Plan, Environment, Research Facilities

Graduate Coursework: Stephanie is an engaged and intellectually energetic student who has thrived in her coursework at Pitt and CMU. At the time of her proposal (April 2018), Stephanie has completed the coursework, teaching assistantship and course grading requirements for the CMU Department of Biological Sciences and CNBC programs. Stephanie has also formed her thesis committee and passed her thesis proposal defense examination to become a PhD candidate. Her completed courses consisted of lectures in advanced cellular neuroscience, statistical models of brain function, and systems-level cognitive neuroscience. Stephanie continues to participate actively in required CMU and CNBC journal clubs, faculty seminars, research retreats, and research ethics seminars.

During medical school, Stephanie completed MSTP-specific coursework in scientific writing, grantsmanship, research design and biostatistics (with focus on achieving sound methods and proper controls, and following NIH guidelines for reporting and reproducibility), as well as medical school coursework on clinical statistical methods and bioethics. *Since all of her graduate program course requirements are complete, Stephanie will be able to devote almost the entire award period to the completion of her proposed aims and thesis*.

Mentorship Resources

Barth Lab: Stephanie's desk is right next to my office and we will continue to meet at least weekly on an individual basis. In these meetings she is encouraged to prepare and present figures in suitable condition for publication whenever possible and discuss these in the context of her ongoing and future research plans. We analyze technical approaches and primary data analysis, and I encourage Stephanie to relate her findings to

published concepts and established knowledge in the field. We also regularly discuss professional development, including upcoming opportunities to attend local and national meetings, establish collaborations with other faculty, and improve Stephanie's skills to better manage data, time, personnel, teaching responsibilities and work-life balance. In discussing work to be published, we try to anticipate how our work may be evaluated by reviewers and the optimal presentation format for a clear and high-impact product.

Stephanie also benefits from weekly lab meetings and journal clubs. Our lab meetings include brief updates on all ongoing projects in the lab as well as in-depth discussion of a particular topic of research or professional development. Stephanie has presented her work at lab meetings every 6-8 weeks since joining the lab, and will continue to do so throughout her time here. In addition, I formally meet with students once a year (and often more) for the sole purpose of discussing long-term career goals and planning. I use an IDP-style mentoring form to facilitate goal-setting, accountability, and follow-up at these meetings and encourage my students to consider how their experimental and professional engagements can serve their long-term career goals.

Thesis Committee: Stephanie completed her PhD Comprehensive Examination in March 2018 with the approval of her dissertation committee, which consists of:
- Alison Barth, PhD (Professor of Biological Sciences, CMU)
- Susanne Ahmari, MD, PhD (Associate Professor of Psychiatry, University of Pittsburgh Medical School)
- Anne-Marie Oswald, PhD (Associate Professor of Neuroscience, University of Pittsburgh)
- Aryn Gittis, PhD (Associate Professor of Biological Sciences, CMU)

Stephanie's committee members are accomplished neuroscientists and a strong resource for basic and translational knowledge of cortical circuitry. Their expertise in advanced biologic imaging and neurophysiology will hone the execution of Stephanie's thesis aims and train her in the planning and execution of experiments in this fast-moving field. Meetings with the entire group will occur formally every 6 months to review Stephanie's progress. Individual members will be available to meet one-on-one as needed for technical, scientific, clinical and career advice.

MSTP mentorship: For outside consultation on professional and scientific development, Stephanie has thus far had regular individual meetings with an informal advisory committee consisting of:
- Teresa Hastings, PhD (MSTP career advisor; Professor, UPMC Movement Disorders Section, University of Pittsburgh Medical School)
- Richard Steinman, MD, PhD (Director, University of Pittsburgh MSTP; Associate Professor, UPMC Hillman Cancer Institute, University of Pittsburgh Medical School)
- Jason Rosenstock, MD (Associate Professor of Psychiatry; Director, University of Pittsburgh Neuroscience Area of Concentration Program, University of Pittsburgh Medical School)

These faculty have a combined 50 years of experience mentoring MSTPs and physician-scientists to achieve their career milestones while maintaining a healthy work-life balance.

Stephanie has already completed an MSTP Professional Development course (in which guest speakers, all MD-PhD physician scientists, presented topics related to scientific writing, presenting, networking, and MSTP-specific career trajectory advice). In addition, she has attended mandatory MSTP monthly seminars on ethical and professional development topics throughout her training thus far, and will continue to participate throughout her graduate and medical training.

Other professional development resources: Stephanie is a member of the Center for the Neural Basis of Cognition (CNBC), a cross-institution, interdisciplinary group of neuroscientists at Carnegie Mellon and the University of Pittsburgh. The CNBC offers weekly research presentations from affiliated graduate students throughout the academic year, as well as travel funds for conference attendance and a yearly retreat for the CNBC community. In addition, Stephanie has participated in seminars on manuscript writing and editing, general career development training lectures, science interpretation and public speaking workshops, and networking sessions offered by the University of Pittsburgh's Biomedical Graduate Student Association, and will continue to use these and similar opportunities to expand her professional networks with peers and faculty and refine her research and professional skills.

Other research and scientific presentation activities will also contribute to Stephanie's professional development. Her participation in the clinical and basic research seminars described above, as well as regular presentation of data, critiques of ongoing projects, and literature presentation at weekly lab meetings and journal clubs will strengthen her scientific reasoning and oral presentation skills. Her skills at efficiently and simply explaining complex science in writing will be developed throughout the training period via research

manuscript and grant proposal preparation with input from me and other lab members and collaborators, as well as her critical review of manuscripts and grant proposals prepared by others in the lab.

Stephanie will also continue to develop her research mentorship skills by training undergraduate research assistants and incoming graduate students in patch-clamp electrophysiology and histological staining techniques, and in developing sound record-keeping and data analysis templates to improve lab efficiency. Since joining the MSTP, Stephanie has been heavily involved in both formal and informal mentorship of junior MD-PhD students as an MSTP Interview Committee member, and she will continue in this capacity as well as mentor an individual student with specific interest in neuroscience as a formal MSTP Peer Mentor.

Training in scientific professionalism

Stephanie has an inquisitive mind, and reads broadly in both the medical and basic science literature. She frequently brings her medical knowledge to bear on scientific issues during our group discussions, and is highly motivated to conceive and carry out rigorous and impactful experiments. She has worked hard in her first year of PhD research to learn the basics of patch-clamp electrophysiology, and her nascent skillset will continue to be developed through the course of the challenging and rigorous experiments that we have designed. The concurrent requirements of the MSTP program will ensure that she learns to manage a complex schedule of clinical and academic responsibilities while concurrently driving an experimental research program. Stephanie's dissertation topic, as it addresses cholinergic modulation of neocortical excitatory and inhibitory circuits, is a dynamic and fast-evolving area of research that is relevant to many fields of anatomical, developmental and functional research in brain plasticity. It is a deep project with implications for neurological and psychiatric disorders that takes advantage of Stephanie's medical training, and one that she has great enthusiasm for.

Stephanie's proposed project will develop her skills in patch-clamp electrophysiology and optogenetics to isolate and manipulate differential contributions of basal forebrain and VIP sources of acetylcholine to cortical information processing through modulation of thalamic input and also inhibitory function. She will build upon her expertise in synaptic analysis from paired cell-recordings to examine the pre- and postsynaptic effects of acetylcholine in the cortical circuit, using anatomically-targeted viral injections, targeted patch-clamp recordings, and dual-wavelength optogenetics. In addition, she will learn new skills for quantitative analysis of ACh release sites. This includes stereotaxic viral injections, immunohistochemistry and confocal imaging, and high-throughput 3D cellular reconstructions for synapse quantification using the image analysis program Imaris, with the support of two postdocs proficient in these techniques. These anatomical projects in particular will develop her skills in data organization and management, and longitudinal experimental planning. Our collaborators at the University of Pittsburgh, Bryan Hooks and Susanne Ahmari, will advise Stephanie in technical aspects of multichannel optogenetics.

Scientific Communication: Stephanie has already published as a first author in her post-baccalaureate training and contributed important data to a manuscript from the lab published earlier this year in *Neuron*. She will improve her written communication skills by drafting manuscripts and posters based on her thesis work. In addition to presentations to our research group, Stephanie is required to formally present her research to the Department of Biological Sciences once a year following the completion of her qualifying exam, which will commence in fall 2018. Stephanie will work with me to develop a clear and logical narrative that frames the scientific questions she is interested in and present her data in an organized and compelling fashion. Depending on her research results, she will also assist me in preparing an R01 proposal to examine behavior-dependent changes in cholinergic synapses. We hope to be able to write a review paper at the end of her PhD summarizing brain state-dependent effects of acetylcholine on cortical networks and cell types.

Intramural Seminars: Stephanie's participation in CMU and CNBC seminars will provide critical and timely exposure to contemporary topics in neuroscience and general biology, and facilitate collaborative, skill-building interactions with peers and experts. Stephanie attends weekly, faculty-facilitated peer journal clubs within CMU and CNBC, weekly visiting faculty lectures, and occasional special lectures by visiting faculty.

Extramural Interactions: Stephanie will seek interactions outside of Pittsburgh to diversify her science exposure, practice scientific communication, and connect with peers and institutions relevant to her training as a physician scientist. At the time of submission, Stephanie has presented her work in my lab as a poster at the 2017 Society for Neuroscience conference in Washington, DC and the 2017 Barrels satellite meeting in Baltimore, MD. She will continue to prepare annual presentation abstracts and posters for the Society for Neuroscience and Barrels conferences (November 2018 and 2019). As a senior PhD student, Stephanie plans to submit an abstract to attend a Gordon Seminar (a postdoc- and graduate-student-specific mentoring

conference) related to her work on cholinergic control of inhibition. These meetings will place Stephanie in close contact with national thought leaders in neuroscience and synapse physiology, and expand her scope of knowledge and collaborative skills. Stephanie will also continue to present her work at CNBC, CMU Biological Sciences and MSTP annual retreats and recruitment events to maintain and foster institutional-level contacts. To facilitate clinical networking and explore specialties of interest, Stephanie plans to attend the American Academy of Neurology (May 2019, Philadelphia, PA) and Biological Psychiatry (April-May 2020, Philadelphia, PA) meetings in the G3 and G4 years of her PhD, or one clinical conference each year during these years.

Clinical Development: Stephanie will maintain her exposure to clinical work in numerous ways during her training period to further her goal of connecting clinical and basic research. Since beginning medical school, Stephanie has been a fixture at clinical rounds in the departments of Psychiatry and Neurosurgery at UPMC, and will maintain this exposure during the award period in addition to her longitudinal clinical clerkships (LCCs). LCCs represent 80-hour 4th-year elective clinical rotations, and will be conducted with clinicians who specialize in neuro-cognitive and psychiatric disorders to provide exposure to clinical fields directly related to and impacted by her research. Stephanie is scheduled to begin an LCC with Edward Burton, MD, a neurologist at UPMC, in the spring of this year. By the beginning of the award period, Stephanie will have begun an additional LCC in outpatient psychiatry at the UPMC Western Psychiatric Institute. The LCCs will help integrate Stephanie's research interests in cortical plasticity with further exploration of its clinical impact.

Stephanie will develop new relationships with her LCC advisors, who are also practicing scientists, as foundation for clinical research projects in her 3rd and 4th years of medical school. In these years Stephanie plans to participate in discrete clinical research projects (such as case reports or retrospective outcomes studies) during an elective 1-month research rotation as well as ad libitum during her clerkships. This will allow her to develop data collection and analysis skills unique to clinical research as a supplement to the laboratory research experience gained in her thesis work.

Ethics Training: Training in the responsible conduct of research and clinical care is a key aspect of Stephanie's training. I teach a course on scientific communication to PhD students in their second year of the CMU Biology program. This course includes modules on research ethics and misconduct, and Stephanie participated and did well in this course. Research ethics training is also a priority at the University of Pittsburgh. This training comprises multiple formats including semiannual monthly seminars conducted as part of the MSTP's mandatory monthly workshops, annual online training modules required to maintain clinical contact at UPMC, and dedicated coursework offered through the MSTP and integrated longitudinally across both medical and graduate training phases. Stephanie has completed a semester-long didactic course on Ethics, Law, and Professionalism as part of the medical school curriculum, as well as an additional month-long didactic/workshop course on Ethics for Medical Scientists through the MSTP program. She will continue to participate in ethics-based seminars twice a year through the MSTP program.

Relevant to Stephanie's clinical research and clerkship training, she has also completed annual, self-directed online training modules on research integrity, human subjects research in biomedical science, conflicts of interest, HIPAA privacy requirements for researchers, and responsible conduct of research. Stephanie also completed a semester-long, non-credit graduate elective course related to technology transfer, intellectual properties and commercialization of scientific research (the Benchtop to Bedside course offered jointly by the Univ of Pittsburgh Innovation Institute and Clinical and Translational Science Initiative), which directly addressed ethical topics of data ownership and monetary conflicts of interest. (Please see the section on "Responsible Conduct of Research" for a detailed description of these training activities.)

D. Intellectual and Lab Environment and Resources

Barth Laboratory: My lab has a long history of training independent scientific thinkers with a strong track-record of high-impact scientific publication. Stephanie is the first MSTP student I have mentored, but several of my past trainees (see section II.B) are involved in clinically-oriented research and the majority have pursued distinguished academic careers.

In my joint appointments at CMU, the University of Pittsburgh and the CNBC, I have been a member of 25 PhD thesis committees including several MSTP students. Stephanie will benefit from my experience gaining and maintaining R01 grant funding, publishing high-impact research papers, and advising successful students. We will discuss these things during our regularly scheduled weekly meetings and our daily interactions in the lab. Stephanie currently meets with me formally to discuss specific components of her research progress, abstract and manuscript preparation, and other career and professional development concerns (see "Training Plan" section II.C.1).

I maintain a close relationship to daily experimental activities in the lab, and lab members have easy access to me, as my office is in the lab and I welcome their informal interactions. The training potential of my group is enhanced by the availability of our dedicated lab technicians, who handle routine aspects of managing our transgenic mouse colonies and provide expertise in cellular staining and microscopy. Our lab also greatly benefits from the expertise of my postdoctoral fellows who are experienced in patch-clamp electrophysiology and quantitative high-throughput synapse detection in brain tissue. Stephanie has also learned stereotaxic surgeries, viral injections and perfusions from senior members in our open lab, and continues to give and receive advice from other trainees from the two directly adjacent research groups that share contiguous lab space (Aryn Gittis and Sandy Kuhlman) with mine as well as two other research groups that share animal resources and experimental expertise (Eric Yttri and Andreas Pfenning).

The research and training potential of my lab is enhanced by our ongoing interactions with the Molecular Biosciences Imaging Core (MBIC) at CMU, as well as the Neuroscience graduate program at the University of Pittsburgh. Both programs are widely recognized as leading academic institutions in the US and have complementary expertise, with CMU specializing in technologies development and computational neuroscience, and the University of Pittsburgh excelling in clinically-relevant and translational basic neuroscience research. The University of Pittsburgh is consistently within the top 10 recipients of overall NIH funding nationwide, and the heavy focus on translational neuroscience research within the graduate department as well as the departments of Psychiatry, Neurology and Neurosurgery at UPMC places our work thematically in the center of the university's research and training interests.

The university's commitment to the research and career development of pre-doctoral MD/PhD trainees is emphasized by the size and academic rigor of the MSTP, which enjoys strong funding support from both the NIH and the School of Medicine and the resources necessary to develop and support a unique, highly-integrated curricular training program tailored specifically for combined medical and graduate research training.

Facilities and Equipment: My lab occupies approximately 2,400 sq. ft. of space on the first floor of the Mellon Institute at CMU. My lab is directly adjacent to the labs of two other neuroscientists studying sensory systems: Sandy Kuhlman (CMU Biology) and Aryn Gittis (CMU Biology). Significant space and equipment are shared among these three labs including several microscopes for fixed tissue imaging, a 2-photon confocal microscope, and multiple computers for data analysis and simulations. The Barth lab is on the same floor as the main offices of the Center for the Neural Basis of Cognition (CNBC), which houses labs for four investigators doing single unit recording in non-human primates. The proximity of the neuroscience labs in the Department of Biological Sciences with faculty and students in the CNBC creates a rich environment for discussion and collaboration.

In the Barth lab, four acute brain-slice electrophysiology rigs designed for fluorescence-visualized, whole-cell patch clamp are present in the lab. Each is equipped with a fixed-stage Olympus BX51 WI microscope with fluorescence capabilities including an XL Fluor4x/340 0.28 N/A objective for fluorescence visualization at low magnification, dual Sutter MP-225 electrode micromanipulators, an Axon Instruments Multiclamp 700B amplifier, and a Retiga CCD camera system for visualized whole-cell recordings. Each rig is also equipped with a Dell desktop PC for data acquisition and analysis. All rigs are currently equipped with a Plexon instrument white-light LED for optogenetic stimulation at multiple wavelengths. The lab also has one in vivo electrophysiology set-up for extracellular single-unit recordings. This set-up consists of a Knopf sterotax with mouse adaptor, an FHC temperature controller, a Zeiss OPMI 1-FC surgical microscope, a CED Micro 1401 A:D board, a Neurolog amplifier and pulse generator, Hitachi V-252 oscilloscope, and a dedicated Dell desktop PC for data acquisition.

The Barth lab also has one dedicated setup for 2-photon-guided targeted patch-clamp and juxtacellular recording in living animals, including a laser (Mai-Tai; Spectraphysics) and integrated microscope system (Femtonics). The Femto2D 2-photon laser scanning microscope is based on an Olympus BX61WI microscope, with laser control software Mai Tai 2.x and data acquisition through MES v.5.2878, and custom adjustments for in vivo imaging and recording. Three recording microelectrodes (Luigs & Neumann SM-7 remote control and SM10) are mounted on a Luigs & Neumann X-Y translator. The system is mounted on a Newport ST series smart table with IQ damping technology. The recording set-up is equipped with a Tektronix DPO 2004B Digital Phosphor Oscilloscope and electrophysiological data are acquired using two, dual-headstage Axon CNS MultiClamp 700B amplifier (Molecular Devices). Imaging and recording data is collected by a system-dedicated Dell desktop PC.

The 2-photon microscope has a dedicated surgical preparation area for making craniotomies, including a digital display Kopf stereotax (Model 923-B), with a specialized mouse anesthesia head mount (Scivena

Scientific anesthesia innovations M3000), a surgical microscope (Carl Zeiss OPMI 1-FC), a high-intensity fiber-optic illuminator (Edmund Optics MI-150), a hand drill, and heating pad to maintain animal temperature (FHC). The lab also possesses a Leica SM 2000R freezing microtome for basic histology, a deli case with electrical supply for immunohistochemistry, and assorted water baths and incubators. Equipment for molecular biology includes heat blocks, shakers, microcentrifuges, incubators, and a -80^{o} C freezer.

The Barth lab maintains ~300 mouse cages in the Mellon Institute Central Vivairum, including more than 10 different transgenic lines. This facility is equipped for BSL-3 level virus work. Also in the facility are set-ups for stereotaxic surgery for viral injections into the brain in both neonate and adult animals.

The Barth laboratory has individual PCs associate with each electrophysiology set-up (4 total), plus additional computers for microscopy and lab use (6 total). The Barth lab has two licenses for the image analysis program Imaris (Bitplane), with two high-performance computers and >10 TB storage space. All lab students and associated personnel have dedicated office space.

E. Number of Fellows/Trainees to be Supervised during the Fellowship

I am mentoring three postdoctoral fellows, with a fourth joining the lab this spring. For graduate trainees, I am mentoring Stephanie and one outgoing senior graduate student who will complete his PhD this summer. I hope to recruit at two additional PhD students during Stephanie's fellowship, for a total of 3 pre-doctoral trainees at different stages of training in the lab.

F. Applicant's Qualifications and Potential for a Research Career

Stephanie and I share common interests in the application of fundamental neuroscience to improving health outcomes, and I was delighted when she selected my lab as a place for her to carry out her thesis research. Stephanie is smart, driven, and experimentally talented, and she has significantly enhanced the quality and productivity of my research group in substantial ways even in the first year or so of her doctoral training. Most notably, Stephanie jumped into a mature project looking at cholinergic modulation of different types of excitatory synapses in the mammalian cortex, discovering a dissociation between pharmacological effects of cholinergic receptor activation and effects from endogenous ACh release on excitatory synapses in the neocortex, efforts that placed her as a coauthor on a manuscript published in *Neuron* before the end of her first year of PhD research. Stephanie's passion for research is underscored by her strong record of research in a post-baccalaureate program at the NIH, and she brings a thoughtfulness and curiosity to the lab that improves the thinking of everyone around her.

Her proposed research project lies at the intersection of detailed cellular microcircuits (a burgeoning field) and complex behavioral processes like attention (a well-studied but poorly understood phenomenon). The discovery that even within a restricted region of the brain, there are different types of neurons that can be molecularly defined and genetically manipulated has revolutionized the kinds of questions that neuroscientists can pose about information processing, learning, and disease progression. Stephanie has independently conceived and begun to implement a well-thought through series of controlled experiments to isolate and dissect the role of different regimes of cholinergic signaling in neocortical function, with particular attention to how different cell types can respond to endogenous ACh release. I am very enthusiastic about where these experiments will lead. Indeed, Stephanie medical training uniquely positions her to be able to make sense of diverse cellular effects that engage different cholinergic receptor subtypes, and I predict her work will be highly impactful.

Stephanie has a wide-reaching intellect and is eager to learn and improve upon her skills. She is an exceptional student, rising to the tippy top of her class in both medical school and in her PhD coursework. She brings this same attention to detail to her experimental life. It has been a great pleasure to see how she has matured scientifically even in the short time she has been in the lab, where she has demonstrated skill not only in carrying out complex patch-clamp experiments but also in organizing her research activities to take advantage of scarce transgenic mice. She has exceptionally high standards for herself, and is dedicated to her training. She will be a fantastic physician-scientist.

Attracting and retaining those with medical training in research careers is an important objective of the NIH. Awarding Stephanie an F30 grant will provide both recognition and reinforcement to her talents in bridging clinical and scientific knowledge, and I support her application with the greatest enthusiasm.

UPMC | University of Pittsburgh Medical Center

Western Psychiatric Institute and Clinic

Susanne Ahmari, MD, PhD
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Department of Psychiatry
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March 26, 2018

Dear Stephanie,

I am pleased to serve on your thesis committee and to consult on the use of optogenetic techniques for your F30 project, “Sources of cholinergic modulation of cortical microcircuits“, with Alison Barth. I have great respect for Alison’s scientific work and mentorship and I have no doubt that your project will lead to an impactful discovery. As a member of your thesis committee, I have vetted your proposed and preliminary work and I think your project has significant promise. As a physician-scientist, I am especially attuned to the unique training requirements and opportunities you face as an MSTP trainee, and am glad to offer this perspective.

As you know, I have significant experience using state-of-the-art tools in mice to investigate the pathophysiology of psychiatric disorders. Ongoing projects in my lab using optogenetics, DREADDs, in vivo microscopy, and in vivo electrophysiology are examining the role of orbitofrontal cortex and striatum in the development and expression of obsessive-compulsive disorder (OCD)-relevant behaviors. Your implementation of dual-color optogenetics to simultaneously study thalamic and cholinergic projections to the sensory cortex is novel and feasible, given Alison’s prior experience in targeting both thalamic and cholinergic inputs separately, and your collaborations with Brian Hooks and myself regarding technical details of this setup.

Your project has exciting implications for understanding cognitive disorders. As you know, I am also an MSTP graduate, and since completing my combined degree training I have pursued a career similar to your goal: integrating basic science rodent work on OCD with a clinical practice specializing in treating patients with the disorder. I am more than happy to offer advice and guidance on this career trajectory, and help you prepare for the next steps.

Since we first met at your interview for the MSTP five years ago, we have met many times to talk about science and I have greatly enjoyed our interactions. As a member of your thesis committee, I have been able to evaluate your graduate student milestones, including your second-year Pre-Proposal exam and Proposal Defense. I was very impressed with your performance on both exams, where you eloquently showed a deep understanding of the primary literature and the design of your project as well as possible outcomes and how you would pivot your approach to make sense of ambiguous data. Given these experiences, I am not surprised that you have prepared a compelling application for this NRSA. I look forward to our next committee meeting and to seeing your thesis come to fruition.

Feel free to contact me at any time should you have questions about your use of optogenetics, your approach to circuit mapping, or more general career advice. Keep up the good work!

Sincerely,

Susanne Ahmari, MD, PhD
Assistant Professor of Psychiatry
Director, Translation OCD Laboratory
University of Pittsburgh School of Medicine

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Stephanie Myal
PhD Student, Biological Sciences, Carnegie Mellon University
MSTP Student, University of Pittsburgh School of Medicine
4400 Fifth Ave.
Pittsburgh, PA 15213

March 27th, 2018

Dear Stephanie,

I am happy to provide advice to help support your use of dual-channel optogenetics tools a means to co-activate thalamic and neuromodulatory inputs to the somatosensory cortex, for your proposed thesis aims and for your F30 NRSA, on "Sources of Cholinergic Modulation of Cortical Microcircuits." Your proposed work is very similar to my prior publication with Karel Svoboda during my postdoctoral work at Janelia Farm. There I used channelrhodopsin concurrently with the red-shifted opsin ReaChR in targeted recordings to activate somatosensory and thalamic (POm) projections to motor cortex (Hooks et al. 2015). Your approach using virus injections of AAV-Chrimson in tandem with transgenic ChAT-ChR2 mice seems feasible to achieve independent activation of these channels, and I will be happy to help you troubleshoot your installation of a second LED and your parameters for stimulation.

I am confident that the preparation you have described for dissecting thalamic and cholinergic influences on cortical neurons will be able to achieve sufficient stimulation with minimal crosstalk and clearly interpretable results. I will be happy to work with you and your laboratory on how to perform proper controls to ensure maximal stimulation with good timing on your equipment. As we share an interest in neocortical connectivity and the roles played by a range of different cell types in sensory areas, I hope this leads to further collaboration between our groups. This is an exciting project, and I am delighted to share our expertise!

Very respectfully,

Bryan (Mac) Hooks, Ph.D.
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Robert E. Kass
Maurice Falk Professor of Statistics
and Computational Neuroscience
kass@stat.cmu.edu
www.stat.cmu.edu/~kass
412-268-8723

March 28, 2018

To Whom It May Concern:

I am happy to offer statistical guidance to Stephanie Myal for her proposed project, "Sources of Cholinergic Modulation of Cortical Microcircuits."

During my 20 years as a faculty member in our CNBC, the Center for the Neural Basis of Cognition, I have provided similar guidance for a large number of students on NRSAs and other training grants, and have served on many PhD thesis committees in Neuroscience and Neurobiology. My goal is always to move students beyond the "find the best test" mentality, and get them instead to think more comprehensively about their data. This perspective was a driving motivation for my 2014 text, *Analysis of Neural Data*, co-written with Uri Eden and Emery Brown.

Stephanie was an attentive and inquisitive student in my class on *Statistical Models of the Brain*, and I have interacted with her primary advisor, Alison Barth, on numerous occasions. The aims of Stephanie's proposed research strike me as highly worthy. I look forward to helping her in any way I can.

Sincerely,

Robert E. Kass
Maurice Falk Professor of Statistics and
Computational Neuroscience
Department of Statistics & Data Science
and Machine Learning Department

Interim Co-Director
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Peter L. Strick, Ph.D.
Scientific Director

Stephanie Myal
Mellon Institute Rm 159D
Department of Biological Sciences
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March 30, 2018

Dear Stephanie,

I am happy to serve as a consultant on your project comparing sources of cholinergic modulation of intra-cortical and thalamocortical synapses. The precise study of neuromodulator activity is a burgeoning area in neuroscience and your proposed research cuts to the heart of some lingering questions in the field, namely where do cholinergic synapses reside, what is the spatial "reach" of neuromodulators released from these synapses, and how do globally released neuromodulators act locally to help specialized brain areas take distinct roles in cognition.

As you know, I have spent the past 40+ years mapping thalamocortical projections to cognitive and motor brain areas in non-human primates. We have developed precise, staged rabies-based tracing methods to identify topography of functionally discrete loops pf projections between the limbic cortex and basal ganglia. Recently, colleagues and I have published papers concerning motor outputs from the posterior parietal cortex; connections between the basal ganglia, cerebral cortex, and cerebellum; and areas, including motor areas of the cerebral cortex, that connect to and influence the adrenal medulla.

I look forward to being an advisor and collaborator on your proposed work, which is exciting but also highly feasible given your lab's foundation of work on synapse-specific thalamocortical and cholinergic signaling. I am available to participate in conference calls and in-person meetings whenever you would like to discuss progress or find technical solutions to roadblocks. Feel free to get in touch at any time if you have questions about specifics of your project, or neuroanatomy more broadly, or for career advice.

I am very glad to hear that you are pursuing a career at the intersection of basic circuit biology and clinical neuroscience. I have been following your progress since you arrived in the MSTP, and I am impressed by your verve for research in this field and your output so far. I have mentored many young scientist-doctors and I see particular promise in you. I think this is an area that is ripe for someone to pursue dual-degree training, and I am looking forward to seeing how your career unfolds.

Sincerely,

Peter L. Strick, Ph.D.
Scientific Director, University of Pittsburgh Brain Institute
Thomas Detre Professor & Chair of Neurobiology

Additional Educational Information

Prepared by Richard Steinman MD PhD, Associate Dean & Director, Pittsburgh MSTP, University of Pittsburgh SOM. Steinman@pitt.edu, 412 623 3237

Description of MSTP

MSTP Structure**.** MSTP students at Pitt complete an MSTP-specific enrichment curriculum beyond the standard courses in medical and graduate school. This consists of 3 summer research rotations, 3 summer professional development courses, one 3-semester weekly journal club featuring research papers whose topics follow the SOM curriculum; one 4-week, case-based ethics course; a monthly program-wide workshop, which students attend throughout all years of the program; 40 weeks of longitudinal clinical clerkships (one half-day per week) during the graduate years; annual special events such as the 2-day MSTP Scientific Retreat and Second Look events; assigned peer mentors from the senior MSTP classes, and assigned MSTP Career Advisors who monitor students' progress longitudinally throughout the program.

Laboratory Research Rotations**.** Research rotations begin the summer prior to the start of medical school. In addition to developing manuscripts and presenting at scientific meetings based on their rotation results, all students turn in a written scientific report that is reviewed by MSTP leadership and present their work at the annual MSTP Scientific Retreat. The choice of thesis laboratories by students is informed by their rotation history and by discussion with their individual Career Advisors.

Professional Development**.** Students take three successive, 10-week Professional Development Courses during the summers prior to starting graduate school. The first course (PD1) focuses on scientific writing and introduces students to biomedical software and key research and analysis methods used by different disciplines. The PD2 course focuses on scientific design and career development strategies, with particular emphasis on reproducibility and biostatistics. The PD3 course focuses on grant review and writing.

Training in Reproducibility in Science**.** The PD2 course focuses on optimizing statistical power and reproducibility of findings, and analyzing data using appropriate statistical tests. Topics for classes include problems arising from non-reproducible work, optimal experimental and reagent documentation and handling, the ARRIVE guidelines for animal work, measurement validity and sources of error, robust hypothesis testing, and a series of sessions on biostatistics including customized problem solving tied to student data.

Biomedical and ethical expertise**.** During MS1 and 2 years, students build biomedical knowledge through a three-semester MSTP literature review course in which students present papers after formal consultation with local faculty experts in the field of that paper. During the G1 year of graduate school, MSTP students take a month-long, weekly, case-based research ethics course. Throughout both medical school and graduate school, all MSTP students meet monthly for student-arranged seminars that pose scientific, logistical, clinical and/or ethical dilemmas. These workshops are presented by students and/or guest faculty experts.

Clinical and Research Integration**.** This is a central focus to better model the physician scientist career.

*Clinical Activities During the Research Years***:** Prior to starting graduate school, all of our MSTP students complete 4 to 8 weeks of required clinical core clerkships. This front-loads requirements once students re-enter medical school post-thesis and enables research engagement in MS3 and 4. MSTP students are required to complete a (credited) minimum of two 20-week long *Longitudinal Clinical Clerkships (LCCs)* during graduate school. For each LCC, students spend a half day per week with a clinician scientist receive one-on-one clinical mentoring in an area of interest chosen by the student with guidance from the MSTP LCC director, Paul Monga, MD. Student objectives for the LCC and write-ups at the end are reviewed by MSTP leadership.

*Transition from Graduate to Clinical Years***.** During our MSTP Clinical Reentry elective, a master clinician mentors the returning students once weekly over the month prior to reentry as they examine, discuss, diagnose and plan treatment for surrogate patients presenting with common outpatient or inpatient ailments.

*Research During Clinical Years***.** Our students continue their research focus after re-entry to medical school generally in four ways: (1) MS3 and MS4 students continue to plan and execute MSTP Workshops that feature research topics and research challenges to be discussed with MSTP peers. (2) Students complete formal reflective and goal-oriented self-assessment evaluations during twice-yearly Career Advisor meetings. (3) Students average 2.8 new publications during the MS3 and MS4 years (at least one first authored), averaging 5-7 papers upon graduation. (4) Most students elect to take 1-2 Research Elective months during

MS4 to extend findings of thesis work and/or build skillsets in a translational area. Another novel feature of our MSTP, the Postdoctoral Fellowship, provides support for 5 months of postdoctoral research prior to residency for MSTP students graduating in December (25% of recent graduates). Applications address research hypotheses and aims, career development aims, planned deliverables, mentor fit and intellectual goals.

Monitoring and Evaluating Student Progress**.** Prior to matriculation, the Program Director assigns each new student a Career Advisor based on matching research interests who help orient and guide the students throughout their careers. Most of a trainee's time in the graduate program is spent in research training under the guidance of their research mentors, program leadership, and eventually their doctoral thesis committee. To customize advice and resource allocation, all MSTP students complete and share individual development plans with the Director and with their Career Advisor. The form allows students to identify specific skills that they want to develop, set technical, intellectual and professional goals, and identify how goals will be achieved and measured. Resources to reach goals and obstacles that could compromise success are enumerated and discussed. Progress toward goals is reviewed regularly with the Advisor and new goals are set.

Career Counseling**.** To better reflect the student's educational experience to prospective residency programs, the MSTP creates an executive summary which describes student evaluations, honors, presentations and participation in the combined degree training, rewarding students who altruistically give their time and demonstrate prowess in working in groups. Six months to one year before completing their doctoral program, students meet with the Program Director and the Career Advisory committee to discuss postgraduate training, residencies, fellowships, and faculty positions and non-academic based positions. Many of the faculty are MD/PhDs and are capable of participating in career planning for third and fourth year medical students.

Program Duration and Outcomes**.** Over the past 5 years, our time from enrollment to graduation has averaged 7.7 years. The Pittsburgh MSTP has 170 alumnae. 89% of graduates from the past 15 years are in the academic pipeline (either still in training or in academic positions). Graduating MSTPs in 2011-16 averaged 6.2 papers/student (3 first authored) and 47% had obtained F-grants.

Description of CMU and CNBC

CMU Biological Sciences and the CNBC have several program milestones with timelines adjusted for expeditious completion by MSTP students. *Stephanie was exempt from all but three PhD courses, which she completed in 2017. She has also completed her requirements to assist the department as an exam grader and teaching assistant.* To maintain good standing in the CNBC, Stephanie must attend its annual retreats and a quota of weekly Brain Bag seminars on scientific and ethics topics. To remain in good standing at CMU, Stephanie will continue to attend weekly departmental seminars until her dissertation. *Stephanie defended her Thesis Proposal on 2/19/2018 (on schedule, as a G2) and is now a PhD candidate.* She will now have regular, biannual committee meetings and will be expected to complete her degree within ~24 months of this proposal.

Stephanie plans to defend her PhD and return to medical School in May of 2020, putting her on track to complete her remaining clinical clerkships and graduate from the MSTP in May of 2022. Thus, from the time of this proposal, Stephanie expects to complete an additional 25 months of research and 24 months of clinical work. Should Stephanie elect the route in which she graduates medical school in December of 2022, she will then undertake a 5-month MSTP Postdoctoral Fellowship, which will be beneficial to her training but is not included in the time of covered support requested in the current application.

Stephanie Myal is a stellar member of our MSTP who matriculated into the program in June 2014 and who is completing her G2 year as a Biology PhD student at CMU. Stephanie completed her MS1 and MS2 coursework and passed Step 1 of the Boards in May 2016. She completed all three MSTP Professional Development courses, laboratory rotations, the RBMK course, the MSTP Ethics course, and a 4-week clinical rotation in Family Medicine. Stephanie began her PhD in September 2016. She has chosen to work in the laboratory of Alison Barth, PhD, where she is investigating cholinergic circuits in the cerebral cortex. CMU Biological Sciences is a highly interdisciplinary department with expert faculty in genetics, evolutionary biology, computational biology and neuroscience. Trainees benefit from extensive additional resources in professional development through CMU's support services for graduate students. Stephanie also maintains full access to facilities and career development resources at Pitt. Stephanie will begin her first LCC in May 2018 with Edward Burton, MD, DPhil, a practicing neurologist-scientist specializing in movement disorders. She plans to complete her second LCC in the Spring of 2019 at the Western Psychiatric Institute and Clinic at UPMC.

PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001 and 0925-0002

Expiration Date: 03/31/2020

Are Human Subjects Involved	❍ Yes ● No
Is the Project Exempt from Federal regulations?	❍ Yes ❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Does the proposed research involve human specimens and/or data	❍ Yes ● No
Other Requested information	

Vertebrate Animals and Procedures

Description of animals and their use: The proposed studies will use transgenic mice (age P21-25). All strains have been successfully bred in our lab and backcrossed to a C57Bl6/J genetic background. These strains are:

- GPR26-Cre (stock no. 036915-UCD; MMRRC)[123]
- Nelf-Cre (Nsmf-Cre; stock no. 036664-UCD, MMRRC)[123]
- ChAT-Cre (stock no. 006410; Jackson Laboratory)[111]
- ChAT-ChR2 (ChAT-COP4-eYFP,stock no. 014546, Jackson Laboratory)[124]
- PV-tdTomato (stock no. 027395; Jackson Laboratory)[125]
- Pv-Cre (stock no. 017320, Jackson Laboratory)[126]
- GIN (Martinotti SST eYFP; stock no. 003718; Jackson Laboratory)[127]
- Vip-Cre (stock no. 010908; Jackson Laboratory)[128]
- Ai3 (flex-YFP; stock no. 007903; Jackson Laboratory)[129]

Additional transgenic mice can be obtained if required for finer-scale targeting of particular cell types (for example, genetic control of layer 5 excitatory neurons). Our mice are typically maintained as homozygote lines, with heterozygous or double-heterozygous transgenics used for experiments. Our lab maintains a colony of 27 mouse strains, including SST-Cre mice which could be substituted as needed for experiments requiring GIN mice. In designing experiments, we have prioritized the use of single-transgenic mice wherever possible, to minimize the number of breeding cages required. We also opted for simple surgical protocols wherever possible, to minimize discomfort of the mice.

Sex and age of mice: Both males and females will be used. The sex of all mice will be recorded and every effort will be made to balance the number of males and females used. All mice used will be prepubescent (P21-25 days). Though our preliminary experiments suggest no sex differences in synapse organization or cortical plasticity at these ages, we will calculate male and female values separately to monitor possible trends.

Stereotaxic injections of AAV-encoded opsins and fluorescent proteins: For viral injections into the cortex of neonatal pups (P1-3), we will anesthetize them using 10-15 minutes of hypothermia in an ice-cooled, moisture-free, well-ventilated chamber until motionless and unresponsive to leg pinch before transfer to a stereotaxic apparatus. For injections in older mice (P11-15), we will initially anesthetize them with isoflurane until unresponsive to foot and tail pinch. They will then be placed in a jaw bar, blunt ear bars and a nosecone for continued delivery of isoflurane (3-5% with 10 PSI oxygen). During injections, animals will be placed on a warm heating pad and monitored for maintained anesthesia. After injections of AAV-encoded DNA constructs, the mice will be treated once with ketofen (5 mg/kg, Sigma-Aldrich), then monitored for full recovery in their home cage, with supplemental ketofen and special diets given as needed. All mice will be group-housed.

Euthanasia: Before tissue collection for experiments, mice will be deeply anesthetized with inhaled isoflurane until they are unresponsive to foot and tail pinch. Once unresponsive, they will either be decapitated to retrieve the brain for electrophysiology experiments, or transcardially perfused with 1% PBS followed by 4% paraformaldehyde fixative and then decapitated to retrieve fixed brain tissue for anatomic experiments. All mice experiencing significant pain or distress prior to experiments will be euthanized by CO_2 inhalation. All procedures have been approved by the Carnegie Mellon University IACUC and are in accordance with American Veterinary Medical Association guidelines for euthanasia.

Justification for use of mice: The results of this work are of potential significance to both clinical and basic science. Our study design was inspired by clinical questions about the role of cortical acetylcholine in cognition and psychiatric disease. Thus, it is important to conduct our studies in a model species that has a cortex and is capable of relatively complex behaviors. Our experiments depend on the layered architecture of the intact brain, which is currently impossible to recreate in cell culture. It is also impossible to carry out the proposed experiments solely using computer models, since many of the basic parameters required for modeling have not yet been described in studies like this one. Mice are an ideal model system to carry out the proposed studies due to highly conserved aspects of brain structure and ion channel function between mice and humans, and the availability of extensive transgenic tools for cell type targeting. Our studies lay the groundwork for behavioral and translational studies of cortical function in complex behaviors without using humans or non-human primates.

Experimental Timeline: The experiments in Aim 1 and Aim 2.1 can be performed concurrently, and should be ~40% complete by the beginning of the award period. The experiments in Aim 2.2 await the setup of our red LED for dual-channel optogenetics, which we will begin this summer. In many cases, *our immunohistochemical staining of cholinergic inputs to various cell types can be performed in tissue that has also been used for electrophysiology*. We have done this successfully in our preliminary experiments with no loss of tissue quality. In this way, we can characterize cortical cholinergic circuits to fullest capacity possible while reducing the number of experimental animals needed for anatomic experiments by up to 60%.

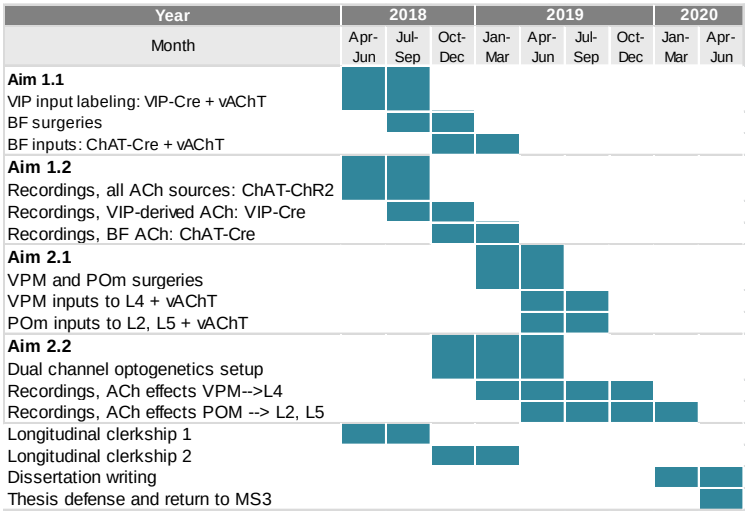

Table 1. Experiment timeline in relation to PhD and MSTP milestones.

Completion of these experiments will be expedited by various support personnel. *The Barth lab has a dedicated technician to assist in animal breeding and colony maintenance, and an additional technician to perform genotyping* and assist in tissue perfusion, cryosectioning, and viral injections of S1 cortex on an as-needed basis.

Justification for number of subjects: To generate a sufficient number of transgenic animals for the proposed experiments, we expect to require **approximately 70 breeding cages** in total to generate the single- and double-transgenic mice necessary to complete the proposed experiments. This includes the number required for maintenance of homozygous stocks of transgenic mice as well as breeding of heterozygous animals for recordings and anatomical studies. Power analyses are discussed in the "Research Strategy."

Patents and resource distribution: No patents or novel transgenes are expected to arise from this work. Our data dissemination strategy is further discussed in the "Resource Sharing Plan."

Select Agent Plan

A subset of the proposed experiments (particularly the comparison of responses evoked by thalamic stimulation in different target cell types) requires use of the select agent and Na channel antagonist tetrodotoxin (TTX), which at high concentrations can be toxic. Stock concentrations of TTX (1 mM in H_2O) will be stored with cautionary labeling in secondary containment at -20^{o}C. Only authorized personnel will be permitted to access, possess, or use stocks and working solutions of TTX. We will use TTX at low concentrations (0.2-1.0 µm) diluted in ACSF and will collect TTX-containing solutions separately for approved disposal. After use, all working solutions and stocks of TTX will be inactivated in a freshly prepared 2.5% Sodium Hypochlorite solution for a period of no less than 30 minutes. Following this treatment, the inactivated solution will be disposed of via a third-party hazardous waste vendor.

Resource Sharing Plan

We are committed to sharing our experimental methods and results with fellow researchers. We plan to publish or present all findings (in the form of raw data files, text, photographs, videos, or multimedia as appropriate) to the neuroscience community. Some of this dissemination will occur in the form of posters and presentations. I have presented, and expect to continue presenting my work at the annual Society for Neuroscience and Barrels meetings. I will also take advantage of local opportunities to share and discuss my findings at lab-wide and institution-wide meetings, as well as additional relevant national meetings (Gordon Conferences, etc.). Other data dissemination will take the form of research publications that I will write as first author. The field of neuromodulation in cortical circuits is rapidly evolving, and we will strive to deliver our results to our peers and the public in a timely manner.

This project will not involve the development of new transgenic technologies, but our lab has a policy of open sharing of transgenic mice and reagents with other labs at Pitt and Carnegie Mellon, as well as national and international collaborators.

We do not anticipate creating any new computer programs using open-source software, but if we do, we will make them publicly available. Our lab is currently exploring platforms for open dissemination of our datasets of 3D neuron reconstructions; possible options include KiltHub (a CMU-wide data archive), bioRxiv, and the Open Science Framework.

Authentication of Biological and Chemical Resources

Overview: The experiments proposed will employ transgenic mice bred in-house on a C57Bl6/J background, and genotyped using primers and protocols recommended by the vendor of each strain. Because all transgenes are used to ultimately achieve fluorescent labeling of neurons, transgene expression will be verified by visual inspection of the tissue under a fluorescence microscope as part of our anatomic and electrophysiologic experiments.

Vertebrate Animals: The strains of transgenic mice to be used in this study are as follows:

--GPR26-Cre (stock no. 036915-UCD; MMRRC)[123]
--Nelf-Cre (Nsmf-Cre; stock no. 036664-UCD, MMRRC)[123]
--ChAT-Cre (stock no. 006410; Jackson Laboratory)[111]
--ChAT-ChR2 (ChAT-COP4-eYFP,stock no. 014546, Jackson Laboratory)[124]
--PV-tdTomato (stock no. 027395; Jackson Laboratory)[125]
--PV-Cre(stock no. 017320, Jackson Laboratory)[126]
--GIN (Martinotti SST eYFP; stock no. 003718; Jackson Laboratory)[127]
--Vip-Cre (stock no. 010908; Jackson Laboratory)[128]
--Ai3 (flex-YFP; stock no. 007903; Jackson Laboratory)[129]

All mice used for experiments will be genotyped using genomic DNA from tail tissue obtained at postnatal days 7-12 using genomic DNA isolated from a 0.5 cm segment cut from the mouse tail. Mice will be genotyped using primers recommended by the vendor (Jackson Laboratories or MMRRC) to test that particular strain.

Recombinant DNA viral constructs: To achieve cell type- or brain-area specific expression of fluorescent markers or light-activated ion channels for optogenetics, we will use the following adeno-associated virus-encoded (AAV) genetic constructs in our stereotaxic injections (all are Biosafety Level 1 pathogens):

--DIO-YFP(rAAV2/EF1a-DIO-eYFP, UNC vector core)
--DIO-mCherry(rAAV2/EF1a-DIO-mCherry, UNC vector core)
--CAG-GFP(rAAV2/CAG-GFP, UNC vector core)
--DIO-ChR2-YFP(rAAV2/EF1a-DIO-hChR2[H134R]-eYFP, UNC vector core)
--DIO-ChR2-mCherry(rAAV2/EF1a-DIO-hChR2[H134R]-mCherry, UNC vector core)
--ChrimsonR-TdT(rAAV2/EF1a-Syn-ChrimsonR-TdTomato, UNC vector core)

Specialty Chemicals: Our electrophysiologic experiments will require the following reagents to block neurotransmitter receptors and ion channels:

--D-AP5 (Tocris, Cat # 0106, batch no. 73A/199002)
--NBQX disodium salt (Tocris, Cat # 1044)
--Picrotoxin (Sigma, Cat # 528105)
--Mecamylamine (Sigma, Cat # M9020)
--Atropine (Sigma, Cat # A0132, batch no. 106K1638)
--4-Aminopyridine (Sigma, Cat # A78403)
--Tetrodotoxin (Tocris, Cat # 1078)

We will validate the action of our working solutions of these chemical in our electrophysiologic experiments by applying them to known cells and synapses that contain the receptors blocked by the toxin.

Antibodies: We will use primary antibodies against the vesicular acetylcholine transporter (rabbit anti-vAChT, Synaptic Systems, Cat # 139-103), which we have successfully used in our published studies[14]. We may require antibodies against YFP to boost the fluorescence of transgenic or weak viral YFP labeling (rabbit anti-YFP, Life Technologies, Lot# 1616593). We will also use the following secondary antibodies: AlexaFluor 488 goat anti-rabbit (Life Technologies, Lot # 1853312), AlexaFluor 546 goat anti-rabbit (Life Technologies, Lot # 1488578), and AlexaFluor 647 goat anti-rabbit (Thermo Fisher, Cat # A21245).

Because all antibodies are used to visualize fluorescent protein expression, we will validate them by visual inspection and comparison to published results of positive labeling, positive control tissue from prior studies in the lab, and patterns of auto-fluorescence in known wild-type tissue.