AWARD NUMBER: W81XWH-16-2-0011

TITLE:

Cortical Photostimulation Technology for Vision Prosthesis

PRINCIPAL INVESTIGATOR:

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CONTRACTING ORGANIZATION:

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REPORT DATE:

May 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
May 2018	Annual	15 Apr 2017 - 14 Apr 2018

4. TITLE AND SUBTITLE
Cortical Photostimulation Technology for Vision Prosthesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-2-0011

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UNIVERSITY OF MARYLAND
220 ARCH ST RM 02148
BALTIMORE MD 21201-1531

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall goal of the project is to evaluate the feasibility of using photochemical stimulation of cortical neurons as the basis for a visual prosthesis. Accomplishments during year 2 of the project include: 1) production of oxNI-Glu, the caged glutamate compound, for in vivo toxicological and in vitro physiological studies; 2) completion of toxicological studies showing that chronic infusion of oxNI-Glu into mouse cortex is benign and well-tolerated; 3) in vitro photoactivation tests in mouse brain slices showing that focal photolysis of oxNI-Glu in situ can photoactivate cortical neurons reliably; and 4) successful training of mice in an automated operant conditioning cage to exhibit conditioned behavior in response to a stimulus.

15. SUBJECT TERMS
Visual prosthesis, photostimulation, photorelease, uncaging, caged glutamate

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	U U		**19b. TELEPHONE NUMBER** *(include area code)*
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1. Introduction

The goal of this project is to develop a photostimulation technology that can serve as the basis of a new visual prosthesis for wounded warriors who have lost their anterior visual system (i.e., the eyes and/or optic nerves). At the core of the technology is a “caged glutamate”, a molecule that is inert (or “caged”) until it is transformed by a flash of light into the active neurotransmitter molecule, glutamate, which then can stimulate neurons in the visual cortex to evoke visual perception. The photochemical transformation that turns caged glutamate into active glutamate is called “uncaging” or “photorelease”. The project comprises three key aims: 1) development of a caged glutamate can be uncaged by visible light, 2) testing of the new caged glutamate in vitro and in vivo, 3) validation of the photostimulation approach in the mouse cortex.

2. Keywords

Visual prosthesis, photostimulation, photorelease, uncaging, caged glutamate

3. Accomplishments

► Major goals of the project (year 2)

Major goals for year 2 of the project, as outlined in the SOW, are summarized in the table below.

	Proposed month of completion	**Actual completion month**
Specific Aim 1: Develop a photoreleasable neurotransmitter optimized for in vivo photostimulation of the cortex		
Major Task 2. Synthesis and characterization of oxNI-Glu		
● ***Milestone*** *Revised synthesis and characterization of oxNI-Glu complete*	7	20
Major Task 3. Scale-up production of caged Glu for in vivo animal studies		
● ***Milestone*** *Scaled up oxNI-Glu synthesis*	10	21
● ***Milestone*** *HPLC acquisition and set up, oxNI-Glu purification*	12	24
Major Task 4. Test in vivo toxicity of oxNI-Glu		
● ***Milestone*** *Brain tissue harvest complete*	12	19
● ***Milestone*** *Histological evaluation complete*	14	24
Specific Aim 2: Validate photostimulation technology in vitro		
Major Task 1. *Determine parameters for safe and reliable photoactivation of neurons*		
● ***Milestone:*** *In vitro stimulus parameters defined*	18	ongoing (35%)
Major Task 2. *Determine spatial resolution of photoactivation*		
● ***Milestone:*** *Spatial resolution of photostimulation defined*	18	ongoing (20%)
Specific Aim 3: Validate photostimulation technology in vivo		

Major Task 1. *Construct automated operant conditioning cage and develop mouse training protocol*		
● ***Milestone:*** *Operant conditioning cage and protocol completed*	17	17
Major Task 2. *Determine optical damage threshold in vivo*		
● ***Milestone:*** *Damage threshold determined*	22	0%
Major Task 3. *Determine in vivo spatial resolution of photoactivation*		
● ***Milestone:*** *Spatial resolution of in vivo photoactivation determined*	24	0%
Major Task 4. Determine if in vivo photostimulation can evoke a percept		
● ***Milestone:*** *Test of photostimulation-evoked behavior complete*	24	0%

► Accomplishments under the major goals/tasks

The accomplishments are 1) revised the synthesis of the caged glutamate, oxNI-Glu, and completed characterization of the compound; 2) stockpile of oxNI-Glu for in vivo studies; 3) in vivo tracer and toxicological studies in the brains of live mice; 4) initiating in vitro photostimulation studies in mouse brain slice preparations; 5) installation of cranial window in mice; and 6) construction of an automated operant conditioning cage and designing a mouse training protocol. These are summarized below.

1,2) As stated in earlier quarterly reports, the original chemical synthesis scheme for oxNI-Glu yielded material that was not bioactive. We revised the scheme (Fig. 1) and were able to synthesize sufficient authentic, bioactive oxNI-Glu, which was used for subsequent studies.

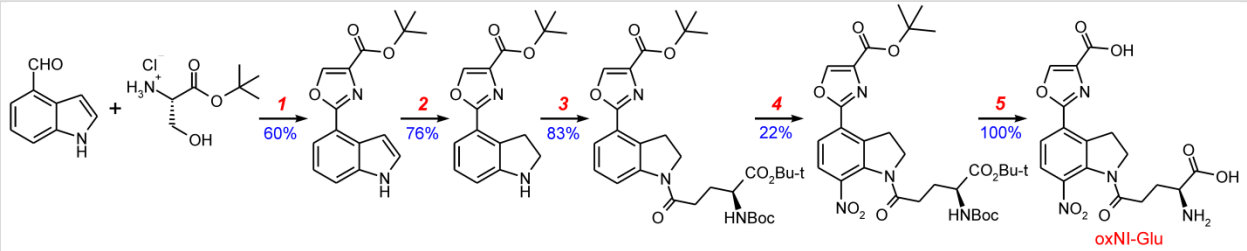


Figure 1. Final revised synthesis of oxNI-Glu. *Reagents and conditions*: ***1. a.*** K_2CO_3, DMA; ***b.*** $CBrCl_3$, DBU; ***2.***$NaCNBH_4$, CH_3CO_2H; ***3***. *N*-Boc-L-Glu-OBu-*t*, EDC·HCl, CH_3CN; ***4.*** $Bi(NO_3)_3$, $(CH_3CO)_2O$, CH_2Cl_2; ***5'.*** CF_3CO_2H. *Abbreviations*: Bu-*t* = *tert*-butyl, Boc = *tert*-butoxycarbonyl, DMA = *N,N*-dimethylacetamide, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, EDC·HCl = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. Percent yield of each reaction step is indicated in blue font.

3) Two groups of mice received long-term (3 – 4 weeks) infusion of either oxNI-Glu or the fixable fluorescent tracer Alexa Fluor 568 hydrazide (AF568H). Each compound was delivered by an osmotic pump in the back of the animal through a cannula implanted into the visual cortex. At the end of the infusion, the animals were transcardially perfused with saline and fixative. The fixed brains were cut into 20-µm-thick sections and examined histologically for damage and by fluorescence microscopy to visualize the spatial extent of the fluorescent tracer; the results are shown in Fig. 2. As evidenced by Fig. 2A, after infusion into visual cortex, the aqueous tracer AF568H showed quite wide spread. This means that highly water-soluble compounds like caged glutamate can be locally infused and reach high concentrations over substantial volumes of

tissue. Cresyl violet-stained sections (Fig. 2B) showed that other than the physical injury caused by cannula insertion, no pycnotic nuclei nor signs of apoptosis and necrosis were visible. No significant differences were observed between AF568H- or oxNI-Glu-infused brains — consistent with oxNI-Glu having no unique toxicity at the cytoarchitectural level. We also attempted immunohistological analysis for the antigens IBA-1 (AIF-1), GFAP, and parvalbumin. Unfortunately, the sections were very poorly stained by the antibodies, which worked well in brain sections from control mice that were not infused. Because the infused brains were sectioned by a novice technician, the sections may not have been cryopreserved properly to retain antigenicity. If time permits, we would like to repeat the infusion experiment to enable immunohistological analysis. Most importantly, the lack of obvious damage as revealed by cresyl violet and the large spatial extent of AF568H permeation suggests that oxNI-Glu is suitable for use in cortical photostimulation.

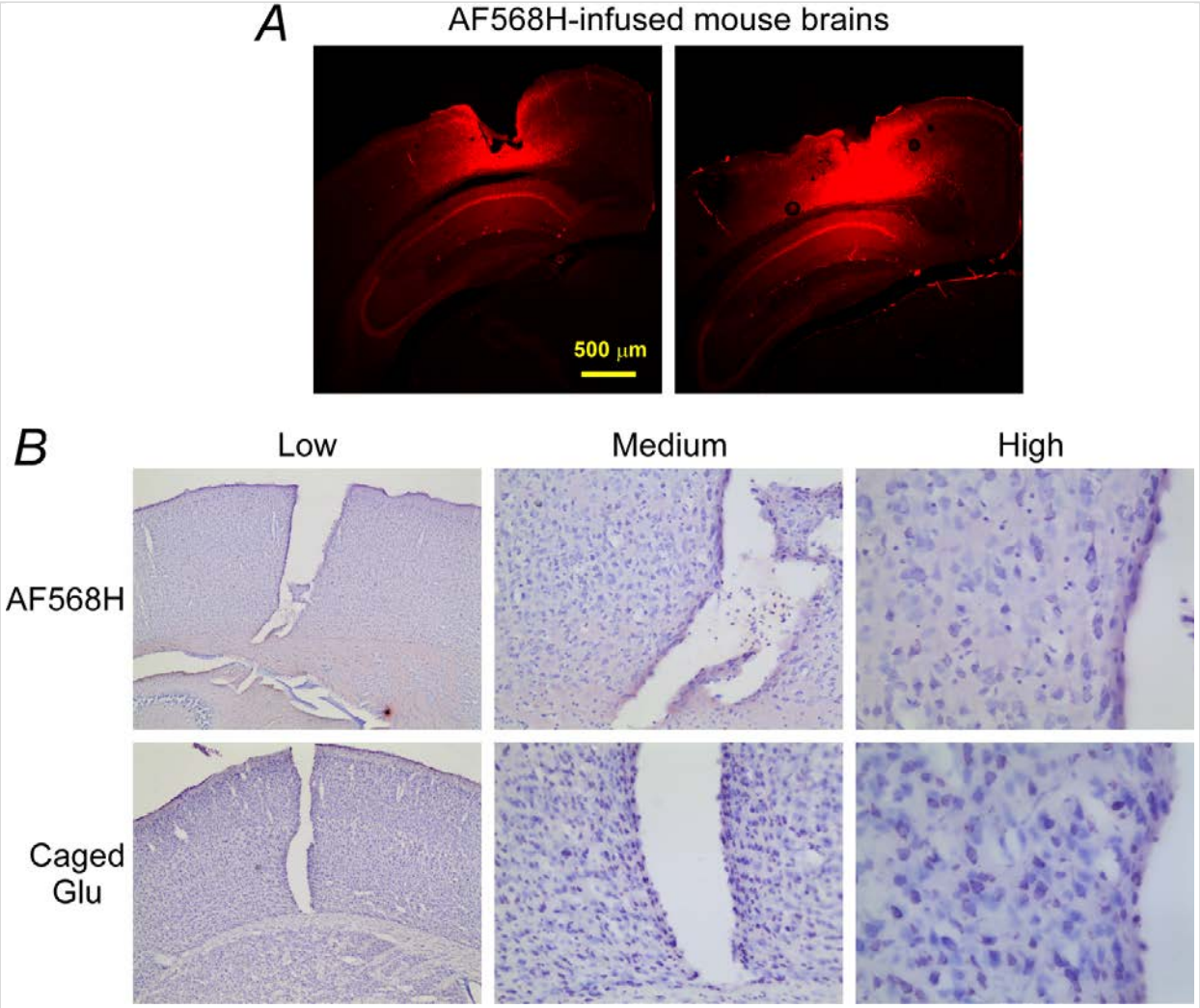


Figure 2. Sections of mouse brains infused with Alexa Fluor 568 hydrazide (AF568H) tracer and oxNI-Glu. ***A.*** Images of sections from two mouse brains showing the spatial spread of AF568H tracer. The divots caused by cannula penetration are clearly visible. ***B.*** Cresyl violet staining of brain sections from two representative animals whose visual cortices were infused with AF568H and oxNI-Glu, respectively. Sections are shown at three magnifications (Low, Medium, and High).

We noted that the infused AF568H was not restricted to the immediate site of

but spread quite widely in the cortex and was present in distal structures in the hippocampus. This could be suggestive evidence that water-soluble molecules in the cerebrospinal fluid (CSF) are swept into diverse brain regions by the endogenous irrigation system that transports CSF throughout the brain. Thus we may be able to deliver caged glutamate or tracers to the cortex by introducing the compounds into an earlier segment of the CSF flow path — e.g., the third ventricle or the cisterna magna. This approach allows us to move the site of cannulation to a part of the brain that is distal to the cortex, so the cannulation hardware would not obstruct the photoactivation hardware. We would like to explore this approach in the coming months.

4) In vitro studies were conducted in brain slices to validate oxNI-Glu for photostimulation. Preliminary measurements in brain slices perfused with oxNI-Glu showed that uncaging of oxNI-Glu can reliably activate pyramidal neurons in the visual cortex (Fig. 3). Judging from our experience, the performance of oxNI-Glu is at least comparable to, and possibly superior to, that of a well-tested caged glutamate, Ncm-Glu, which we have used extensively in photostimulation experiments (Muralidharan et al. 2016. *PLoS One* 11(10):e0163937). The measurements on oxNI-Glu were performed with a 355-nm laser. A 405-nm laser has been purchased and set up for testing oxNI-Glu in slice preparations; the set-up is being debugged.

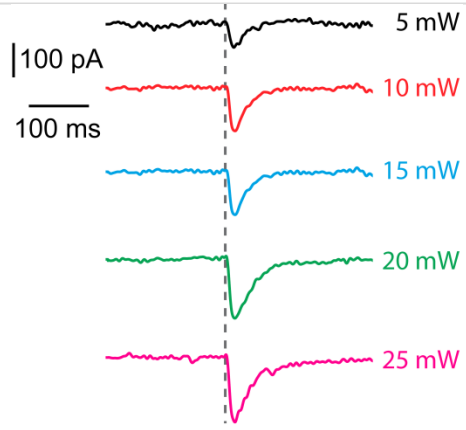


Figure 3. Photoactivation of pyramidal neuron in a mouse brain slice. A brain slice was perfused with 1.18 mM oxNI-Glu in artificial cerebrospinal fluid. A photolysis beam at 355 nm was directed onto a pyramidal neuron, which was patch-clamped. Photolysis pulses of ~1 msec duration were delivered at various power levels to evoke graded excitatory post-synaptic currents (EPSCs). Dashed vertical line marks the time of the laser pulse.

5) As stated in the previous quarterly report, we can install a cranial window to enable long-term optical observation and manipulation of the cortex of a live mouse (Fig. 4). This allows us to do the key experiment in Aim 3 — to test whether photostimulation of the cortex of a live, behaving mouse could evoke a conditioned response.

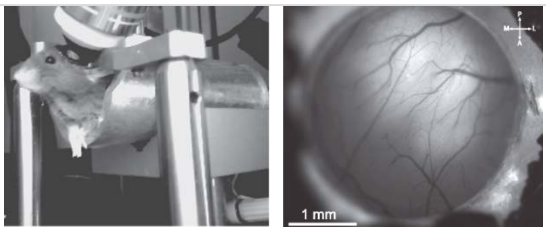


Figure 4. Mouse cranial window. *Left*: Awake, restrained mouse with a chronic cranial window. *Right*: View of the brain through the cranial window; rostral-caudal and medial-lateral directions are indicated.

6) An automated operant conditioning cage for mice was designed and prototyped at the end of year 1. This cage incorporates an electronically controlled water spout and loudspeaker, can be placed under video surveillance, and is run by custom software. The cage was used to train mice to lick a water spout (and be rewarded with an aliquot of water) upon hearing a target tone. A representative example of conditioned mouse behavior evoked by a target stimulus is shown in Fig. 4. Under this protocol, mice are generally conditioned after about two weeks of training. The success in training means that we will have properly conditioned mice to use in the key major task of the third Specific Aim — to determine if photostimulation of the "correct" part of the tonotopic map can evoke a conditioned behavior from a trained mouse *as if* the mouse had heard the physical tone.

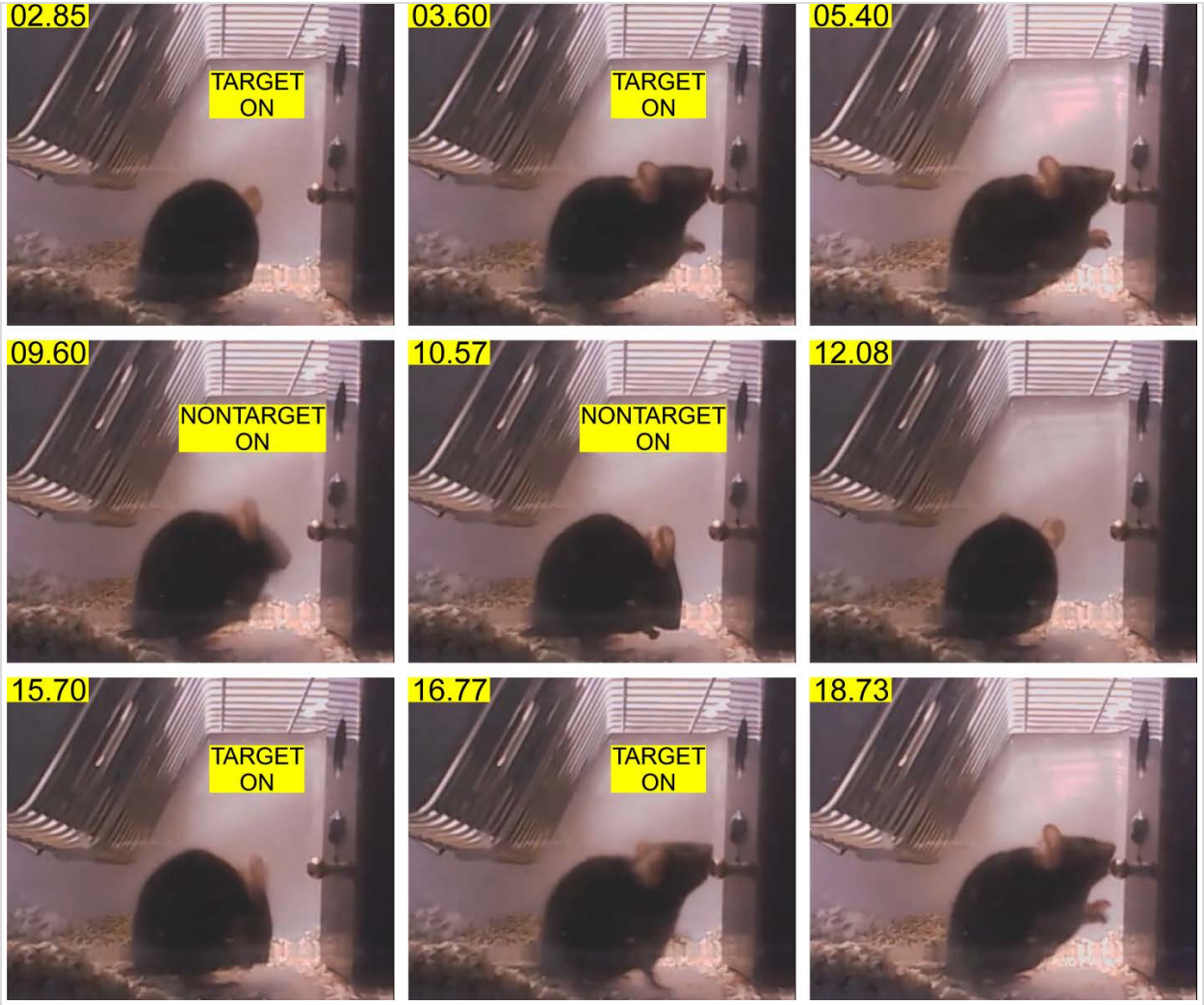


Figure 5. Mouse in an operant conditioning cage was trained to lick a water spout upon hearing a target tone. The mouse was trained in the cage for two weeks during which it learned to lick the water spout upon hearing a target tone of 1-sec duration. A temporal sequence of frames is shown; the time in seconds after the start of the recording is shown in the upper left of each frame. The mouse responds to the target stimulus, but not to the nontarget, by exhibiting the conditioned behavior.

► Opportunities for training and professional development

Nothing to report.

► Dissemination of results to communities of interest

Nothing to report.

► Planned activities in the next reporting period

To achieve the goals of the project, the following key tasks will be performed in the next reporting period.

1. Complete in vitro testing of oxNI-Glu photostimulation: specifically, to determine the stimulus parameters and spatial resolution of photoactivation in brain slices.
2. Test delivery of tracers and oxNI-Glu by introducing the compounds into the third ventricle and/or the cisterna magna.
3. Determine in vivo photostimulation parameters (power-response relation and spatial resolution) using oxNI-Glu in live animals.
4. Determine if photostimulation of the "correct" part of the tonotopic map can evoke a conditioned behavior in a mouse that was trained in the operant conditioning cage.

4. Impact

► Impact on the development of the principal discipline(s) of the project

- Development of oxNI cage and a reliable chemical synthesis of oxNI-caged glutamate is a significant advance, because we show that 1) light absorption by the NI family of cages can be red-shifted into the visible wavelength range through rational design of the molecular structure of the cage, and 2) production of useful quantities of oxNI-Glu is feasible (if not straightforward — the chemistry having taken more than a year to iron out). Such red-shifted cages make a visual prosthesis based on caged glutamate photochemistry increasingly feasible.
- The fact that cortical neurons in brain slices can be reliably photoactivated by uncaging oxNI-Glu show that the new caged glutamate fulfills its intended function.
- The finding that long-term (3 – 4 weeks) oxNI-Glu infusion into the cortex 1) does not affect the health and behavior of the mouse, and 2) does not cause damage to brain tissue is remarkable for two reasons. First, long-term infusion of caged compounds into animals has never been performed; therefore the effects of such infusion were unknown until the present study. Second, the tolerance of brain tissue to chronic oxNI-Glu exposure suggests that this family of caged glutamates may safely serve as the photochemical platform for a prosthesis.
- The tracer studies with Alexa Fluor 568 hydrazide (AF568H) showed that this tracer was not spatially confined to the immediate neighborhood of the site of infusion; rather, it was distributed over substantial volumes of brain tissue. This implies that water-soluble molecules may be widely distributed through brain tissue by an endogenous flux of cerebrospinal fluid (CSF). This opens the door to introducing caged glutamate into early segments of the CSF flow path (e.g., third ventricle or cisterna magna) and allow the CSF flux to bring the compound to the visual cortex. Such a method of delivery could simplify delivery mechanics and would eliminate the need to disrupt cortical tissue by cannula implantation.

● The success of the automated operant conditioning protocol means that mice to be used for the behavioral study in Aim 3 can be trained readily and independently without intervention by the human experimenter. Training can proceed during the night, when the mice are most active. This makes the “production” of conditioned mice much less laborious than it otherwise would be.

► Impact on other disciplines

Nothing to report.

► Impact on technology transfer

Nothing to report.

► Impact on society beyond science and technology

Nothing to report.

5. Changes/Problems

► Changes in approach and reasons for change

There was no significant change in approach. The chemical synthesis scheme for oxNI-Glu was revised and optimized as part of standard chemical practice.

► Actual or anticipated problems or delays and actions or plans to resolve them

The chemical synthesis and production oxNI-Glu proved more complex than originally anticipated, and this delayed biological studies that depended on using the compound. We were ultimately successful in producing oxNI-Glu for subsequent studies. The histological studies proved more protracted than initially planned, but

► Changes that had a significant impact on expenditures

Nothing to report.

► Significant changes in use/care of vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. Products

Nothing to report.

7. Participants & other collaborating institutions

► Individuals who worked on the project

Name	Joseph P. Y. Kao
Project Role	P.I.
Researcher identifier	eRA Commons: KAOJOESPH
Person months worked	2
Contribution	Overseeing and coordinating project, synthesis and characterization of caged glutamates
Funding support	This award (W81XWH-16-2-0011)

Name	Patrick O. Kanold
Project Role	Site P.I. at Univ. of Maryland, College Park
Researcher identifier	eRA Commons: Pkanold
Person months worked	1
Contribution	Design of automated operant conditioning cage, set up of in vitro photostimulation instrumentation
Funding support	This award (W81XWH-16-2-0011)

Name	Nathaniel D. A. Dirda
Project Role	Chemical technician
Researcher identifier	
Person months worked	7
Contribution	Performed organic synthesis, production, purification, and characterization of oxNI-Glu under the direction of Dr. Kao.
Funding support	This award (W81XWH-16-2-0011)

Name	Eric A. Legenzov
Project Role	Graduate student
Researcher identifier	eRA Commons: ELEGENZOV
Person months worked	2
Contribution	In vivo toxicology and microscopy for histology
Funding support	This award (W81XWH-16-2-0011)

Name	Rongkang Deng
Project Role	Graduate student
Researcher identifier	
Person months worked	2
Contribution	Electrophysiology and photoactivation in brain slice preparations
Funding support	This award (W81XWH-16-2-0011)

► Change in the active other support of the PD/PI(s) or senior/key personnel

No change from previous report.

► Other organizations that were involved as partners

Nothing to report.

8. Special reporting requirements

None.

9. Appendices

None.

Cortical Photostimulation Technology for Vision Prosthesis

MR152030
W81XWH-16-2-0011

PI: Joseph P. Y. Kao **Org:** UNIVERSITY OF MARYLAND, BALTIMORE **Award Amount:** 410,000.00 (total cost, incl. $100,000 plus-up)

Study/Product Aim(s)

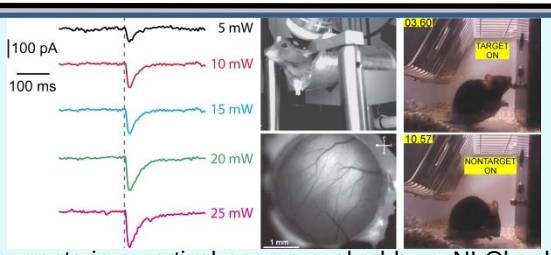
- (1) Develop a photoreleasable (caged) neurotransmitter optimized for in vivo photostimulation of the cortex
- (2) Validate the photostimulation technology in vitro
- (3) Validate the photostimulation technology in vivo

Approach

(1) Chemical synthesis, and characterization of product compounds by physico-chemical techniques. Toxicological tests of the new photoreleasable neurotransmitter in mice.

(2) Optical and electrophysiological techniques to determine optimal stimulation parameters and the achievable spatial resolution of photostimulation.

(3) Use fiberoptic delivery of light to photorelease caged neurotransmitter in the brain of behaviorally trained mice to determine if photostimulation can elicit a behavior response.



Left: Inward currents in a cortical neuron evoked by oxNI-Glu photolysis. *Middle*: Awake mouse with chronically installed cranial window and view of brain through the window. *Right*: Conditioned mouse licking water spout in response to target, but not non-target, stimulus

Year 2 accomplishments:

1) Revised oxNI-Glu synthesis & produced oxNI-Glu for in vitro & in vivo studies.
2) Completed toxicology & histology of tracer- & oxNI-Glu--infused brains.
3) Performed UV photostimulation with oxNI-Glu in mouse brain slices.
4) Developed capability to install chronic cranial windows in mice.
5) Use automated operant conditioning cage successfully to train mice to exhibit conditioned behavior in response to auditory stimuli.

Timeline and Cost

Activities (Aims as above) CY	16	17	18
Aim 1			
Aim 2			
Aim 3			*
* reflects no-cost extension			
Estimated Budget ($K)	**$137,007**	**$141,380**	**$131,613**

Updated: April 15, 2018

Goals/Milestones

CY16 Goals– Develop photoreleasable neurotransmitter (caged Glu)
☒Chemical synthesis of new caged Glu
☒Characterization of new caged Glu
☒ Scale-up production of caged Glu

CY17 Goals – Validate photostimulation technology in vitro
☒ Test in vivo toxicity of caged Glu
☒ Determine photostimulation parameters (355 nm done, 405 nm ongoing)
☒ Determine maximum photostimulation frequency (ongoing)
☒ Determine spatial resolution of photostimulation in vitro (355 nm done)
☐ Assess damage threshold in vivo
☐ Determine achievable spatial resolution of photostimulation in vivo
☒Test if in vivo photostimulation can evoke a percept (operant conditioning & cranial window complete)

Comments/Challenges/Issues/Concerns Noticed some issues with consistency of photostimulation at 405 nm; diagnosis ongoing.

Budget Expenditure to Date

Projected Expenditure: $320,000
Actual Expenditure: $330,000 (est.)