

MEMORY ENCODING IN AMYGDALA

by

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for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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ABSTRACT

Memory Encoding in Amygdala

Chen Yan

Doctor of Philosophy

Institute of Medical Science

University of Toronto

2016

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GLOSSARY

A/P anteroposterior

AFC auditory fear conditioning

Arc activity-regulated cytoskeleton-associated protein

CA1 cornu ammonis area 1

cDNA complimentary DNA

CMOS complimentary metal-oxide-semiconductor

CMV cytomegalovirus

CPP cocaine-conditioned place preference

CREB cyclic adenosine monophosphate response binding protein

D/V dorsoventral

DC direct current

DIG digoxigenin

DNA deoxyribonucleic acid

EDTA ethylenediaminetetraacetic acid

FDM fused deposition modelling

FISH flourescent *in situ* hybridization

FITC flourescein isothiocyante

GFP green fluorescence protein

GRIN gradient-index

HRP horseradish peroxidase

HSV herpes simplex virus

IEG immediately-early gene

i.p. intraperitoneal

LA lateral amygdala

LED light-emitting diode

M/L mediolateral

NAc nucleus accumbens

PBS phosphate-buffered saline

PFA paraformaldehyde

PLA polylactic acid

RNA ribonucleic acid

SSC buffer saline sodium citrate buffer

TRITC tetramethylrhodamine

TSA tyramide signal amplification

USB universal serial bus

TODO LIST

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1

INTRODUCTION

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LITERATURE REVIEW

50+ pages

2.1 Hypothesis and Research Aims

2-5 pages

3

GENERAL METHODS

3.1 Mice

Wildtype C57/BL6 \times 129 F1 mice of 3-months age were used in the experiments. All animals were caged in groups of 4 or 5, with a 12-hour light/dark cycle. All experiments are performed during the light phase of the cycle. Food and water are provided *ad libitum* to all animals. All procedures are approved by the Animal Care and Use Committee in the Hospital for Sick Children.

3.2 Viral Infusion

Each animal received intraperitoneal (i.p.) injection of atropine (0.1 mg kg^{-1}) and chlorohydrate (400 mg kg^{-1}) before being secured on a stereotaxic frame. An incision was made on the scalp and the skin was pulled to the side to reveal the skull. Holes were drilled above lateral amygdala (LA) on the skull for micropipette injection. Virus was loaded into a glass micropipette and gradually lowered to target coordinate. $1.5 \mu\text{l}$ of virus were injected on each side at a rate of $0.12 \mu\text{l/min}$, aiming at LA (anteroposterior (A/P) -1.4 mm , mediolateral (M/L) $\pm 3.5 \text{ mm}$, dorsoventral (D/V) 5.0 mm from Bregma). The micropipette was left in the brain for an extra 10 min before slowly retracted. The incision was sutured and treated with antibiotics. Each animal then received subcutaneous injection of analgesic (ketoprofen, 5 mg kg^{-1}) before returned to a partially heated clean cage for recovery. All behaviour experiments are conducted at least 3 days after surgery.

3.3 Histology

Placement of implants and extent of viral infections was determined by green fluorescence protein (GFP) expression. After all experiments, animals were transcardially perfused with

first phosphate-buffered saline (PBS) then 4% paraformaldehyde (PFA). The brains were dissected and kept in 4% PFA overnight, and washed with PBS. The brains were then sliced coronally on a vibrotome () to 50 μm thickness. Slices containing LA were then mounted on gelatin-coated glass slides with a hardening mounting media (Permaflour) and assessed under an epi-flourescence microscope(Nikon).

vibrotome
info

permaflour
info

Nikon info

3.4 Behavioural Experiments

3.4.1 Auditory fear conditioning

The auditory fear conditioning (AFC) chamber (Med Associates) consists of two plexiglass walls and two metal walls, with an overhead camera for recording animal behaviour and a metal grid floor for shock delivery. The chamber was cleaned with water before AFC training. The animals were placed in the chamber and allowed for a 2-minute habituation. A tone (2800 Hz, 85 dB) was played for 30 s and co-terminated with a 2-second shock (0.7 mA). The animals were returned to their home cage 30s after the shock.

The AFC chamber is modified for testing by inserting a plexiglass board horizontally to cover the metal grids, and two more boards vertically to meet at the rear of the chamber, creating a triangular space. The testing chamber is washed with 70% ethanol. During testing, animals were placed in the triangular space. A 1 min tone was played 2 min after the animal was put in the chamber. The amount of freezing was assessed.

3.4.2 Cocaine-conditioned place preference

The cocaine-conditioned place preference (CPP) boxes are composed of two chambers with a removable shutter that can be switched to allow animals to move between the chambers. One chamber has white walls, textured floor and is sprayed with water, the other chamber has striped walls, smooth floor and is sprayed with 2% acetic acid. Animals were first placed in the box, allowed to travel between two chambers for 10 min, and the amount of time spent in each chamber is recorded as a measure for pre-conditioning preference. During training, animals received saline (i.p.), and confined to one of the chambers for 15 min. In the next day, the animals received cocaine injection (30 mg kg^{-1} , i.p.), and paired to the other chamber for 15 min. The animals are kept in home cage for one day and put back to the CPP boxes and allowed to move freely between chambers for 10 min. The amount of time spent in each chamber were recorded. The difference in time spent between cocaine and saline conditioned chamber were calculated as cocaine preference.

insert cpp
image here

4

MEMORY TRACES IN AMYGDALA

4.1 Introduction

4.2 Methods

4.2.1 Animals

Wildtype C57/BL6 \times 129 F1 mice of 3-month age were used in the experiment. All animals were caged in groups of 4 or 5, with a 12-hour light/dark cycle. Food and water are provided *ad libitum* to all animals.

4.2.2 Viral vectors

p1005 plasmid expresses GFP under cytomegalovirus (CMV) promoter. CREB-p1005-GFP plasmid was constructed by inserting cyclic adenosine monophosphate response binding protein (CREB) complimentary DNA (cDNA) into p1005 plasmid, under the control of herpes simplex virus (HSV) IE4/5 promoter. Plasmids were packaged with a replication-defective HSV helper virus as previously described (Neve et al., 2005). All virus was concentrated by sucrose gradient, diluted in 10% sucrose and stored in -80°C . A typical titer of the virus is 1×10^7 infectious unit per ml.

4.2.3 Surgery

See general methods.

refer to
general
methods

4.2.4 Auditory fear conditioning

See general methods.

refer to
general
methods

4.2.5 Cocaine-conditioned place preference

See general methods.

refer to
general
methods

4.2.6 Fluorescent *in situ* hybridization

description
why cat-
fish

Tissue preparation

After behavioural experiments, animals were anaesthetized with isoflurane and decapitated. The brains were quickly dissected, frozen on dry ice, and stored in -80°C . At least 1 day after, the brains were taken out for slicing and equilibrated in the cryostat at the optimal cutting temperature (typically -20°C , with blades, brain matrix, mold, and mounting base. The brains were then cut on the brain matrix into 1 cm trunks containing the structure of interest. A thin layer of embedding medium () was added to the mold, and 4-5 brain trunks from different conditions were quickly mounted in the mold. The brains are sliced into $20\text{ }\mu\text{m}$ coronal slices and melt-mounted on Superfrost Plus slides (VWR). The slices were kept in -80°C until hybridization.

info about
OCT com-
pound

right way
to put this

Riboprobe synthesis

Template deoxyribonucleic acid (DNA) plasmids were linearized by restriction enzyme digestion at the 5' end of the target gene and purified. Riboprobes were synthesized using commercially available *in vitro* transcription kit () with digoxigenin (DIG) or fluorescein labeling mix (). Turbo DNase were then added to the mixture, and incubated at 37°C for 15 min to remove the DNA template. The DNase was inactivated by 0.05 M ethylenediaminetetraacetic acid (EDTA). The riboprobes were purified by a ribonucleic acid (RNA) purification column (). A small sample of the riboprobes were taken out for quantification of the yield on a spectrophotometer (Absorbance at A_{260}) and run on a denaturing gel for confirming the integrity of the probe. The rest of the probes were stored in -80°C .

in vitro
transcrip-
tion detail

labeling
mix detail

RNA col-
umn info

Hybridization and staining

A selection of slides evenly distributed in A/P were selected to cover LA. The slides were thawed on a filter paper and loaded on an autoclaved metal rack. The slides were lightly fixed in cold 4% PFA for 10 min, then carried through fresh acetic anhydride solution (10 min), $2\times$ saline sodium citrate buffer (SSC buffer) (5 min), 1:1 methanol/acetone (5 min), and $2\times$ SSC buffer (5 min). The slides were then treated with prehybridization buffer (Sigma) for 30 min. The riboprobes were diluted to $0.67\text{ ng }\mu\text{l}^{-1}$, heated to 90°C for

prehyb
info

7 min and cooled on ice. Each slide was applied with 150 μ l riboprobe solutions, coverslipped, and incubated in a humid chamber overnight at 56 °C for hybridization.

After hybridization, slides were treated with 1 ng ml⁻¹ RNase A at 37 °C for 30 min, washed with 2× SSC buffer, incubated 2 times with 0.5×SSC buffer at 56 °C for 30 min each, and washed with 2×SSC buffer. the slides were then incubated with blocking solution at room temperature for 30 min and then treated with Alexa-488 conjugated anti-GFP (1:100,) and horseradish peroxidase (HRP)-conjugated anti-DIG antibodies (1:300,) for 2 h to detect hybridized GFP and activity-regulated cytoskeleton-associated protein (Arc) RNA probes. The Arc signals were amplified using tyramide signal amplification (TSA)-biotin kit and stained by Alexa 568-conjugated streptavidin (1:300). The nuclei were counter-stained with Hoechst 33258.

Ab info

Ab info

Analysis

Images were taken on a laser-scanning microscope. The parameters were optimized for Arc signal and kept constant across slides. Confocal stacks of 1 μ m were taken by 40x objectives. Small-uniform nuclei from glial cells and nuclei on the top or bottom stacks were excluded from the counting. Nucleus immediately-early gene (IEG) signals are characterized by a double puncta that overlaps with nuclei counter-staining.

4.3 Results

4.4 Discussion

5

CONSTRUCTION OF A MINIATURE EPI-FLUORESCENCE MICROSCOPE

5.1 Introduction

One of the major technological limitation in neuroscience research is recording neural activity in model animals. Traditional techniques such multi-unit recordings give excellent temporal resolution, however the spatial resolution — as measured by the number of cells simultaneously recorded — is limited. Moreover, it is very hard to distinguish cell subpopulations within the same region from the recording. Neural activity can also be inferred by post-hoc staining of neural activity markers, such as *cfos* or *arc*. This method give excellent spatial resolution, however the temporal resolution is very poor, where the time window of neural activity lasts from minutes to hours.

Live calcium imaging gives the best of both method. By labelling the cell of interest with a calcium indicator, neural activity can be inferred in milli-second resolution. Hundreds of cells can be simultaneously recorded, and specific subpopulations can be distinguished by fluorescence in different colour channels. However, traditional live calcium imaging requires the animals' head firmly fixed under a microscope stage. This requirement is incompatible with most well established behaviour assays, and at the same time introduce significant stress to the animal, potentially confounding the behavioural result. Moreover, due to light scattering in the opaque brain tissue, most of the studies have focused only on cortical areas, while techniques to image deep brain tissue on a standard two-photon microscope is still under development and not widely adopted (Barretto and Schnitzer, 2012).

In vivo calcium imaging in behaving animals is first demonstrated by Mark Schnitzer's group in Stanford (Ghosh et al., 2011). The authors constructed a miniature epifluorescence

edit mi-
croscope
intro

microscope which is chronically implanted in the brain to image the fluorescence from region of interest. In a follow-up paper (Ziv et al., 2013), the authors demonstrated that the miniature microscope can image GCaMP3 calcium signals from hippocampal cornu ammonis area 1 (CA1) place cells for more than a month. However, there has been several limitations of their design: first their design incorporates an objective lens of 1 mm in diameter, which is impractical to reach deep brain tissue; second, their mini-microscope was only able to identify GCaMP signals, and therefore unable to distinguish different cells types within the population (Ghosh et al., 2011; Ziv et al., 2013).

In the current project, we aim to tackle the above mentioned limitations by building a head-mount miniature microscope which is able to image calcium signals in deep brain structures, while also able to image a separate fluorescence colour channel, allowing to distinguish difference cell type. The mini-microscope, once developed, will be used for imaging LA neurons to investigate mechanisms of fear memory encoding.

5.2 Material and Methods

5.2.1 Design of the mini-microscope

The mini-microscope has the same design as an general single-photon epifluorescence microscope except for the size constraints. Excitation light is emitted from a light-emitting diode (LED) light source, filtered, reflected by a dichroic mirror and evenly illuminate the sample. The fluorescent light from the sample is collected by the objective, passes through the dichroic mirror, filtered and focused on the camera. To fit the size constraints, we chose the use a high-intensity LED as the light source, a gradient-index (GRIN) lens as an objective, and a miniaturized complimentary metal-oxide-semiconductor (CMOS) camera to capture the image.

The optical design of the microscope is aided with Zemax software (Zemax Development Corporation) to optimize the lens and filter configuration. The casing of the microscope is modelled using OpenSCAD software.

maybe a
illustration

Lens configuration

The lenses configuration consists a GRIN objective lens and a ocular cube lens forming a 4F system, where the distance between the thin-lens equivalent of the two lenses equals to the sum of the focal length. Potential lenses for emission light path are selected from modelling and calculation to give a working distance of 100 μm in water, a magnification of 6x and a back focal length of 6 cm. During prototyping, the lenses are purchased and installed into custom-made mounts on a two-arm stereotaxic frame. The distance of the lenses are then

optimized against a fibre bundle light source close to the GRIN lens. A drum lens is used to collect light from the LED. The drum lens is tested in a similar manner and selected to give diverging light after GRIN lens. We have chosen to use an achromatic doublet ($F=15$ mm, Edmund Optics). We used a 1.8 mm diameter 0.25-pitch GRIN lens (64–537, Edmund Optics) as objective for hippocampus imaging. To minimize brain damage for deep brain imaging, the objective lens was a home-assembled doublet of a 0.5 mm diameter, 1-pitch GRIN relay lens () and a 2 mm diameter 0.25-pitch GRIN lens (). The details of doublet assembly is described in .

GoFoton
lens infoGoFoton
lens info

Filter selection

The filters are selected to cover the excitation and emission spectrum of the genetic encoded calcium sensor GCaMP6s (Chen et al., 2013), and further screen for high bandwidth and low overlap. The fit the size constraints, the excitation and emission filters had dimensions of $5\text{ mm} \times 5\text{ mm} \times 1\text{ mm}$, and the dichroic mirror $7.1\text{ mm} \times 5\text{ mm} \times 1\text{ mm}$. We have chosen to use a fluorescein isothiocyanate (FITC) filter set for gCaMP6 imaging (). For dual colour imaging, we took advantage of significant long tail of the red retrobeads spectrum, and use the same blue light to excite the red fluorescent. In these experiments, a TRITC/FITC filter set was used ().

chroma
filter infochroma
filter info

Electronics

The image sensor are selected to have a packaged size of less than $1.5\text{ cm} \times 1.5\text{ cm}$. We used a commercially available analogue camera module (HD1313BW, Ruishibao) that gives satisfactory sensitivity and dynamic range. The camera board is connected to a 5 V power regulator. After the power regulator, the wires were connected through a slip ring () was used to avoid tanglement of the wires during animal behaviour. After the slip ring, the wires are connected to a 12 V direct current (DC) power source and a universal serial bus (USB) analogue video capture card (). The video capture card is controlled by custom software for synchronized video capture (See code in).

slipring
infoVideo cap-
ture info

A monochrome, high-intensity blue LED (, Lumiled) was used as the light source. The LED wires join the video camera wires through the slip ring, and then connected to a variable DC power source (). During recording, the LED was driven at a current between 20 mA and 100 mA.

reference
code

led info

DC power
source info

Casing and assembly

The casing model is produced by 3D printing using PolyJet technology with VeroBlackPlus material (Stratasys). This gives a rigid, opaque and black casing with highest resolution

for details. The microscope body is screwed onto the camera holder via M8 thread to allow easy change of the focus plane. A side M2×2 mm nylon screw () is used to lock the camera holder on the microscope body.

info side
screw

A metal nut was glued to the bottom of the microscopy body concentric to the light path opening using a fast-curing epoxy glue (). The nut allows to easily and firmly lock the microscope onto the baseplate.

microscope
body nut
spec

Unlike the microscope body, the base plate was 3d printed using fused deposition modelling (FDM) with polylactic acid (PLA) material. A threaded tube () was heated with a soldering iron and inserted into the center of the base plate, and further fixed with epoxy glue. The objective lens was inserted into the center of the threaded tube, and fixed with crazy glue on the bottom. For objective that are thinner than the inner diameter of the threaded tube, an additional stainless steel tube was inserted inbetween to aid alignment of the lens.

epoxy
info?

baseplate
thread
tube info

Objective for deep brain imaging

5.2.2 Implantation of the mini-microscope

Two weeks after viral infusion, animals are anesthetized and head-fixed on a stereotaxic frame as described in . Three screws were placed around the viral injection site for anchoring the microscope.

ref general
methods

For implantation targetting CA1, a circular craniology of 2 mm was performed above the viral injection site. The dura was pierced and lifted with a fine tweezer to expose the brain. The brain is then constantly irrigated with artificial cerebral-spinal cord fluid to remove the blood., a 27 gauge aspiration needle was used to remove cortex, to expose CA1. For implantation targetting amygdala after anchoring the screws, a 27-gauge needle was lowered to the target coordinate, left for 5 min, and slowly retracted.

The mini-microscope is then fixed on the stereotaxic frame and gradually lowered to the target coordinates (CA1: LA:). Opaque black dental acrylic was used to secure the microscope baseplate to the skull. Once the dental acrylic cured, the microscope body was detached from the baseplate and replaced with a cap. Animals were given 5 mg kg⁻¹ ketoprofen for analgesia.

coordinate
for ca1
and amygdala

5.2.3 In vivo mini-microscope testing

The animals were kept in the home cage for two weeks before the first image session. This time allows the optical window to clear up. The animals were scruffed, the cap was removed and replaced with the microscope body. A typical imaging session lasts for 5 min. After the imaging session the microscope body was removed, and the animal was recappeded.

5.2.4 Image analysis

Individual cell calcium signals were extracted from the movie as previously described (Mukamel et al., 2009). Briefly, we first estimate the number of cells in the movie, and reduced the number of temporal dimension to roughly number of cells using principle component analysis. The resulting principle components were then subjected to independent component analysis, where the spatial filter for individual cells were extracted from the components, and the calcium signal of the corresponding cell was extracted from the mixing matrix. The time-course calcium signal was then aligned with behaviour recordings to identify neural activity patterns.

5.3 Results

With the design from Ghosh et al. (2011) as a guide, we started to make our own epifluorescence mini-microscope. Currently we have constructed working prototypes weighing less than 3 g, and can be bounded in a $25\text{ mm} \times 16\text{ mm} \times 11\text{ mm}$ box. The light source is a high intensity blue LED (LXML-PB01-0023, Lumileds). The illumination light is collected by a drum lens (45–549, Edmund Optics) and then filtered by a blue bandpass filter (ET470/40x, Chroma). The filtered illumination is then reflected by a dichroic mirror (T495lpxr, Chroma) on to the sample. Fluorescence is collected by a 1.8 mm GRIN lens (64–537, Edmund Optics), filtered with a green bandpass filter (ET525/50m, Chroma), then focused by an achromatic lens (49–277, Edmund Optics) onto a 600 tv-line analogue CMOS camera sensor (ASX340, Aptina). The analogue signal is then converted by a consumer video capture device (MyGica) at resolution of 720×576 and a frame rate of 25 frames per second.

An image of the mini-microscope is shown in Figure ???. The resolution of the microscope is better than $2\text{ }\mu\text{m}$, as shown in Figure ??? when it is tested against USAF resolution target (Lines in group 7 element 6 have width of $2.07\text{ }\mu\text{m}$ and are clearly visible). We have tested the prototype on a perfused brain with GFP signals. And as shown in ???, the GFP cells are clearly identified, with some of the neural processes visible.

To test *in vivo* imaging capability of the microscope, we first implanted the microscope above the cortex, and injected $150\text{ }\mu\text{l}$ of fluorescein-dextran (molecular weight 120 kDa). The fluorescein-dextran will fill the blood vessels and have similar excitation and emission wavelength to GCaMP. As expected, after fluorescein-dextran injection, the blood vessels are clearly visible when the microscope is implanted (Figure ???).

We have first tested GCaMP6s fluorescence *in vitro*. HEK–293 cells were transfected with pGP–CMV–gCAMP6s (Addgene), and imaged the next day. During imaging session,

edit mi-
croscope
result

we challenged the cell with 10 nmol ATP, which is known to up-regulate intra-cellular Ca^{2+} level (*e.g.* Lee et al., 2004). The result is shown in Figure ???. The GCaMP6s gives minimal background but bright fluorescence when the intracellular Ca^{2+} is induced.

To test GCaMP6s expression *in vivo*, we infused AAV-syn-GCaMP6s-WPRE into CA1 hippocampus of animals. After AAV expression plateaued, we aspirated cortical tissue above the viral infusion site and implanted the microscope baseplate. After two weeks when the animals recovered and the cranial window was cleared, the microscope was re-attached to the implanted baseplate. The animal were placed in a novel environment to explore for 5 min, during which GCaMP6 fluorescence were recorded. The maximum projection of the GCaMP6 fluorescence in a 5-minute session is shown in Figure ???. More than 200 cells are clearly identifiable.

We used a previously established method to extract Ca^{2+} signals from the movie (Mukamel et al., 2009). Briefly, we used principle component analysis to reduce the temporal dimension, and then independent component analysis to extract the spatial location of cells and their corresponding Ca^{2+} signals. Figure ?? shows a sample independent component that represents a cell and it's activity. The extracted cells are random coloured in Figure ??.

The timecourse of the identified cells were mapped back to the behaviour of the animal. Figure ?? shows Ca^{2+} activity of potential place cells as they respond to specific location in the environment the animal is in.

This design of the miniature microscope incorporates an objective lens of 1.8 mm in diameter. This lens is both too thick and too short to reach deep brain structures such as amygdala. We have modified the design and attached a 4.8 mm long 0.5 mm diameter relay GRIN lens (ILW-050-P050, GoFoton) to the objective lens. Attaching the relay lens does not significantly alter the imaging ability of the microscope, however allows the lens to reach deep brain regions without extensive damage. With this configuration, we are able to visualize activity from more than 40 cells in lateral amygdala and track them over time (Figure ??).

To enable us to identify different cell types in a population, we decided to add a second colour channel in the microscope. We have switched the filter set to a FITC/TRITC dual band set (Chroma 59004), and also from grayscale camera to an RGB camera chip. Figure ?? shows the two-colour microscope against perfused brain expressing GFP and TdTomato. Both fluorophore can be clearly seen. We have also tested the red channel *in vivo*, where we infused red retrobeads (LumaFlour) in nucleus accumbens (NAc) and implanted the mini-microscope in LA. The retrobeads travels retrogradely, and will label amygdala neurons that have connection to NAc. These cells can be clearly identified under the mini-microscope in the red channel, with no interference to the green channel (Figure ??,??).

5.4 Discussion

6

DYNAMICS OF NEURAL NETWORK IN AMYGDALA DURING MEMORY FORMATION

7

GENERAL DISCUSSION

20 pages

8

CONCLUSIONS AND FUTURE DIRECTIONS

4-5 pages

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APPENDIX

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