

# MEMORY ENCODING IN AMYGDALA

by

Chen Yan

A thesis submitted in conformity with the requirements  
for the degree of Doctor of Philosophy  
Institute of Medical Science  
University of Toronto

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# ABSTRACT

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## Memory Encoding in Amygdala

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Doctor of Philosophy

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2016

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# ACKNOWLEDGEMENTS

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# GLOSSARY

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**A/P** anteroposterior.

**AFC** auditory fear conditioning.

**Arc** activity-regulated cytoskeleton-associated protein.

**CA1** cornu ammonis area 1.

**cDNA** complimentary DNA.

**CMV** cytomegalovirus.

**CPP** cocaine-conditioned place preference.

**CREB** cyclic adenosine monophosphate response binding protein.

**D/V** dorsoventral.

**DIG** digoxigenin.

**FISH** fluorescent *in situ* hybridization.

**FITC** fluorescein isothiocyanate.

**GFP** green fluorescence protein.

**GRIN** gradient-index.

**HRP** horseradish peroxidase.

**HSV** herpes simplex virus.

**i.p.** intraperitoneal.

**LA** lateral amygdala.

**LED** light-emitting diode.

**M/L** mediolateral.

**RNA** ribonucleic acid.

**TSA** tyramide signal amplification.

# TODO LIST

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# 1

## INTRODUCTION

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# 2

## LITERATURE REVIEW

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# 3

## GENERAL METHODS

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### 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 ( $0.1 \text{ mg kg}^{-1}$ ) and chlorhydrate ( $400 \text{ mg kg}^{-1}$ ) before being placed 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.  $1.5 \mu\text{l}$  of virus were injected on each side at a rate of  $0.12 \mu\text{l/min}$ , aiming at LA (anteroposterior (A/P)  $-1.4 \text{ mm}$ , mediolateral (M/L)  $\pm 3.5 \text{ mm}$ , dorsoventral (D/V)  $5.0 \text{ mm}$  from Bregma). Each animal received subcutaneous injection of analgesic (ketoprofen,  $5 \text{ mg kg}^{-1}$ ) when injection of one side is finished. The animals were allowed recovery in home cage for 3 days before they were subjected to behaviour experiments.

### 3.3 Auditory Fear Conditioning

Auditory fear conditioning was done in the middle of the animals' light cycle on the 3rd day after surgery. The animals were placed in a training 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 allowed to rest in their home cage until early in their next light cycle when they were sacrificed for analysis.

complete  
methods  
for fear  
testing

# 4

## MEMORY TRACES IN AMYGDALA

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### 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 green fluorescence protein (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  $-80^{\circ}\text{C}$ . A typical titer of the virus is  $1 \times 10^7$  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  
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#### 4.2.5 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 ( $30 \text{ mg kg}^{-1}$ , 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

#### 4.2.6 Fluorescent *in situ* hybridization

The animals were sacrificed 2 h after the beginning of the light cycle on the day following the auditory fear conditioning. The brains were quickly dissected, frozen on dry ice, and stored in  $-80^{\circ}\text{C}$ . The brains were then sliced into  $20 \mu\text{m}$  coronal sections using a cryostat and mounted onto gelatine-coated slides. Fluorescent *in situ* hybridization (FISH) were carried out as previously described (Guzowski et al., 1999). digoxigenin (DIG)-labeled activity-regulated cytoskeleton-associated protein (Arc) and fluorescein isothiocyanate (FITC)-labeled GFP antisense ribonucleic acid (RNA) probes were prepared and hybridized to the slices overnight. Alexa-488 conjugated anti-GFP (1:100) and horseradish peroxidase (HRP)-conjugated anti-DIG (1:100) antibodies were used to detect hybridized GFP and Arc RNA probes. The Arc signals were amplified using tyramide signal amplification (TSA)-biotin kit and visualized by Alexa 568-conjugated streptavidin. The nuclei were counter-stained with Hoechst 33258.

#### 4.2.7 Imaging and cell counting

Images were taken on a laser-scanning microscope. The parameters were optimized for Arc signal and kept constant across slides. Confocal stacks of  $1 \mu\text{m}$  were taken by 40x objectives. Small-uniform nuclei from glial cells and nuclei on the top or bottom stacks were excluded from the counting. Arc and GFP transcription foci were examined for each included nuclei. Total number of cells, Arc positive cells (Arc+), Arc and GFP double positive cells (Arc+/GFP+) were counted. Arc ratio was calculated by dividing Arc+/GFP+ with GFP+.

## 4.3 Results

## 4.4 Discussion

# 5

## CONSTRUCTION OF A MINIATURE EPI-FLUORESCENCE MICROSCOPE

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### 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 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 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 lateral amygdala neurons to investigate mechanisms of fear memory encoding.

## 5.2 Material and Methods

### 5.2.1 Construction of the mini-microscope

#### Microscope design

Optical design of the microscope is aided using Zemax software (Zemax Development Corporation) to optimize the lens and filter configuration. The casing of the microscope is modelled using Rhinoceros 3D software (Robert McNeel & Associates) and OpenSCAD software.

#### Lens configuration

Potential lenses for emission light path are selected from modelling and calculation to give a working distance of less than 50  $\mu\text{m}$ , a magnification of about 2–5x and a focal length of less than 3 cm. 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 gradient-index (GRIN) lens. A drum lens is used to collect light from the light-emitting diode (LED). The drum lens is tested in a similar manner and selected to give diverging light after GRIN lens.

#### 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 size of the filters are custom-made to fit the size constraints.

### Image sensor

The image sensor are selected to have an packaged size of less than  $1.5\text{ cm} \times 1.5\text{ cm}$ . The sensor with highest sensitivity is then used.

### Casing

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.

#### 5.2.2 Implantation of the mini-microscope

Two weeks after viral infusion, animals are anesthetized and head-fixed on a stereotaxic frame. Three screws were placed around the viral injection site for anchoring the microscope. A circular craniotomy 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. For cornu ammonis area 1 (CA1) experiments, a 27 gauge aspiration needle was used to remove cortex, to expose CA1. The mini-microscope is then fixed on the stereotaxic frame and gradually lowered to the target coordinates. 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  $5\text{ mg kg}^{-1}$  ketoprofen for analgesia.

#### 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  $720 \times 576$  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  $120\text{ kDa}$ ). 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, we challenged the cell with  $10\text{ nmol}$  ATP, which is known to up-regulate intra-cellular  $\text{Ca}^{2+}$  level (*e.g.* Lee et al., 2004). The result is shown in Figure ???. The GCaMP6s gives minimal background but bright fluorescence when the intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 nuclear accumbens (NAc) and implanted the mini-microscope in lateral amygdala. 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

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Nulla mattis luctus nulla. Duis commodo velit at leo. Aliquam vulputate magna et leo. Nam vestibulum ullamcorper leo. Vestibulum condimentum rutrum mauris. Donec id mauris. Morbi molestie justo et pede. Vivamus eget turpis sed nisl cursus tempor. Curabitur mollis sapien condimentum nunc. In wisi nisl, malesuada at, dignissim sit amet, lobortis in, odio. Aenean consequat arcu a ante. Pellentesque porta elit sit amet orci. Etiam at turpis nec elit ultricies imperdiet. Nulla facilisi. In hac habitasse platea dictumst. Suspendisse viverra aliquam risus. Nullam pede justo, molestie nonummy, scelerisque eu, facilisis vel, arcu.

Curabitur tellus magna, porttitor a, commodo a, commodo in, tortor. Donec interdum. Praesent scelerisque. Maecenas posuere sodales odio. Vivamus metus lacus, varius quis, imperdiet quis, rhoncus a, turpis. Etiam ligula arcu, elementum a, venenatis quis, sollicitudin sed, metus. Donec nunc pede, tincidunt in, venenatis vitae, faucibus vel, nibh. Pellentesque wisi. Nullam malesuada. Morbi ut tellus ut pede tincidunt porta. Lorem ipsum dolor sit amet, consectetur adipiscing elit. Etiam congue neque id dolor.

Donec et nisl at wisi luctus bibendum. Nam interdum tellus ac libero. Sed sem justo, laoreet vitae, fringilla at, adipiscing ut, nibh. Maecenas non sem quis tortor eleifend fermentum. Etiam id tortor ac mauris porta vulputate. Integer porta neque vitae massa.

Maecenas tempus libero a libero posuere dictum. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Aenean quis mauris sed elit commodo placerat. Class aptent taciti sociosqu ad litora torquent per conubia nostra, per inceptos hymenaeos. Vivamus rhoncus tincidunt libero. Etiam elementum pretium justo. Vivamus est. Morbi a tellus eget pede tristique commodo. Nulla nisl. Vestibulum sed nisl eu sapien cursus rutrum.

Nulla non mauris vitae wisi posuere convallis. Sed eu nulla nec eros scelerisque pharetra. Nullam varius. Etiam dignissim elementum metus. Vestibulum faucibus, metus sit amet mattis rhoncus, sapien dui laoreet odio, nec ultricies nibh augue a enim. Fusce in ligula. Quisque at magna et nulla commodo consequat. Proin accumsan imperdiet sem. Nunc porta. Donec feugiat mi at justo. Phasellus facilisis ipsum quis ante. In ac elit eget ipsum pharetra faucibus. Maecenas viverra nulla in massa.

Nulla ac nisl. Nullam urna nulla, ullamcorper in, interdum sit amet, gravida ut, risus. Aenean ac enim. In luctus. Phasellus eu quam vitae turpis viverra pellentesque. Duis feugiat felis ut enim. Phasellus pharetra, sem id porttitor sodales, magna nunc aliquet nibh, nec blandit nisl mauris at pede. Suspendisse risus risus, lobortis eget, semper at, imperdiet sit amet, quam. Quisque scelerisque dapibus nibh. Nam enim. Lorem ipsum dolor sit amet, consectetur adipiscing elit. Nunc ut metus. Ut metus justo, auctor at, ultrices eu, sagittis ut, purus. Aliquam aliquam.

Etiam pede massa, dapibus vitae, rhoncus in, placerat posuere, odio. Vestibulum luctus commodo lacus. Morbi lacus dui, tempor sed, euismod eget, condimentum at, tortor. Phasellus aliquet odio ac lacus tempor faucibus. Praesent sed sem. Praesent iaculis. Cras rhoncus tellus sed justo ullamcorper sagittis. Donec quis orci. Sed ut tortor quis tellus euismod tincidunt. Suspendisse congue nisl eu elit. Aliquam tortor diam, tempus id, tristique eget, sodales vel, nulla. Praesent tellus mi, condimentum sed, viverra at, consectetur quis, lectus. In auctor vehicula orci. Sed pede sapien, euismod in, suscipit in, pharetra placerat, metus. Vivamus commodo dui non odio. Donec et felis.

Etiam suscipit aliquam arcu. Aliquam sit amet est ac purus bibendum congue. Sed in eros. Morbi non orci. Pellentesque mattis lacinia elit. Fusce molestie velit in ligula. Nullam et orci vitae nibh vulputate auctor. Aliquam eget purus. Nulla auctor wisi sed ipsum. Morbi porttitor tellus ac enim. Fusce ornare. Proin ipsum enim, tincidunt in, ornare venenatis, molestie a, augue. Donec vel pede in lacus sagittis porta. Sed hendrerit ipsum quis nisl. Suspendisse quis massa ac nibh pretium cursus. Sed sodales. Nam eu neque quis pede dignissim ornare. Maecenas eu purus ac urna tincidunt congue.

Donec et nisl id sapien blandit mattis. Aenean dictum odio sit amet risus. Morbi purus. Nulla a est sit amet purus venenatis iaculis. Vivamus viverra purus vel magna. Donec in justo sed odio malesuada dapibus. Nunc ultrices aliquam nunc. Vivamus facilisis

pellentesque velit. Nulla nunc velit, vulputate dapibus, vulputate id, mattis ac, justo. Nam mattis elit dapibus purus. Quisque enim risus, congue non, elementum ut, mattis quis, sem. Quisque elit.

Maecenas non massa. Vestibulum pharetra nulla at lorem. Duis quis quam id lacus dapibus interdum. Nulla lorem. Donec ut ante quis dolor bibendum condimentum. Etiam egestas tortor vitae lacus. Praesent cursus. Mauris bibendum pede at elit. Morbi et felis a lectus interdum facilisis. Sed suscipit gravida turpis. Nulla at lectus. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Praesent nonummy luctus nibh. Proin turpis nunc, congue eu, egestas ut, fringilla at, tellus. In hac habitasse platea dictumst.

Vivamus eu tellus sed tellus consequat suscipit. Nam orci orci, malesuada id, gravida nec, ultricies vitae, erat. Donec risus turpis, luctus sit amet, interdum quis, porta sed, ipsum. Suspendisse condimentum, tortor at egestas posuere, neque metus tempor orci, et tincidunt urna nunc a purus. Sed facilisis blandit tellus. Nunc risus sem, suscipit nec, eleifend quis, cursus quis, libero. Curabitur et dolor. Sed vitae sem. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Maecenas ante. Duis ullamcorper enim. Donec tristique enim eu leo. Nullam molestie elit eu dolor. Nullam bibendum, turpis vitae tristique gravida, quam sapien tempor lectus, quis pretium tellus purus ac quam. Nulla facilisi.

Duis aliquet dui in est. Donec eget est. Nunc lectus odio, varius at, fermentum in, accumsan non, enim. Aliquam erat volutpat. Proin sit amet nulla ut eros consectetur cursus. Phasellus dapibus aliquam justo. Nunc laoreet. Donec consequat placerat magna. Duis pretium tincidunt justo. Sed sollicitudin vestibulum quam. Nam quis ligula. Vivamus at metus. Etiam imperdiet imperdiet pede. Aenean turpis. Fusce augue velit, scelerisque sollicitudin, dictum vitae, tempor et, pede. Donec wisi sapien, feugiat in, fermentum ut, sollicitudin adipiscing, metus.

Donec vel nibh ut felis consectetur laoreet. Donec pede. Sed id quam id wisi laoreet suscipit. Nulla lectus dolor, aliquam ac, fringilla eget, mollis ut, orci. In pellentesque justo in ligula. Maecenas turpis. Donec eleifend leo at felis tincidunt consequat. Aenean turpis metus, malesuada sed, condimentum sit amet, auctor a, wisi. Pellentesque sapien elit, bibendum ac, posuere et, congue eu, felis. Vestibulum mattis libero quis metus scelerisque ultrices. Sed purus.

Donec molestie, magna ut luctus ultrices, tellus arcu nonummy velit, sit amet pulvinar elit justo et mauris. In pede. Maecenas euismod elit eu erat. Aliquam augue wisi, facilisis congue, suscipit in, adipiscing et, ante. In justo. Cras lobortis neque ac ipsum. Nunc fermentum massa at ante. Donec orci tortor, egestas sit amet, ultrices eget, venenatis eget, mi. Maecenas vehicula leo semper est. Mauris vel metus. Aliquam erat volutpat. In

rhoncus sapien ac tellus. Pellentesque ligula.

Cras dapibus, augue quis scelerisque ultricies, felis dolor placerat sem, id porta velit odio eu elit. Aenean interdum nibh sed wisi. Praesent sollicitudin vulputate dui. Praesent iaculis viverra augue. Quisque in libero. Aenean gravida lorem vitae sem ullamcorper cursus. Nunc adipiscing rutrum ante. Nunc ipsum massa, faucibus sit amet, viverra vel, elementum semper, orci. Cras eros sem, vulputate et, tincidunt id, ultrices eget, magna. Nulla varius ornare odio. Donec accumsan mauris sit amet augue. Sed ligula lacus, laoreet non, aliquam sit amet, iaculis tempor, lorem. Suspendisse eros. Nam porta, leo sed congue tempor, felis est ultrices eros, id mattis velit felis non metus. Curabitur vitae elit non mauris varius pretium. Aenean lacus sem, tincidunt ut, consequat quis, porta vitae, turpis. Nullam laoreet fermentum urna. Proin iaculis lectus.

Sed mattis, erat sit amet gravida malesuada, elit augue egestas diam, tempus scelerisque nunc nisl vitae libero. Sed consequat feugiat massa. Nunc porta, eros in eleifend varius, erat leo rutrum dui, non convallis lectus orci ut nibh. Sed lorem massa, nonummy quis, egestas id, condimentum at, nisl. Maecenas at nibh. Aliquam et augue at nunc pellentesque ullamcorper. Duis nisl nibh, laoreet suscipit, convallis ut, rutrum id, enim. Phasellus odio. Nulla nulla elit, molestie non, scelerisque at, vestibulum eu, nulla. Ut odio nisl, facilisis id, mollis et, scelerisque nec, enim. Aenean sem leo, pellentesque sit amet, scelerisque sit amet, vehicula pellentesque, sapien.

# 7

## GENERAL DISCUSSION

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Suspendisse vitae elit. Aliquam arcu neque, ornare in, ullamcorper quis, commodo eu, libero. Fusce sagittis erat at erat tristique mollis. Maecenas sapien libero, molestie et, lobortis in, sodales eget, dui. Morbi ultrices rutrum lorem. Nam elementum ullamcorper leo. Morbi dui. Aliquam sagittis. Nunc placerat. Pellentesque tristique sodales est. Maecenas imperdiet lacinia velit. Cras non urna. Morbi eros pede, suscipit ac, varius vel, egestas non, eros. Praesent malesuada, diam id pretium elementum, eros sem dictum tortor, vel consectetur odio sem sed wisi.

Sed feugiat. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Ut pellentesque augue sed urna. Vestibulum diam eros, fringilla et, consectetur eu, nonummy id, sapien. Nullam at lectus. In sagittis ultrices mauris. Curabitur malesuada erat sit amet massa. Fusce blandit. Aliquam erat volutpat. Aliquam euismod. Aenean vel lectus. Nunc imperdiet justo nec dolor.

Etiam euismod. Fusce facilisis lacinia dui. Suspendisse potenti. In mi erat, cursus id, nonummy sed, ullamcorper eget, sapien. Praesent pretium, magna in eleifend egestas, pede pede pretium lorem, quis consectetur tortor sapien facilisis magna. Mauris quis magna varius nulla scelerisque imperdiet. Aliquam non quam. Aliquam porttitor quam a lacus. Praesent vel arcu ut tortor cursus volutpat. In vitae pede quis diam bibendum placerat. Fusce elementum convallis neque. Sed dolor orci, scelerisque ac, dapibus nec, ultricies ut, mi. Duis nec dui quis leo sagittis commodo.

Aliquam lectus. Vivamus leo. Quisque ornare tellus ullamcorper nulla. Mauris porttitor pharetra tortor. Sed fringilla justo sed mauris. Mauris tellus. Sed non leo. Nullam elementum, magna in cursus sodales, augue est scelerisque sapien, venenatis congue nulla arcu et pede. Ut suscipit enim vel sapien. Donec congue. Maecenas urna mi, suscipit in, placerat ut, vestibulum ut, massa. Fusce ultrices nulla et nisl.

Etiam ac leo a risus tristique nonummy. Donec dignissim tincidunt nulla. Vestibulum rhoncus molestie odio. Sed lobortis, justo et pretium lobortis, mauris turpis condimentum

augue, nec ultricies nibh arcu pretium enim. Nunc purus neque, placerat id, imperdiet sed, pellentesque nec, nisl. Vestibulum imperdiet neque non sem accumsan laoreet. In hac habitasse platea dictumst. Etiam condimentum facilisis libero. Suspendisse in elit quis nisl aliquam dapibus. Pellentesque auctor sapien. Sed egestas sapien nec lectus. Pellentesque vel dui vel neque bibendum viverra. Aliquam porttitor nisl nec pede. Proin mattis libero vel turpis. Donec rutrum mauris et libero. Proin euismod porta felis. Nam lobortis, metus quis elementum commodo, nunc lectus elementum mauris, eget vulputate ligula tellus eu neque. Vivamus eu dolor.

Nulla in ipsum. Praesent eros nulla, congue vitae, euismod ut, commodo a, wisi. Pellentesque habitant morbi tristique senectus et netus et malesuada fames ac turpis egestas. Aenean nonummy magna non leo. Sed felis erat, ullamcorper in, dictum non, ultricies ut, lectus. Proin vel arcu a odio lobortis euismod. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Proin ut est. Aliquam odio. Pellentesque massa turpis, cursus eu, euismod nec, tempor congue, nulla. Duis viverra gravida mauris. Cras tincidunt. Curabitur eros ligula, varius ut, pulvinar in, cursus faucibus, augue.

# 8

## CONCLUSIONS AND FUTURE DIRECTIONS

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Suspendisse vitae elit. Aliquam arcu neque, ornare in, ullamcorper quis, commodo eu, libero. Fusce sagittis erat at erat tristique mollis. Maecenas sapien libero, molestie et, lobortis in, sodales eget, dui. Morbi ultrices rutrum lorem. Nam elementum ullamcorper leo. Morbi dui. Aliquam sagittis. Nunc placerat. Pellentesque tristique sodales est. Maecenas imperdiet lacinia velit. Cras non urna. Morbi eros pede, suscipit ac, varius vel, egestas non, eros. Praesent malesuada, diam id pretium elementum, eros sem dictum tortor, vel consectetur odio sem sed wisi.

Sed feugiat. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Ut pellentesque augue sed urna. Vestibulum diam eros, fringilla et, consectetur eu, nonummy id, sapien. Nullam at lectus. In sagittis ultrices mauris. Curabitur malesuada erat sit amet massa. Fusce blandit. Aliquam erat volutpat. Aliquam euismod. Aenean vel lectus. Nunc imperdiet justo nec dolor.

Etiam euismod. Fusce facilisis lacinia dui. Suspendisse potenti. In mi erat, cursus id, nonummy sed, ullamcorper eget, sapien. Praesent pretium, magna in eleifend egestas, pede pede pretium lorem, quis consectetur tortor sapien facilisis magna. Mauris quis magna varius nulla scelerisque imperdiet. Aliquam non quam. Aliquam porttitor quam a lacus. Praesent vel arcu ut tortor cursus volutpat. In vitae pede quis diam bibendum placerat. Fusce elementum convallis neque. Sed dolor orci, scelerisque ac, dapibus nec, ultricies ut, mi. Duis nec dui quis leo sagittis commodo.

Aliquam lectus. Vivamus leo. Quisque ornare tellus ullamcorper nulla. Mauris porttitor pharetra tortor. Sed fringilla justo sed mauris. Mauris tellus. Sed non leo. Nullam elementum, magna in cursus sodales, augue est scelerisque sapien, venenatis congue nulla arcu et pede. Ut suscipit enim vel sapien. Donec congue. Maecenas urna mi, suscipit in,

placerat ut, vestibulum ut, massa. Fusce ultrices nulla et nisl.

Etiam ac leo a risus tristique nonummy. Donec dignissim tincidunt nulla. Vestibulum rhoncus molestie odio. Sed lobortis, justo et pretium lobortis, mauris turpis condimentum augue, nec ultricies nibh arcu pretium enim. Nunc purus neque, placerat id, imperdiet sed, pellentesque nec, nisl. Vestibulum imperdiet neque non sem accumsan laoreet. In hac habitasse platea dictumst. Etiam condimentum facilisis libero. Suspendisse in elit quis nisl aliquam dapibus. Pellentesque auctor sapien. Sed egestas sapien nec lectus. Pellentesque vel dui vel neque bibendum viverra. Aliquam porttitor nisl nec pede. Proin mattis libero vel turpis. Donec rutrum mauris et libero. Proin euismod porta felis. Nam lobortis, metus quis elementum commodo, nunc lectus elementum mauris, eget vulputate ligula tellus eu neque. Vivamus eu dolor.

Nulla in ipsum. Praesent eros nulla, congue vitae, euismod ut, commodo a, wisi. Pellentesque habitant morbi tristique senectus et netus et malesuada fames ac turpis egestas. Aenean nonummy magna non leo. Sed felis erat, ullamcorper in, dictum non, ultricies ut, lectus. Proin vel arcu a odio lobortis euismod. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Proin ut est. Aliquam odio. Pellentesque massa turpis, cursus eu, euismod nec, tempor congue, nulla. Duis viverra gravida mauris. Cras tincidunt. Curabitur eros ligula, varius ut, pulvinar in, cursus faucibus, augue.

Nulla mattis luctus nulla. Duis commodo velit at leo. Aliquam vulputate magna et leo. Nam vestibulum ullamcorper leo. Vestibulum condimentum rutrum mauris. Donec id mauris. Morbi molestie justo et pede. Vivamus eget turpis sed nisl cursus tempor. Curabitur mollis sapien condimentum nunc. In wisi nisl, malesuada at, dignissim sit amet, lobortis in, odio. Aenean consequat arcu a ante. Pellentesque porta elit sit amet orci. Etiam at turpis nec elit ultricies imperdiet. Nulla facilisi. In hac habitasse platea dictumst. Suspendisse viverra aliquam risus. Nullam pede justo, molestie nonummy, scelerisque eu, facilisis vel, arcu.

Curabitur tellus magna, porttitor a, commodo a, commodo in, tortor. Donec interdum. Praesent scelerisque. Maecenas posuere sodales odio. Vivamus metus lacus, varius quis, imperdiet quis, rhoncus a, turpis. Etiam ligula arcu, elementum a, venenatis quis, sollicitudin sed, metus. Donec nunc pede, tincidunt in, venenatis vitae, faucibus vel, nibh. Pellentesque wisi. Nullam malesuada. Morbi ut tellus ut pede tincidunt porta. Lorem ipsum dolor sit amet, consectetur adipiscing elit. Etiam congue neque id dolor.

Donec et nisl at wisi luctus bibendum. Nam interdum tellus ac libero. Sed sem justo, laoreet vitae, fringilla at, adipiscing ut, nibh. Maecenas non sem quis tortor eleifend fermentum. Etiam id tortor ac mauris porta vulputate. Integer porta neque vitae massa. Maecenas tempus libero a libero posuere dictum. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Aenean quis mauris sed elit commodo



placerat. Class aptent taciti sociosqu ad litora torquent per conubia nostra, per inceptos hymenaeos. Vivamus rhoncus tincidunt libero. Etiam elementum pretium justo. Vivamus est. Morbi a tellus eget pede tristique commodo. Nulla nisl. Vestibulum sed nisl eu sapien cursus rutrum.

Nulla non mauris vitae wisi posuere convallis. Sed eu nulla nec eros scelerisque pharetra. Nullam varius. Etiam dignissim elementum metus. Vestibulum faucibus, metus sit amet mattis rhoncus, sapien dui laoreet odio, nec ultricies nibh augue a enim. Fusce in ligula. Quisque at magna et nulla commodo consequat. Proin accumsan imperdiet sem. Nunc porta. Donec feugiat mi at justo. Phasellus facilisis ipsum quis ante. In ac elit eget ipsum pharetra faucibus. Maecenas viverra nulla in massa.

Nulla ac nisl. Nullam urna nulla, ullamcorper in, interdum sit amet, gravida ut, risus. Aenean ac enim. In luctus. Phasellus eu quam vitae turpis viverra pellentesque. Duis feugiat felis ut enim. Phasellus pharetra, sem id porttitor sodales, magna nunc aliquet nibh, nec blandit nisl mauris at pede. Suspendisse risus risus, lobortis eget, semper at, imperdiet sit amet, quam. Quisque scelerisque dapibus nibh. Nam enim. Lorem ipsum dolor sit amet, consectetur adipiscing elit. Nunc ut metus. Ut metus justo, auctor at, ultrices eu, sagittis ut, purus. Aliquam aliquam.

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# A

## APPENDIX

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Nulla malesuada porttitor diam. Donec felis erat, congue non, volutpat at, tincidunt tristique, libero. Vivamus viverra fermentum felis. Donec nonummy pellentesque ante. Phasellus adipiscing semper elit. Proin fermentum massa ac quam. Sed diam turpis, molestie vitae, placerat a, molestie nec, leo. Maecenas lacinia. Nam ipsum ligula, eleifend at, accumsan nec, suscipit a, ipsum. Morbi blandit ligula feugiat magna. Nunc eleifend consequat lorem. Sed lacinia nulla vitae enim. Pellentesque tincidunt purus vel magna. Integer non enim. Praesent euismod nunc eu purus. Donec bibendum quam in tellus. Nullam cursus pulvinar lectus. Donec et mi. Nam vulputate metus eu enim. Vestibulum pellentesque felis eu massa.

Quisque ullamcorper placerat ipsum. Cras nibh. Morbi vel justo vitae lacus tincidunt ultrices. Lorem ipsum dolor sit amet, consectetur adipiscing elit. In hac habitasse platea dictumst. Integer tempus convallis augue. Etiam facilisis. Nunc elementum fermentum wisi. Aenean placerat. Ut imperdiet, enim sed gravida sollicitudin, felis odio placerat quam, ac pulvinar elit purus eget enim. Nunc vitae tortor. Proin tempus nibh sit amet nisl. Vivamus quis tortor vitae risus porta vehicula.

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Suspendisse vel felis. Ut lorem lorem, interdum eu, tincidunt sit amet, laoreet vitae, arcu. Aenean faucibus pede eu ante. Praesent enim elit, rutrum at, molestie non, nonummy vel, nisl. Ut lectus eros, malesuada sit amet, fermentum eu, sodales cursus, magna. Donec eu

purus. Quisque vehicula, urna sed ultricies auctor, pede lorem egestas dui, et convallis elit erat sed nulla. Donec luctus. Curabitur et nunc. Aliquam dolor odio, commodo pretium, ultricies non, pharetra in, velit. Integer arcu est, nonummy in, fermentum faucibus, egestas vel, odio.

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Morbi luctus, wisi viverra faucibus pretium, nibh est placerat odio, nec commodo wisi enim eget quam. Quisque libero justo, consectetur a, feugiat vitae, porttitor eu, libero. Suspendisse sed mauris vitae elit sollicitudin malesuada. Maecenas ultricies eros sit amet ante. Ut venenatis velit. Maecenas sed mi eget dui varius euismod. Phasellus aliquet volutpat odio. Vestibulum ante ipsum primis in faucibus orci luctus et ultrices posuere cubilia Curae; Pellentesque sit amet pede ac sem eleifend consectetur. Nullam elementum, urna vel imperdiet sodales, elit ipsum pharetra ligula, ac pretium ante justo a nulla. Curabitur tristique arcu eu metus. Vestibulum lectus. Proin mauris. Proin eu nunc eu urna hendrerit faucibus. Aliquam auctor, pede consequat laoreet varius, eros tellus scelerisque quam, pellentesque hendrerit ipsum dolor sed augue. Nulla nec lacus.

Suspendisse vitae elit. Aliquam arcu neque, ornare in, ullamcorper quis, commodo eu, libero. Fusce sagittis erat at erat tristique mollis. Maecenas sapien libero, molestie et, lobortis in, sodales eget, dui. Morbi ultrices rutrum lorem. Nam elementum ullamcorper leo. Morbi dui. Aliquam sagittis. Nunc placerat. Pellentesque tristique sodales est. Maecenas imperdiet lacinia velit. Cras non urna. Morbi eros pede, suscipit ac, varius vel, egestas non, eros. Praesent malesuada, diam id pretium elementum, eros sem dictum tortor, vel consectetur odio sem sed wisi.