PROPOSAL FOR DISSERTATION

DOCTOR OF PHILOSOPHY IN COGNITIVE NEUROSCIENCE

COLLEGE OF ARTS AND SCIENCES

DEPARTMENT OF PSYCHOLOGY

Locomotor Behavior and Hippocampal Activity in Pig Models of Alzheimer's Disease

Valentina Roldan[1], Timothy A. Allen[1]

Keywords: Electrophysiology; Pigs; Alzheimer's Disease; Behavior; Cognition; Learning; Memory; Brain Trauma.

[1]Cognitive Neuroscience Program, Department of Psychology, Florida International University, Miami, FL 33199, USA

I propose to the Major Professor (Dr. Timothy Allen) and Committee Members (Dr. Anthony Dick, Dr. Aaron Mattfeld, and Dr. Jamie Theobald) a study of the following topic to be conducted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology with an emphasis in Cognitive Neuroscience: **Locomotor Behavior and Hippocampal Activity in Pig Models of Alzheimer's Disease.**

BACKGROUND AND THEORY

Introduction/Statement of Problem and Aims

Alzheimer's Disease (AD) is the most common type of dementia. Specifically, AD can often develop in people over 65 years old with memory decline as the disease progresses. However, in less than 1% of cases, this onset can occur at earlier ages due to genetic factors, which are referred to as familial AD (FAD). Importantly, AD can only be diagnosed post-mortem based on a variety of factors such as the presence of traditional molecular biomarkers like amyloid beta (Aβ) plaques and Tau proteins, neurodegeneration, and axonal swelling (Braak & Braak, 1991a, 1991b). Thus, the field continues to search for translational FAD models that allow experimenters to observe these pathologies at different disease stages.

The pig has emerged as a key translational model due to the many genetic, anatomical, and physiological similarities to humans. Pigs are often used to study traumatic brain injuries (TBI) since their large body and brain size allow for accurate modeling of these insults with uniform shock distribution (Cullen et al., 2016). AD pig models have been developed through genetic modifications to get more insight into the disease (Jakobsen et al., 2013; Kragh et al., 2009; Uh et al., 2024). In addition to the characteristic cognitive decline, AD patients have shown alterations in ambulatory behavior and gait (Duan et al. 2023). However, the literature on ambulatory aberrations in AD and their implications is limited, especially in large translational models, such as the pig.

Similarly, little work has been done to examine the anatomy of the porcine brain at the neural level using electrophysiology (Ulyanova et al., 2018, 2019). No studies have examined the pig AD brain using invasive electrophysiology in the same manner as with smaller animal models, such as rodents.

In this project, we seek to examine AD-related pathologies in pig AD models at molecular, behavioral, and physiological levels to provide key insight into AD hallmarks and signatures that can be taken into consideration for diagnoses and treatments.

Aim 1: Test for AD-related motor changes in 5xFAD pigs. We will use video tracking to investigate the locomotor and ambulatory-related behavioral differences between wild-type (control) and AD (5xFAD and *PSEN1*) pigs performing our validated automated T-maze task and the treadmill task.

Aim 2: Validate the 5xFAD pig model at the protein level. We will quantify increased AD-related protein levels (tau and Aβ) in 5xFAD pigs compared to controls using quantitative approaches (e.g., Western Blotting and/or ELISA techniques).

Aim 3: Test for AD-related electrophysiological changes in the brain. We will obtain and compare hippocampal and cortical recordings in anesthetized control and 5xFAD pigs. We will record cardiovascular vitals and brain pulsation simultaneously. We will investigate local field potential (LFP) signals for potential electrophysiological AD signatures such as frequency slowing, aberrant synchrony, phase-amplitude coupling (PAC), and frequency composition.

Research Question(s)/Objectives/Hypothesis

Cognitive decline and abnormal ambulatory behavior are notable characteristics of AD patients (Andrade-Guerrero et al., 2024). Additionally, in AD patients and AD rodent models, non-invasive electrophysiology has been used to identify changes in oscillations, decoupling, and other alterations that serve as signatures of the disease state (Calvin-Dunn et al., 2025; Scott et al., 2012). Therefore, it is crucial to examine the pathologies, the cognitive and behavioral decline, and the electrophysiological aberrations associated with AD in a suitable translational model. Here, we focus on the pig as a translational model to assess AD from perspectives that are not practical in mice, rats, or humans.

Hypothesis 1: I hypothesize that AD pigs will show decreased walking speeds, disturbed gait, and overall changes in ambulatory activity compared to controls.

Hypothesis 2: I hypothesize that the HC of the AD group will show increased levels of Aβ and tau proteins compared to the HC of control pigs. Additionally, I hypothesize that these increases will negatively correlate with the ambulatory aberrations in AD pigs observed in experiment #1.

Hypothesis 3: I hypothesize that the hippocampal LFP of AD pigs will show increases in delta and theta frequencies and decreases in alpha and beta rhythms compared to oscillatory activity in the HC of the control group.

METHODS

Experimental Approach

For our project's first aim, we will run two experiments to assess locomotor behavior. The first part of this experiment will use three experimental groups consisting of wild-type pigs (controls, n = 7), one AD group expressing a *PSEN1* knockout (n = 6), and one group expressing the 5xFAD combination of mutations (n = 4). We will examine discrete ambulatory behaviors across groups in two different regions of the maze. Each region will allow for different analyses, such as ambulatory behavior in the delay box and ambulatory behavior at the decision point. For the second part of this experiment, we will use control (n = 2) and 5xFAD pigs (n = 2). We will train both experimental groups to use a standard treadmill and obtain recordings from different perspectives to assess gait. More details on behavioral variables for this experiment are described in the *Behavioral Tracking and Analyses* section.

For our second aim, we will assess flash-frozen brain hemispheres of control pigs (n = 2) and of 5xFAD pigs (n = 2). We will quantify the levels of phosphorylated tau proteins and Aβ deposits or plaques using immunoassays, such as Enzyme-linked immunosorbent assay (ELISA) and Western Blotting. We will compare these levels across the two experimental groups.

For our third aim, we will obtain anesthetized recordings from the pigs in experiment #1: two control pigs and two 5xFAD pigs. Pigs will undergo terminal endpoint surgeries to record LFP activity. We will use two linear probes with 32 channels each to aid source localization. We will record from various HC and cortical regions and relate activity to vitals during surgery. We will compare the electrophysiology and cardiovascular couplings across experimental groups.

Animals

Animals will be sourced from our partners at the National Swine Research & Resource Center (NSRRC) at the University of Missouri and held at the Porcine Neuroscience Facility (PNF) located at the Owa Ehan building in the FIU MMC campus (Miami, FL). All breeding and targeted genetic manipulations will be done at the NSRRC prior to shipping. We will use wild-type domestic pigs (*Sus scrofa domesticus*) and genetically modified pigs to express presenilin 1 (*PSEN1*) gene deletions (axon 9 or axons 9-12) (Uh et al., 2024), and/or additional APP mutations (i.e., double Swedish mutation) for 5xFAD models. Notably, 5xFAD mutations are done to observe the highest levels of traditional molecular AD pathology accumulation and at the earliest onsets. Pigs of approximately two months of age will be ordered in cohorts of two or four pigs, depending on availability. Shipments will be sent from Columbia, MO, under USDA conditions. Importantly, similarly to non-human primate and other research involving large mammals, the number of subjects is limited due to housing, cost, and ethical restrictions.

Pigs will be fed twice a day as per the Guide for the Care and Use of Laboratory Animals recommendations and will have access to water throughout the day. Pigs will be housed in individual adjacent pens such that pigs can socialize. They will be maintained under a 12-hour light-dark cycle starting at 5:30 am. Pigs will be kept in quarantine for at least one week. After this period, they will be supervised daily and interact with experimenters on schedule for up to a week to allow for proper habituation prior to behavioral training. Habituation procedures will include providing housing enrichment, introducing experimenters' tools and PPE for sniffing, introducing food rewards, and acclimating them to the automated T-maze behavioral apparatus. Logs will be maintained daily to account for pigs' weights and any additional health information.

All experiments proposed will be conducted with the approval of the FIU Animal Care and Use Committee (IACUC), and the Guide, and in compliance with their guidelines.

Automated T-Maze Behavioral Apparatus

The popular T-maze configuration has been adapted and validated for use with domestic pigs (L. M. Allen et al. 2023). Briefly, the pig T-maze consists of a delay box (or "start" area), where pigs are initially located, and a connecting decision region (or the "stem" area) where they continue until the choice point and must turn to the left or right wing of the maze. The pigs learn to alternate sides between trials with food rewards automatically dispensed after the correct

choice. A trial is rewarded if the pig turns to the side opposite to the last correct side. After learning the alternation task, a set of random delays is introduced. Pigs are then held in the delay box before the start of each trial for 5, 60, 120, or 240 seconds. The T-maze has been configured to automatically open and close the gates that keep the pig in the corresponding region during the task. For instance, the gate from the start to the stem area opens once the delay period is completed. The maze is cleaned after each session to avoid contamination between pigs.

Behavioral Tracking and Analyses

T-maze

While basic behavioral measures, such as trial accuracy, will not be the focus of this project, the locomotor behavior of the pigs will be tracked during behavioral sessions using overhead cameras in four locations. For this project, we will use the recordings from the two main behavioral locations of the T-maze: the delay box and the decision region. Locomotor behavior will be tracked using the pose estimation software DeepLabCut. We will obtain x-y coordinates for each labeled body part of the control and AD pigs throughout the video frames. We will analyze ambulatory variables depending on the maze area and compare them across the three experimental groups. In the delay box, we will measure stationary and walking variables, including the number and duration of both types of bouts, head and body direction, walking speeds, and acceleration. In the decision region, we will measure other specific variables such as the number of head turns and time before the decision, lateral bias or prediction, and walking speed and accelerations.

Treadmill

We will use a research-grade treadmill to evaluate gait in the pigs. Pigs will be habituated to the use of the treadmill in the presence of at least two experimenters. Habituation to the treadmill will begin with exposure to the treadmill on day one by leading the pig to get on the apparatus after regular habituation is complete. We will start with low velocities to allow for walking or running speeds that are amenable to the pig. Two cameras will record walking activity on the treadmill, including an overhead camera and a lateral-view camera. We will use DeepLabCut to label and track the relevant body parts, such as legs and trotters, to obtain y-z coordinates throughout the session from both perspectives. We will analyze gait-related variables, such as the number of steps and stances (gait cycles) per minute, and stride length.

Surgical Procedures

All surgical procedures will occur in the large surgical suite in the Academic Health Center 4 (AHC4) building (5^{TH} floor), located at the FIU Animal Care Facility at the MMC campus. This suite is maintained by the Office of Laboratory Animal Research (LAR) with on-site veterinary services. All tools will be autoclaved before surgery, and the surgical suite will be cleaned and sterilized accordingly. The surgical suite is equipped with an adapted large stereotaxic device to hold pigs during surgery and custom parts from the Allen Lab (US Patents 9,707,049, 10,251,722, 10,492,882).

Briefly, terminal surgical procedures begin after pre-anesthesia is administered to each pig in their pen or preparation room using Ketamine/Xylazine (Ketamine 20 mg/kg/Xylazine 2 mg/kg IM) or Telazol while maintaining their body temperature and vital signs. Anesthetized state will be maintained with isoflurane. We will use an IV catheter to administer any necessary medications and fluids to the pig. Prior to the brain surgery, the head area will be shaved, and antiseptic agents will be applied (e.g., betadine). The pig will be administered Atropine (.02-.05 mg/kg). The pig will then undergo endotracheal intubation through dorsal or ventral recumbency to avoid fluid accumulation throughout the pharynx while under anesthesia. The head area or surgical site will be sterilized with a surgical preparation solution and alcohol and dried with sterile gauze sponges. The area surrounding the surgical site and the pig's body will be covered with sterile drapes while the pig is lying on the heated surgical table. We will also administer intravenous fluids to maintain homeostasis (10-15 ml/kg/hour). The pig will be monitored for a continuous anesthetized state, and regular EKG signals, pulse, body temperature, muscle reflexes, blood pressure, heart rate, brain pulsations, and breathing will be recorded. An incision will be made on the surgical site to expose the dorsal view of the skull. Craniotomies will be done to remove the dura mater based on bregma and lambda coordinates, further confirmed by the sulci locations. Probes will be driven to contact the pia mater and fixed with dental cement and bone screws.

Emergency equipment and supplies will be available on site should any cardiac, anoxia, acidosis, or alkalosis complications occur. All anesthetic and surgical procedures will be reflected on the corresponding logs, including administered medications/fluids and all vital signs.

At the end of all data collection procedures, pigs will be overdosed with isoflurane and Euthesol, and the brains will be removed for further processing.

Electrophysiology

We will obtain recordings during terminal surgeries of pigs under anesthesia with two 32-channel linear probes and digital headstage processors. We will acquire wide-band neural data using the Plexon Omniplex recording system with A/D conversion at 40 kHz (16-bit). The wide-band data will be passed by our digital head-stage 256-channel data acquisition system and referenced against the metal screws. This data will be stored using the PlexControl data acquisition software. We will down-sample our wide-band data as needed to obtain our LFP recordings. Wide-band data will later be passed through a high-pass filter to obtain spike and LFP data.

Brain Processing

We will use standard procedures to obtain the brain after a double-carotid perfusion (see Surgical Procedures section) and prepare the tissue. Briefly, after careful removal from the body, the brain will be halved to divide the two hemispheres. One half will be flash frozen and sent to Virginia Tech for protein and RNA assessment, while the other will be kept in a 4% paraformaldehyde solution for at least two weeks for histology.

Histology

The brain hemisphere in the paraformaldehyde solution will be moved to a sucrose solution at 10% to allow for descent over 2-4 days. The concentration of sucrose will be increased to 20 and 30% until the hemisphere sinks to the bottom and is ready for slicing. We will fix and block the tissue corresponding to the areas of interest. We will mount the tissue on the microtome for freezing and subsequent slicing in 40μm thick slices. These slices will be stored in 24-well plates with PBS solution and will be kept refrigerated until processing. We will use Cresyl violet to stain the tissue and observe it under the microscope to locate the recording marks and confirm target sites.

Protein Analyses

One brain hemisphere from each pig will be flash frozen using dry ice and expedited shipped to our colleagues at Virginia Tech. Specifically, we will look for Aβ deposition and plaques, as well as phosphorylated tau and neurofibrillary tangles. Our collaborators at Virginia Tech will perform protein analyses using ELISA and Western Blotting methods

ELISA: ELISA is a simple and fast immunoassay method used to quantify and qualify Aβ and tau proteins in brain tissue. This assay requires 96-well polystyrene absorbent plates and the appropriate antibodies (primary and/or secondary) for these proteins. These antibodies are used to coat the plates, followed by a substrate that shows the protein in color for quantification. Specifically, to extract soluble or total Aβ, we can mix the brain homogenates with diethylamine and sodium chloride, or with cold formic acid, respectively, and further centrifuge and neutralize them using tris(hydroxymethyl)aminomethane (Tris) (Scholtzova et al. 2014). Phosphorylated tau can also be extracted using a blocking buffer solution and commercially available Invitrogen Human ELISA kits (Scholtzova et al. 2014).

Western Blotting: The tissue will also be analyzed using Western Blotting assays to quantify these two proteins with more accurate results. Brain slices will be homogenized using lysis buffer, and the proteins will be separated using gel electrophoresis based on their molecular weights. A membrane with the appropriate antibodies will be used to transfer the resulting proteins. To separate Aβ oligomers using Western Blotting, we can use a Tricine buffer and Tris-tricine polyacrylamide gels (Scholtzova et al. 2014). Similarly, to isolate phosphorylated tau, we can use bicinchoninic acid and polyacrylamide gels (Scholtzova et al. 2014).

Data and Statistical Analyses

Electrophysiology

Electrophysiology recordings will be preprocessed using Plexon's Offline Sorter to band-pass signals and obtain spikes and LFP signals. We will also evaluate the coupling of electrophysiology with measured cardiovascular vital signs during surgery, including brain pulsations, breathing, and heart rate.

Single Unit Analyses (SUA): We will use MATLAB custom scripts to analyze spiking data from isolated units. These analyses will include epoch-specific firing rate differences across units from spikes in 50 ms time bins and 250 ms epochs for statistical analyses. We will perform *k*-means clustering of unit groups based on waveform characteristics (i.e., width, valley-to-peak slope, frequency, and others), and the type of activity.

LFP Analyses: We will examine the LFP recorded from HC from control and AD pigs. Specifically, LFP will be band-passed filtered at delta (1-4 Hz), theta (5-7 Hz), alpha (8-12 Hz), beta (13-30 Hz), and gamma (>30 Hz) frequencies, and averaged to reduce noise. Phase will be determined using a Hilbert transform. We will use perievent spectrograms to visualize LFP oscillatory power changes, as well as perform power-spectral density analyses, PAC, and spike-phase analyses. Our PAC analyses across frequencies will be tested using an adaptation of the Kullback-Leibler distance (Tort et al. 2010) and an 'oscillation-triggered coupling' technique for short epochs (Dvorak and Fenton 2014).

Ensemble analyses: Groups of neurons will be assessed per physiological event by creating ensemble matrices. We will use population vector (PV) correlational analyses (Allen et al. 2016) and clustering algorithms to compare experimental groups across trials and phases. We will determine neurons' phase preferences using the distribution of spiking phases per frequency, and we will further sort them by phase to determine sequential neuron activation.

Statistics

Statistical approaches for electrophysiological measures will be done with two-tailed tests with significance below 0.05 after corrections. We will generate probability distributions and use inferential statistics, including t and F ratios to account for variance, and classify epochs based on corresponding bins. Importantly, before reporting these results, all plots will be visually assessed to avoid interpretation and systematic errors. For data that does not meet normal distribution assumptions, we will use non-parametric permutation t-tests and ANOVAS (1000 permutations), such as in unit firing rates. Repeated measures ANOVA on frequencies across groups will be done.

TIMELINE TO DEGREE COMPLETION/SCHEDULE OF WORK

2025:

- **August:**
  - Ordering histology materials.
  - Working with collaborators for qualifying paper journal publication.
  - Ongoing data analysis for locomotor dissertation chapter.
  - Preparation of electrophysiological and physiological tools.
- **September:**
  - Receiving the first pig cohort.
  - Running treadmill and T-maze task.
  - Ordering histology materials.
  - Submitting qualifying paper to journal for publication.
- **October:**
  - Surgery and recordings of the first pig.
  - Reviewer's feedback and work for qualifying paper publication.
- **November:**
  - Surgery and recordings for the rest of the pigs.
  - Histology for the first cohort.
  - SfN 2025 conference on the locomotor chapter's work.
- **December:**
  - Histology for the second cohort.
  - Data preprocessing.

2026:

- **January:** Data preprocessing, analyses and writing.
- **February:** Data analyses and writing.
- **March:** Data analyses and writing.
- **April:** Data analyses and writing.
- **May:** Data analyses and writing; SfN 2026 abstract submission.

- **June:** Writing and editing.
- **July:** Writing and editing.
- **August:** Writing and editing.
- **September:** Chapters editing.
- **October:** Submitting dissertation to UGS.
- **November:** Oral Defense; SfN 2026.
- **December:** Graduation.

REFERENCES

Allen, Leila M., Murphy, Deirdre, Roldan, Valentina et al. 2023. “Testing Spatial Working Memory in Pigs Using an Automated T-Maze.” *Oxford Open Neuroscience* 2 (February): kvad010. https://doi.org/10.1093/oons/kvad010.

Allen, Timothy A., Daniel M. Salz, Sam McKenzie, and Norbert J. Fortin. 2016. “Nonspatial Sequence Coding in CA1 Neurons.” Articles. *Journal of Neuroscience* 36 (5): 1547–63. https://doi.org/10.1523/JNEUROSCI.2874-15.2016.

Andrade-Guerrero, Jesús, Humberto Martínez-Orozco, Marcos M. Villegas-Rojas, et al. 2024. “Alzheimer’s Disease: Understanding Motor Impairments.” *Brain Sciences* 14 (11): 1054. https://doi.org/10.3390/brainsci14111054.

Braak, H., and E. Braak. 1991a. “Alzheimer’s Disease Affects Limbic Nuclei of the Thalamus.” *Acta Neuropathologica* 81 (3): 261–68. https://doi.org/10.1007/BF00305867.

Braak, H., and E. Braak. 1991b. “Neuropathological Stageing of Alzheimer-Related Changes.” *Acta Neuropathologica* 82 (4): 239–59. https://doi.org/10.1007/BF00308809.

Calvin-Dunn, Kirsten N., Adam Mcneela, A. Leisgang Osse, et al. 2025. “Electrophysiological Insights into Alzheimer’s Disease: A Review of Human and Animal Studies.” *Neuroscience & Biobehavioral Reviews* 169 (February): 105987. https://doi.org/10.1016/j.neubiorev.2024.105987.

Cullen, D. Kacy, James P. Harris, Kevin D. Browne, et al. 2016. “A Porcine Model of Traumatic Brain Injury via Head Rotational Acceleration.” *Methods in Molecular Biology (Clifton, N.J.)* 1462: 289–324. https://doi.org/10.1007/978-1-4939-3816-2_17.

Duan, Qi, Yinuo Zhang, Weihao Zhuang, et al. 2023. “Gait Domains May Be Used as an Auxiliary Diagnostic Index for Alzheimer’s Disease.” *Brain Sciences* 13 (11): 1599. https://doi.org/10.3390/brainsci13111599.

Dvorak, Dino, and André A. Fenton. 2014. “Toward a Proper Estimation of Phase–Amplitude Coupling in Neural Oscillations.” *Journal of Neuroscience Methods* 225 (March): 42–56. https://doi.org/10.1016/j.jneumeth.2014.01.002.

Jakobsen, Jannik Ejnar, Marianne G. Johansen, Mette Schmidt, et al. 2013. “Generation of Minipigs with Targeted Transgene Insertion by Recombinase-Mediated Cassette Exchange (RMCE) and Somatic Cell Nuclear Transfer (SCNT).” *Transgenic Research* 22 (4): 709–23. https://doi.org/10.1007/s11248-012-9671-6.

Kragh, Peter M., Anders Lade Nielsen, Juan Li, et al. 2009. “Hemizygous Minipigs Produced by Random Gene Insertion and Handmade Cloning Express the Alzheimer’s Disease-Causing Dominant Mutation APPsw.” *Transgenic Research* 18 (4): 545–58. https://doi.org/10.1007/s11248-009-9245-4.

Scholtzova, Henrieta, Peter Chianchiano, Jason Pan, et al. 2014. “Amyloid β and Tau Alzheimer’s Disease Related Pathology Is Reduced by Toll-like Receptor 9 Stimulation.” *Acta Neuropathologica Communications* 2 (September): 101. https://doi.org/10.1186/s40478-014-0101-2.

Scott, Liam, Jianlin Feng, Tamás Kiss, et al. 2012. “Age-Dependent Disruption in Hippocampal Theta Oscillation in Amyloid-β Overproducing Transgenic Mice.” *Neurobiology of Aging* 33 (7): 1481.e13-1481.e23. https://doi.org/10.1016/j.neurobiolaging.2011.12.010.

Tort, Adriano B. L., Robert Komorowski, Howard Eichenbaum, and Nancy Kopell. 2010. “Measuring Phase-Amplitude Coupling Between Neuronal Oscillations of Different Frequencies.” *Journal of Neurophysiology* 104 (2): 1195–210. https://doi.org/10.1152/jn.00106.2010.

Uh, Kyungjun, Kaylynn Monarch, Emily D. Reese, et al. n.d. “Impaired Skeletal Development by Disruption of Presenilin-1 in Pigs and Generation of Novel Pig Models for Alzheimer’s Disease.” *Journal of Alzheimer’s Disease* 101 (2): 445–61. https://doi.org/10.3233/JAD-231297.

Ulyanova, Alexandra V., Carlo Cottone, Christopher D. Adam, et al. 2019. “Multichannel Silicon Probes for Awake Hippocampal Recordings in Large Animals.” *Frontiers in Neuroscience* 13: 397. https://doi.org/10.3389/fnins.2019.00397.

Ulyanova, Alexandra V., Paul F. Koch, Carlo Cottone, et al. 2018. “Electrophysiological Signature Reveals Laminar Structure of the Porcine Hippocampus.” *eNeuro* 5 (5): ENEURO.0102-18.2018. https://doi.org/10.1523/ENEURO.0102-18.2018.