CURRICULUM VITAE

Miriam N. Weiss, PhD, BSN, RN

Department of Pain and Translational Symptom Science

University of Maryland Baltimore School of Nursing

Department of Pain and Translational Symptom Science

655 West Lombard Street, Room 746

Baltimore, Maryland 21201

Email: miriamnweiss@gmail.com

EDUCATION

2019-2023	University of Maryland Baltimore School of Nursing, Baltimore, MD Doctor of Philosophy
2017-2018	University of Maryland Baltimore School of Nursing, Baltimore, MD Bachelor of Science in Nursing
2014-2016	Community College of Baltimore County, Catonsville, MD

RESEARCH EXPERIENCE

2019-2023	Graduate Research Assistant University of Maryland Baltimore School of Nursing, Baltimore, MD
2017-2018	Undergraduate Research Assistant University of Maryland Baltimore School of Nursing, Baltimore, MD

INTERNSHIPS

2018	Summer Intern National Institutes of Health, Bethesda, MD
2016	Electrophysiology Research Intern Franklin Medstar Hospital, Baltimore, MD

FEDERAL SERVICE

2020-2021 NINR Pathways Working Group – Bethesda, MD
National Institutes of Health, Bethesda, MD

FUNDED GRANTS

2021 Biology and Behavior Across the Lifespan Research Facilitation Award

2020-2023 Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship

HONORS AND AWARDS

2018 Summa cum laude
University of Maryland Baltimore School of Nursing, Baltimore, MD

2017 Dean's List
University of Maryland Baltimore School of Nursing, Baltimore, MD

2017 Joanne and William Conway Scholarship Program
University of Maryland Baltimore School of Nursing, Baltimore, MD

2017 Seely Merit Scholarship
University of Maryland Baltimore School of Nursing, Baltimore, MD

2015 Dean's List
Community College of Baltimore County, Catonsville, MD

PROFESSIONAL MEMBERSHIPS

2020-2023 Center to Advance Chronic Pain Research
University of Maryland Baltimore School of Nursing, Baltimore, MD

2019-2023 Biology and Behavior Across the Lifespan,
University of Maryland Baltimore School of Nursing, Baltimore, MD

2018-2020 Sigma Theta Tau
University of Maryland Baltimore School of Nursing, Baltimore, MD

2015-2016 Honors Program
Community College of Baltimore County, Catonsville, MD

2015-2016 Phi Theta Kappa
Community College of Baltimore County, Catonsville, MD

PUBLICATIONS

Weiss, M., Mocci, E., Zhu, S., Davenport, M., English, E., Dorsey, S., Renn, C. (2023). Nociceptive and Transcriptomic Responses in a Swine Diabetic Wound Model Treated with a Topical AT1R Antagonist. *Nursing Research.* DOI: 10.1097/NNR.0000000000000704

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PRESENTATIONS

Weiss, M., Mocci, E., Zhu, S., Davenport, M., English, E., Dorsey, S., Renn, C. (2023). Nociceptive and Transcriptomic Responses in a Swine Diabetic Wound Model Treated with a Topical AT1R Antagonist. National Institutes of Health, Pain in Animals Workshop (PAW), Bethesda, MD.

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Weiss, M., Mocci, E., Zhu, S., Davenport, M., English, E., Dorsey, S., Renn, C. (2022). Nociceptive and Transcriptomic Responses in a Swine Diabetic Wound Model Treated with a Topical AT1R Antagonist. University of Wisconsin-Madison, Swine in Biomedical Research Conference, Madison, WI.

Moore, S., Aycock, D., Wolfe, J., Phillips, J., Jones, R., Gonzalez-Guarda, R., Minissian, M., Howard, M., Hetland, B., Davidson, P., Butlin, J., **Weiss, M.**, Vasbinder, A., Gill, J., Banks, D., Tamez, P., Rasooly, R., Bryan, Y., Rosenbaum, L. (2021). Training Pathways Working Group Report. NINR National Advisory Council, Bethesda, MD.

Wickersham, K.E., Crothers, M., Puth, D., **Weiss, M.**, Powell, K. (2018). Targeted therapy use in adults with cancer ≥ 85 years of age. Gerontological Society of America Annual Meeting, Boston, MA.

Weiss, M., Cashion, A., Dorsey, S., Joseph, P. (2018). Genomic profiles in chronic pain research. National Institutes of Health, Summer Intern Poster Day, Bethesda, MD.

Wickersham, K.E., Crothers, M., Puth, D., **Weiss, M.**, Powell, K. (2017). Targeted therapy use in adults with cancer ≥ 85 years of age. University of Maryland, Center for Advancement of Chronic Pain Research/Biology and Behavior across the Lifespan.

University of Maryland, Baltimore, Trainee Poster Session (Trainee Poster Award), Baltimore, MD.

Wickersham, K.E., Crothers, M., Puth, D., **Weiss, M.**, Powell, K. (2017). Targeted therapy use in adults with cancer ≥ 85 years of age. Gerontological Advanced Practice Nurses Association (GAPNA) Annual Meeting, (GAPNA Research Project Award), Nashville, TN.

ABSTRACT

Dissertation Title: Nocifensive Behavior and Transcriptomic Biomarkers in a Translational Pig Model of Spared Nerve Injury

Miriam N. Weiss, Doctor of Philosophy, 2023

Dissertation Directed By: Eun-Shim Nahm, PhD, RN, FAAN, FGSA
Professor and Associate Dean, PhD Program
School of Nursing
University of Maryland, Baltimore

Background: Neuropathic pain is a prevalent condition that significantly impacts quality of life. Translational animal models are needed to understand the mechanisms and develop novel treatments. The spared nerve injury (SNI) model is a well-validated neuropathic pain model that causes allodynia and hyperalgesia. While the SNI was originally developed in rats, rodent models have translational limitations. Pigs, by contrast, share many characteristics with humans, making them uniquely suitable for modeling painful conditions.

Objective: The goal of this study was to test the feasibility and efficacy of a translational pig model of SNI.

Methods: The left common peroneal nerve of SNI pigs was ligated, while the nerve remained untouched in sham pigs. Mechanical and dynamic allodynia, thermal hyperalgesia, pressure, and gait alterations were assessed over four weeks. Video footage was recorded during testing and analyzed for a pig grimace scale. Blood and spinal cord

tissue were harvested and sequenced to determine the presence of differentially expressed genes and associated enriched pathways.

Results: Surgical ligation of the common peroneal nerve was feasible and safe. However, there were significant floor and ceiling effects for mechanical and dynamic allodynia and thermal hyperalgesia testing. Females showed lower pressure tolerance than males over time at the medial left ($p=0.003$), medial right ($p=0.003$), lateral left ($p=0.041$), and lateral right ($p=0.003$) testing locations. The left side had a lower threshold tolerance than the right side for both medial ($p<0.001$) and lateral ($p=0.03$) locations, although this difference was not significant over time. There was no significant difference in threshold tolerance between the sham and SNI groups. There were no significant changes in gait. The grimace scale showed substantial interrater reliability for ear position ($kappa=0.73$) and moderate interrater reliability for snout tension ($kappa=0.43$). Several pathways related to inflammation and wound healing were differentially expressed between the SNI versus sham groups and left versus right sides of the SNI animals.

Conclusion: While the pig model of SNI is feasible, special considerations are needed when acclimating animals and calibrating study equipment to avoid floor and ceiling effects. Relevant enriched pathways may be targets of interest for future neuropathic pain research.

Nocifensive Behavior and Transcriptomic Biomarkers in a Translational Pig Model of Spared Nerve Injury

by
Miriam N. Weiss

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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DEDICATION

To my loving and supportive parents, Chaim and Sara Weiss

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List of Abbreviations

BDNF	Brain-Derived Neurotrophic Factor
CGRP	Calcitonin Gene-Related Peptide
DEG	Differentially Expressed Gene
FAU	Facial Action Unit
ICC	Interclass Correlation Coefficient
NF1	Neurofibromatosis Type 1
PNT	Peripheral Neuritis Trauma
SNI	Spared Nerve Injury
TPI	Total Pressure Index
ZYMND12	Zinc Finger MYND-Type Containing 12

CHAPTER I. INTRODUCTION AND BACKGROUND

1.1 Background and Problem

A. Neuropathic Pain

(See appendix for glossary)

Neuropathic pain is a debilitating and often chronic pain condition caused by damage to the somatosensory nervous system[1–4]. It has been estimated that 10% of Americans[5] and between 3-17% of the global population[3,6] suffer from neuropathic pain, with woman, minorities, and people over 50 years of age disproportionately affected[3,7,8]. Symptoms include sensations of numbness, pins and needles, shooting, pricking, burning, and electrical pain[2,7].

Neuropathic pain is characterized by spontaneous and stimulus-evoked pain[2]. Spontaneous pain is pain that occurs in the absence of any sensation. Stimulus-evoked pain includes allodynia, i.e., pain resulting from a stimulus that does not normally cause pain, and hyperalgesia, i.e., hypersensitivity to a painful stimulus[2,3]. Patients report that neuropathic pain has a significant impact on their quality of life, affecting mobility, mental health, and sleep[7,9]. NSAIDs and opioids, which are frequently used in treating nociceptive pain, are often ineffective in combating neuropathic pain[10].

Unlike nociceptive pain, which serves a protective role by alerting the body to the presence of a harmful stimulus, neuropathic pain does not appear to have any biological function and is itself a disease state[11]. The pathology of neuropathic pain is complex and often related to the underlying condition[1,10], including autoimmune disorders such as multiple sclerosis, metabolic disorders such as diabetes, viral diseases such as HIV, inflammatory conditions such as Guillain-Barre syndrome, trauma to the nervous system

such as spinal cord injury, and chemotherapy-induced peripheral neuropathy[1,3]. In some patients, the exact cause of neuropathy is unknown[12].

Peripheral sensitization plays a key role in the development of neuropathic pain[13]. It involves decreased threshold to noxious stimulation and increased sensitivity of primary afferent nociceptors. The release of inflammatory mediators from synaptic terminals and the presence of inflammatory cytokines sensitizes nociceptors and decreases activation thresholds. In addition, ectopic discharge, in which unprovoked electrical nerve impulses fire from an injured nerve or a nerve located near the site of injury, is thought to play a role in spontaneous pain[10].

Central sensitization is another component of the neuropathic pain process. It is caused by heighted sensitivity of nociceptive central nervous system neurons to afferent signals[10]. When repeated nociceptive input occurs, the sensitization of the second-order neurons to stimulation increases, contributing to allodynia and hyperalgesia[14]. In addition, glial cells release cytokines and chemokines that contribute to neuroinflammation[15].

B. Pig Models

Despite the critical need for more research in this area, conducting neuropathic pain studies in humans presents challenges. Recruiting participants and controlling for confounding variables is difficult and it is unethical to induce neuropathic pain in healthy participants[16,17]. The use of animal models is vital to improving scientific understanding of neuropathic pain, identifying potential therapeutic targets, and testing novel treatments[16–19].

Testing and developing treatment for neuropathic pain necessitates an accurate, highly translational model. Traditionally, rodent species have been favored in pain research for their ease of handling and cost effectiveness. However, rodents have limited translational ability because their anatomy and physiology differ significantly from that of humans[20,21]. Drugs that appear to be effective in rodents often fail to show therapeutic benefits in human clinical trials[20,21]. Conversely, pigs have myriad characteristics comparable to those of humans, including similarities in skin[22,23], metabolism[24], nervous system physiology[25,26], and genetic homology[27]. Their larger size minimizes the scaling effect that arises in rodents, wherein calculations need to be made to adjust for the size differences between humans and animals when translating findings[27]. Furthermore, pigs, like humans, exhibit highly individualized nocifensive responses[28,29], rendering them ideal candidates for neuropathic pain research.

C. Methods of Pain Assessment in Pigs

When assessing pain in humans, the gold standard of pain assessment is patient report[28,30]. Since this is not feasible in animals, alternative methods of pain assessment must be employed. These include nociceptive threshold testing, spontaneous behavioral scoring, and measurement of biomarkers.

Measurement of nociceptive threshold is a common method of pain assessment in animals[31]. This involves the application of a stimulus to a location on the body until a behavioral response is elicited. A response can consist of vocalization, limb withdrawal, muscle twitching, or other signs of discomfort in the animal. The point at which the animal responds is considered its pain threshold[31]. While the nociceptive threshold is a

measure of sensitivity, not pain, it still enables assessment of allodynia and hyperalgesia via response threshold to stimuli[28].

Mechanical allodynia testing in pigs can be conducted using von Frey filaments, wherein graded nylon filaments are applied to the skin until the point where they begin to bend[32,33]. This is repeated five times, and the number of responses is recorded. If there are fewer than three out of five responses, the next highest diameter of filament is used until at least three responses are noted. If the initial filament yields three or more responses, a lower diameter filament is used until the lowest filament at which at least three responses is observed is identified[33,34].

A pressure algometer can be used to assess mechanical hyperalgesia[31,35]. For this test, a probe is positioned at a peripheral angle to the limb of interest and applied with steadily increasing pressure. When a nocifensive response is noted, the probe is withdrawn and the pressure required to elicit the response is recorded. In the absence of a response, the probe is removed when a predetermined cutoff is reached to avoid tissue damage[31,35]. The procedure is repeated five times, and the mean pressure is considered the nociceptive threshold.

Determination of thermal hyperalgesia employs the application of heat to the testing site. This can be conducted by using a laser or other heat source and recording the temperature or amount of time to withdrawal[36–38]. As with mechanical hyperalgesia testing, the process is repeated five times and the mean time or temperature considered the threshold.

Another method of testing nociception in pigs is through the assessment of spontaneous behaviors[28]. A variety of scales have been developed for use in pig models

of pain, most notably for castration and tail docking, routine husbandry procedures[39]. These scales involve the grading presence and degree of normal and pain-related behaviors in pigs to determine pain severity and possible need for analgesia. These behaviors include body position, activity, pain-related behaviors, and interaction with other pigs[40].

Another form of spontaneous behavioral assessment is the facial grimace scale. Used in nonverbal humans and animals, this scale measures specific facial movements called facial action units (FAUs)[30]. FAUs in pigs can include expressions such as ear position, orbital tension, or snout bulge[30,41]. These are usually graded on in an ordinal manner using scores of 0-2 to represent "not present," "moderately present," and "obviously present" for each FAU[30]. Grimace scales in large animals have displayed interobserver reliability (ICC 0.41-8.0) and validity (68.2-80.0%)[30].

Assessment of pain-associated biomarkers can inform about the presence of pain. The presence of pain biomarkers can be measured in a wide variety of tissue, including blood, saliva, spinal cord, and dorsal root ganglion[42]. Dysregulations in pain-relevant biomarkers, such as calcitonin gene-related peptide (CGRP)[36,43], substance P[36,44], and brain-derived neurotrophic factor (BDNF)[20], have been identified in pig models of pain. Validation of biomarkers can be a useful tool for developing targeted treatments[42].

D. Spared Nerve Injury

The spared nerve injury (SNI) model is an animal model of neuropathic pain that involves the ligation of the common peroneal and tibial branches of the sciatic nerve while keeping the sural branch intact[45–48]. The SNI leads to alterations in neuronal signaling, ectopic discharge, central and peripheral sensitization, and structural

reorganization[19,45]. The SNI is marked by rapidly developing (<24 hours) mechanical and thermal hypersensitivity to normally innocuous stimuli including von Frey filaments[45,47,48], heat, and cold testing[19,45] that persists for up to 6 months[45].

A pig model of common peroneal nerve injury displayed mechanical allodynia, thermal hyperalgesia, and increased spontaneous pain behavior, as well as demyelination of the ligated nerve[33], indicating their suitability for translational neuropathic pain experimentation. While this study showed promising results, testing was only conducted at a single timepoint seven days after injury induction[33]. Since neuropathic pain is often chronic in nature, it is advantageous to evaluate whether the pain in a SNI model persists for a longer period of time. Establishment of an SNI model in pigs could pave the way for future treatment studies.

1.2. Purpose of Study

Neuropathic pain is a significant problem in healthcare, necessitating research into its causes and potential treatments. Creation of accurate animal models enables thorough investigation of pathophysiological mechanisms and testing of therapeutics. Pigs are ideal candidates for translational research because of their physiological and genetic overlaps with humans.

The purpose of this work is to discuss the feasibility and preliminary effects of an SNI model of neuropathic pain pigs. Feasibility is operationally defined as the practicality of conducting the SNI procedure and carrying out the nocifiensive and transcriptomic testing. A pig model of spared nerve injury can be an asset to pain research by providing translational model for the future testing of treatments for neuropathic pain.

The specific aims of this dissertation research were:

1. To determine feasibility of an SNI model in pigs, including conducting SNI and sham surgery, conducting nocifensive testing, and collecting data.
    a. Feasibility of an SNI model will be demonstrated through ability to:
        a. Maintain health of pigs as demonstrated by a retention of at least 90% of the animals for the duration of the study
        b. Successfully conduct the SNI and sham procedures with no morbidity or mortality directly related to the surgery
        c. Conduct the nocifensive behavior testing with no more than 10% missing data for any of the tests
2. Determine preliminary effects of SNI model in pigs on nocifensvie behavior.
    a. Hypothesis: SNI procedure will increase nocifensive behavior compared to sham surgery.
3. To examine blood and spinal dorsal horn tissue for activation or repression of pain-associated biomarkers and biological signaling pathways that are linked to spared nerve injury pain.
    a. Hypothesis: Spared nerve injury in pigs will affect the biomarkers and biological signaling pathways.

1.3. Theoretical Framework

The conceptual model used as the basis for this research design (Figure 1) is adapted from the pharmacokinetic/pharmacodynamic (PK/PD) model, which stresses the importance of use of accurate indicators to enable identification of surrogate endpoints in pre-clinical trials and drug development[49]. Here, a surrogate endpoint is defined as a

marker that is used to substitute for a clinical endpoint[50]. This is of particular importance when clinical endpoints cannot ethically or feasibly be obtained[49]. In this case, the primary problem of interest is neuropathic pain. The gold standard for pain assessment is patient report, which cannot be measured in pigs[28]. Thus, limb withdrawal threshold and facial grimace in response to a noxious stimulus, as well as identifiable transcriptomic biomarkers that differ between animals with or without pain will be used as surrogate endpoints to evaluate model efficacy. These surrogates are evaluated as support for the validity of and translational ability of pig models and can be used to inform clinical endpoints such as pain assessment techniques and possible targets for treatments.

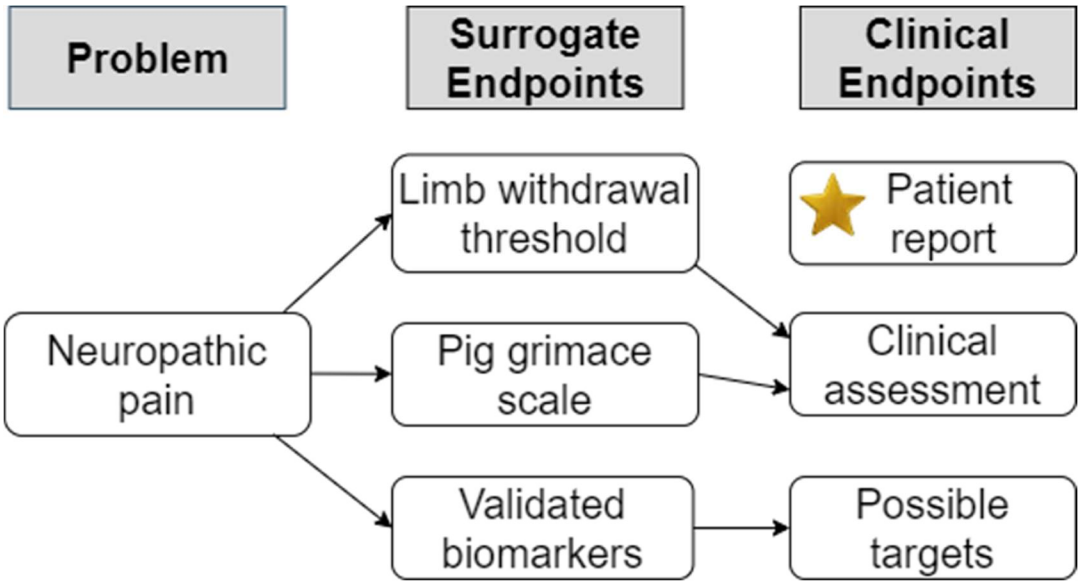


Figure 1: The theoretical framework for a pig SNI model

CHAPTER II. LITERATURE REVIEW OF PAIN ASSESSMENT IN PIGS

Assessing pain in nonverbal animals, such as pigs, is challenging in both research and agricultural settings. There are many surrogate endpoints for measuring the presence and degree of pain in pigs. This chapter reviews the current literature on pain assessment methods in pigs with a focus on studies that combine nocifensive behavior testing with tissue-based pain-associated biomarker analysis.

A REVIEW OF NOCIFENSIVE AND BIOMARKER-BASED PAIN ASSESSMENT METHODS IN PIGS

ABSTRACT

Background: Patient report is the gold standard for pain assessment in humans. Because pigs cannot communicate pain verbally, their pain cannot be measured directly. Therefore, it is necessary to identify surrogate methods of pain assessment that can be applied in both translational research and agricultural settings. These methods can include identification of nocifensive behaviors and pain-associated biomarker analysis.

Methods: A literature review was conducted to examine pig studies that assess both nocifensive behaviors or evoked pain-like responses and pain-associated biomarkers in translational models or agricultural-based research.

Results: Twenty-two studies combined both methods of assessment. Scoring spontaneous nocifensive behaviors was the most common method of measurement. However, there has been no standardized scale developed for this purpose. Evoked assessment of pain-like behaviors, such as von Frey or thermal testing were also used. Biomarker analysis

primarily focused on cortisol, but other pro-inflammatory and pain-associated markers were also assessed.

Conclusion: Measuring pain in pigs presents a challenge to researchers because the gold standard of self-report is not possible. A variety of surrogate methods have been used to assess the presence of nociception in pigs, but there is no standardized technique. Combining nocifensive behavioral analysis, evoked pain-like responses, and pain-associated biomarker measurement may be the most valid and reliable way to improve model accuracy and prevent suffering in pigs used for research and agriculture.

2.1 Introduction

Pain is defined as, “an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage”[51]. It is highly prevalent and the most common reason for seeking medical attention (Katz 2002). Pain can have a significant impact on quality of life and can lead to disability, inability to perform activities of daily living, and can affect mental and emotional health[52,53]. There is strong evidence to suggest that animals experience pain, as well[54]. Pain in animals is defined as “an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species-specific behaviour, including social behaviour”[55]. When animals are experiencing pain, they avoid the noxious stimulus and display protective behavioral changes to prevent further injury[56].

Because not all pain research can be ethically or feasibly conducted on humans, animal models are often used as surrogates for understanding pain and developing treatments. While rodent models have traditionally been used for pain research because of their low cost and convenience, their translational ability is limited by the differences in anatomy and physiology between rodents and humans[21]. Indeed, many drugs that have shown promise for pain reduction in mice have failed to translate to humans[21,57]. Conversely, pigs share myriad functional characteristics with humans, including genomic overlap[58], body and organ size[27], and similarities of the nervous[25], integumentary[22], metabolic, and cardiovascular systems[27]; Use of pig surgical models is positively correlated with translation to clinical trial studies[59]. A peroneal nerve injury model in pigs found that the responses of the animals to nocifensive testing was comparable to that of humans with

neuropathic pain[33]. Furthermore, the time course of postoperative spontaneous pain behaviors in pigs is similar to that seen in patients following surgery[60]. As such, reliable and valid measurements of pain in pigs are essential for furthering scientific understanding and treatment development.

In addition to their value as research models, pigs are abundant in the agricultural sector. There are 72.9 million pigs being raised on farms in the United States alone[61] and pork is the most popular meat product globally[62]. Despite this, the welfare of farm pigs is often neglected and painful husbandry procedures, such as castration and tail docking, are typically done without analgesia or anesthesia[63]. Male pigs are castrated to prevent boar taint, an unpleasant taste and odor in the meat, as well as to reduce aggression[64]. Tail docking is conducted to prevent pigs from biting the tails of littermates, which can cause infection[65]. Conducting these procedures without pain relief presents a welfare concern [66], as well as affecting growth and weight gain[67]. Furthermore, pigs given analgesia before painful procedures experience faster recovery[68]. Thus, for both ethical and economic reasons, pain should be controlled in farm pigs. This necessitates the accurate techniques for pig pain assessment.

The gold standard for pain assessment in humans is patient report. However, as it is not possible to obtain this measurement in pigs, surrogate endpoints must be used[28]. A surrogate endpoint is a biomarker, or an objective physiological, behavioral, or molecular indicator of health status[69], that is used in place of a clinical endpoint when that endpoint cannot be ethically or feasibly obtained[49].

Surrogate endpoints for behavioral pain assessment in pigs include quantitative sensory testing, spontaneous behavior measurement, physiological parameters, lameness

and gait assessment, and vocalization. These results of these assessments can be compared at baseline and after a painful stimulus, between pigs who received the painful stimulus and sham or control pigs, between pigs who did or did not receive analgesia, or by seeing if pain behaviors increase when the painful stimulus is more intense[28].

Another surrogate endpoint for pain assessment is the identification of pain-associated molecular biomarkers found in blood or tissue. Expression of these biomarkers in pigs can provide an indication of distress or discomfort. Furthermore, biomarkers can help inform about the translational ability of a model and provide targets for novel treatments. Biomarkers are a good way to infer pain in both translational and agricultural research and are important in improving animal welfare and developing new treatments.

The purpose of this scoping review is to analyze studies that assess both pain behaviors and pain-associated biomarkers in translational and agricultural research studies. The use of both methods of pain assessment provides stronger evidence to support conclusions about the existence of pain in the animals as well as the efficacy of the model or treatment, and how to likely minimize pain in pigs and other animals for ethical reasons.

2.2 Methods

A. Search Strategy

PubMed, Scopus, Embase were searched on March 22, 2023 for all relevant peer-reviewed papers from the past twenty years available in the databases. The search terms used included “pig,” “swine,” “porcine,” “pain,” “nociception,” “neuropathy,”

"neuropathic," "biomarker," "marker," "translational," "model". References of relevant studies and reviews were then manually checked to identify additional papers.

B. Eligibility Criteria

Studies included in this review were those that involved assessment of nociceptive behavior and pain-associated tissue-based molecular biomarker analysis in pigs experiencing pain. Studies could be translational research models of pain or agricultural studies in which pigs were undergoing a painful procedure. Reviews and conference abstracts were excluded, as were studies that did not assess both pain assessment and biomarker analysis.

C. Data Collection and Analysis

All search results were uploaded to Covidence, an online literature review tool that organizes, categorizes, and allows for efficient title/abstract and full-text screenings and data extraction[70]. Study titles and abstracts were screened, and relevant articles were read in full. Information regarding pain type, sample information, treatment (if applicable), nocifensive testing and behavioral analysis, tissue type used for biomarker analysis, biomarkers of interests, and findings were extracted.

2.3 Results

A. Search Results

The literature search identified 243 records. Following duplicate removal, 179 records remained. After title and abstract screening, the 72 remaining articles were examined for relevance. A total of 22 articles met the eligibility criteria and were included in the final review (Figure 1). There were sixteen relevant agricultural studies

and six translational model studies (Table 1). Agricultural studies focused on castration, tail docking, farrowing, lameness and prolapse, and vaccination. Translational model studies investigated thermal pain, peripheral neuritis trauma (PNT), neurofibromatosis type 1 (NF1), and postoperative pain.

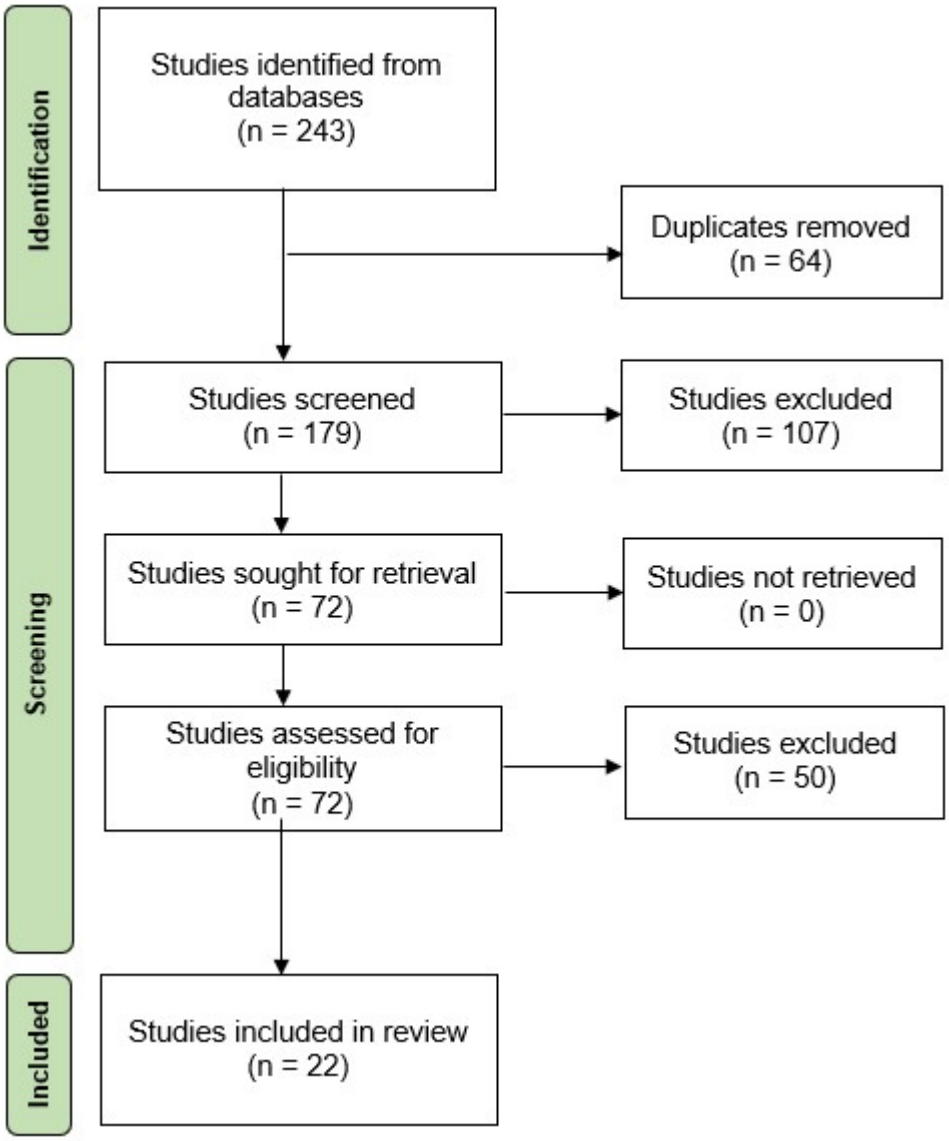


Figure 2: PRISMA diagram for the review of the literature

B. Spontaneous Pain Assessment

The most reliable method of measuring pain in pigs is through observation of spontaneous pain behaviors[28]. Out of the 22 studies reviewed here, only two did not use this assessment technique[36,71]. Despite its prevalence, there is no standardized tool for this purpose. Instead, a wide variety of scales and ethograms were used for spontaneous pain behavior assessment across studies, complicating the comparison and translation of findings.

These methods generally involved using an ethogram to measure specific behaviors associated with pain and discomfort[20,38,40,66,72–75]. Behaviors were often grouped into categories, such as pain-related, non-specific, body position, or social[20,38,40,73,76]. Pain-related behaviors included activities such as trembling, huddling, rump scratching, or tail movement[40,73,76]. Nonspecific behaviors focused on walking, sleeping, playing, or nursing[40,73,76]. Body position involved standing, sitting, kneeling, and lying laterally or ventrally[38,40,73,74,76]. Social behaviors related to amount and type of interaction with penmates[20,38,40,73,76]. The behaviors were measured with either a numerical scale where higher numbers indicated increased severity or frequency of the behavior or by recording the amount of time spent in each activity.

Two studies used wearable devices attached to the pigs' necks to measure sleep and activity[38,77]. However, Khanna et al. (2019) noted that some pigs would remove and destroy the devices[38]. In addition to the loss of study data and equipment, this also presents a potential hazard for the animals, so supervision and precautions should be put into place when using such devices.

While the majority of studies monitored pigs in their home pens, a number of agricultural studies measured behavior and vocalization during painful procedures, namely castration and tail docking[44,63,64,66,71,73,75,78,79]. Two studies used a decibel meter to gauge intensity of vocalizations during the procedures[63,73]. Hansson et al. (2011) also used a visual analog scale to score resistance movements during castration[73]. Similarly, Keita et al. (2010) measured castration pain with a global behavior score and a description of the type of vocalization[75]. Temple et al. (2020) scored the presence or absence of retreat attempts and high-pitched vocalizations during vaccination[79]. Recordings of vocalizations during castration were analyzed for high-frequency calls, which are associated with stress and pain, in several castration studies[44,64,66,71,78].

C. Evoked Pain Assessment

Several studies used measures of evoked pain, such as von Frey filaments to assess mechanical allodynia[20,21,38,43] or a laser to measure thermal hyperalgesia[36,38]. Use of a feather to assess tactile sensitivity was successful with 9-10-week-old pigs[20,21], but did not elicit a response from 7-9-month-old pigs[38], suggesting that the skin of older pigs is too thick to feel the sensation. Response to bright light and air puff tests did not differ between groups with or without a painful condition[38].

D. Biomarkers

Blood was most frequently collected for biomarker analysis. Several translational models extracted nervous system tissue, such as the dorsal root ganglion, spinal cord, and nerves. Other tissue collected included saliva, skin, interstitial fluid, and urine.

All studies with the exception of two[63,66] assessed more than one biomarker, with cortisol being the most popular. In most cases, it was measured in the blood, but two studies examined its concentration in saliva[72,74] and one in urine[40]. Elevated cortisol levels are associated with pain[40] and stress[72], but they can also be present in during periods of increased activity in pigs. Increased salivary cortisol was present in pregnant sows in the pre-farrowing period in conjunction with nest building and lactation onset[74]. Therefore, cortisol alone is not a specific and consistent biomarker for assessing the presence of pain[27].

A variety of pro-inflammatory biomarkers were investigated. These included C-reactive protein (CRP), which was measured in agricultural studies in relation to lameness and prolapse, farrowing, castration, and vaccination[44,72,74,76,79]. Other inflammatory biomarkers included TNF-α, IL-1β[20,74,76], and serum amyloid A[72,76,79].

Several studies measured biomarkers that have been more specifically and consistently associated with pain. For example, CGRP[20,21,36,43] is strongly implicated in pain behavior[20]. Three studies measured substance P, a neurotransmitter that has associated with post-surgical pain[36,43,44]. Castel et al. (2016) studied BDNF, which plays a vital role in in neuropathic pain[80].

E. Treatment and Techniques

Fourteen studies assessed nocifensive behavior and evoked pain-like responses following administration of treatments, three involved techniques to reduce pain, and five did not use any treatments or techniques. Of the latter, two were translational studies focused on model development: NF1[38] and PNT[21].

A variety of treatments were employed by studies, including opioids, gabapentin, aprepitant, resiniferatoxin (RTX), abobotulinumtoxinA (aboBoNT-A), and bupivacaine. Opioid treatments were associated with higher activity levels, appetite, and weight gain following abdominal surgery[81] and decreased sensitivity to stimuli and spontaneous pain behavior in pigs with PNT[20]. In the abdominal surgery model, blood cortisol levels were higher in pigs given placebo treatment as compared to those treated with opioids[81]. Lowered sensitivity to stimuli and pain behavior was similarly seen in PNT pigs treated with gabapentin, but not aprepitant[20]. CT-guided resiniferatoxin injections lowered sensitivity to thermal pain and decreased pain associated biomarkers transient receptor potential cation channel subfamily V member 1 (TRPV1), substance P, and CGRP in the dorsal root ganglion[36]. AboBoNT-A and bupivacaine reduced allodynia, but CGRP and substance P were unaltered[43].

Many agricultural studies focused on the use of analgesia for pain relief during routine castration and tail docking. Meloxicam was the most frequently used for this purpose, but lidocaine, flunixin, NSAIDs, and CO2 anesthesia were also investigated. During the painful procedures, there was no difference in behavior or vocalizations between pigs who did or did not receive meloxicam; however, with the exception of Reiner et al. (2012), they showed fewer pain behaviors following castration than untreated pigs. One study found that pigs treated with meloxicam had lower mortality levels after castration and tail docking than those given a placebo only[63], but another castration-only study did not identify any difference[71]. The effect of meloxicam on cortisol levels was inconclusive.

Ketoprofen was not found to affect pain behaviors or biomarkers in farrowing sows[74], but ketoprofen- and flunixin-treated pigs had higher levels of activity following castration than those given meloxicam or saline[77]. However, only flunixin-treated pigs had lower blood cortisol concentrations than those given saline[77].

Technique studies were exclusively agricultural in nature. One examined the effects of a local anesthetic delivered to the scrotal sac via a needle versus a needle-free system that administered the drug via compressed gas. Pigs given the anesthetic with the needle-free system had decreased tail flicking and lower behavior scores during injection than those administered with a needle[78]. Similarly, piglets vaccinated with a needle-free intradermal Porcilis®PCV ID device had fewer high-pitched vocalizations and retreat attempts, as well as lower levels of blood C-reactive protein and haptoglobin, than those vaccinated with a needle[79].

2.4 Discussion

A. Findings

Pain is a multidimensional experience that involves physical, psychological, and emotional suffering[52,53]. While humans can verbally convey discomfort, animal pain must be evaluated through use of surrogate endpoints. These endpoints can be used to assess animal welfare during routine painful procedures and inform the development of translational models and treatments. Pig models are used to scientific understanding of pain conditions and pigs in agricultural settings are routinely subjected to painful procedures. Thus, it is imperative that validated methods of pain assessment in pigs be established.

The limitations of pain measurement in pigs necessitates a combination of methods for accurate pain assessment [28]. The correlation of behavioral assessment and physiological biomarkers helps with validation of findings. Many translational and agricultural studies have employed one or the other as a technique for pain assessment, but few have measured both behavioral assessment and physiological biomarkers in any given experiment.

B. Suggestions

Because pain in pigs cannot be measured directly, it is best to assess spontaneous, evoked, and physiological measurements when studying pain in pigs[27,28]. More agricultural than translational studies incorporated multiple techniques for nocifiensive testing. In order to improve the translational potential of pig studies, there is a need for incorporation of a combination of pain measurements.

Spontaneous behavior measurements are an optimal method of pain assessment. However, there is no standardized tool for assessing it. Instead, a wide variety of scales and ethograms were used for spontaneous pain behavior assessment across studies, complicating the comparison and translation of findings. The development of a standardized scale would be a valuable tool for both agricultural and translational research. An optimal scale would include categories that capture the full spectrum of pig behavior, such as pain-specific and nonspecific behaviors, body position, location, and interaction with other pigs. Forming a scientific consensus on the most valid and reliable of these tools should be prioritized for improvement of pig welfare and enhanced model translation.

Once possible method is the UNESP-Botucatu Pig Composite Acute Pain Scale (UPAPS), a well-validated and reliable tool that was developed to determine pain levels and the need for analgesia following castration[82]. It demonstrates intra- and interrater reliability (ICC≥0.57), predictive and concurrent criterion validity (Spearman correlation≥0.85), internal consistency (Cronbach's α>0.80), and specificity (>95%). Pig facial grimace scales have also been developed for use in castration and tail docking[41,83] and farrowing[84], and cryptorchidectomy[85]. These scales involve the use of FAUs, such as orbital tension, nose bulge, or ear position to assess the presence and level of pain in pigs[30]. These scales show high levels of interrater reliability (ICC=0.73-0.97) and are easy to implement[30]. Standardizing the use of one of these or another similar measure would improve interpretation of results in both translational and agricultural studies.

When using any ethogram to score pain behaviors, there is a risk of observer bias[27]. For this reason, it is important to establish inter- and intra-rater reliability. Inter-rater reliability can be measured by having more than one observer and comparing their scores. Intra-rater reliability can be measured by taking video footage of pain behaviors and having the same observer score them at different time points[86].

Farm pigs routinely undergo painful procedures without administration of pain relief. Based on the findings, there is strong evidence to suggest that the use of analgesia, such as meloxicam, gabapentin, and lidocaine, improves well-being and comfort of pigs undergoing routine painful husbandry procedures. Widespread adoption of analgesia should be implemented to improve pig welfare and outcomes.

Age is an important consideration when designing a study to assess pain in pigs. As pigs grow, their sensitivity to mechanical testing decreases[20], so unless there is a

specific need for older pigs, use of juvenile animals is optimal. Proper training and socialization of pigs is also necessary to incorporate into the study protocol. Pigs are prey animals[87], which means that they are naturally cautious around humans. They have a tendency react to stressful situations with vocalization and escape attempts[88]. Thus, they need to be carefully acclimated to study staff and environments prior to experimentation to avoid undue stress and associated behaviors that may be a confounding variable when interpreting results[88].

2.5 Conclusion

Pigs are widely used in both translational studies of pain and in agricultural settings where they frequently experience pain for a variety of reasons. While there are many techniques for assessing pain-like behavior and biomarker expression in pigs, there is no gold standard measurement due to lack of self-report. Spontaneous pain behavior assessment is currently the most valid method, but standardization of a pig behavior scale would improve the field. Combining spontaneous and evoked behavioral assessments with biomarker analysis appears to be the optimal method for determining presence and degree of nociception in pigs. Although direct measurement of pain in pigs is not possible, valid and reliable pain surrogate measures are necessary to improve model accuracy in translational research and prevent unnecessary suffering in laboratory and agricultural settings.

CHAPTER III. METHODS

Translational animal models are necessary for understanding and developing treatments for neuropathic pain. Because of their anatomical and physiological similarities to humans, pigs are optimal for this purpose. The SNI procedure induces neuropathic pain-like symptoms in animals, including mechanical allodynia and thermal hyperalgesia. Determining the feasibility and efficacy of a pig model of SNI can be a valuable asset for future translational research.

DEVELOPMENT OF A PIG MODEL OF SPARED NERVE INJURY

ABSTRACT

Neuropathic pain is a condition resulting from damage to the somatosensory nervous system. It causes considerable suffering and has a major impact on quality of life. Animal models are necessary to understand the mechanisms that cause the condition and to develop and test novel treatments. The spared nerve injury (SNI) model is a well-validated neuropathic pain model that involves the surgical ligation of the common peroneal and tibial branches of the sciatic nerve, while sparing the third branch, the sural nerve. While the SNI was originally developed in rats, rodents have translational limitations because of their physiological differences from humans. Pigs share many characteristics with humans, making them uniquely suitable for modeling painful conditions. In this study, we implemented a variation of the SNI model in pigs by ligating the common peroneal nerve. We then tested for the presence of mechanical and dynamic allodynia, thermal hyperalgesia, and gait alterations over the course of four weeks. At the conclusion of the study, blood and nervous system tissue was harvested and sequenced to determine the presence of differentially expressed genes and associated enriched

pathways. The purpose of this study was to assess the feasibility and efficacy of a pig model of SNI for future translational research.

3.1 Introduction

Neuropathic pain is defined as "pain caused by a lesion or disease of the somatosensory nervous system" [1]. Unlike nociceptive pain, which serves a protective role by providing feedback in the presence of an actual or potentially damaging stimulus, neuropathic pain is a pathological and often chronic pain disorder[2,11]. It can arise from various conditions, such as nerve injury, diabetic peripheral neuropathy, or HIV[13]. Patients with neuropathic pain complain of burning, shooting, pricking, and electric shock-like pain[2,7]. These symptoms affect quality of life, such as sleep, metal health, and ability to work and perform activities of daily living[7,9]. Pharmacological treatments such as tricyclic antidepressants, anticonvulsants, non-steroidal anti-inflammatory drugs, and opioids have limited efficacy[10,89].

Improving scientific understanding of neuropathic pain, identifying therapeutic targets, and testing novel treatments necessitates accurate animal models. Mice and rats have traditionally been used for pain research, but their translational ability is limited by the considerable physiological differences between rodents and humans[20,21]. One promising alternative to rodents is the use of pig models. Pigs and humans share metabolic[24], genomic[27,58], integumentary[22], and nervous system[25,90] characteristics. A surgical pig model exhibited spontaneous pain behavior in a manner consistent with postoperative patients[60], indicating suitability of pigs as translational pain models.

One well-validated method of inducing neuropathic pain in animal models is the spared nerve injury (SNI). Originally developed in rats, the SNI is a partial nerve injury that involves the ligation of two of the three branches of the sciatic nerve, the tibial and common peroneal, while leaving the third, the sural, unaffected[45,91]. The SNI leads to the

development of robust mechanical allodynia and thermal hyperalgesia within 24 hours of injury that persists for up to six months[45]. Because it approximates the symptoms reported by patients with neuropathy[91], the SNI model is widely used in neuropathic pain research.

Development of a pig model of SNI has the potential to improve understanding and treatment of neuropathic pain. While a pig model of common peroneal nerve injury has been developed, mechanical and thermal tolerance thresholds was only tested at a single time point seven days after injury induction[33]. Because neuropathic pain is often chronic, there is a need for a study that looks at development of neuropathic pain in a pig model over a longer period of time. In this study, we assessed the feasibility and efficacy of a pig SNI model, as well as the resulting alterations in pain-associated biomarker expression.

3.2 Model Development

A. Power Analysis

An a priori power analysis was conducted to determine the number of animals needed based on repeated measures ANOVA using G*Power. Assuming a medium effect size of f=0.25, a two-sided α of 0.05, a power of 0.8, two groups, and five measurements over time, the sample size needed was 22. Accounting for the possibility of 10% attrition and unusable data, a total of n=24 animals (n=12 male, n=12 female) were obtained for this study.

B. Animal Subjects

Sinclair miniature swine (Sinclair Bio Resources, Auxvasse; MO) were singly housed in a temperature- and humidity-controlled environment with a 12-hour light:dark cycle. They were allowed access to water *ad libitum* and were given a measured volume of high protein swine chow (S-9 Miniature Swine Feed; Sinclair Bio Resources) twice daily. All animal care and procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore and conducted in coordination with university's the Veterinary Resources department.

C. Habituation and Training

Pigs were allowed to habituate to the facility for one week after arrival. This was followed by a two-week habituation and training process during which pigs were introduced to the room and study equipment. Pigs were acclimated to the restraint sling used for pain assessment by placing them in it for increments of time increased by five mintes every day until they were able to remain restrained for thirty minutes without struggling. They were also trained to walk in a straight line down the center of the gait sensor mat (GAITRite; Franklin, NJ).

D. Spared Nerve Injury Surgery

Pigs were randomized to either SNI or sham groups. Those in the SNI group underwent surgical ligation of the common peroneal nerve in their left leg, while the sham group had a left leg incision only with no ligation. The common peroneal nerve was selected for its role in pain sensation[92]. The right leg of both groups was unaffected.

Prior to surgery, buprenorphine (0.01-0.05 mg/kg) was administered subcutaneously for pain relief. Animals were sedated with Telazol (4-5 mg/kg)/Xylazine

(1.8-2.2 mg/kg)/Midazolam (0.2-0.4 mg/kg) prior to intubation. Maintenance anesthesia consisted of isoflurane 1-3% in 100% O_2. Blood samples were drawn via venous catheter. After a local subcutaneous injection of bupivacaine (0.25-0.5%, ≤6 mg/kg), a 4 cm incision was made in the thigh proximal to the knee to expose the common peroneal nerve. For the pigs in the spared nerve injury group, the nerve was then ligated with two sutures placed 1-2 cm apart near the patella. For pigs in the sham group, the incision was made to expose the nerve, but no sutures were placed. The fascia and tissues were then repaired with sutures and skin was stitched closed. The wound area was cleaned and dressed with triple antibiotic ointment and covered by Tegaderm.

Carprofen (2-4 mg/kg) was administered subcutaneously at surgical recovery and then every day for 4 days. Buprenorphine (0.01-0.05 mg/kg) was administered subcutaneously twice daily for pain relief for four days after surgery. Wounds were assessed daily for signs of infection or complications. Dressing was changed at 72 hours after surgery and then after each daily wound check until the incision sutures were removed at seven days post-surgery.

3.3 Nocifensive Testing

A. Overview

Baseline nocifensive behavioral assessment testing was conducted one week before surgery. Beginning one week after surgery, pigs were tested once a week for four weeks. Testing was conducted in two locations on each hind leg: the dorsal surface just above the hoof (medial) and the external side of the leg midway between the hoof and knee (lateral).

B. von Frey Testing

Mechanical allodynia was assessed with a series of calibrated von Frey filaments (Touch Test Sensory Kit, North Coast Medical; Morgan Hill, CA) with bending forces ranging from 60g to 300g. The filaments were applied perpendicularly to the leg, depressed slowly until they bent, and held in place for five seconds. Each von Frey filament was applied five times at intervals of three seconds. The mechanical threshold was determined by the smallest filament that produced three or more responses (i.e. leg withdrawals or kicking) out of five trials and the gram force of the filament was recorded. The average filament intensity per location was calculated as the outcome measure for each testing location.

C. Thermal Testing

Mechanical hyperalgesia was assessed using a pressure algometer designed for large mammals (Bio-Vetalgo; Bioseb; Pinellas Park, FL). The algometer was applied with steadily increasing pressure over the course of ten seconds. When a response was elicited, or, in the absence of a reaction, when the pressure reached the 5000g cutoff, the pressure was immediately removed. The gram force at the time of the reaction or the max cutoff was recorded. Each location was tested five times and the average gram force per location was calculated as the outcome measure for each testing location.

Heat hyperalgesia and cold allodynia were assessed using a thermode (Pathway, Medoc, Advanced Medical Systems; Ramat Yishai, Israel) connected to a computer that allowed a gradual change in temperature. For heat hyperalgesia testing, the baseline starting temperature was 32°C with a gradual ramp of the temperature increasing by 1°C

per second until it reached the cutoff of 50°C or until a reaction was noted. The temperature at the time of the behavioral response was recorded. The heat stimulus was repeated for three trials with a one-minute break between trials. The average scores of the three trials were calculated as the outcome measure for each testing location.

For cold allodynia testing, the baseline starting temperature was 32°C with a gradual decrease in the temperature by 1°C per second until it reached the cutoff of 0°C or until a reaction was noted. The temperature at the time of the behavioral response was recorded. The cold stimulus was repeated for three trials with a one-minute break between trials. The average scores of the three trials were calculated as the outcome measure for each testing location.

D. Dynamic Allodynia

Dynamic allodynia was assessed by stroking the leg with a paintbrush. This was repeated five times and the number of responses was calculated as the outcome measure for each testing location.

E. Pig Grimace Scale

During nocifensive testing, the pigs were placed in slings facing a digital video camera (Digital USB 3.1 CMOS Camera; Stoelting; Wood Dale, IL) to record facial expression and body movement. Still frames from the video footage were used to develop a Pig Grimace Scale for adult pigs based on ones designed for piglets[41,83]. Images were scored by two blinded observers based on two FAUs: ear position and snout tension. Ear position was graded as relaxed (0) or folded back (1). Snout tension was graded as

absence of tension (0) or presence of tension (1). The scores were compared to assess the inter-rater reliability of the scale.

F. Gait Analysis

Gait was assessed by having the pigs walk in a straight line along a 13-foot sensor mat (GAITRite; Franklin, NJ). Pigs repeated the walk until there were three straight, uninterrupted walks. The GAITRite software was used to analyze the total pressure index (TPI) for the hind legs. TPI is a measure of the percentage of the animals' weight carried by each limb. The average TPI scores of the three walks were calculated as the outcome measure for each hind leg.

G. Statistical Methods

Statistical analysis was conducted using IBM SPSS Statistics (IBM; Armonk, NY). Descriptive statistics were computed to characterize the data. Assumptions (e.g., normality) were checked for each outcome variable. The outcome variables that had majority ceiling or flooring effects (>50%) were dichotomized into "met or exceeds threshold" or "does not meet or exceed threshold." Linear or generalized linear mixed models were used to compare changes in all behavioral test measurements along time by experimental group. In addition, potential differences by sex and sides of the above effects were also explored by including three-way interaction and corresponding main effects and two-way interactions in the model. The final parsimonious model was achieved for each outcome.

3.4 RNA-Sequencing and Differential Gene Expression

Blood samples were collected immediately prior to SNI surgery and at the conclusion of the four weeks of testing. At the conclusion of the four weeks of testing, the pigs were euthanized and the spinal dorsal horn was harvested and preserved in Trizol at -80°C. Tissue was sent to University of Maryland School of Medicine Institute for Genome Sciences for RNA-sequencing analysis.

Using the classic alignment method, sequences were aligned to the Sus_scrofa.Sscrofa11.1.dna.fa reference genome using Spliced Transcripts Alignment to a Reference (STAR), an alignment software[93]. Gene counts estimates were conducted via HT-seq using Sus_scrofa.Sscrofa11.1.108.gtf. Gene counts were normalized to perform quality control (QC) checks at sample-level and gene-level, and then analyzed for differential expression across sham versus SNI and right versus left tissue using Limma-Voom[94].

Genes with a $p<0.05$ were considered as significantly differentially expressed genes (DEGs). Pathway analyses of significantly expressed DEGs were conducted using Metascape Gene Ontology. Because the Metascape platform does not include specific genomic information for sus scrofa, homo sapiens were used as the reference genome for the unbiased pathway analyses[95]. The genomes of homo sapiens and sus scrofa are 84.1% matched[58]. For tissue comparisons with enriched pathways relevant to pain, Cytoscape was used to create a network of enriched terms, each represented by a colored node that identifies its cluster ID[96].

3.5 Conclusion

Neuropathic pain is a prevalent and significant health issue that causes considerable suffering and decreases quality of life. The purpose of this study was to develop and test a novel pig model of spared nerve injury. After surgically inducing an injury in the common peroneal nerve, nocifensive testing was conducted to assess pain-associated behaviors. Then, blood and nervous system tissue was analyzed using RNA-sequencing to determine differential gene expression in injured pigs. This study has potential to validate a novel translational model for use in neuropathic pain research.

CHAPTER IV: RESULTS AND DISCUSSION

This chapter discusses the results and implications of the SNI pig model. It examines the findings, feasibility, and efficacy of the surgical procedure and the nocifensive and transcriptomic testing.

FEASIBLITY AND EFFICACY OF A SPARED NERVE INJURY MODEL IN PIGS

ABSTRACT

The purpose of this study was to examine the feasibility and efficacy of a spared nerve injury (SNI) model in pigs. Surgical ligation of the common peroneal nerve was feasible and safe. However, there were significant floor and ceiling effects for von Frey, thermal, and dynamic allodynia testing that prevented the determination of significant differences between the sham and SNI pigs. Females had an overall lower tolerance threshold than males across all time points at the lateral left ($p=0.041$) and lateral right ($p=0.007$) locations. This interaction was also significant over time, with females showing lower pressure tolerance than males at the medial left ($p=0.003$), medial right ($p=0.003$), lateral left ($p=0.041$), and lateral right ($p=0.003$) testing locations. The left side had an overall lower threshold tolerance than the right side for both medial ($p<0.001$) and lateral ($p=0.03$) locations, although this difference was not significant over time. There was no significant difference in tolerance between the sham and SNI groups. The grimace scale showed substantial interrater reliability for ear position (kappa=0.73) and moderate interrater reliability for snout tension (kappa=0.43). Several pathways of interest related to inflammation and wound healing were differentially expressed in blood and nervous system tissue. While pigs show promise as translational neuropathic pain models, special considerations are needed when conducting this research.

4.1 Background

Translational models of neuropathic pain are necessary for understanding and treating the condition. The purpose of this study was to test a spared nerve injury (SNI) in pigs to assess the resulting nocifensive behavior and pain-associated biomarkers. To create this model, the common peroneal nerve in the pigs' left hind legs was surgically ligated and the behavior and biomarker expression was compared to sham pigs. The feasibility and efficacy of this model can inform future studies and contribute to the development of novel treatments that will improve patients' quality of life.

4.2 Findings

A. Animal Subjects

Although 24 pigs were obtained for the study, one died of preexisting complications following surgery. Therefore, a total of 23 pigs were included, with 12 males and 11 females. There were 11 pigs in the sham group and 12 in the SNI group. At baseline, the average age of the pigs was 6.87 months (SD=0.51), and the average weight was 16.48 kg (SD=1.84) (Table 1).

Variable	Mean	SD	Frequency	Percentage
Group				
Sham			11	47.8
SNI			12	52.2
Sex				
Male			12	52.2
Female			11	47.8
Age (months)	6.87	0.51		
Weight (kg)	16.48	1.84		

Table 1: Characteristics of animal subjects at baseline

B. Nocifensive Testing

There was a large ceiling effect for von Frey, with 75.7% of outcomes at or above the cutoff (skewness=-1.48, SD=93.06), and heat, with 68% (skewness=-3.54, SD=1.89). There was a large floor effect for cold (skewness=4.61, SD=3.04) and dynamic allodynia (skewness= 4.35; SD=0.70), with outcomes at or below cutoff at 83.7% and 90.1%, respectively. Only pressure had fewer than 50% of outcomes at or above cutoff, at 16.2% (skewness=-0.56; SD=994.94). Therefore, datapoints for all variables except pressure were dichotomized to "met or exceeds threshold" or "does not meet or exceed threshold."

The results of the linear mixed model showed that there was no significant difference between the SNI and sham group for von Frey, heat, cold, or dynamic allodynia tolerance thresholds. Following surgery, the right side had a significantly lower tolerance threshold than the left side at the lateral location ($p=0.011$), but there was no difference between the sham and SNI pigs.

Pressure tolerance threshold showed a significant difference between males and females over time at the medial left ($p=0.003$) (Figure 1a), medial right ($p=0.003$) (Figure 1b), lateral left ($p=0.041$) (Figure 1c), and lateral right ($p=0.003$) (Figure 1d) locations. Females had an overall lower tolerance threshold than males across all time points at the lateral left ($p=0.041$) and lateral right ($p=0.007$) locations. The left side had a significantly lower overall tolerance threshold than the right side at both the medial ($p < 0.001$) and lateral ($p=0.03$) locations. However, side by time interactions were not significant for medial (Figure 2a) or lateral locations (Figure 2b). There was no significant difference between the tolerance thresholds of the sham and SNI groups at any of the testing locations (Figure 3a-d).

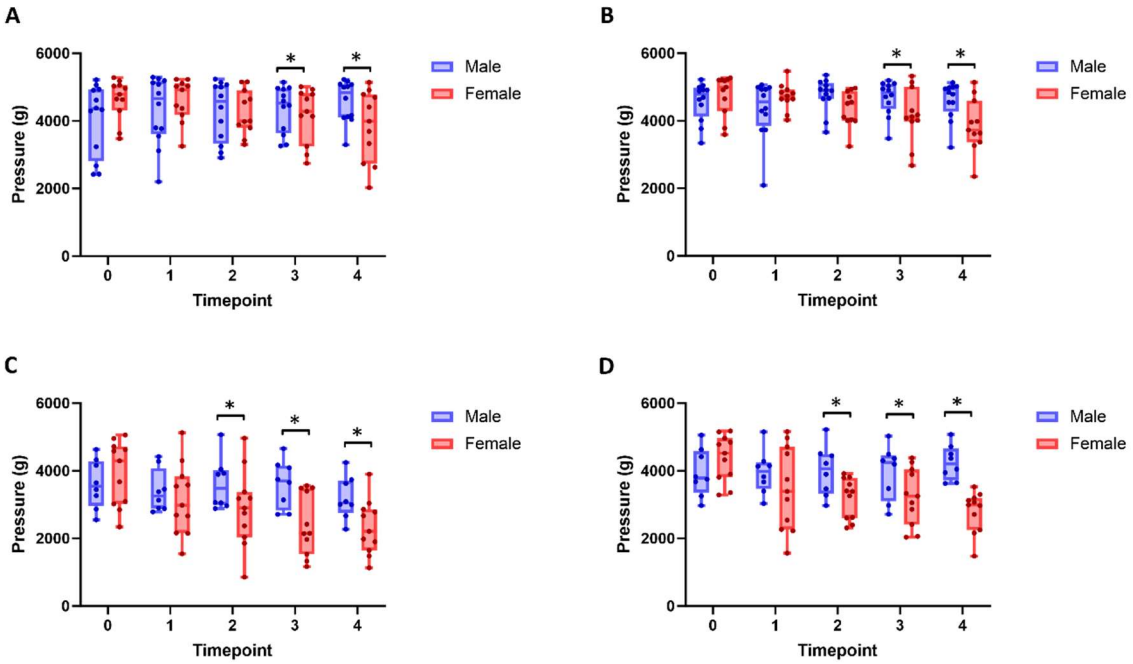


Figure 3: Time by sex interactions A. Time by sex interactions for medial left pressure testing location B. Time by sex interactions for medial right pressure testing location C. Time by sex interactions for lateral left pressure testing location D. Time by sex interactions for lateral right testing location

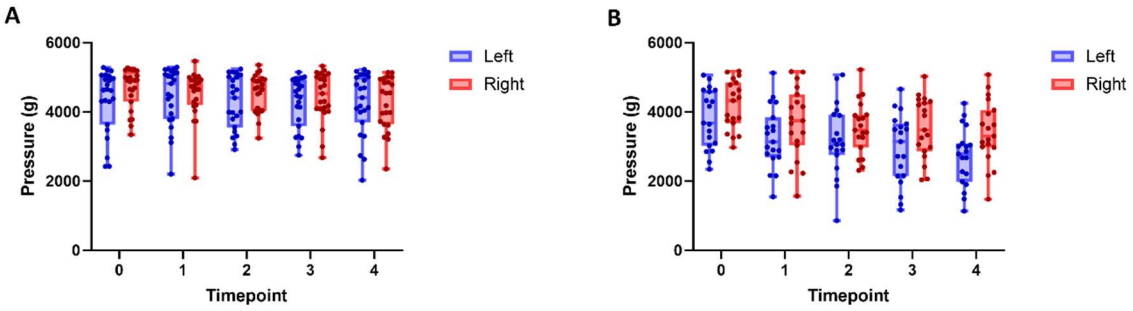


Figure 4: Time by side interactions A. Time by side interaction for pressure testing of medial locations B. Time by side interactions for pressure testing of lateral locations

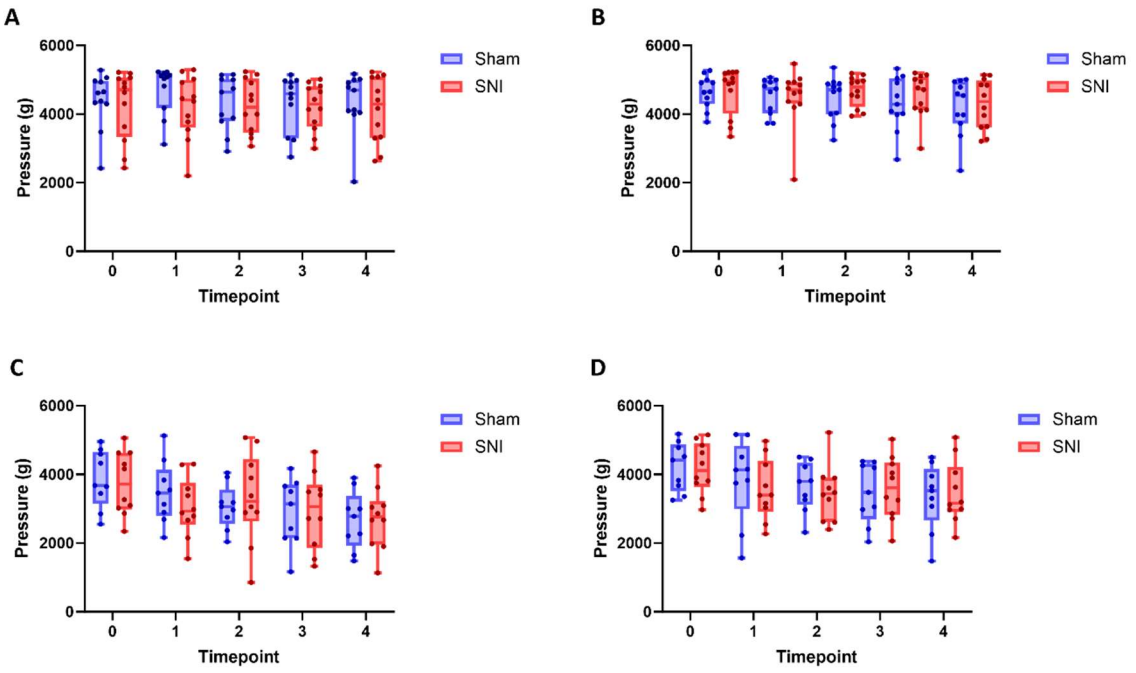


Figure 5: Time by group interactions A. Time by group interactions for medial left pressure testing location B. Time by group interactions for medial right pressure testing location C. Time by group interactions for lateral left pressure testing location D. Time by group interactions for lateral right testing location

C. Pig Grimace Scale

The Cohen's kappa for ear position was 0.73, indicating substantial interrater agreement[97]. When no painful stimulus was present, Observer 1 reported relaxed ears in 44% of the images, while Observer 2 reported relaxed ears in 56% of the images. When a stimulus was present, Observer 1 reported relaxed ears in 46% of the images, while Observer 2 reported relaxed ears in 38% of the images. The Cohen's kappa for snout tension was 0.43, indicating moderate interrater agreement[97]. When no painful stimulus was present, Observer 1 reported no snout tension in 46% of the images, while Observer 2 reported no snout tension in 30% of the images. When a stimulus was present, Observer 1 reported no snout tension in 61% of the images, while Observer 2 reported no snout tension in 46% of the images.

D. Gait Analysis

There were no significant differences between the right and left hind leg TPI for group, time, or side, or any two- or three-way interactions.

E. RNA-Sequencing and Pathway Analysis

Spinal dorsal horn tissue and blood were sequenced and assessed for differential gene expression between the SNI and sham groups and the left and right leg of the SNI group. The percentage of reads mapped to the reference genome was 88.29% for the spinal dorsal horn and 75.03% for the blood. Genes with a p-value of <0.05 were analyzed using a cluster analysis in Metascape (Figure 3).

There were 282 upregulated and 200 downregulated DEGs in the left spinal dorsal horn of the SNI pigs versus the sham pigs. Metascape analysis showed 56 enriched pathways for upregulated DEGs and 6 enriched pathways for downregulated DEGs. The left spinal dorsal horn of the SNI pigs had 134 upregulated and 222 downregulated DEGs as compared to the right side. There were 13 enriched pathways for upregulated DEGs and 9 enriched pathways for downregulated DEGs. Blood drawn at the study endpoint had 47 upregulated and 31 downregulated DEGs in SNI versus sham pigs. There were 9 enriched pathways for upregulated DEGs and 3 enriched pathways for downregulated DEGs. As compared to blood drawn at baseline, blood from the SNI pigs at the study endpoint had 6 upregulated and 29 downregulated DEGs. Because of the low number of DEGs, pathway analysis was not done for this comparison.

In the spinal dorsal horn tissue of SNI versus sham pigs, pathways related to regulation of inflammatory response, response to wounding, and wound healing were

upregulated (Figure 3a). The left spinal dorsal horn had enriched pathways related to positive regulation of inflammatory response and high-affinity igE receptor (FCERI)-mediated nuclear factor kappa-light-chain-enhancer B (NF-kB) activation versus the right side (Figure 3b). In blood collected at the study endpoint, the sensory perception of mechanical stimulus pathway was enriched (Figure 3c).

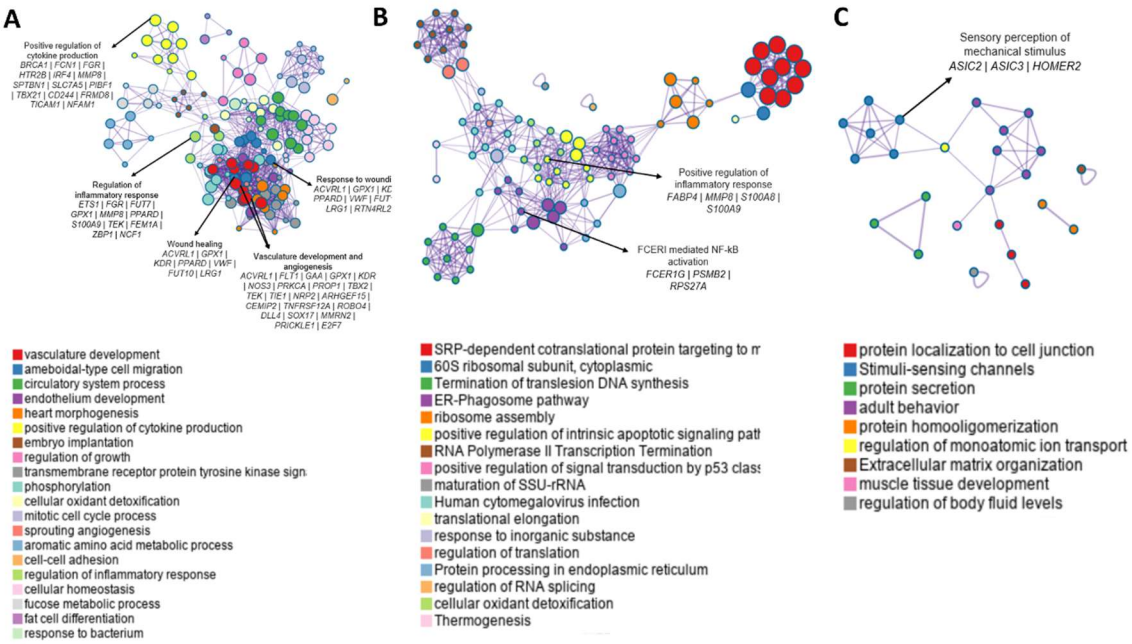


Figure 6: Metascape pathway analyses of DEGs A. Pathway network analysis for SNI left versus sham left spinal dorsal horn B. Pathway network analysis for SNI left versus right spinal dorsal horn C. Pathway network analysis for SNI versus sham blood

Only one gene, Zinc Finger MYND-Type Containing 12 (ZMYND12) had an adjusted p-value of <0.05 (p-adjusted=0.03). ZMYND12 was downregulated in the left spinal dorsal horn of the SNI pigs versus the sham pigs (logFC=-1.24).

4.3 Discussion

A. Study Feasibility

All study procedures were feasible and could be carried out without risks to the health and safety of the pigs or the study staff. There were no complications or infections

from the surgery and no mortality outside of preexisting conditions. While pigs could be successfully trained to stay in the sling and walk along the gait mat, they became agitated and irritable when hungry. For this reason, it was important that pigs be fed before any experiments were conducted.

B. Nocifensive Testing

The lack of significance for von Frey, heat, cold, and dynamic allodynia testing may be attributable to the floor and ceiling effects. In most cases, the pigs tolerated the maximum testing threshold without exhibiting any reaction. Thus, any changes in sensitivity between sham and SNI pigs were likely masked.

Von Frey testing has been successfully conducted in previous pig studies using filament ranges from 1g to 60g[43,60]. However, the pigs in these studies ranged in age from seven to ten weeks, while our pigs were five to seven months old. Since mechanical tolerance threshold increases with age and corresponding weight gain[20], it is possible that the ceiling effect can be attributed to the age of the pigs used in our study. Di Giminiani et al. used an electronic von Frey device with a maximum cutoff of 1,500g[98], which may be more suitable for older pigs.

The results for heat, cold, and dynamic allodynia tolerance testing may be similarly influenced by age. In a study by Hellman et al., heat up to 50°C was applied to seven- to eight-week-old pigs with a common peroneal nerve injury. They found that there was a significant decrease in temperature tolerance following injury induction[33]. Khanna et al. used a pigeon feather to assess dynamic allodynia in a seven- to nine-month-old pig model of NF1. They found that the pigs did not respond to the testing,

likely because their skin was too thick. However, they were able to successfully conduct von Frey and thermal testing on the inner part of the ear[38]. In contrast to the leg skin, the ear skin of the pig is close in thickness to human skin and is thus better suited for pain assessments[99].

The pressure data yielded more significant results, but there was no difference between the sham and SNI groups. Overall, the left hind leg, which received the surgery, was more sensitive than the right leg, which was unaffected, for both the medial and lateral testing sites. This suggests that the pigs were still experiencing acute pain from the surgical incision. A longer study timeline may have been advantageous in distinguishing acute postoperative pain from chronic neuropathic pain.

The overall tolerance threshold for the females decreased over time, likewise regardless of group. It is possible that this is not a result of sensitivity, but rather that the females learned that they could stop the testing early by withdrawing as soon as they felt the stimulus. To avoid this possibility, future studies should incorporate false pressure tests that reach the maximum threshold regardless of the pigs' reactions.

C. Pig Grimace Scale

The grimace scale results indicate that blinded observers were able to identify ear position with substantial agreement and snout tension with moderate agreement. However, this does not necessarily translate directly into identification of painful expression. While calm pigs theoretically have relaxed ears and no snout tension, pigs in states of both agitation and pain tended to exhibit folded back ears and snout tension.

For the purposes of this study, agitation and pain were differentiated by the presence or absence of a stimulus. Pigs were considered to be in pain if they were reacting to the application of a stimulus. Reactions included kicking, vocalization, limb withdrawal, or retreat attempts. Agitation was determined by the presence of these behaviors in the absence of a stimulus.

The grimace scale was not sensitive in that it was unable to reliably detect the presence painful expressions when a stimulus was present. There was little to no consistency between the presence of a stimulus and how the observers scored each FAU. It was also not specific, as it designated pained and agitated pigs as exhibiting these same FAU expressions. Thus, the scale, while demonstrating interrater reliability, does not show validity.

Because the sensitivity and specificity of the pig grimace scale likely depend on a variety of factors, such as the method of evoked nocifensive testing, the pain model being used, and the age and temperament of the pigs, further analyses will be necessary to assess the utility and generalizability of the pig grimace scale. This may involve calculating the percentage of times the pigs exhibit tension for each FAU during periods when the stimulus is being applied versus when the stimulus is absent.

D. Gait Analysis

No difference in TPI was detected between the sham and SNI group or right and left legs. This may be partially attributable to weight distribution. Pigs naturally place approximately 58% of their weight on their front hooves and 42% on their hind

hooves[100]. An injury to the front leg may have been more apparent in gait analysis, as pigs rely on these limbs more heavily.

E. RNA-Sequencing and Pathway Analysis

Metascape pathway analysis revealed several upregulated pathways in the left spinal dorsal horn of the SNI pigs versus the sham pigs. Positive regulation of cytokine production and regulation of inflammatory response are both involved in the inflammatory pain process[101]. Pathways related to wound healing and response to wounding were also significantly expressed. Moreover, pathways related to vasculature development and angiogenesis, important components of tissue repair [102], were upregulated, as well. The overexpression of the genes involved in these pathways may imply a higher degree of inflammation and response to injury in the SNI pigs.

Similarly, a comparison of the left versus right sides of the spinal dorsal horn in wounded pigs showed an upregulation of genes involved in positive regulation of inflammatory response. FCERI-mediated NF-kB activation, which is involved in release of inflammatory cytokines and development of neuropathic pain[103], is also upregulated. This suggests that, within the wounded animals, there was increased inflammation in the side with the nerve injury.

RNA-sequencing analysis revealed one gene, ZMYND12, that had a significant adjusted p-value and was downregulated in the left spinal dorsal horn. ZMYND12 is a paralog of Programmed Cell Death Protein 2-Like (PDCD2L), the upregulation of which suppresses NF-kB activation[104,105]. Similarly, ZYMND11 has been implicated in negative NF-kB expression[106]. The upregulation of NF-kB-related pathways and the decrease

ZMYND12 suggest NF-kB involvement in heightened inflammation and pain on the affected side.

Blood collected at the experimental endpoint showed enrichment of the sensory perception of mechanical stimulus and detection of external stimulus pathways in the SNI versus the sham samples. These pathways include genes involved in sensory perception of pain and temperature stimulus[107]. Upregulation of these pathways may imply an increased sensitivity to stimuli in the injured group.

F. Other considerations

Determining the presence of pain in pigs is difficult. As prey animals, pigs naturally conceal pain[108]. They are also easily stressed by handling and restraint[88] and may respond with agitated behavior, which is difficult to distinguish from pain. Furthermore, pigs are social and intelligent animals[88], so seeing or hearing another pig undergoing discomfort or pain can cause agitation, avoidance, or aggression in the observing animals. Therefore, it is important to conduct any potentially distressing procedures in a separate room from the other pigs. During periods when the pigs are not being used for experimental procedures, they should be housed in a manner that allows for visual, auditory, and olfactory interaction[88].

Possible side effects and adverse events should also be considered when conducing the SNI procedure. Animals may develop hypersensitivity in the unoperated, contralateral limb[109]. Sural nerve injury during surgery can cause paralysis of the affected leg[109]. Following surgery, pigs may be more aggressive. As such, they should not be housed together during the recovery period to avoid damaging the incision area[88].

4.4 Conclusion

Translational pig models are vital to understanding and treating neuropathic pain. The SNI is a robust model that produces mechanical allodynia and thermal hyperalgesia, simulating the symptoms experienced by patients with neuropathic pain. SNI was originally developed in rodents, but pigs are a more suitable model for translation to human patients. Therefore, this study focused on testing the feasibility and efficacy of an SNI model in pigs. While the model was feasible, the results indicate that conducting effective pain research in pigs requires special considerations. Findings from this study can be used to develop future valid and reliable translational models of neuropathic pain in pigs.

CHAPTER V: CONCLUSIONS AND IMPLICATIONS

5.1 Problem Overview

Neuropathic pain has a serious impact on quality of life and is estimated to affect up to 17% of adults worldwide[3]. It can result from a variety of conditions that cause damage to the somatosensory nervous system, such as diabetes, HIV, or trauma to the nervous system[3]. Patients report symptoms of burning, shooting, and shock-like pain that have a significant impact on their quality of life[2]. To compound the issue, neuropathic pain is often refractory to traditional pain treatments[10].

Understanding the mechanisms and developing treatments for neuropathic pain requires the use of accurate animal models. While rodents have traditionally been used for this purpose, their translational ability is limited and results often fail to translate to human clinical trials[17,20]. Thus, a more suitable animal model is needed. Pigs share many anatomical and physiological similarities with humans[27] and their use as models is associated with translation to clinical trials[59].

Despite these advantages, measuring pain in pigs requires special considerations. The gold standard for pain assessment is verbal report, which is not possible in pigs[28]. Therefore, surrogate measures, such as nocifensive behavior and expression of pain-associated biomarkers, must be used to validate models and treatments.

5.2 Study Summary

A surgical ligation of the common peroneal was performed on the left hind leg of 12 juvenile pigs. These SNI pigs were compared to 11 sham pigs who had nerve exposure surgery at the same location, but no ligation was made. Baseline nocifensive testing was conducted prior to injury and for four consecutive weeks beginning one week after

surgery. Nocifensive testing consisted of von Frey, pressure, heat, cold, and dynamic allodynia testing. Testing was conducted on both legs the medial surface above the hoof and the lateral surface below the incision point. All stimuli were applied until a nocifensive reaction, such as kicking or withdrawing the limb, was evoked, or until a predetermined cutoff was reached. A video camera was used to record facial expression during testing. Gait analysis was conducted by having the pigs walk down a walkway with pressure sensors to detect the percentage of pressure placed on each limb.

At the end of the testing period, the animals were euthanized and tissue from the spinal dorsal horn was harvested. Blood was collected at baseline, prior to nerve injury surgery, and at the study endpoint, immediately prior to euthanization. Blood and tissue were sequenced and analyzed using Metascape software.

There were no significant differences between the tolerance thresholds of the sham and SNI pigs for von Frey, heat, cold, or dynamic allodynia testing. Pressure testing showed a significant difference between males and females, with the tolerance threshold of females decreasing over time for all testing locations (medial left: $p=0.003$; medial right: $p=0.003$; lateral left: $p=0.041$; lateral right: $p=0.003$). Overall, the females had a significantly lower tolerance to pressure at the lateral testing points (left: $p=0.041$; right: $p=0.007$). The left side had a consistently lower tolerance to pressure stimulation than the right side for both the medial ($p < 0.001$) and lateral ($p = 0.03$) testing locations, but there was no change over time. There was no significant difference between pressure tolerance for the sham and SNI groups. Gait analysis did not reveal any significant differences between side, group, or time interactions.

Still images from the testing footage were rated by blinded observers for creation of a Pig Grimace Scale. Images were scored for ear position and snout tension. Ear position showed substantial interrater agreement (kappa=0.73) [97] and snout tension showed moderate interrater agreement (kappa=0.43) [97]. However, because the folded back ear position and the presence of snout tension occur both when the pigs are agitated, as well as when they are experiencing painful sensations, the scale cannot be validated for detection of nocifensive behavior.

RNA-sequencing analysis detected the expression of several genes associated with pathways related to inflammation and wound healing. These pathways were upregulated in SNI versus sham blood and spinal dorsal horn tissue, as well as spinal dorsal horn tissue on the left versus the right side of the SNI pigs.

5.3 Strengths and Limitations

The SNI is a partial denervation model that exhibits mechanical allodynia and thermal hyperalgesia[46,91], key symptoms reported by patients with neuropathic pain[3,110]. The SNI was originally developed in rats[45] and, while rodent species remain popular models for preclinical research, their translational ability has been called into question. There is a push for use of large animal models that more accurately represent human conditions[111]. This study uses pigs, which have many similarities to humans[27], to test a model of SNI.

Despite these advantages, pigs cannot covey pain verbally. This means that pain can only be assessed through surrogate measures, which limits translational ability. Furthermore, the pigs were frequently agitated during testing, making it difficult to tell the difference between nocifensive behavior and escape attempts.

This study was further limited by the lack of a naïve group. Ideally, the sham and SNI groups would be compared to a group that did not receive any surgery. This would address the possibility that the nocifensive testing was measuring acute pain from the surgical incision, rather than neuropathic pain from the nerve injury.

An additional limitation was the testing order. The right medial and lateral locations were always tested before the corresponding left locations. Time by side comparisons for pressure showed that the right-sided locations had higher tolerance thresholds than the left-sided ones. It is possible that the pigs became increasingly sensitized to the stimulus as the testing progressed, causing the left side to appear more sensitive than the right. In the future, it would be advantageous to randomize the testing order of the sites.

RNA-sequencing analysis was conducted using the Metascape platform. Metascape does not have a reference genome for sus scrofa, so the DEG- were matched to homo sapiens[95]. While the genomes of homo sapiens and sus scrofa are closely matched[58], the pathways that were identified may not correlate directly between the species.

5.4 Implications for Future Research

Pigs are good candidates for translational research, although more testing is needed to validate models of neuropathic pain conditions. The spared nerve injury model is feasible in pigs, but conducting pain research in this species requires special considerations. In future studies, younger pigs should be used, since their comparatively thinner skin is more suitable for nocifensive testing. Animals should be allowed to acclimate to the environment and study equipment for several weeks prior to baseline

testing. In addition, care should be taken to calibrate the equipment appropriately to avoid floor and ceiling effects. Neuropathic pain research in pigs presents unique challenges, but also has great potential for translation.

APPENDIX A: Glossary

Biomarker – an objective, quantifiable indicator of health status that can be accurately and reliably measured

Nociception – the process by which the nervous system detects a noxious stimulus

Nocifensive behavior – the reaction of an organism in response to a noxious stimulus

Spared nerve injury – an animal model of neuropathic pain induced by ligation of the common peroneal and/or tibial branches of the sciatic nerve, while leaving the sural nerve intact

Surrogate endpoint – a biomarker used to substitute for a clinical endpoint when that clinical endpoint cannot be ethically or feasibly obtained

RNA-sequencing – a technique for identifying and quantifying the transcriptome of a tissue sample using next-generation sequencing

Transcriptome – all RNA transcripts present in a given cell

APPENDIX B: Literature Review Table

First Author (Year)	Type of Pain	Sample	Treatment	Behavior	Tissue Type	Biomarkers	Findings
Brown (2015)	TRPV1-mediated thermal pain transmis-sion	Female farm pigs (n=9)	Computed tomograp-hy (CT)-guided resiniferat-oxin (RTX) injections	Thermal pain withdrawal threshold, lameness, and gait	DRG, spinal cord dorsal horn	TRPV1, substance P, CGRP	Pigs who received CT-guided RTX injections in the lumbar DRGs had a dose-dependent increased withdrawal latency to thermal pain There were no changes in lameness or gait TRPV1 mRNA, TRPV1-positive cells, and substance P-expressing IR cell bodies were reduced in the treated DRG CGRP-IR cells in treated DRG and CGRP-IR terminals in treated L5 DH decreased
Castel (2016)	Periphe-ral neuritis trauma (PNT)	9–10-week-old male Large White x Landrace (n=30)	Group 1: Saline at day 28 and 48 hours later Group 2: Gabapentin (3 mg/kg IV at day 28 and 6 mg/kg IV 48 hours later) Group 3: Aprepitant (2 mg/kg IV at day 28) Group 4: Morphine (0.1 mg/kg IV at day 28 and 0.3 mg/kg IV 48 later)	von Frey mechanical sensitivity, feather stroke tactile sensitivity, composite behavior scale to assess spontaneo-us pain behavior (solitary and social), mobility	Spinal cord, sciatic nerve	CGRP, BDNF, TNF-α, IL-1β, C-X3-C motif chemokine receptor 1 (CX3CR1), β-actin	Gabapentin and morphine decreased sensitivity to stimulation and spontaneous pain behavior in pigs with PNT Spinal cord CX3CR1 and BDNF mRNA expression were upregulated on study days 10 and 28, respectively Sciatic nerve CGRP, BDNF, TNF-α, and IL-1β mRNA levels were increased at day 10 and BDNF levels remained elevated, although decreased from day 10, at day 28
Contre-ras-Aguilar (2019)	Lamene-ss and prolapse	2-3-month-old male Large	N/A	Perception of pain, distress, and discomfort	Saliva, blood	CRP, cortisol, sAA, TEA, BChE, lipase,	Saliva from pigs with lameness and prolapse with pain had increased cortisol,

		White pigs (n=66)		(PPD) assessment, lameness score		ADA1, ADA2, uric acid, TEAC, CUPRAC, FRAS, AOPP, H_2O_2	sAA, TEA, BChE, and ADA isoenzymes Saliva from prolapsed pigs with pain also had increased FRAS, AOPP, and H_2O_2 The PPD score was positively correlated with serum CRP and salivary cortisol, TEA, BChE, FRAS, AOPP, and ADA isoenzymes
Cornet (2022)	Post-operative pain	8-10-week-old male Danish Landrace x Large White pigs (n=30)	AbobotulinumtoxinA (aboBoNT-A) (100, 200, or 400 U/pig), vehicle (saline), or wound infiltration of extended-release bupivacaine	Mechanical allodynia (von Frey), distress behavior score (DBS), approaching test (latency to approach investigator), open field test	Skin, DRG, spinal cord	Cleaved synaptosomal-associated protein 25 kD (SNAP25), glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor protein-1(Iba1), CGRP, substance P (SP)	AboBoNT-A and bupivacaine reduced both allodynia, but only aboBoNT-A normalized distress and approaching test scores Cleaved SNAP25 was present in the ipsilateral dorsal horn of the spinal cord of aboBoNT-A pigs, but not the skin or DRG GFAP and Iba1 were reduced in the spinal cord of aboBoNT-A-treated pigs, but the levels of CGRP and SP were unaltered
Hansson (2011)	Castration	1-7-day-old male piglets (n=557)	Meloxicam (0.5 ml per testicle), lidocaine with epinephrine (ml)	During castration, a decibel meter was used to gauge vocal intensity and a visual analog scale was used for resistance movements; after castration, pain behaviors were scored with an ethogram	Blood	Serum Amyloid A (SAA)	Lidocaine-treated piglets had lower intensity vocalizations and less resistance movements during castration; meloxicam-treated piglets had less pain behaviors following castration; piglets treated with meloxicam and lidocaine + meloxicam had lower SAA concentration than controls and those treated with lidocaine only

Hay (2003)	Castrati-on	5-day-old male piglets (n=84)	N/A	Daytime scan sampling and nighttime continuous observation of pain-related, social, movement, nursing, and social behaviors for 5 days after castration	Urine	NE, EPI, cortisol, cortisone, creatine	Castrated piglets displayed behavioral changes suggesting the presence of pain for up to 4 days after the procedure as compared to sham and naïve piglets, but there was no significant difference in biomarker expression between conditions and growth performance was not affected
Ison (2018)	Farrowi-ng	Large White x Landrace sows (n=56)	Ketoprofen (3 mg/kg bodyweight)	Continuous video observation of posture and spontaneo-us pain behaviors during farrowing	Saliva, plasma	Cortisol, C-reactive protein (CRP), IL-1β, IL-6, TNF-α	There was no significant difference in pain behaviors, salivary cortisol, CRP, IL1β, IL6, or TNFα between treated and untreated farrowing pigs; sows had increased pain behavior and TNFα levels as compared to gilts, while gilts had increased CRP as compared to sows; salivary cortisol was higher in sows during farrowing and in gilts 3 days after farrowing
Keita (2010)	Castrati-on	<1 week old male piglets (n =180)	Meloxicam (0.4 mg/kg^{-1})	Global behavior score (GBS) and vocalizati-on during castration and after castration	Blood	Cortisol, ACTH, haptoglobin	Cortisol and ACTH were lower in piglets treated with meloxicam versus placebo but there was no significant difference in haptoglobin levels; GBS and vocalization were not significantly different between groups during castration, but a greater proportion of treated piglets had no behavioral alterations at 2 and 4 hours after castration
Khanna (2019)	Neurofib romatos-is type 1 (NF1)	7-9-month-old male and female Yucatan	(S)-lacosamide (applied to dissected DRG only)	von Frey mechanical testing, laser thermal testing,	DRG	Collapsin response mediator protein 2 (CRMP2), calcium	8-month-old NF1 pigs displayed mechanical allodynia and thermal hyperalgesia (females only), but no

		miniatu-re swine (wild type, NF1 mutation with no tumor, NF1 mutation with tumor)		bright light, air puff, feather, spontaneo-us pain and sleep quality via observation and FitBark2 devices			sensitivity to light, air, or feather tests There were no significant differences in spontaneous pain behaviors between any of the groups Male NF1+ with tumors and females had decreased sleep quality and increased time resting Calcium influx was increased in NF1 DRG as compared to WT, but decreased when (S)-lacosamide, a CRMP2 phosphorylation inhibitor, was applied Three CRMP2 phosphorylation sites were found to be modified in NF1 pigs: Serine 27, 627, and 647
Kluive-rs-Poodt (2012)	Castrati-on	3-5-day-old male piglets (n=160)	Group 1: Castration without interventi-on (CAST) Group 2: Lidocaine (1.0 ml per testicle) (LIDO) Group 3: Meloxicam (0.4 mg/kg) (MELO) Group 4: Lidocaine and meloxicam (L+M) Group 5: Sham castration (SHAM)	Vocalizati-on during castration	Blood	Cortisol, lactate, glucose, creatine kinase	CAST piglets had the highest intensity vocalizations of all groups, followed by MELO; the CAST and MELO groups had a significantly larger increase in post-castration cortisol levels than the LIDO group; lactate levels increased from baseline in all groups except for the LIDO group, in which it was lower; glucose and creatine kinase levels did not differ significantly between groups; growth and mortality were not affected by any of the treatments
Llamas Moya (2008)	Castrati-on	5-day-old male piglets (n=40)	N/A	Scan sampling of pain-related behaviors after castration	Blood	Cortisol, TNF-α, IL-1β, CRP, SAA, haptoglobin	Castrated pigs demonstrated pain-related behaviors and had higher cortisol levels than sham pigs, but there was no significant difference in TNF-α, IL-1β, CRP,

							SAA, or haptoglobin levels
Malav-asi (2006)	Abdomi-nal surgery	7-week-old male and female Swedish Landrace x Yorkshi-re pigs (n=10)	Group 1: Morphine (0.1 mg/kg) and fentanyl patches (50mg/kg/h) Group 2: Epidural saline and placebo patches	Video recordings of behavior	Blood	Cortisol, β-endorphin	At 12 hours following abdominal surgery, the placebo pigs showed decreased activity and appetite from baseline, while treated pigs had no change, but both groups had lower activity levels from 12-60 hours postoperatively In the 48-hour period after surgery, treated gained weight while placebo pigs lost weight The placebo group had significantly higher blood cortisol levels than the treated group in the immediate postoperative period, but there was no significant between-group difference in β-endorphin
Nixon (2021)	Castrati-on and tail docking	6-day-old male Yorkshi-re x Landrace piglets (n=40)	Group 1: Meloxic-am (MEL; 0.4mg/kg) Group 2: Flunixin (FLU; 2.2mg/kg) Group 3: Ketoprof-en (KETO; 3.0mg/kg) Group 4: Saline (SAL PROC) Group 5: Saline with sham handling only (SAL SHAM)	Activity monitors placed on neck from 24 hours before drug administrat-ion to 48 hours after	Blood Intersti-tial fluid	Cortisol Prostagland-in E2 (PGE2)	At 24-48 hours post-procedure, the FLU and KETO groups had higher activity levels than the SAL PROC and MEL groups At 2-4 hours post-procedure, there were lower levels of cortisol in the blood of the FLU and SAL SHAM groups as compared with the SAL PROC and lower levels of PGE2 in the ISF of all NSAD-treated groups as compared with the SAL PROC group
Reiner (2012)	Castrati-on	4-day-old Pietrain x	Group 1: Sham castrated (SHAM)	Vocalizati-on during castration and scoring	Blood	Cortisol	Vocalization during castration did not differ significantly

		Landrace male piglets (n=150)	Group 2: Castration without analgesia (CAS) Group 3: Flunixin (5 mg) 30 minutes prior and 0.9% NaCl (0.1 ml) immediate-ly before castration (FLU-30) Group 4: 0.9% NaCl (0.1 ml) 30 minutes prior and flunixin (5 mg) immediate-ly before castration (FLU-0) Group 5: .9% NaCl (0.1 ml) 30 minutes prior and meloxicam immediate-ly before castration (MET-0)	of pain behaviors from video recording after the procedure			between the castrated groups FLU-30, FLU-0, and MET-0 spent more time lying down than the CAS groups and had reduced teat stimulation at 24 hours CAS spent more time walking than FLU-0 The MET-0 group had increased tail wagging Cortisol levels were lower in pigs in the FLU-30 and FLU-0 groups as compared to the CAS group, but they were not significantly different in MET-0
Rice (2019)	Periphe-ral neuritis	9-week-old male Danish Landrace x Large White pigs (n=38)	N/A	Mechanical sensitivity (von Frey), tactile stimulation (feather), spontaneo-us pain behavior (solitary and social) 10-min observation period for 7 parameters of solitary and social behavior graded 0-2, motor function	Skin	CGRP, Nav1.7, endothelin-1 receptor A (ETA), endothelin-1 receptor B (ETB)	Full nerve crush (FC), partial nerve crush (PC), and peripheral neuritis trauma (PNT) pigs displayed sensitivity to von Frey filaments and responded to tactile stimulation, but PNT pigs showed mechanical sensitivity earliest and it persisted for the duration of the study Spontaneous pain behaviors were present in FC, PC, and PNT Nav1.7 and CGRP expression was higher

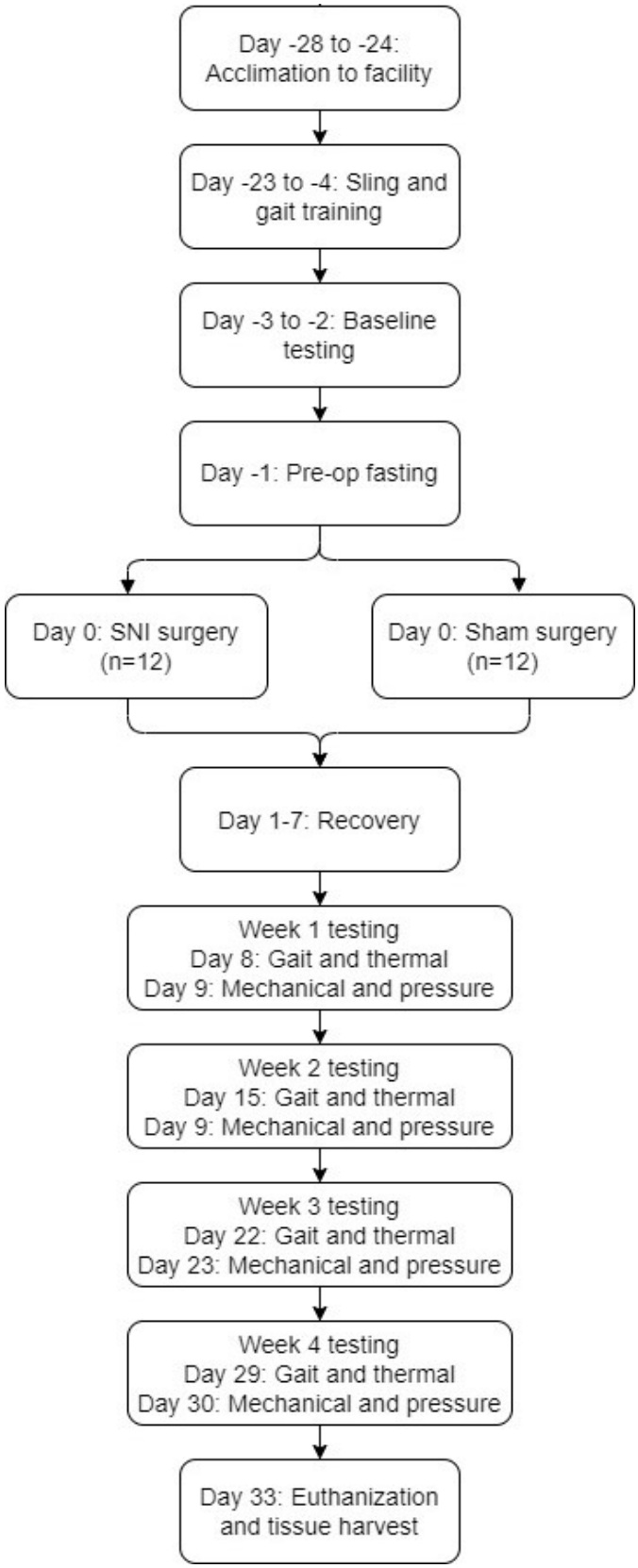
							and ETB expression was lower in PC and PNT pig keratinocytes as compared to sham ETA expression was higher in upper keratinocytes and lower in the lower keratinocytes in PNT as compared with sham pigs
Suther-land (2008)	Tail docking	6-day-old male and female piglets (n=80)	Cautery iron, blunt trauma cutters	Scan sampling of spontaneo-us pain behavior	Blood	White blood cells, cortisol	There was no significant difference between the spontaneous pain behaviors or white blood cell count of piglets docked with a cautery iron versus blunt trauma cutters, but blood cortisol levels were elevated in piglets docked with blunt trauma cutters as compared to sham docked piglets and those docked with a cautery iron
Suther-land (2012)	Castrati-on	3-day-old male piglets (n=70)	Group 1: Sham castration (CON) Group 2: Castration (CAS) Group 3: NSAID before castration (NSAID) Group 4: CO2 anesthesia before castration (CO2) Group 5: NSAID and CO2 before castration (BOTH)	Scan sampling of pain behaviors during castration and handing, stress vocalizati-on during drug administra-tion	Blood	Cortisol, CRP, substance P	Vocalizations were highest in the CAS and NSAID groups For 30 minutes after castration, the CAS group spent more time lying alone than the treated groups and the CO2 group had higher levels of pain behavior than all other groups After 30 minutes, the CO2 group spent more time lying alone than the NSAID and BOTH groups Cortisol, CRP, and substance P levels were not decreased in the treated groups as compared to the CON group
Suther-land (2010)	Castrati-on	2-4-day-old male and female	Group 1: Sham castration (CON)	Vocalizati-on during castration and	Blood	Cortisol, white blood cells, neutrophils,	All castrated groups had an increased percentage of vocalizations as

		Cambor-ough-22 piglets (n=76)	Group 2: Castration (CAS) Group 4: Castration with short-acting topical anesthetic (Cetacaine ®) (SHORT) Group 5: Castration with long-acting topical anesthetic (Tri-Solfen) (LONG)	behavior for 3 hours after		lymphocytes, leukocytes	compared with the CON group After castration, the CAS group spent more time lying alone than the SHORT and LONG groups Cortisol levels were elevated in all castrated groups as compared with the CON group at 30 and 60 minutes post-procedure There was no significant difference between blood cell counts in any of the groups
Sutherl and (2011)	Tail docking	2-4-day-old male and female Cambor-ough-22 piglets (n=120)	Group 1: Sham docking (CON) Group 2: Conventio-nal docking (CUT) Group 3: CUT docking with local anesthetic injection (LA) Group 4: CUT docking with short-acting topical anesthetic (Cetacaine ®) (SHORT) Group 5: CUT docking with long-acting topical anesthetic (Tri-	Scan sampling of pain behavior and posture for 2 hours after procedure	Blood	Cortisol, white blood cells, neutrophils, lymphocytes, leukocytes	CUT, SHORT, and LONG groups had increased percentage of vocalizations as compared to baseline Cortisol levels were elevated in CUT, LA, and LONG groups as compared with SHORT and CON groups at 30 minutes post-procedure; WBC counts were lower in LONG and CO_2 groups than the CUT group The ratio of neutrophils to lymphocytes was higher in CUT, LA, SHORT, and LONG groups as compared with the CON group, There was no significant difference between leukocyte levels in any of the groups

			Solfen) (LONG) Group 6: CUT docking with CO_2 anesthesia (CO_2)				
Suther-land (2017)	Castrati-on	2-4-day old male Cambor-ough-22 piglets (n=76)	Group 1: Sham castration (CON) Group 2: Castration (CAS) Group 3: Castration with local anesthetic using needle (NED) Group 4: Castration with local anesthetic using needle-free injection system (NF)	Stress behavior and vocalizati-on during treatment and behavior for 3 hours after	Blood	Cortisol, white blood cells, neutrophils, lymphocytes, leukocytes	The NF group had decreased tail flicking and pain behavior scores than the NED group during anesthetic administration, but all castrated groups had higher percentages of vocalizations, tail movements, and pain behavior scores than the CON group during handling and castration After castration, the NED and NF groups spent less time lying alone than the CAS group Cortisol concentrations were significantly higher in all castrated groups than the CON group, but there was no significant difference in complete blood cell counts between groups
Temple (2020)	Vaccina-tion	28-day-old male and female piglets (n=339)	Group 1: Intradermal Porcine Circovirius type 2 vaccination using a needle-free Porcilis®PCV ID device (IDAL) Group 2: Intramuscu-lar Porcine Circovirius type 2 vaccination using Porcilis®P-	Retreat attempts and high-pitched vocalizati-on during vaccination ; scan sampling of social, resting, and general activity at baseline and following vaccination	Saliva, blood	Saliva: Salivary alpha-amylase(sAA) and Chromogran in-A (CgA) Blood: C-reactive protein levels, haptoglobin	IM piglets displayed higher levels high-pitched vocalizations and retreat attempts than IDAL and CONT piglets during vaccination and lower negative social behavior and rope toy exploration the day following vaccination as compared with CONT piglets Blood from IDAL piglets had lower levels of CRP and haptoglobin as compared to CONT and IM piglets, but salivary biomarkers

			CV with needle (IM) Group 3: Handled only did not receive vaccine (CONT)				sAA and CgA were not significantly different between groups There was no difference between the growth or rectal temperature between groups
Tenbe-rgen (2014)	Castrati-on and tail docking	5-7-day-old male and female piglets (n=2888)	Meloxicam (0.4 mg/kg)	Vocalizati-on intensity via decibel meter, observation of pain behaviors	Blood	Cortisol	Piglets given a placebo had higher isolation scores and cortisol levels than those treated with meloxicam; among piglets nursing from sows with higher parity, those given a placebo had 4.4 times higher mortality than those treated with meloxicam

APPENDIX C: Study Timeline



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