Towards a More Ethical Animal Model in Biomedical Research

by

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B. Eng Chemical Engineering
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Submitted to the Program in Media Arts and Sciences,
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Abstract

Since the early twentieth century, mice have emerged as the standard mammalian model organism for biomedical research. When pain relief is provided during experimentation, it typically comes in the form of transient and sometimes ineffective analgesics or anesthesia. This thesis proposes an alternative to the current method of research in the form of an engineered mouse model in which pain sensing can be ablated before an experiment. An ERT2–inducible Cre recombinase under the Wnt1 promoter was designed to be combined with a floxed Nav1.7 ion channel mouse model. When a 4-hydrotamoxifan class small molecule is fed to the mouse, Cre recombinase expression in the peripheral nervous system will disrupt function of the ion channel involved in inflammatory and mechanosensory pain. Additional designs for floxed Nav1.6 ion channel and Nax ion-like channel were made to explore disruption of peripheral cancer-induced neuropathic pain. In parallel with mouse model development, a survey was conducted to understand the potential for adoption of this new animal model by researchers. The survey was sent to IACUC members questioning if this model was needed, as well as how it may be regulated under the existing protocol approval framework. Results indicated that there is a both a need and desire for further refinement strategies within animal research, and that this inducible pain-free mouse model could be categorized as alternative analgesic upon sufficient characterization and peer-reviewed publications. Additional input was provided that will shape testing done on the generated animals to assure that this model can mitigate animal suffering while still recapitulating important biological processes investigated in biomedical research.

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Thesis Overview

This thesis (fig.1) is meant to contextualize the past and present state of animal suffering in US research institutions, and propose a refinement to the system in the form of an inducible pain-free mouse model.

First, I explore the evolution of human opinion on animals and their ability to sense and perceive stimuli in their environment.

I will then consider the state of animal welfare within the US and whether treatment of animals has developed at the same pace as animal industrialization.

Thereafter, I will review the molecular basis for pain sensing within vertebrates, with a focus on the particular ion channels of interest for modification.

Following this, I will describe the design for the proposed inducible pain-free mouse model. This will include the initial designs for the system, as well as the final constructs made and the status of animal models for testing.

Then, I will describe the survey that was designed and sent to IACUC members that questions whether there is a need for further refinement within the animal, and if so, how the inducible pain-free mouse model could be used as a refinement method after sufficient characterization.

Finally, I will discuss the next steps intended for the project.

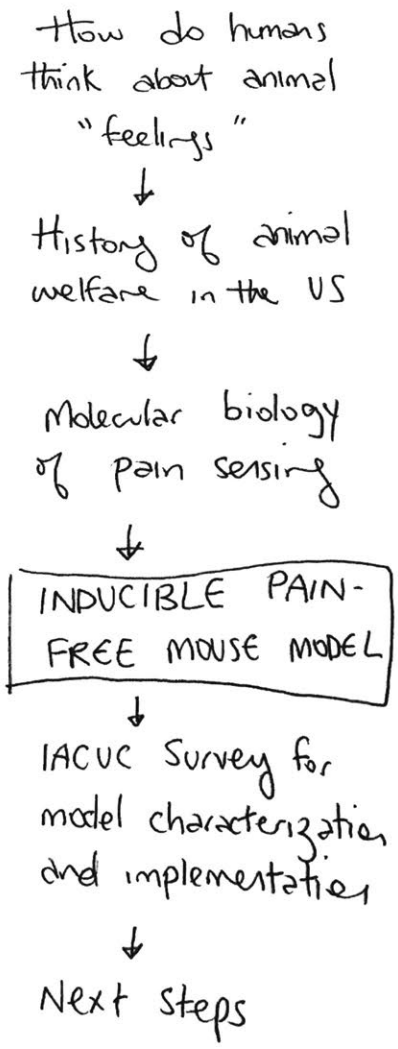

Figure 1: Thesis flow chart

"Correspondent to discovery and improvement in the natural world, is reformation in the moral"

-Preface, *A fragment on government*, Jeremy Bentham

I. A Brief History of Animals in Philosophy

Humans have used animals for their benefit since domestication allowed for the rapid development of civilization, but it took many years for humans to recognize these animals as have any capacity for pain and pleasure. The analysis of the ethics around our treatment of these non-human animals has developed over time. This section will highlight key philosophers whose opinions on animal sentience challenged the societal norms that existed in their time. This will help situate this thesis and the imperative behind the inducible pain-free mouse project within a moral and ethical context.

I.I Michel de Montaigne

Michel de Montaigne (1533-1592), a French Lord and Renaissance philosopher and statesman known for his humility, curiosity, and humorous approach to life is held as a true progressive of his time. He influenced thinkers ranging from René Descartes and Blaise Pascal to Jean Jacques Rousseau and Ralph Waldo Emerson. Montaigne lived during the Renaissance, a time when classical Greek and Roman thinking was being rediscovered. Consequently, many of his contemporaries took pride in touting the

superiority of human intellect over all of nature. Montaigne was not so convinced. His publications were titled "*Essais,*" which translates to "Attempts", popularizing the style of Essay writing through his seminal works. In his essay *Of Cruelty*, Montaigne poignantly notes,

> *"But when, amongst the more moderate opinions, I meet with arguments that endeavour to demonstrate the near resemblance betwixt us and animals,* ***how large a share they have in our greatest privileges****, and with how much probability they compare us together, truly I abate a great deal of our presumption,* ***and willingly resign that imaginary sovereignty that is attributed to us over other creatures****. But supposing all this were not true,* ***there is nevertheless a certain respect, a general duty of humanity, not only to beasts that have life and sense, but even to trees, and plants****. We owe justice to men, and graciousness and benignity to other creatures that are capable of it; there is a certain commerce and mutual obligation betwixt them and us."*
>
> - Of Cruelty, *Essays of Michel de Montaign*

In a similar vein, he writes in his essay *Apology for Raymond Sebond*:

> *'Tis by the same vanity of imagination that he equals himself to God, attributes to himself divine qualities, withdraws and separates himself from the the crowd of other creatures, cuts out the shares of the animals, his fellows and companions, and distributes to them portions of faculties and force, as himself thinks* ***fit How does he know, by the strength of his understanding, the secret and internal motions of animals****?—from what comparison betwixt them and us does he conclude the stupidity he attributes to them?... The defect that hinders communication betwixt them and us, why may it not be in our part as well as theirs? 'Tis yet to determine where the fault lies that we understand not one another,—for we understand them no more than they do us****; and by the same reason they may think us to be beasts as we think them****."*
>
> -Apology for Raymond Sebond, *Essays of Michel de Montaigne*

Montaigne makes in clear in his statements that he is going against the commonly held "presumption" that humans have dominion over all other creatures. At the beginning of the statement on animals made in *Apology to Raymond Sabond*, Montaigne claims that presumption of man is "our natural and original disease." It is no coincidence that the word comes up twice in the context of humans' treatment of animals. This presumption is what has allowed humans to attribute "stupidity" to these animals, without any concrete proof support the claim. Furthermore, Montaigne points to the vanity of man as the reason for not giving animals "portions of faculty and force." He does not go as far as to claim that animals and humans are equal in their metal faculties, but acknowledges that they have something similar that, though hard to understand, does exist.

These statements are progressive for a writer in the 19th century, let alone the 16th. More than that, Montaigne maintains that there a societal *duty* for humanity to make sure that things that have "life and sense" as well as other living things, to be respected. Humanity owes "benignity" to creatures, though he does not elaborate on what that would look like. It would not be out of line to say that Montaigne would believe that respecting the lives of these creatures and not causing them undue suffering is within the realms of "benignity."

I.II Descartes

René Descartes (1596-1650) is largely considered the father of Western philosophy. Descartes was a French natural scientist and mathematician, but above all a rationalist. For his life-long foray into philosophy, Descartes focused on that which could be confirmed through the senses. Influenced by Montaigne's notion that humans should study themselves before they buy into into the thoughts of previous thinkers, he was uninterested in believing in something simply because someone intelligent in the past had said it. Instead, Descartes desired to re-derive every philosophical tenet he believed in.

He coined the phrase "*je pense, donc je suis,"* which is translated to "I think, therefore I am" (Descartes 1969). Descartes believed in a soul-body separation which made man unique among other living things.
This belief can be seen in his opinion on humans and animals in Part V of *Discourse on the Method*, entitled "Physics, the heart, and the soul of man and animals." In it, he states:

> *"Such persons will look upon this body as a machine made by the hands of God, which is incomparably better arranged, and adequate to movements more admirable than is any machine of human invention... but if there were machines bearing the image of our bodies, and capable of imitating our actions as far as it is morally possible, there would still remain two most certain tests whereby to know that they were not therefore really men."*
>
> - Part V, *Discourse on the Method*

Descartes is stating that both human and animal anatomy can be thought of as highly complex machines. He brings up the key difference between the two in his two tests that distinguish humans above animals. The two tests are:

1. Animals do not have the ability to arrange any combination of "words or other signs" in order to declare their thoughts
2. Though there are animals that have the physical capability to perform many actions well, it would eventually become obvious that these actions the animal is performing are not from their own knowledge or reason but just a "disposition of their organs"

Where does that leave our understanding on Descartes' conception of the faculties of animals? Can an animal have any type of "feeling," and if so, could it process and understand that feeling without a soul? This is the premise of a long-standing argument regarding Descartes' beliefs on the automata-like nature animals implying that animals are incapable of feelings of any kind, termed the "monstrous thesis."

An oft-quoted piece of a letter to Henry More sent in February 1649 is brought as further proof that Descartes believed in the "monstrous thesis":

> *"... it seems reasonable since art copies nature, and men can make various automata which move without thought, that nature should produce its own automata much more splendid than the artificial ones.* ***These natural automata are the animals.***"
>
> -Letter to Henry More February 5, 1649

Many philosophers use this assignment of animals as "bête machine," or animal machines, to jump to the conclusion that Descartes believes in the monstrous thesis, though Descartes beliefs are more unclear than the strict divide he has implied.

Reading through *Discourse* it is clear that Descartes thinks animals can respond to stimuli, even if they do not possess the ability to reason and rationalize. He notes:

> *"And, in the last place, what above all is here worthy of observation, is the generation of the* ***animal spirits****, which are like a very subtle wind, or rather a very pure and vivid flame which, continually ascending in great abundance from the heart to the brain,* ***thence penetrates through the nerves into the muscles, and gives motion to all the members****...and we ought not to confound speech with the natural movements which indicate* ***the passions,*** *and can be imitated by machines as well as manifested by animals."*

So the question becomes not whether an animal can respond to a painful stimulus and respond, but whether it matters that they are responding if it is an automatic response that does not have thought or reason behind it. In his analysis of Descartes, John Cottingham points out that in the letter to John More, Descartes clarifies the difference between the concepts of sensation (*sensus*) and thought (*cogitatio*). (Cottingham 1978) (Descartes 1970). Descartes states:

> *"...though I regard it as established that we cannot prove there is any thought in animals, I do not think it is thereby proved that there is not, since the human mind does not reach into their hearts."*
>
> -Letter to Henry More February 5, 1649

The above statement certainly takes a more Montaigne-esqe approach than we have seen so far, which is not surprising given Descartes was influenced by his writing. Both Descartes and Montaigne seem to err on the side of caution when considering animals, though Montaigne seemed to believe "conscious unless proven otherwise" whereas Descartes believed "unconscious unless proven otherwise," as given by his two tests.

When considering what Descartes' final stance is on the matter of the feelings of animals, it is still unclear. Peter Harrison's analysis emphasizes the habit of Descartes to employ skepticism whenever unsure of something (Harrison 1992). But does this uncertainty about the animal soul mean that Descartes did not think animals could feel pain? He certainly agrees that animals have the capability of sensing and responding to pain. And if he does not think animals have a soul and can feel physical stimuli in any meaningful way, does that mean he may encourage animal cruelty? There is no indication of the sort.

I.III David Hume

David Hume (1711-1776) is seen as one of the most important thinkers of his time for eschewing Descartes' will to put mind over all, instead prioritizing human feeling. He believed that most humans act not on rationality, but on their passions. Hume focused on understanding the similarities between non-human and human animals in contrast to the Cartesian method of the differences between the two. It should be noted that while Descartes believed in God, and therefore a divine soul, it is debated whether Hume was even a Theist, so the soul was not necessarily a meaningful divider to him between man and beast.

In his essay "Of the Reason of Animals," Hume confidently states:

"Next to the ridicule of denying an evident truth, is that of taking much pains to defend it; ***and no truth appears to me more evident, than that beasts are endow'd with thought and reason as well as men.*** *The arguments are in this case so obvious, that they never escape the most stupid and ignorant...*
Nature may certainly produce whatever can arise from habit: Nay, **habit is**

nothing but one of the principles of nature, and derives all its force from that origin"

-Part III Section XVI, *A Treatise of Human Nature*

Hume points out that the commonality between behaviors in human and non-human animals cannot be denied. In his opinion, the most likely scenario is that both are derived from a common source. This opinion comes a century before the ideas of natural evolution started taking hold in society. It speaks to Hume's foresight that this statement sounds obvious, even while being quite a radical thought at the time.

I.IV Jeremy Bentham

Jeremy Bentham (1748-1832) is considered to be the father of Utilitarianism. Bentham was hugely impacted by Hume and his focus on the passions as the most important aspect of the human experience, which drove a lot of his work. As one who completed his studies at Oxford and was called to the bar by the age of 21, Bentham had intimate knowledge of a legal system he quickly came to despise. While writing about reforms for the British legal system he realized he wanted much more than just reform in one area of society (Crimmins 2018). Bentham set out to write a complete code of laws, a "pannomiom" as he referred to it, according to his own moral philosophy. Bentham's fundamental axiom was "*it is the greatest happiness of the greatest number that is the measure of right and wrong*" (Bentham 2001).

Bentham focus strongly on the axis of pleasure and pain, creating a "felicific calculus" for those who were interested in quantifying any given experience. In Chapter Four of *Introduction to the Principles of Morals and Legislation*, seven different variables are given which can define any experience on the pain/pleasure axis (Bentham 1996). Those are:
(1) its intensity
(2) its duration
(3) its certainty or uncertainty
(4) its nearness or remoteness.
(5) its fecundity
(6) its purity

(7) its extent

Bentham felt these principles to be so important that he even came up with a mnemonic doggerel, a catchier way to remember his felicific calculus:

> "*Intense, long, certain, speedy, fruitful, pure—*
> Such marks in *pleasures* and in *pains* endure.
> Such pleasures seek if *private* be thy end:
> If it be *public*, wide let them *extend*
> Such *pains* avoid, whichever be thy view:
> If pains *must* come, let them *extend* to few."

Bentham did not reserve the right to a felicific life to humans alone. He was more outspoken than his predecessors on the rights of animals, and any sentient being for that matter, to the right of avoiding pain and increasing pleasure throughout their lifetime.

> *"It may one day come to be recognised that the number of the legs, the villosity of the skin, or the termination of the os sacrum, are reasons equally insufficient for abandoning a sensitive being to the same fate. What else is it that should trace the insuperable line? Is it the faculty of reason, or perhaps the faculty of discourse? But a full-grown horse or dog is beyond comparison a more rational, as well as a more conversable animal, than an infant of a day, or a week, or even a month, old.* ***But suppose they were otherwise, what would it avail? The question is not, Can they reason? nor Can they talk? but, Can they suffer****?"*
>
> -Chapter XVII, Section I, *The Principles of Morals and Legislation*

Bentham's philosophy of "*the greatest good for the greatest number*" means that the systematic nature of millions of animals today suffering under the control of humans is extremely problematic. The suffering of those animals is significant, just as significant as human suffering. To him, it is social obligation to mitigate this suffering and increase the quality of life of these sentient creatures.

I.V Charles Darwin

Charles Darwin (1809-1882), considered the father of evolution, had much to say about animal welfare. As someone who saw humans and monkeys as close cousins, it is not hard to believe that Darwin would have much sympathy for the pain and suffering of animals. Lingering feelings within the scientific elite that held humans as far superior to animals was shattered with the development of the theory of evolution. In addition to publishing *On the Origin of Species*, he had written *The Expression of the Emotions in Man and Animals*. This book is a fascinating look at how humans analyzed both themselves as well as non-human animals during this period. Going against the theories of Sir Charles Bell and Descartes who believed that true emotion can only be expressed through a divinely-imbued aspect to the person, Darwin took a more evolutionary approach. He studied both human and non-human animal expressions, and tried to understand how one may have been derived from the other. In the introduction the the book Darwin notes:

> *"Consequently, when I read Sir C. Bell's great work, his view, that man had been created with certain muscles specially adapted for the expression of his feelings, struck me as unsatisfactory.* ***It seemed probable that the habit of expressing our feelings by certain movements, though now rendered innate, had been in some manner gradually acquired.*** *But to discover how such habits had been acquired was perplexing in no small degree. The whole subject had to be viewed under a new aspect, and each expression demanded a rational explanation."*
>
> -Introduction, *The Expression of the Emotions in Man and Animals*

Darwin employs a methodical approach to the development of emotions, using the same law of parsimony he employs when understanding all aspects of the development of animals. In reference to the emotion of pain, Darwin states:

> *Great pain urges all animals, and has urged them during endless generations,* ***to make the most violent and diversified efforts to escape***

> ***from the cause of suffering****...As the muscles of the chest and vocal organs are habitually used, these will be particularly liable to be acted on, and loud, harsh screams or cries will be uttered. But the advantage derived from outcries has here probably come into play in an important manner;* ***for the young of most animals, when in distress or danger, call loudly to their parents for aid, as do the members of the same community for mutual aid****.*
>
> -Chapter III, *The Expression of the Emotions in Man and Animals*

Darwin emphasizes the similarity both in physical and emotional responses between human and non-human animals when physical pain is inflicted upon them. He uses words like "distress" to describe an animal's response to pain, and if employing Bentham's concept of a felicific calculus, it is clear that animals are in a state they would rather not be in if given the choice. Why then, is so much of human pleasure built upon a framework on systematic animal suffering?

I.VI Peter Singer

Peter Singer (1946-) is one of the most well-known contemporary utilitarian thinkers as well as a leader in the Animal Liberation movement. Grounded in Bentham's utilitarianism, Singer believes in addressing as well as making strides to decrease the suffering of all sentient beings. Bentham's famous question question "*Can they reason*? nor, *Can they talk*? but, *Can they suffer*?" drives much of Singer's animal welfare work. It informs his views on who has power and how that power is used to increase good for the most. His book *Animal Liberation*, first published in 1975, is one of the seminal boots associated with the animal rights movement.

Singer points out that humans practice speciesism, a term coined by animal activist Richard Ryder (Ryder 1998). Speciesism is a bias where members of a species have a preference towards one another over other species. This can be seen in the human attitude towards animals, putting their pleasure over the suffering of animals and taking moral issue with killing even the most mentally impaired human over the most sentient non-human animal.

Singer does not emphasizes equality in all aspect between humans and animals, but equality in *consideration* (Singer *et al.* 1998). In reference to the current animal experimentation practices, Singer states:

> "*The experimenter, then, shows a bias in favor of his own species whenever he carries out an experiment on a nonhuman for a purpose that he could not think justified him in using a human being at an equal or lower level of sentience, awareness, ability to be self-directing etc. No one familiar with the kind of results yielded by most experiments on animals can have the slightest doubt that if this bias were eliminated the number of experiments performed would be a minute fraction of the number performed today.*"

Singer feels that if humans would not use an infant or a person who has severe mental disabilities that prevent him or her from understanding and making active choices in his or her own life, that humans should not put preference to these individuals over non-human animals that have the same or possibly higher cognitive abilities.

This opinion is in contrast to the opinions held by Descartes, who himself has stated in the *Discourse* Part V,

> *"it is incredible that the most perfect ape or parrot of its species, should not in this be equal to the most stupid infant of its kind or at least to one that was crackbrained, unless the soul of brutes were of a nature wholly different from ours."*

Singer's statements are meant to make humans uncomfortable. His intention is to force us into questioning our place within this world and be confronted by the face that humans clearly place their pleasure at the expense of the suffering of millions of sentient beings.

I.VII David Foster Wallace

David Foster Wallace (1962-2008), is a writer known for his frank analysis and excessive use of footnotes. His essay on the Main Lobster Festival,

Consider the Lobster, Wallace brings up may points that have roots in the philosophers just quoted (Wallace 2005).

Within his analysis, DFW first notes the differences in the nervous system of lobsters compared to humans and organisms that have cerebral cortices with which to process feelings of emotion. This lobster nervous system of course does not apply to the vertebrates that are currently used as models in biomedical research, but it harkens back to some of the Cartesian ideas around the ability for animals to process the stimuli they experience (though in his argument it was due to a lack of a soul instead of higher order brain function).

DFW goes on to explain the complexity of trying to understand pain, which in itself is a challenge because it is a subjective mental experiences. He notes:

> *"The fact that even the most highly evolved nonhuman mammals can't use language to communicate with us about their subjective mental experience is only the first layer of additional complication in trying to extend our reasoning about pain and morality to animals."*

This statement has traces of Montaigne, recognizing that the fundamental difficulty in humans properly understanding animals should not preclude animals from having their own complex way of thinking, feeling, or experiencing. Humans should not be so vain or *presumptuous* as to jump from not understanding animals to imply that they do not equal us in other ways outside of verbal communication. In this regard though, Descartes would disagree with Montaigne and DFW. He would stand by the idea that though animals might have bodily passions and respond to stimuli, it is in fact akin to a cog moving that would produce a mechanical response in an automaton. Animals do not have a soul, which is required for processing and reasoning aspects within humans. So though he may agree that an animal should not be put in a painful situation, he would certainly not do it on the basis of an animal's "mental experience."

DWF gets more into the details of how ethicists decide whether there is a moral imperative to respond to imposed suffering on organisms, with the criteria being:

> *"One is how much of the neurological hardware required for pain-experience the animal comes equipped with—nociceptors, prostaglandins, neuronal opioid receptors, etc. The other criterion is whether the animal demonstrates behavior associated with pain."*

These two conditions have roots in both the Animal Liberation movement as well as overarching utilitarian belief systems. Singer, Bentham, and Hume would all agree with these criteria as important points to consider when determining pain and pleasurable states for an organism to exist within. Introducing the concept of pain and pleasure as perceived by the animal indicates that there is a preferred state of that organism. This becomes a moral obligation for society to try and make sure we are maximizing the pleasure experienced by the most for all organisms that demonstrate a preference away from suffering, like what the above two criteria do define.

Wallace ends with a statement that is as applicable to the field of biomedical research as it is to the morals of human animal consumption:

> *"Is it not possible that future generations will regard our own present agribusiness and eating practices in much the same way we now view Nero's entertainments or Aztec sacrifices? My own immediate reaction is that such a comparison is hysterical, extreme—**and yet the reason it seems extreme to me appears to be that I believe animals are less morally important than human beings**; and when it comes to defending such a belief, even to myself, I have to acknowledge that (a) I have an obvious selfish interest in this belief, since I like to eat certain kinds of animals and want to be able to keep doing it, and (b) I have not succeeded in working out any sort of personal ethical system in which the **belief is truly defensible instead of just selfishly convenient**.*

This point drives home that philosophy can only go so far in incentivizing humans to make personally uncomfortable changes in their life. Using animals is convenient. Mice are well characterized and bred in numbers that make them an extremely appealing option within biomedical research and a standard set within the scientific community. Similar to Wallace, who confesses "my own main way of dealing with this conflict has been to avoid

thinking about the whole unpleasant thing," many a researcher dissociates the animals they work with during their interactions because of the emotional toll. Even at a point when it is absolutely morally indefensible, humans usually need more than philosophy, and at times even lived experience, to bring about systemic change.

"All the arguments to prove man's superiority cannot shatter this hard fact: in suffering the animals are our equals"

-Peter Singer

II. Research Animal Welfare in the United States

The history of animal welfare in the United States has been a multi-century push to protect animal exploitation and unnecessary suffering. Though there is a long history of protection against animal cruelty for domesticated animals, particularly ones raised for slaughter and consumption, this section will focus on the history of animal welfare as it pertains to those non-human animals used for research purposes. It will then question whether the current state of the field where it should be, and if not, what can be done.

II.I Anti-Vivisection Movement

One of the first movements supporting the welfare of research animals used in the United States was created in solidarity with the Anti-vivisection movement in England. Vivisection is the practice of performing operations on living animals, commonly done in animal research. The organization first fought to regulate the use of animals in science but then pivoted to pushing for the complete abolition of live animal experimentation in the US. The Gallinger Bill was brought before Congress in 1900, mirroring the British

Cruelty to Animal Bill of 1876, which requested regulation on scientific experimentation (Gallinger, 1900). Unfortunately, the bill did not pass and the movement did not stir the same response in the US as it did in England to further protect animals from experimentation.

II.II *The Principles of Humane Experimental Technique*

In 1954, biomedical research in the US was moving at a rapid pace. At the same time within the United Kingdom, the Universities Federation for Animal Welfare (UFAW) had commissioned a research study on how to advance more humane techniques within the field of animal experimentation (National Research Council (US) Committee to Update Science 2004). The two men assigned to the study were William Russel, a zoologist, and Rex Burch, a microbiologist. Together, they toured labs all over the UK and analyzed how animals were used in research experiments. They report they generated for the UFAW was turned into *The Principles of Humane Experimental Technique* (1959).

Within this book, the two discuss the current state of pain and distress experienced by animals, as well as "how humanity can be promoted without prejudice to scientific and medical aims" (Russel, Burch, and Hume 1959). They proposed the "3 R's" within animal welfare: replacement, reduction, refinement. Replacement aims to find alternatives to using vertebrates for research whether it be cell culture or a microorganism. Reduction aims to limit the number of vertebrates that will be used through strategic planning of experiments, proper controls, and careful design. Finally, refinement aims to assure that the procedures and animal choice are the best fit for the experiment and will be the least inhumane method of performing the research. This includes techniques that are the noninvasive, that provide appropriate anesthetics and analgesics, and providing species-appropriate enrichment for the animals.

Though this survey was done in the United Kingdom, it has become an internationally set approach for the human treatment of animals and has made strides by providing a framework that can adapt over time with new techniques (Fenwick, Griffin, and Gauthier 2009).

II.II Laboratory Animal Welfare Act of 1966

In early 1966, a piece in LIFE magazine caused the necessary uproar needed to make changes to a grossly unregulated field (Cosgrove 2014). The article, titled *Concentration Camps for Dogs* and focused on a dog named Pepper who had disappeared in Pennsylvania only to turn up euthanized in New York. What followed was an exposé on the cruel and horrifying black market that existed for dogs (stolen or bred) used for vivisections experiments in medical research. This article prompted an outcry from the public, with letters flooding Washington that demanded change.

In response, the Office of Animal Laboratory Welfare (OLAW) was established in 1966 upon the signing of the Laboratory Welfare Act. The United States Department of Agriculture (USDA) and Animal and Plant Health Inspection Service (APHIS) were put as the agencies that oversaw this bill. This piece of legislation is the only Federal law in the United States that regulates animals for uses such as research, transport, an exhibition ("Animal Welfare Act", n.d.). It covers any cat, dog, hamster, rabbit, nonhuman primate, guinea pig, and any other warm blooded animal determined by the Secretary of Agriculture, living or dead. Notable exclusions from this law include rats, mice, birds, cold-blooded animals, and farm animals.

Many concepts adopted within this regulation come from the framework that Russel and Burch has designed. The regulations emphasize the replacement, reduction, and refinement of animal experimentation whenever possible. While this law was an important first step towards improving the welfare of animals, it has only set the minimum level of acceptability within the US and leaves much to be desired.

Notably though, a 1986 amendment to this law established the institutional animal care and use committee (IACUC) to be required in all federally funded institutions performing animal research. This amendment allows for a critical analysis of all research experimentation on animals taking place in an institution and creates a formal structure for research protocol approval

as well as reporting. It is certainly a necessary step towards proper oversight within experimentation.

II.III Animal Liberation Movement

The Animal Liberation Movement was started at Oxford University in the early 1970's by a group of philosophy students. The movements have roots in much of the original Anti-Vivisection protesting that was done in the 1800's around animal experimentation. These students spent their time discussing points with each other and pulling from past thinkers to assure that their arguments were both powerful and rational. A core ideal in the movement is moving away from the "speciesism" that takes place in current human-non/ human animal interactions. The group has done work uncovering violations in animal rights laws by corporations, boycotted companies and produces that do not properly care for animals, and performed both symbolic and direct actions to further their mission.

Amendments made to the Animal Welfare Act reflecting the need for a more robust protection for animals is due in part to the work of the Animal Liberation movement. These amendments expanded the animals included under the policy as well as required the use of analgesics and anesthetic to be used during experimentation.

II.IV PHS Policy

Though the Animal Welfare Act aimed to improve the conditions of animals within the US, research fields were still in need of further regulation. The vast majority of animal research is performed in mouse models, with estimates being as high as 100 million mice used per year ("Mice and Rats in Laboratories" | PETA). Under the initial AWA regulations there was no way to regulate both the the number and conditions of these animals.

In 1985, the Health Research Extension Act implemented referred to as "Animals in Research" was passed. This piece of legislations is a requirement for the National Institute of Health (NIH), not US citizens individually. As a government funding agency, the NIH invests tens of billions of dollars in

research money every year to public and private research institutions ("PHS Policy on Humane Care and Use of Laboratory Animals," n.d.). This law requires that there be compliance in reporting on all vertebrate species for those awarded funding through the NIH through IACUC committees. This means that all mouse and rat experiments must be reported.

II.V Changing the System

Though animal welfare has made strides through the establishment of regulations attempting to prevent undue suffering within the animal community, it does not mean that these laws are necessarily effective. It has been shown that fewer researchers, not more, have been seen to implement the legal standards set for the animal experimentation field (Balcombe, Ferdowsian, and Briese 2013). Furthermore, though there are requirements for the delivery of analgesics, most are delivered at the time of surgery and there is little to no post-operative care or treatment of persistent research-related pain (Stokes, Flecknell, and Richardson, 2009).

Additionally, the PHS policy covering all vertebrates is not applicable to any private research institutions. Though there are methods of outside assessment such as AALAC accreditation and state regulation requiring stricter reporting, there are still many ways for animals to fall through the reporting cracks. There is a need for a stronger force encouraging the refinement and replacement of animal research methods within biomedical research.

Researchers must have institutional incentives to assure that they are not merely complying with regulations on animal care, but trying to be at the cutting edge. Incentivizing the design of novel pain management techniques for researchers to implement could provide a constant push to encourage ever more ethical treatment of research animals.

"There are no gains, without pains"

-Benjamin Franklin, *The Way to Wealth*

III. Pain Sensing

Though pain sensing happens quite quickly, there is an extremely complex procession of events happening between the time stimulus is sensed and responded to. There is still work yet to be done before the scientific community can even properly understand how pain perception functions both in human as well as non-human animals (Twilley, 2018, Ingraham, 2018). This chapter will focus on an overview of the nociceptive pathways that initiate the pain sensation as well as methods that have been employed to more closely understand how pain sensing is controlled in the body.

III.I Sodium Ion Channels

Pain signaling caused by an external stimulus is a process that begins at the peripheral pain receptors and leads to the brain (Fig. 2). External nociceptors are found in nerve endings within the epidermis, cornea, and mucosa. The cell bodies of these peripheral nerve endings are primarily located in bundles within the dorsal root ganglia. When a noxious stimulus is received by a

nociceptor, it activates the opening of a sodium ion channel specific to that pain modality. This influx of sodium ions from the ion channel creates an action potential that travels quickly through the myelinated Aδ-fibers and enters the dorsal horn at the anterior of the spinal cord. The signal is then sent from the first-order neuron though a synapses to the second order neurons that cross from the anterior to the lateral spinothalamic tract. It then ascends the spinothalamic tract to the thalamus, the section of the brain that processes these stimuli. From there the signal can be translated into a response at the site of initial nociception if determined appropriate.

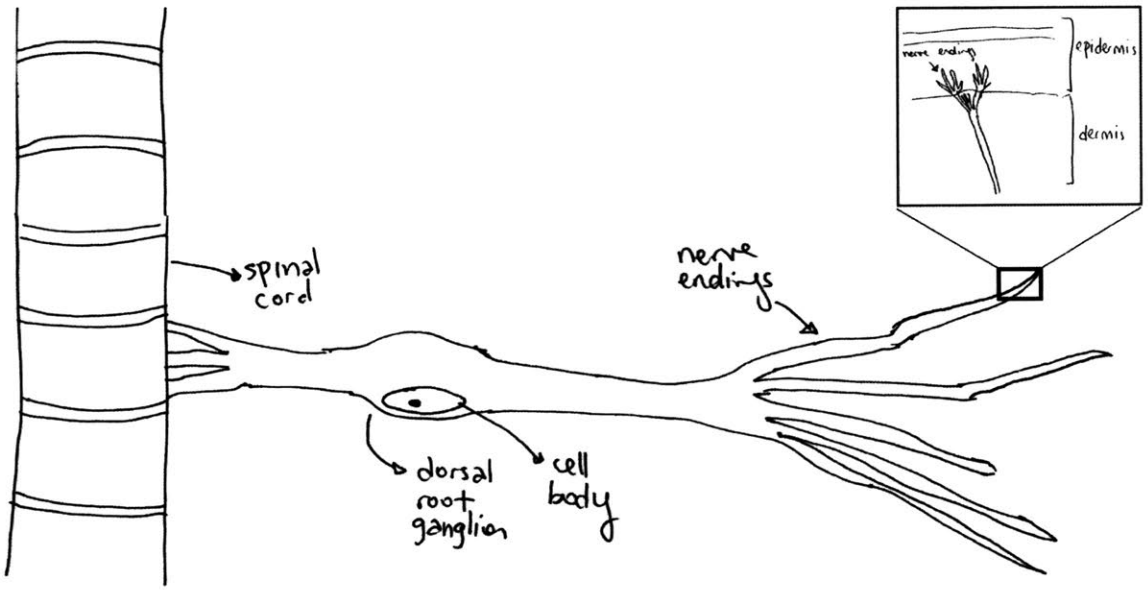

Figure 2: A sketch of the nociceptive pathway of nerve endings

Sodium ion channels were chosen as the area for modification for this project because they are at the very beginning of pain sensation. Sodium ion channels were first discovered in 1952 through Hodgkin and Huxley's voltage clamp technique which elucidated action potentials across the neuronal membranes of giant squid axons (Hodgkin and Huxley, 1952). These foundational experiments showed that sodium ion channels have voltage-dependent activation, rapid inactivation, and selective ion conductance. Sodium ion channels are a family of highly conserved

The family of voltage-gated sodium ion channels have been extensively studied, with all but $Na_v1.5$ being found within the central and peripheral nervous system (Table 1).

Mammalian Sodium Channel α -Subunits			
Type	**Gene Symbol**	**Chromosomal Location**	**Primary Tissue**
$Na_v1.1$	*SCN1a*	Mouse 2 Human 2q24	CNS neurons
$Na_v1.2$	*SCN2a*	Mouse 2 Human 2q23-24	CNS neurons
$Na_v1.3$	*SCN3a*	Mouse 2 Human 2q24	CNS neurons
$Na_v1.4$	*SCN4a*	Mouse 11 Human 17q23-25	SkM
$Na_v1.5$	*SCN5a*	Mouse 9 Human 3p21	Uninnervated SkM, heart
$Na_v1.6$	*SCN8a*	Mouse 15 Human 12q13	CNS neurons
$Na_v1.7$	*SCN9a*	Mouse 2 Human 2q24	PNS neurons
$Na_v1.8$	*SCN10a*	Mouse 9 Human 3p22-24	DRG neurons
$Na_v1.9$	*SCN11a*	Mouse 9 Human 3p21-24	DRG neurons
Na_x	*SCN7a, SCN6a*	Mouse 2 Human 2q21-23	uterus, astrocytes, hypothalamus

Table 1: List of Nav ion channel family. Adapted from (Catterall, 2000)

The structure of the α subunit of these channels is highly conserved, with the proteins forming a selective pore for the sodium ions to travel through. Each of the α subunits are around 260kDa and contain four homologous domains (I-IV) as well as a reentrant loop within the transmembrane, with each domain containing six α-helical transmembrane subunits (S1-6) (Fig. 3) (Catterall, 2000). The fourth subunit (S4) is the voltage sensor, containing hydrophobic amino acids with a positively charged amino acid in every third. There is a P loop between S5 and S6 which acts as the selectivity filter for the ion size, assuring that only the sodium ion enters. Inactivation of the pore is controlled through a loop of hydrophobic amino acids between S6 of domain III and S1 of domain IV, known as the IFM domain. IFM is a reference to the three hydrophobic amino acids that make up the "hinge", isoleucine, phenylalanine, and methionine. This is the tethered "hinge" within the ion channel, allowing voltage-dependent movement through the relative accessibility of key residues.

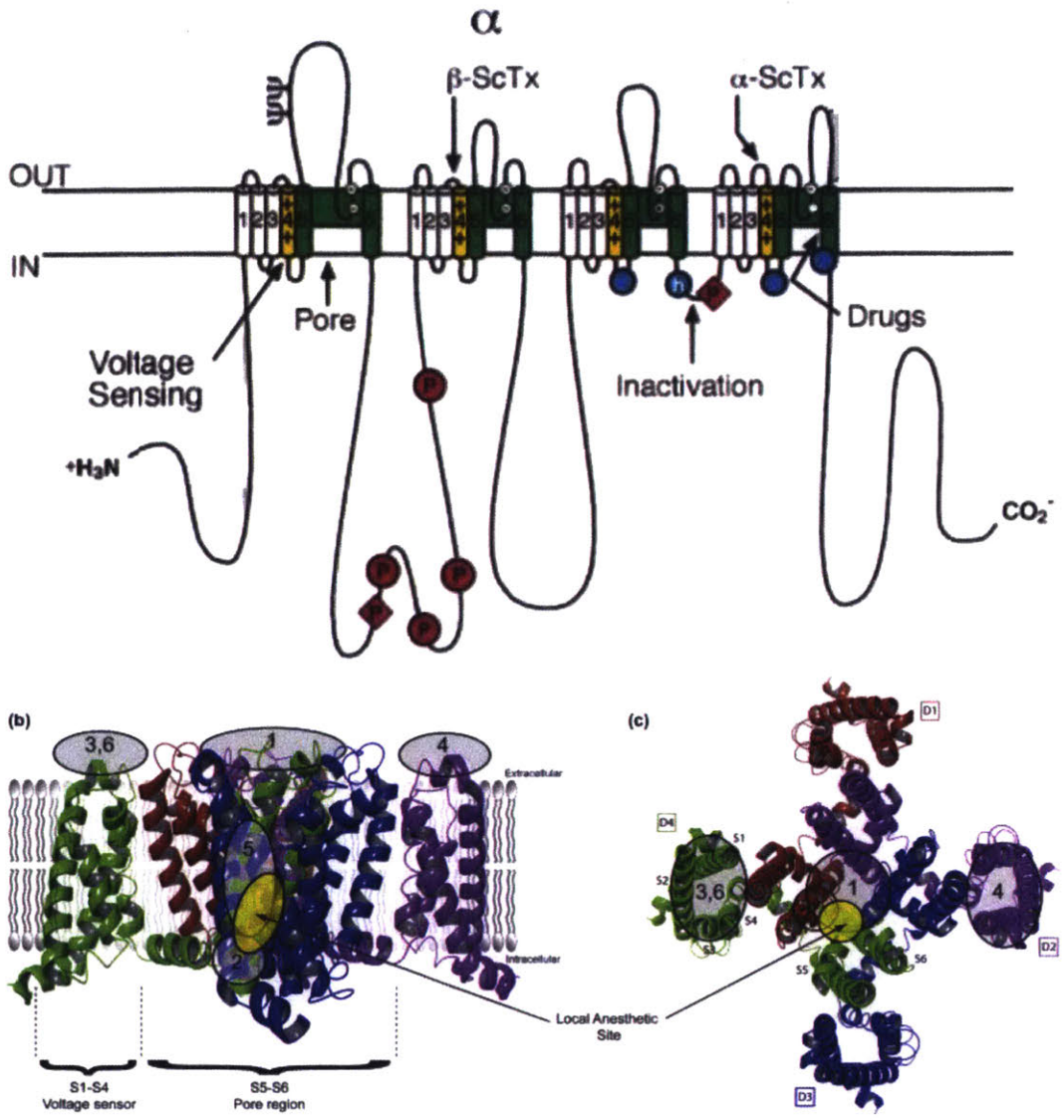

Figure 3: Na_v Channel Structure. a) 2-D structure of the ***α subunit*** *of Na_v ion channels, adapted from Catterall 2000 and b.c) 3-D structure of voltage-gated sodium ion channel cross-section and pore. adapted from and Bagal et al. 2014*

There are many members of the sodium-ion channel family implicated in nociception, but this project is focused on common pain modalities experienced by research animals. Therefore, the three ion channels that will be analyzed for inducible ablation are $Na_v1.7$, $Na_v1.6$, and Na_x (Table 2).

Peripheral Sodium Channels and Pain Pathways		
Pain Modality	**Essential Sodium Channel**	**Peripheral Neuronal Subpopulation**
Classical nociceptive pain pathways		
Acute mechanical pain	$Na_v1.7$	Sensory neurons
Inflammatory hyperalgesia	$Na_v1.7$	Sensory neurons
Nociceptive pain pathways		
Neuropathic cold allodynia	$Na_v1.7$	Sensory neurons
Sympathetically maintained pain	$Na_v1.7$	Sympathetic neurons
Atypical peripheral pain pathways		
Oxaliplatin-evoked allodynia	$Na_v1.6$	A-fiber associated neurons
Cancer-induced bone pain	Na_x	A-fiber associated neurons

Table 2: List of pain types in peripheral nerves. Adapted from Minett et al., 2014

III.I.I Nav1.7

The *SCN9A* gene encodes for the alpha subunit of the $Na_v1.7$ voltage gated ion channel, a pathway shown to be active following initial nociception within sensory and sympathetic neurons as well as in olfactory neurons, pancreatic tissue, and the hypothalamus (Nassar *et al.*, 2004). Initial experiments that induced a global knockout of the gene found that the mice died soon after birth. Upon further investigation, the role of the *SCN9a* within olfactory pathways was implicated as the reason mice died, as destroying the gene from birth resulted in mice that did not feed properly and died early on if not individually fed (Nassar *et al.*, 2004, Gingras *et al.*, 2014).

Subsequent experiments employed localized knockouts in sensory or sensory and sympathetic neurons for a more targeted approach at understanding $Na_v1.7$'s role in pain perception (Minett *et al.*, 2012, 2014, 2015). These

experiments demonstrated that when the ion channel is knocked out in only sensory neural tissue (Advillin), cold and mechanical allodynia pain perception is still present. But when a sensory and sympathetic neural tissue promoter (Wnt1) was used, mechanosensory, inflammatory, thermal, and some neuropathic pain perception are lost (Table 3). This is consistent with the expression of $Na_v1.7$ being in the peripheral nervous system, which encompasses both the sensory and sympathetic neuronal populations.

Distinct Neuronal Subpopulations and Mechanisms Underlying Different Neuropathic Pain Models							
		Chronic Constriction Injury		Spinal Nerve Transection		Oxaliplatin-Induced Pain	
	Deleted from	Cold Allodynia	Mechanical Allodynia	Cold Allodynia	Mechanical Allodynia	Cold Allodynia	Mechanical Allodynia
Nav1.7	Nociceptors	normal	normal	normal	normal	normal	normal
	Sensory Neurons	lost	lost	normal	normal	normal	normal
	Sympathetic and sensory neurons	lost	lost	lost	lost	normal	normal

Table 3: List of pain modes lost in Nav1.7. Adapted from (Minett et al. 2014)

Yet, a *SCN9A* knockout alone does not eliminate all pain states experienced by mouse models used in research. Animals with loss-of-function mutations in the peripheral nervous system are still susceptible to neuropathic pain, such as that caused by oxaliplatin, cancer-associated pain, and acute cold (Minett *et al.,* 2014).

III.I.II Nav1.6

Currently, there is very little understanding of neuropathic pain, and even less about how to mitigate it (Hansson, 2003, Jose L. Ochoa, 2018). *SCN8a,* which encodes for the Nav1.6 sodium ion channel, has been linked to both chronic neuropathic pain and cold sensing in the central and peripheral nervous system (Sittl *et al.,* 2012, Deuis *et al.*, 2013). As with many sodium ion channels, global null mutations were an ineffective method to study this

gene. Global null mutations of the *SCN8a* gene caused ataxia, progressive paralysis, and lethality by three weeks (Meisler *et al.* 2001).

A floxed mouse model of the *SCN8a* gene was then made to further analyze the gene while attempting to avoid the lethality associated with a null mutation from birth (Levin and Meisler, 2004). The tissue-specific knockout was done in cerebellar purkinje neurons and granule cells (Levin *et al.*, 2006). It is clear through these experiments that the floxed allele has mitigated the lethality of the global mutation while allowing tissue-specific knock-out analysis to be done. There has yet to be a published model showing a conditional knockout of the *SCN8a* in either sensory or peripheral neurons. As seen with *SCN9a*, there may be different pain types that are disrupted with *SCN8a*, so tissue-specific promoters for both sensory or peripheral neurons will be analyzed.

III.I.III Na_x

Na_x is an atypical sodium channel that is involved in osmoregulation through extracellular sodium sensing (Gorter *et al.* 2010). Expression within the nervous system has been identified in in the Dorsal Root Ganglia astrocytes, Schwann cells as well as the hypothalamus (Watanabe *et al.* 2002, García-Villegas *et al.* 2009). While little is known about this gene, RNAi knockdown experiments have demonstrated that reducing SCN7a/Nax expression has reversed some of the bone-cancer induced neuropathic pain (Ke *et al.* 2012). There has yet to be a published floxed model of *SCN7a,* or much analysis on tissue-specific knock-outs thereof. Oncological studies are a major source of animal suffering, which puts blocking pain resulting from tumor growth and chemotherapy-induced neuropathy as a high priority.

There is much to be learned about attempting inducible tissue-specific disruptions of *SCN9a*, *SCN8a*, *SCN7a* individually, particularly for further elucidation on chronic and cancer-induced neuropathic pain as well as crossing those that show promise to each other, could begin to address the multiple chronic pain types that arise in animal models during experimentation.

III.II Tissue-Specific Expression

When designing a transgenic system that requires precise localization and expression of a protein, the key is finding the right promoter. This is important primarily to assure that your protein of interest is restricted to be expressed in the correct tissue. Additionally, it is important for the level of expression to be appropriate to the protein that you want to be expressing in a given region. This section will discuss the rationale behind the tissue-specific promoters that were chosen for this project as it pertains to the desired recombinase and nuclease expression.

While many of these nociceptor genes are primarily expressed in sensory cells, they serve other functions as well. The peripheral nervous system is the area of broad interest, as it is where the nociceptors are primarily localized. But within the peripheral nervous system there are different populations of neuronal cells that can be targeted.

III.II.I *Advillin* Promoter

The standard promoter that had been used within the field of nociception has been the *Advillin* promoter (Hasegawa *et al.* 2007). *Advillin* is a gene found primarily in peripheral sensory neurons and is a member of the gelsolin/villin family of actin regulatory proteins (Marks *et al.* 1998). As seen in the previous section, many of the Na_v channels are expressed within DRG populations, which is where sensory neurons can be found. Therefore, it is a good strategy to study a tissue-specific knock-out of a Na_v channel using the *Advillin* sensory neuron-restricted promoter.

Furthermore, an inducible Advillin Cre-ERT2 system has already been made and characterized (Lau *et al.* 2011). This model can be purchased and mated with the floxed mouse models being developed to understand the pain phenotype associated with the *SCN8a* and *SCN7a* genes, neither of which have any published results using this promoter.

III.II.II *Wnt1* Promoter

The *Wnt* family of genes are well characterized for their expression within

the brain and nervous system (Fig.4). Specific attention has been paid to its uses within understanding brain development, including midbrain development and neural crest migration. The *Wnt1* gene in particular has a consistent expression throughout development, so a *Wnt1*-Cre mouse model was made in the 1990's to be mated with a *lacZ* reporter strain. (Danielian *et al.* 1997).

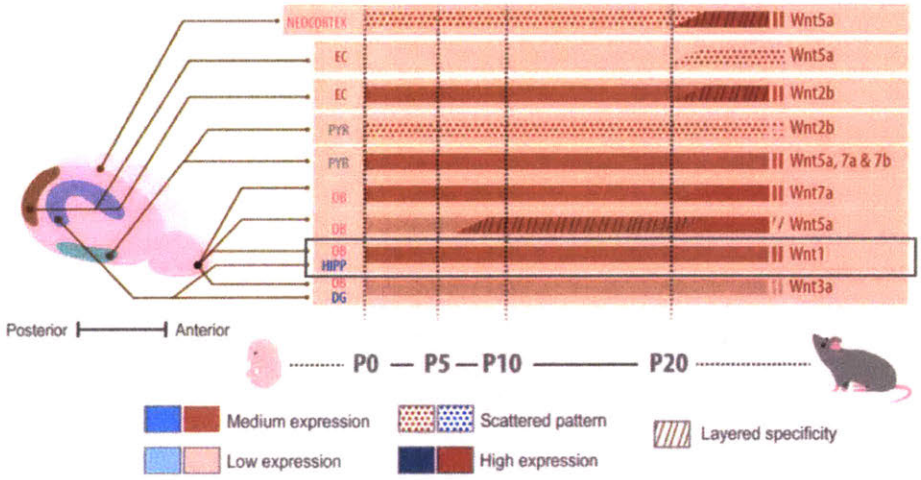

Figure 4: Wnt expression patterns. Adapted from (Oliva, Montecinos-Oliva, and Inestrosa 2018)

Unfortunately, ectopic expression of the Cre made it an unattractive option for use because of midbrain enlargement. The model was successfully modified to limit the ectopic expression found within the system (Lewis *et al.* 2013, 1). This promoter has been shown to give the most robust ablation of pain perception for the *SCN9a* floxed models. Because *SCN8a* is restricted to the CNS neurons, it will be interesting to see whether there is a difference in the pain using the *Wnt1* model in comparison to trying a promoter restricted to the CNS. Similarly, *SCN7a* has not been selectively knocked out in different neuronal populations. Therefore, it will be interesting to see how the addition of sympathetic neuronal expression will impact the pain perception of the mouse model.

"...endless forms most beautiful and most wonderful have been, and are being evolved"

-Preface, *The Origin of Species*, Charles Darwin

IV. Inducible Pain-Free Mouse Model

When designing this system, it is important to note that this was not intended to be novel in technique, but in intent. The experiments done in the Wood Lab were an important foundation used to steer the direction of the design of this project. Studies have shown that sensory and sympathetic pain from birth has impacts on the general well-being of animals due to a failure to learn self-preservation behaviors. For example, humans born without functional *SCN9A* often incur injuries such as broken bones and chewed tongues due to the failure to acquire pain avoidance strategies early in life (Weiss *et al.*, 2011) (Drenth and Waxman, 2007). Therefore, we designed the system so that mice will be raised with pain perception intact to learn pain avoidance and decrease chances of accidental self-inflicted injuries. Then, before with pain perception ideally eliminated just before the mice are scheduled for an experiment.

When considering which inducer to use for the system, it was important to consider how it will impact the system's overall usability as a research model. A promoter induced by a tetracycline-class molecule seemed to be the ideal promoter system, because there are a few tetracycline-class molecules that have been shown to be biologically inert, including anhydrotetracycline and 4-epidoxycycline(Nelis and De Leenheer, 1981). This assures that induction of the system will had minimal impact on experimental conditions and results. However, some reports have indicated that induction with tetracycline-class promoters does not function in all neurons due to silencing of the Tet-responsive element promoter (Qin *et al.*, 2010).

Consequently, an orthogonal system proven to be effectively expressed within neurons was developed as well. 4-hydrotamoxifen class promoters have already been published to function within sympathetic and sensory neurons (Minett *et al.* 2012). Because this is a hormone-associated pathway, it might not be best for all research types. The hope was that between the two systems there would be sufficient options for researchers to try.

Finally, considering conditions associated with animal experimentation, is important to consider the genetic background of the system in order to assure that the mouse will be useful for a broad range of research uses. C57Bl/6 mice have been estimated to comprise ~50-80% of all mice used in medical research and are reportedly unusually sensitive to pain while being resistant to common analgesics (Mogil *et al.*, 1999). Therefore, we chose this genetic background for the initial inducible pain-free system design ("C57BL/6", 2016).

V.I Early Designs

The system was initially designed to be made via embryonic stem cell selection. Three different system designs were considered for gene disruption in an attempt to understand which model would provide the best special and temporal control of the gene disruption. I will describe these initial designs because they were not abandoned due to flaws in the system, but due to switching methods of integration and simplifying experimentation.

V.I.I Cre-ERT2 Inducible Mouse

This mouse model will be identical to previous Cre-based SCN9A knockout lines save that Cre will be inducible in *Wnt1*-expressing cells upon delivering a 4-hydrotamoxifan class molecule rather than expressed in the peripheral nervous system from birth (Minett *et al.* 2012, 2015). The DNA cassette was designed with modified lox sites to be integrated into the HPRT locus, a common integration site for ES cell selection, using a modified targeting vector with complimentary lox sites. The system is designed such that once the Wnt1-CreERT2 in integrated, the lox sites will be dead to assure that any future Cre expression will not impact the cassette (Fig. 5).

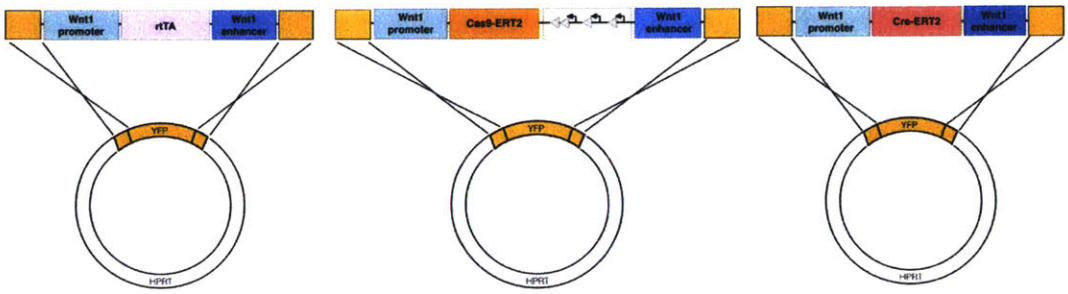

Figure 5: HPRT Integration. A depiction of the Wnt1 cassettes integrating into the modified HPRT locus targeting vector via lox sites

To maximize efficiency, loxP sites will be inserted around exons 14 and 15 of the mouse *SCN9A* gene encoding the Nav1.7 ion channel. Single-guide RNAs (sgRNAs) will direct Cas9 to cut upstream of the targeted exons, leading to insertion of a repair template with the floxed exons and an FRT-site flanked neo/kan selectable marker (Fig. 6). After selection, the neo/kan selectable marker will then be removed using FLP recombinase(Buchholz, Angrand, and Stewart 1998) A similar approach will be employed to flox key exons in *SCN7a* and *SCN8a*.

After cells have gone through rounds of selection for integration of both the Cre recombinase and the floxed exons, induction of the generated mice by a 4-hydrotamoxifan class molecule should result in Cre activity and initiate the gene knockouts in both sensory and sympathetic neurons (Fig.7).

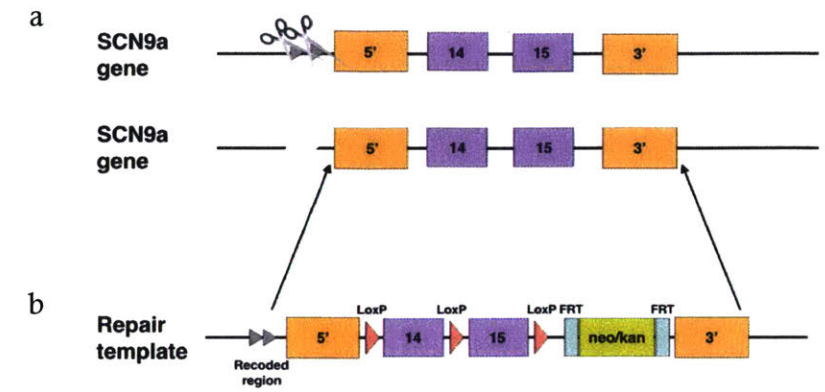

Figure 6: Floxed SCN9a. a) The SCN9A gene will be recoded using CRISPR/Cas9 gene editing to place lox sites around exons 14,15, and 16 as well as a neo/kan selectable marker for east of ES cell selection. b) After the cells are selected for the floxed exon repair template, the neo/kan selectable marker will be removed using a flippase.

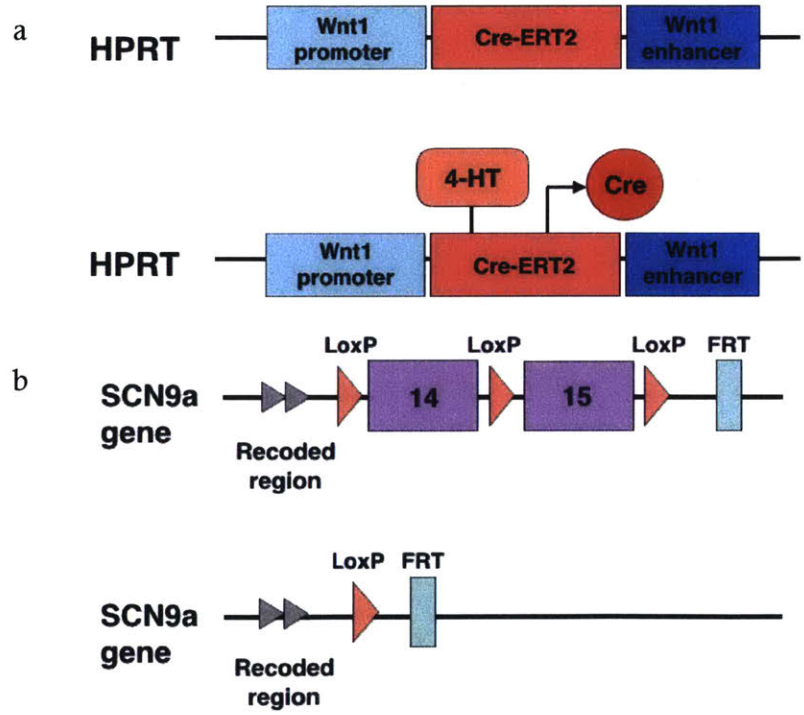

Figure 7: Cre ERT2. a) The Cre-ERT2 construct within the tissue-specific Wnt1 promoter incorporated into the HPRT locus b) Once 4-hydrotamoxifenis introduced into the system, Cre recombinase will be expressed in peripheral nervous tissue and will excise exons of the SCN9A gene.

V.I.II Cas9-ERT2 Inducible Mouse

This mouse model will employ the Cas9 endonuclease and an array of CRISPR sgRNAs targeting key exons within *SCN9a* under the control of a ERT2 inducer within *Wnt1*-expressing cells to disrupt target genes in a tissue-specific manner. The cassette will be integrated into the HPRT locus using the same modified targeting vector discussed previously. (Fig. 8a).

Once integrated, induction of the generated mice by a 4-hydrotamoxifan class molecule should result in expression of the Cas9 endonuclease which will complex with expressed sgRNAs and direct *SCN9a* gene ablation in both sensory and sympathetic neurons of mice (Fig. 8b).

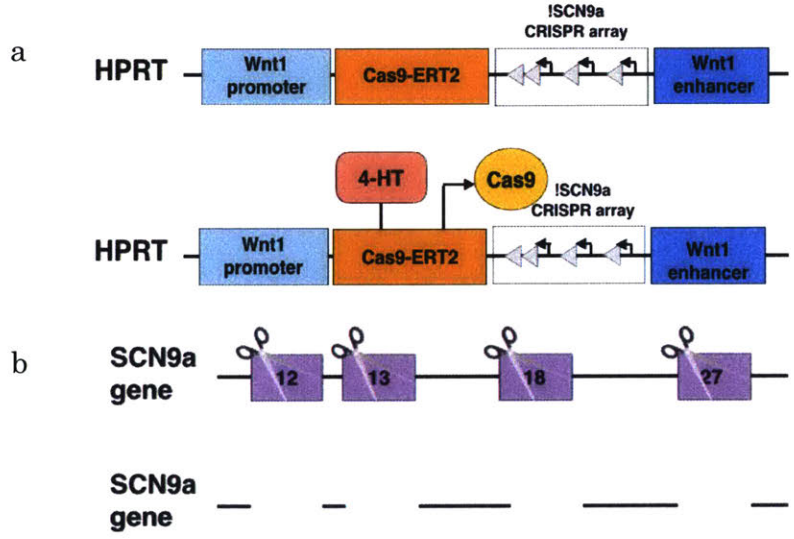

Figure 8: Cas9-ERT2. a) The Cas9-ERT2 construct and anti-SCN9A array within the tissue-specific Wnt1 promoter incorporated into the HPRT locus b) Once 4-hydrotamoxifenis introduced into the system, Cas9 endonuclease and sgRNAs will be expressed in peripheral nervous tissue and will cut key exons in SCN9A.

V.I.III Cas9-Tetracyclin Inducible Mouse

Similar to the previous model, this mouse model will employ a Cas9 endonuclease and the same CRISPR sgRNA array targeting *SCN9a* exons to ablate the target genes. The difference lies in the Cas9 and method of activation, with this system employing a split Cas9 where the N and C terminus will be separately expressing and self-assemble upon activation of a tetracycline-responsive activator (Zetsche, Volz, and Zhang 2015).

A TRE3G-bidirectional promoter along with the *SCN9a* CRISPR array and an FRT-flanked neo/kan selectable marker will be inserted in the ROSA26 locus. This will be done using a modified targeting vector similar to what was designed for the HPRT locus, which has previously been used for tet-dependent gene expression (Rideout *et al.* 2000). The reverse tetracycline-controlled transactivator (rtTA) required for TRE3G expression in the presence of tetracycline will be placed under the *Wnt1* promoter along with the CRISPR array using the modified HPRT targeting vector (Fig. 9a).

Once integrated, induction of the system with a doxycycline-class compound is fed to the mouse, rtTA expression will activate the TRE3G promoter which will express both halves of the Cas9 endonuclease. These will self-assemble into an active nuclease, localize with sgRNAs, and direct *SCN9a* gene ablation in both sensory and sympathetic neurons of mice (Fig. 9b).

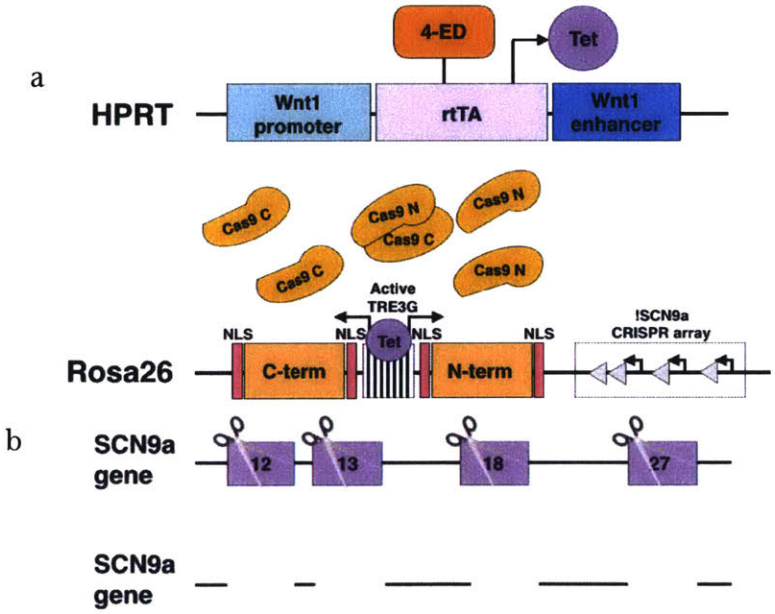

Figure 9: Split-Cas rtTA. a) The rtTA construct within the tissue-specific Wnt1 promoter incorporated into the HPRT locus b) The split Cas system under TRE3G bidirectional tet promoter with the anti-SCN9A array and a neo/kan selectable marker flanked by FRT sites incorporated into the ROSA26 locus c) Once 4-hydrotamoxifenis introduced into the system, Cas9 endonuclease and sgRNAs will be expressed in peripheral nervous tissue and will cut the exons of the SCN9A gene and break the Nav1.7 ion channel.

V.II Final Designs

Upon further consideration of the goals of the project, we decided that using CRISPR gene editing for integrating of the DNA constructs into mice through pronuclear injection (PNI) over embryonic stem cells selection methods (Horii *et al.* 2014) (Fig.10). In a CRISPR PNI, a fertilized zygote is isolated before the first genetic doubling and microinjected with a cocktail of endonuclease pre-complexed with the RNA guide as well as the repair template. This switch in methods allowed for less time lost in breeding.

With ES cell selection, most integrations will produce mosaic animals due to cell cleavage. This requires more breeding to get pure mice and also creates a risk of an integration that is not passed to the germ cells. However, there is typically no instances of mosaicism being found for CRISPR PNI when pre-complexed endonuclease injected. Therefore, all founders that have the correct integration will be ready to be bred with the additional integrations needed for the system to function.

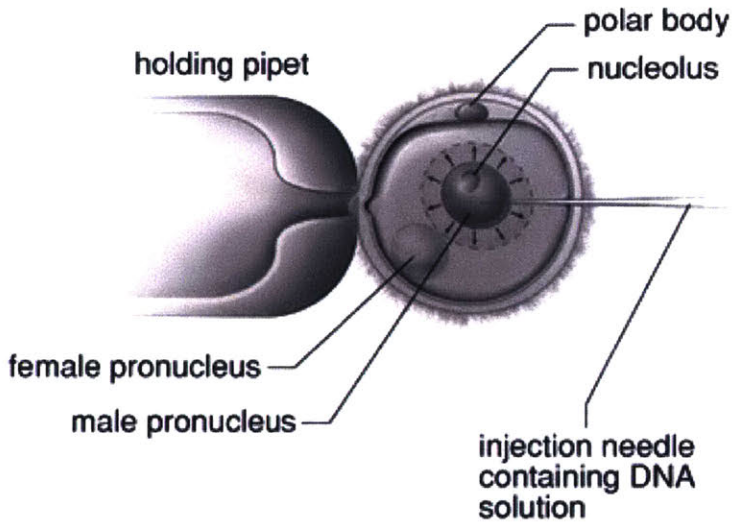

Figure 10: Pronuclear Injection ("Wiley Press Images. 2012")

After we decided to switch to a CRISPR PNI procedure, the DNA construct designs needed modification to accommodate this new method. Firstly, there was no antibiotic selection needed for this method. Secondly, designs were

modified to give asymmetric homology to the homology arm, which has been shown to increase the chances of proper integration of the construct (Wang *et al.* 2018). Because each mouse line would need to be made, genotyped, and bred, we decided that making the three designs discussed above in parallel would be difficult.

We chose the Cre-ERT2 inducible mouse with floxed exons as the first model to be made, because the *Wnt1* promoter has already been proven to work in an non-inducible fashion and the ERT2 inducer has been proven to work in a sensory neuron-restricted context (Lau *et al.* 2011, Minett *et al.* 2012). We will therefore develop a *Wnt1* Cre-ERT2 mouse strain and floxed strains of mice for all of the sodium ion channels of interest. When the system is induced through a 4-HT class molecule, the floxed genes will be excised, which will cause a loss-of-function mutation for the gene going forward (Fig.11).

The Cas9-ERT2 and Split Cas9 mice will be designed and produced, in that order, if the Cre-ERT2 mouse does not produce the desired pain-free phenotype. Primers and full plasmid sequences the final designs can be found in Appendix I.

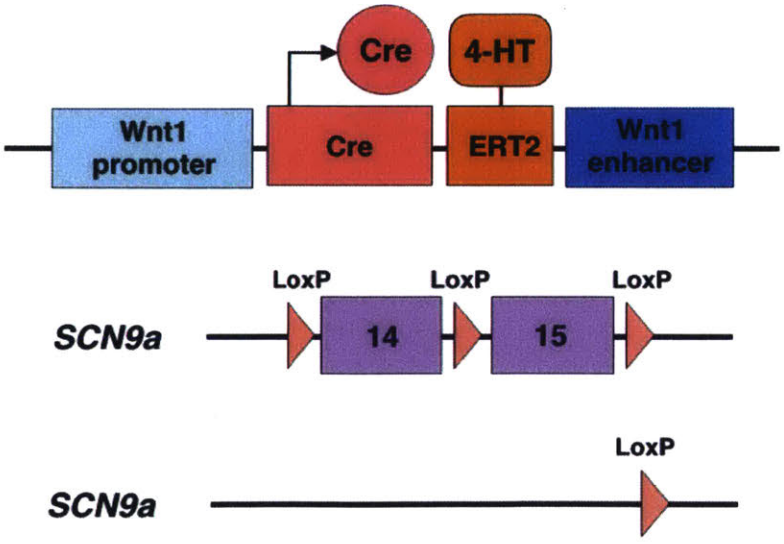

Figure 11: Wnt1 Cre-ERT2 system. When a 4HT-class molecule is fed to the mouse (top), it will induce a recombination at the lox sites which will delete exons 14 and 15 from the gene (bottom).

V.II.I Wnt1 Cre-ERT2 Construct

Once we decided on using CRISPR PNI, the initial *Wnt1* Cre-ERT2 construct design was modified because there is an existing mouse model with Cre recombinase under the Wnt1 promoter (Lewis *et al.* 2013). This mouse model is kept in the Jackson Laboratory mouse facility under B6 Wnt1-Cre2 (Jax #022501).

The exact sequence of the initial Cre recombinase construct was obtained from Prof. Jeffrey Bush to be ordered as gblocks for homology arms. An ERT2 sequence was isolated from the pCAG Cre-ERT2 plasmid (Addgene #14797) to be added between the Cre recombinase sequence and terminator. The plasmid, named "DM Analgesia," can be seen below, along with images of the PCR products and final assembled plasmid. More information on the design can be seen in Appendix I (Fig.12-13, Table 4).

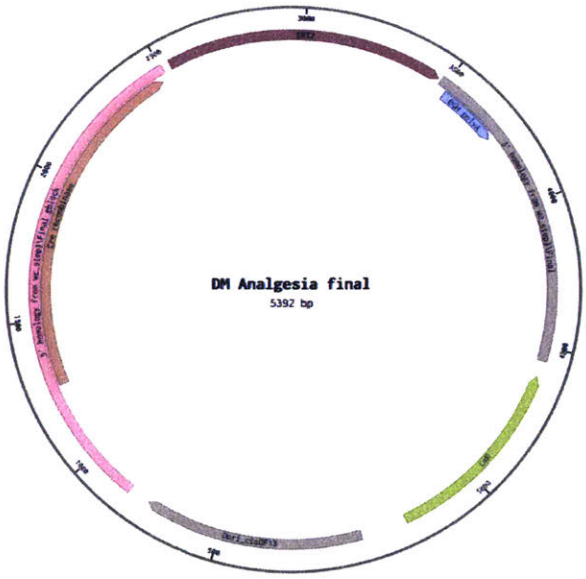

Figure 12: DM Analgesia plasmid design

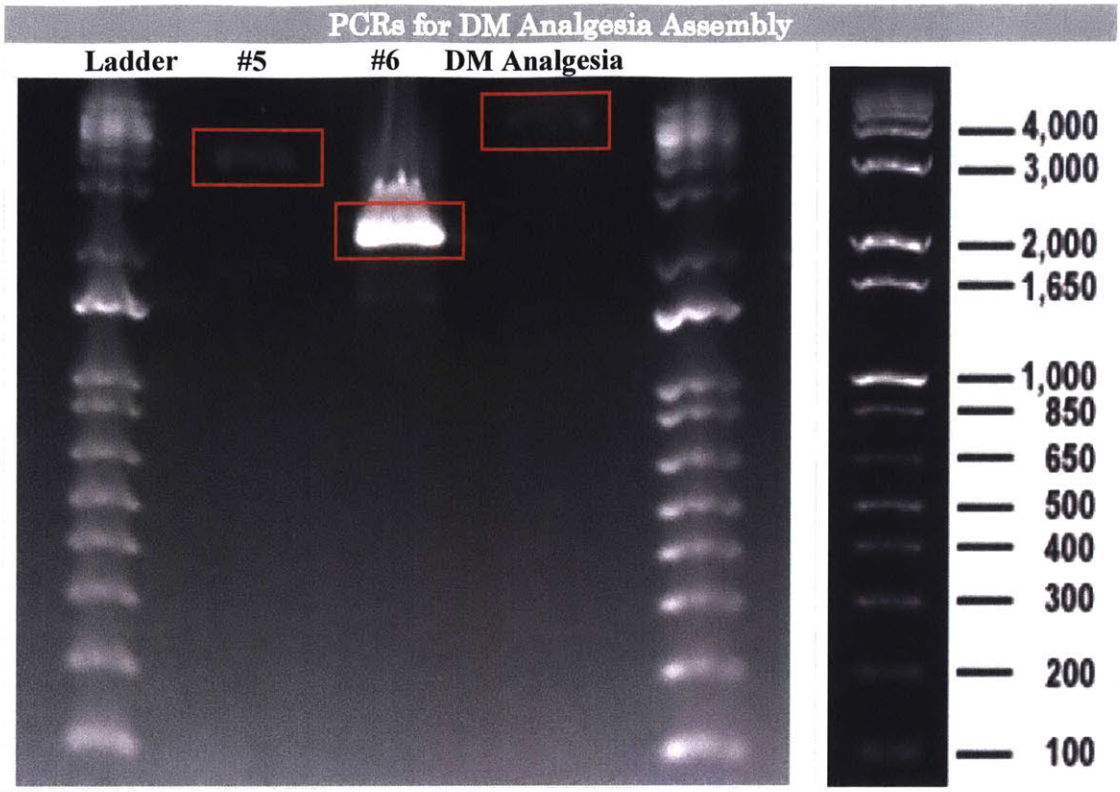

Table 4: DM Analgesia Assembly.

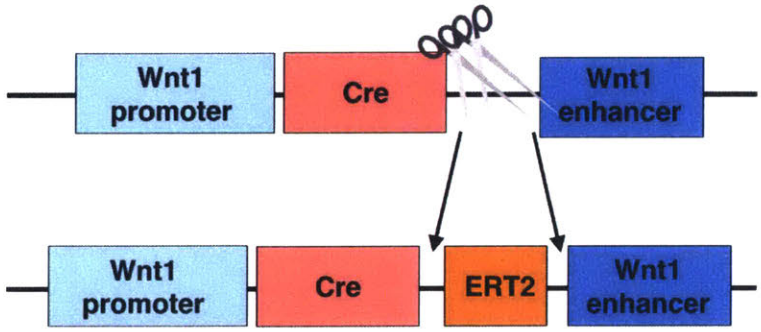

Figure 13: ERT2 insertion. The guides cut between the Cre recombinase and the terminator and the repair template contains the ERT2 addition.

The transgenic facility at Boston Children's Hospital performed the PNI along with four RNA guides pre-complexed with Cas9 before injection for construct insertion. The injection took place on August 13, 2018 and 22 pups were born from the litter. The mice will be genotyped and then bred with the floxed mouse line discussed below.

V.II.II Floxed Ion Channel Constructs

$Na_v1.7$

We designed the floxed *SCN9a* construct to delete exons that are confirmed to disrupt function of the gene. Based on initial work by the Wood lab, we chose to place loxP sites around exon 14 and 15 (Minett *et al.* 2012). Lox sites were place far enough from the spice regions within the introns so as to not disturb function of the gene before the system is induced. We used mouse genomic DNA from a C57Bl/6 background to design this constructs and lox sites were added with primers.

The transgenic facility at Boston Children's Hospital performed the PNI along with four RNA guides pre-complexed with Cas9 before injection for construct insertion. The injection took place on May 25, 2018 and 31 pups were born from the litter. None of the mice were confirmed to have integrated the template with the lox sites. Another guide has been ordered and another round of CRISPR PNI is scheduled for the near future.

The plasmid, "floxed SCN9a," can be seen below, along with images of the PCR products and final assembled plasmid. More information on the design can be seen in Appendix I (Fig.14-15, Table 5).

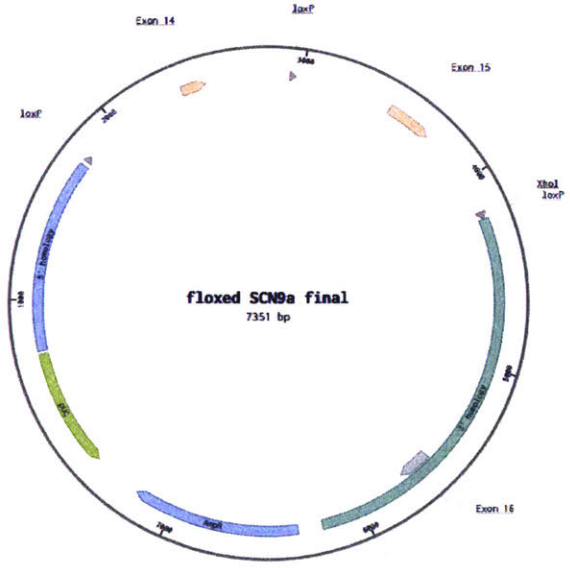

Figure 14: floxed SCN9a plasmid design

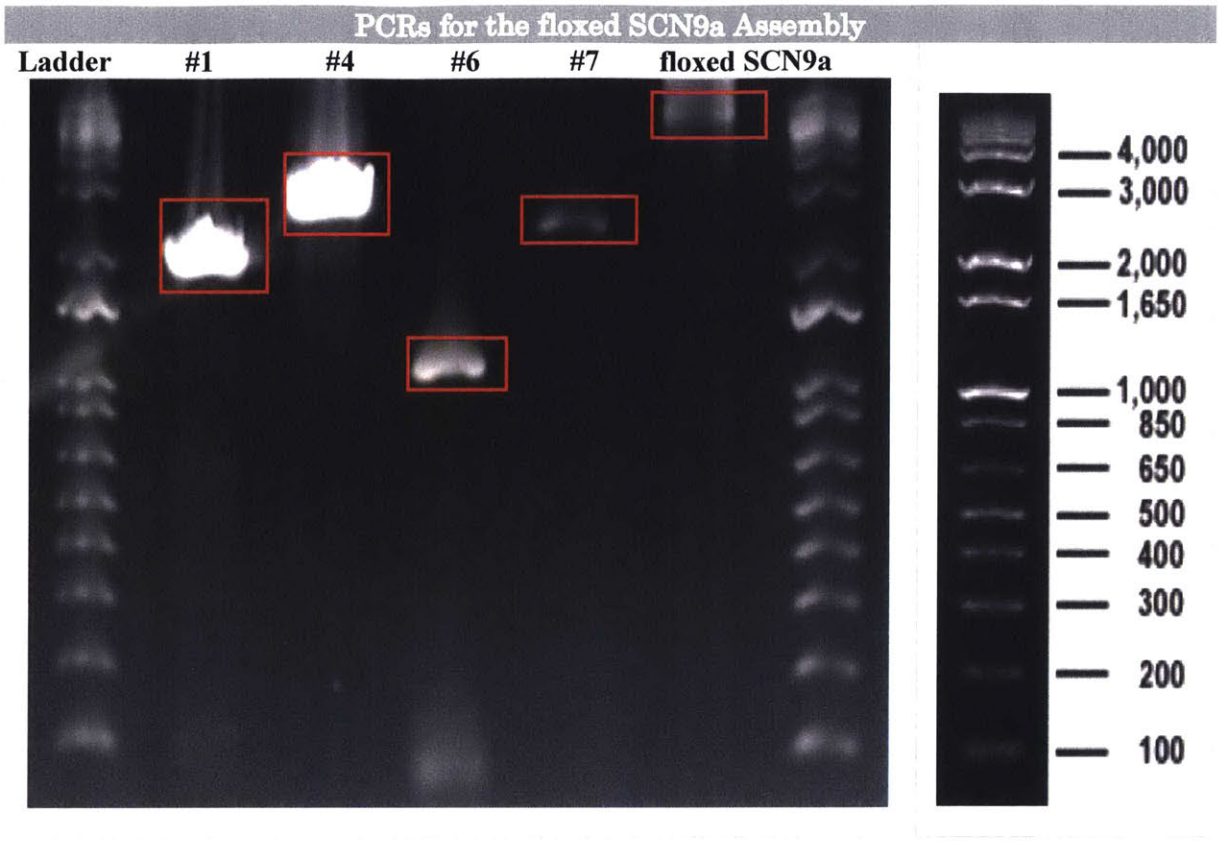

Table 5:Floxed SCN9a Assembly.

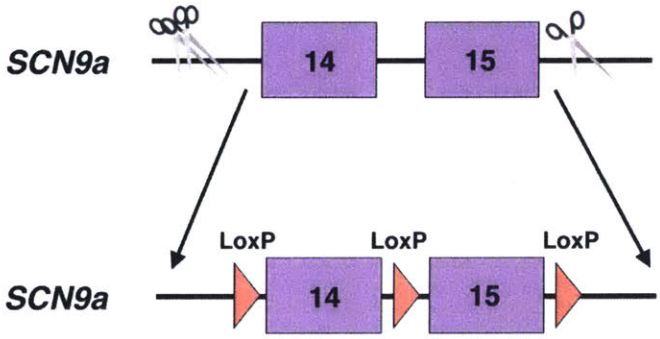

Figure 15: SCN9a loxP insertion. The guides cut proceeding exon 14 and following exon 15, with the repair template containing the loxP sites

$Na_v1.6$

We had made preliminary designs to make a floxed mouse for *SCN8a* encoding for the $Na_v1.6$ ion channel. But upon further investigation we discovered that the Meisler lab still has a colony of the floxed *SCN8a* mouse that they generated for their studies (Levin and Meisler 2004). We hope to acquire these mice upon further discussions with the lab to mate with the inducible Cre strains.

Na_x

There is no floxed model for Na_x that could be found. Therefore, similar to *SCN9a*, we designed placed lox sites far enough from the spice regions within the introns so as to not disturb function of the gene before the system is induced. We used mouse genomic DNA from a C57Bl/6 background to design this constructs and lox sites were added with primers.

The transgenic facility at Boston Children's Hospital has received the guides and plasmid for this construct and the CRISPR PNI is scheduled for the near future.

The plasmid, "floxed SCN7a," can be seen below, along with images of the PCR products. More information on the design can be seen in Appendix I (Fig.16-17, Table 6).

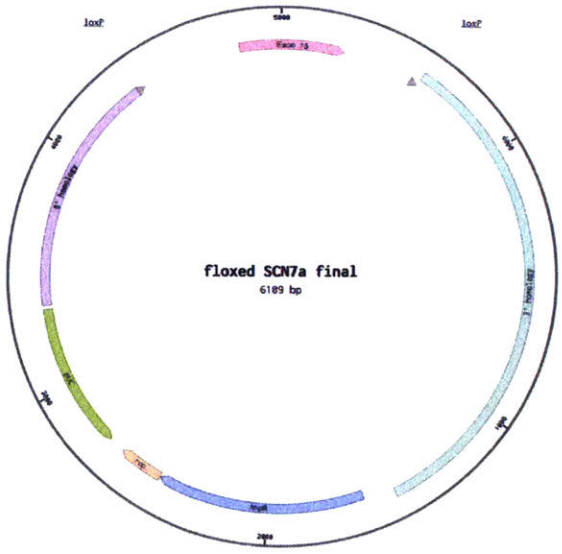

Figure 16: floxed SCN7a plasmid design

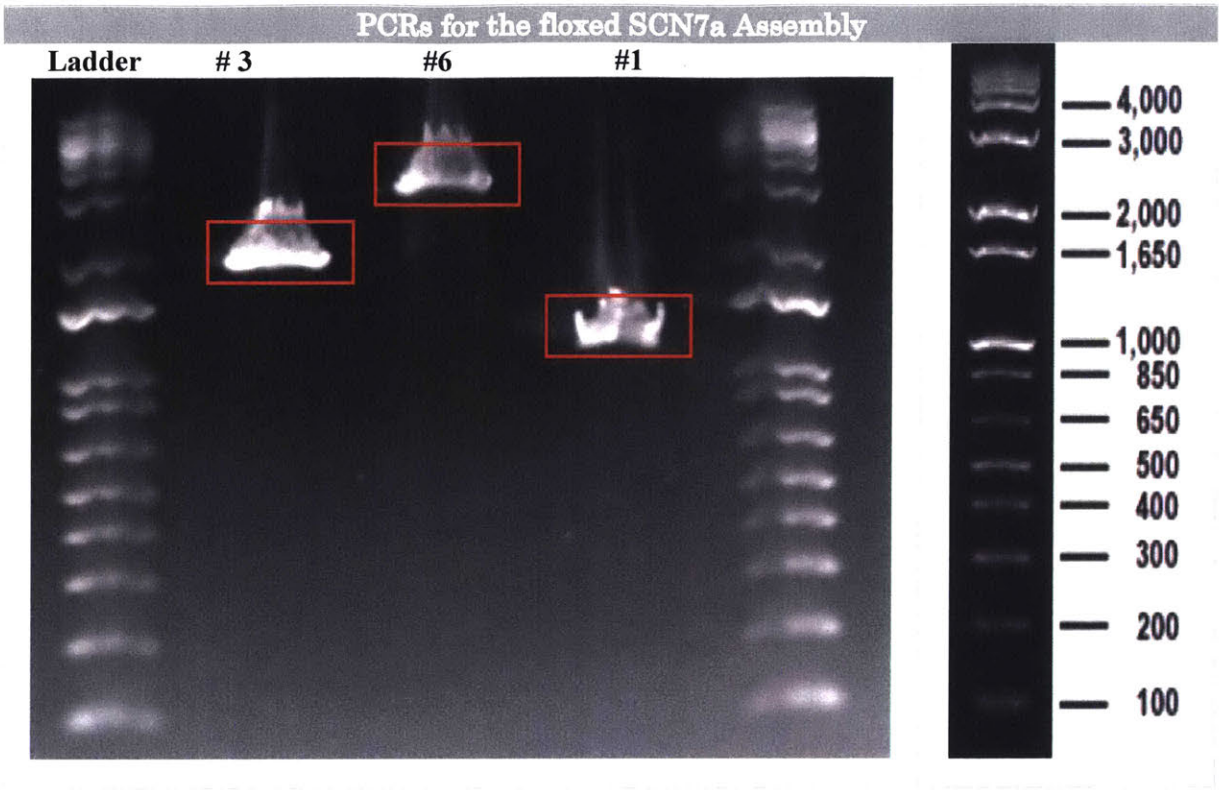

Table 6:Floxed SCN7a Assembly.

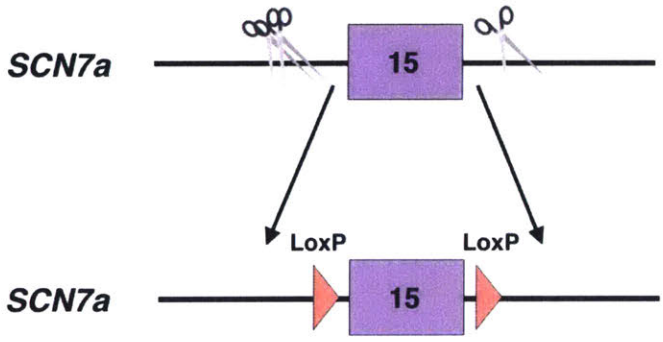

Figure 17: SCN7a loxP insertion. The guides cut proceeding and following exon 15, with the repair template containing the loxP sites

"This subtle matching of procedure to species, and species in turn to objectives, is more significant than appears at first sight for the humanity of technique."

- *The Principles of Humane Experimental Technique*, W.M.S. Russell and R.L. Burch

V. IACUC Survey

To better understand the broader bioethical implications of the pain-free mouse system, as well as its potential adoption by the research community, we designed a survey for IACUC (The Institutional Animal Care and Use Committee) members around the country. The survey seeks to clarify how the framework established by the USDA and OLAW impacts the decisions of IACUC members when approving an animal protocol. There was particular emphasis on understanding the appropriate pain category the animal would be placed in when being reported on a protocol. Additionally, we gathered more information on understanding the level of characterization needed on the system such that IACUC members would feel comfortable approving or even recommending it when researchers submit protocols. The full set of questions for the survey along with the survey logic can be found in Appendix II.

VI.I Survey Approval

Given that this survey was intended to be sent to 'human subjects,' it required review by MIT's Institutional Review Board, called the Committee on the Use of Humans as Experimental Subjects (COUHES). Because the survey posed was anonymous and that did not ask any questions that would reveal sensitive material from participants, the survey was submitted to COUHES requesting an exemption. The request was granted under the conditions that survey taker digitally content to participating in the survey and being notified that they can stop the survey at any time without penalty.

VI.II Survey Development

The survey started as a conversation between myself and Prof. Peter Singer in mid-August of 2017. We had discussed the possible negative outcomes that could arise from the inducible pain-free mouse model outlined in this thesis. He had brought up the possibility of this mouse model being used as a loophole by researchers in order to perform painful research that may otherwise not be approved by an IACUC. It is clear that there is a level of interpretation by individual IACUC members in the process of interpreting some of the regulation and recommendations in place (Tannenbaum and Bennett 2015). Therefore, in an effort to explore the possible negative impacts of the model, I created a survey for IACUC members aimed at understanding how the mouse model might be seen within the USDA's regulatory framework as well as the opinion of the members themselves towards the idea in relation to how things are currently being done.

VI.II.I IACUC Metadata

According to the OLAW requirements, there must be at least 5 members on an IACUC committee, with at least one in each of the following categories: veterinarian, practicing scientist that is experienced in animal research, designated non-scientist affiliated with the institution, and non-institutionally affiliated member that represents the community the

institution resides within. But there is currently no public accounting of how many members any given IACUC has.

Similarly, the OLAW provides two different methods for reviewing protocols, full committee review or designated committee review. In the case of a full committee review, every protocol is discussed and decided upon by all of the members at their meetings in the presence of a quorum. In the case of a designated committee review, the protocol is given to a qualified committee member who is designated by the chair to review and decide upon the protocol with the exception that a disapproval would require a full committee review. There is similarly no accounting for which method of review each IACUC implements.

The first part of the survey therefore collected the role of the survey taker, the number of IACUC members on the committee they are affiliated with, and the preferred method of analysis by their committee. Additionally, this is informative material for the IACUC community at large to learn about itself and will be presented back to the community for their use.

VI.II.II Institutional Animal Experimentation and Pain

The next section focused on clarifying the current state of painful animal experimentation within these institutions. If there is not much experimentation that uses Category D or Category E animals, or if the IACUC members feel that pain is being properly managed within the protocols they approve, there is not much grounds with which to propose a new system a problem that does not exist. To review, Category D animals are classified as animals subjected to potentially painful or stressful procedures for which they receive appropriate anesthetics, analgesics and/or tranquilizer drugs, and Category E animals are classified as animal use activities that involve accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used or delayed for scientific purposes. Scientific justification has to be provided.

The questions first probed how many animals per month on protocols submitted to the IACUC were Category D or Category E animals. Additional questions on whether new refinement strategies were needed within experimentation using Category D & E animals as well as whether the survey taker felt that pain was being properly managed in experiments involving Category D animals (the answer is obviously no for Category E animals by definition) was determined.

VI.II.III The Inducible Pain-free System

After determining whether there is a systemic need for new refinement strategies, questions regarding a possible inducible pain-free system were presented. Questions on whether an inducible pain-free system could be considered a "refinement" strategy for animal experimentation, whether the animals would be categorized in the same way that an animal receiving analgesics or anesthetics would be, how many peer reviewed papers on the system would be sufficient for the committee members to feel comfortable approving, recommending, or even requiring the use of the modified animals.

At the end of the survey, a long-form response was available for survey takers to respond with final comments or recommendations regarding the survey and the proposed system.

VI.III Survey Results

Almost all of the questions presented in the survey were multiple choice to minimize the grey areas that form when open responses are collected. I will first present the data from the survey, and then discuss the long-form responses and methods for improvement for the next iteration of the survey and possibly the project as a whole.

There were 48 survey responders, with 43 responding to any question past the initial consent form and about 36 respondents that completed the whole survey. Below is an in-depth analysis of the survey results. The full survey results can be seen in Appendix II.

VI.III.I IACUC Metadata Results

Question 3: Role on IACUC

Of all of the possible roles that the respondents could be, about 35% were IACUC administrators. IACUC Administrators are not part of the IACUC committee, they are involved in most aspects of IACUC protocol review and approval, and are therefore a huge resource of information with regard to IACUC statistics and committee member sentiments. About equal numbers of those who identify as the Veterinarian or Practicing scientist responded to the quiz, each making up about 20% of the respondents. Chairpersons made up about 14% of respondents. The "Other" category had four responses, one who identified as "Statistician", one as "Compliance", one as "Coordinator," and the final one as "and chair."

Question 3: Role on IACUC	
Answer	%
Veterinarian	20.93%
Non-scientist	2.33%
Non-affiliated member (representing community)	0.00%
Practicing scientist experienced in animal research	18.60%
Chairperson	13.95%
Administrator	34.88%
Other	9.30%

Table 7: Response to Role within the IACUC

Question 4: IACUC size

There was a range from 5-30 members on an IACUC reported, with an average number of members per committee found to be 13 members.

Question 5: Protocol Review Method

In response to the question on the preferred method of protocol review, it is about split between those who use both methods and those who mainly or only choose one. Full committee review seems to be preferred over designated member review, with further analysis showing that designated member review or both methods being more likely to be chosen by a respondent whose IACUC has an above average number of members.

Question 5: Protocol Review Method	
Answer	%
Designated Member Review (mainly or only)	20.93%
Full Committee Review (mainly or only)	32.56%
Both depending on circumstances	46.51%

Table 8: Method of protocol review

VI.III.II Institutional Animal Experimentation and Pain Results

Question 6 and 7:

In response to the questions regarding the number of Category D and E animals investigators submit for review in their experimental protocols, it is clear that protocols involving category D animals are very common. Almost half of respondents indicated that somewhere between 60% to over 80% of protocols they receive involve category D animals, meaning the vast majority of all animal experiments run involve some painful event that the investigator is typically required to mitigate with analgesics or anesthetics. There was a comparative lack in proposed Category E animals used. Over two thirds of respondents indicated that less than 20% of protocols involve category E animals. This is unsurprising, because experiments that are approved with Category E animals typically require extensive reasoning for their pain category choice due of the confirmed pain and distressed the animal will experience. But it should be emphasized that even a few animals in Category E is too many. If there is a method of recapitulating the phenotype needed during painful experiments, typically inflammatory,

without needing the animal to be experiencing the full extent of the pain then steps should be taken to reach that.

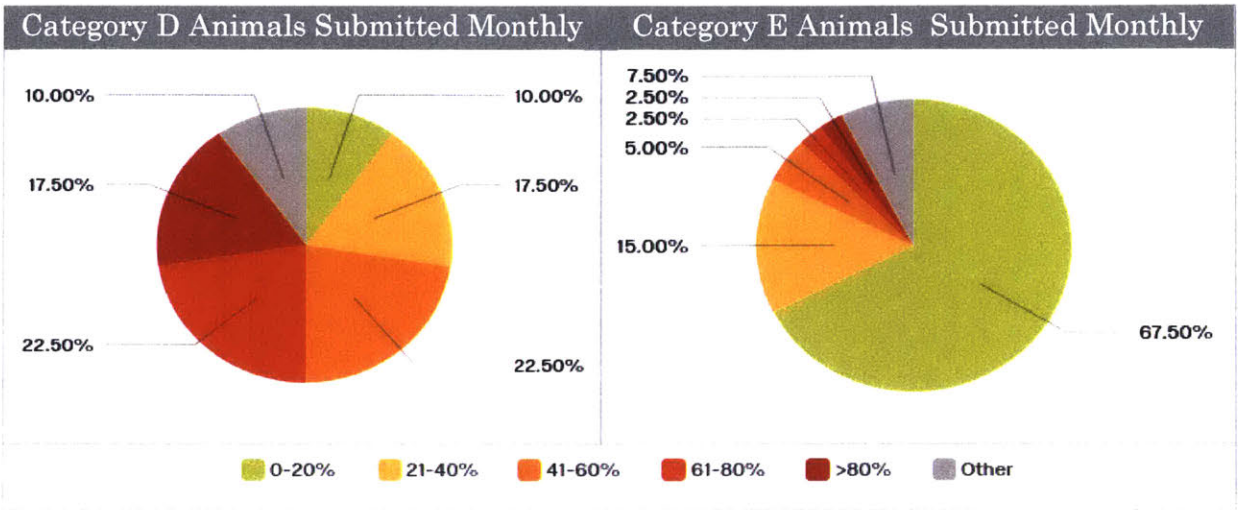

Table 9: Monthly use of Category D & E animals

Question 8:

In response to the question regarding whether there is a need for further refinement within experimentation around Category D and E animals, 80% of respondents agreed. It is a regulatory requirement for IACUCs to find ways to avoid experimentation on animals as well as more refined experimentation of the experiments cannot be avoided, but it is nonetheless an important confirmation that there is always further work that needs to be done within experimental refinement as long as animal experimentation continues.

Question 8: Further refinement needed in Category D&E animal research	
Answer	**%**
Yes	79.49%
No	12.82%
Other	7.69%

Table 10: Further refinement needed in research

Question 9:

In response to whether the respondent feels there is effective main management in the experiments being done under their IACUC, there was a mixed response. About two thirds of the respondents indicated that the issue of pain is moderate, with the intention of controlling the pain as best as institutionally possible. It is important to note that almost one third of respondents felt that the issue of pain was well managed, while about 12% responded that there was no need for further refinement.

Question 9: Rating animal pain in Category D animal experiments institutionally	
Answer	**%**
Low (Either procedures are not painful or the pain is well controlled.)	32.43%
Moderate (Painful procedures are limited, and pain is controlled the best we can, but animals do sometimes experience some pain.)	62.16%
High (Procedures are very painful and controlling pain is a challenge.)	5.41%

Table 11: Animal pain in Category D animals

It is interesting to note that 12 respondents chose that pain was well managed in their institution, only 5 responded that they did not feel there was a need for refinement within Category D&E research. It is clear from this that even if pain management seems to be well established, highlighting that there is always a need for further refinement within experimental practices.

VI.III.III The Inducible Pain-free System Results

Question 10:

In response to whether an inducible pain-free mouse model would be considered a method of "refinement" within the research landscape, most respondents agreed that it would be an appropriate method of refinement.

This indicates that the proposed mouse model would be seen by IACUCs as an improvement in experimental technique with regards to animal welfare.

Question 10: Inducible Pain-Free as a refinement strategy	
Answer	%
Yes	86.11%
No	13.89%
Total	100%

Table 12: Inducible pain-free mouse as refinement

Question 11

In response to whether the mouse model would be classified similar to an analgesic or anesthetic, almost three quarters of respondents said that they would classify it similarly. Within the free response section there were respondents that noted that they would have given a different response had the question be worded to compare it only to an analgesic, not an anesthetic. Anesthesia is used as a method of animal restraint and therefore the pain-free model would not be sufficient in replacing anesthesia. It is possible there were other respondents who felt similarly.

Question 11: Inducible Pain-free animal classified as an analgesic	
Answer	**%**
Yes	73.53%
No	26.47%
Total	100%

Table 13: Inducible pain-free mouse as an analgesic

Question 12-14

In response to whether they would be willing to approve the use of Category D and Category E animals, it seems that preliminarily almost 90% of respondents would approve Category D and over 75% would approve the use

of Category E experiments. This is useful to consider when designing the initial characterization of the system.

Question 12: Approve Category D use for model validation		Question 13: Approve Category E use for model validation	
Answer	**%**	**Answer**	**%**
Yes	88.89%	Yes	77.14%
No	11.11%	No	22.86%
Total	100%	Total	100%

Table 14: Using Category D & E animals for model validation

In response to how many peer-reviewed publications the respondents would like to review in order to feel comfortable approving the model being used for a similar application, a bit over half indicated that 5+ papers would be required. This too is important to consider when designing the tests for characterizing relevant pain modes that may be be best suited for this mouse model.

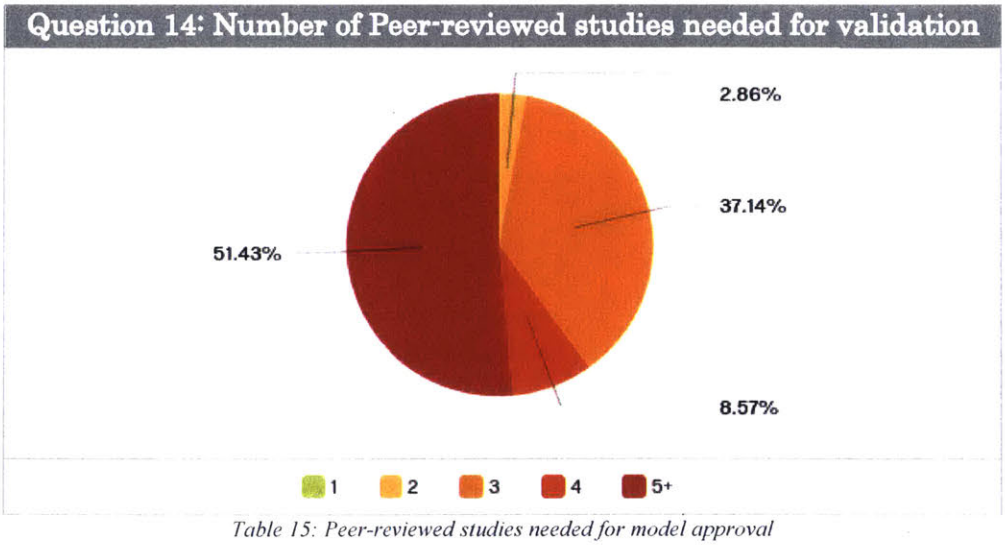

Table 15: Peer-reviewed studies needed for model approval

Question 15

Finally, the in response to whether the IACUC members would require the use of the the inducible pain-free model given enough characterization indicated that, as with most refinement strategies, this model could be a

recommendation made by the IACUC member to the investigator. One respondent further elaborated in the free response section that they do not feel it is their position to tell an investigator which animal model to use. Another noted that labs generate or already have strains recapitulating the biological conditions they are trying to study. Trying to encourage the use of the inducible pain-free mouse model on top of the other requirements of could be a big ask for researchers.

Question 15: With enough characterization, would you require the use of this model in research studies?	
Answer	%
Yes	16.67%
Yes, if the investigators cannot use traditional analgesics or anesthesia	16.67%
No, but I could recommend it as a further refinement strategy	63.89%
No	2.78%

Table 16: Inducible pain-free mouse as a requirement

VI.III.IV Free Response Results

Allowing respondents to answer each question of the survey in their own words would make the results of this survey difficult to analyze, which is why multiple choice was used for many of the responses. But getting the opinions of individual respondents was important both to understand how to improve any further interactions with IACUC members as well as to give the respondents a place to further clarify or qualify their multiple choice responses. All of the responses can be seen in Appendix II, but I will highlight some of the responses that I found to be particularly helpful.

Firstly, there were quite a few very positive responses to the system While it is possible that this is not indicative of the entire IACUC community, but it is encouraging nonetheless.

Many respondents indicated concern over the wellbeing of the inducible pain-free mouse because pain-free phenotypes have been a fitness disadvantage to

mice who do not learn critical pain-avoidance habits. Similarly, there were those who noted that while an animal may not feel physical pain, there is still an ability for those animals to be in a state of distress. The goal of this system is to increase the overall wellbeing of these animals, so the fitness and distress levels are both components on the induced mice that will be characterized in comparison to unindicted mice as well as wild type mice. Additionally, further studies can be done on how inducing the system at various times in mouse development might impact their overall fitness, which could indicate at which stages the mice are most likely to learning pain avoidance habits.

One respondent mentioned that anesthesia is more commonly administered to animals to immobilize the anima during experimented than to reduce pain during a procedure. Therefore, it was suggested that this system only be proposed as an alternative to analgesics. This comment is important to consider, as I had not known that this was a common method of animal restraint. Understanding anesthetics role in animal experimentation can be further researched to determine whether this system should be aimed to replace only analgesics in comparison to also considering it an effective alternative for some anesthetic purposes.

Many other respondents noted the complexity inherent in many animal model systems. Factors involving strain, sex, and specific pain phenotypes or pathologies are all factors that have to be seriously considered in the context of characterization of the pain-free mouse system as well as any eventual implementation thereof. Therefore, it may be in the best interest to understand which areas of research are best suited for this inducible pain-free model and work with investigators in those fields to assure that the system design and characterization is in line with any specifications they may have.

"Our work is never over"

- Daft Punk, *Harder, Better, Faster, Stronger*

VI. Discussion and Next Steps

VII.II Discussion

This thesis presented a holistic approach on understanding animal suffering through the lens of philosophy, history, and biology. It proposed an optimization to the current methods of painful animal experimentations used by researchers in the form of an inducible pain-free mouse model. Finally, it analyzed the regulatory landscape the model would exist within and assured that this model is considered both necessary and needed as a method of further refinement within animal research. Below are the next steps for characterizing and implementing the inducible pain-free mouse project will be outlined.

VII.II Mouse Model Evaluation

Firstly, we will genotype the mouse strains that are being generated and breed to homozygosity in the case of the floxed *SCN9a* gene. Genotyping will be done by using PCR, subcloning the products, and sequencing for

confirmation of insert. Once this is complete, we will mate the Wnt1 Cre-ERT2 mouse with the floxed mouse in order to produce a mouse containing both genetic edits. After further genotyping for confirmation of the final model, we will perform testing the pain threshold of the mice. In collaboration with the Woolf Lab at Boston Children's Hospital / Harvard Medical School, we will set up blinded tests to determine the pain perception capabilities of our transgenic mice in comparison to wild-type littermate control mice. We will also compare the the pain perception capabilities of the transgenic mice with and without induction. The tests that will be performed on the mice can be seen in in Table 17.

Pain Testing	
Pain Type	Test
high-threshold mechanical sensitivity	pinched with calibrated forceps
heat sensitivity	radiant and contact heat latencies will be administered to the mice.
inflammatory pain,	unilateral, intraplantar or intraknee injection of complete Freund's adjuvant
mechanical allodynia	thresholds will be determined using von Frey filaments
cold allodynia	acetone will be evaporated on the paw of the mice
neuropathic pain	surgically induced spare nerve injury and then be tested for mechanical and thermal related neuropathic pain (SNI) post-surgery
oxaliplatin-induced pain	mice will receive oxaliplatin intravenously by tail vein injection
cancer-induced bone pain	metastatic bone pain cancer mice will receive an intrafemoral injection of LL/2 lung carcinoma cells and all spontaneous movement-evoked pain response will be measured to evaluate pain behavior.

Table 17: Pain Testing

Additional tests will be conducted to understand the quantify the quality of life of the mice with and without the system being induced. This will be done

primarily by monitoring the extent of nesting and the grooming habits of the mice.

If initial characterization of the inducible pain-free system performs well, we will characterize the pain states of mouse models that have multiple sodium ion channels floxed to better understand how to mitigate multiple pain states. Additionally, we hope to partner with those working in fields where mice experience chronic neuropathic pain, such as many oncological models, and understand how our model can be best adopted for their research.

VII.III IACUC Survey

In the future, the survey will be sent out to the PRIM&R community to try and gather more responses to the survey. This will be done by a posting on Ampersand, the PRIM&R blog. This will allow for a larger response and give a clearer idea of how the model is perceived by the regulatory system.

With respect to representation within the survey, there is certainly work to be done. There were almost no responses to the survey that represented the non-scientist or non-affiliated members of the IACUC. It would be useful in understanding what about they survey deterred these members from initially participating. Their opinion on animal suffering as well as current and future methods of refinement in animal experimentation is important to the success of this project, and should be looked at further.

Furthermore, there were many points brought up in the responses at the end of the survey that would be good to follow up on. It is yet to be decided whether this will be done through an additional survey or through contacting members of an IACUC or the USDA's OLAW, or both, to clarify important points.

Finally, it would be useful to converge on a particular pain modality and field of research that is best suited for one of these models. The model can then be further characterized to the specifications of the particular field in addition to

the IACUC requirements to assure the model can be implemented as soon as possible.

References

"Animal Welfare Act." n.d. USDA National Agricultural Library. Accessed August 27, 2018. https://www.nal.usda.gov/awic/animal-welfare-act.

Bagal, Sharan K., Mark L. Chapman, Brian E. Marron, Rebecca Prime, R. Ian Storer, and Nigel A. Swain. 2014. "Recent Progress in Sodium Channel Modulators for Pain." *Bioorganic & Medicinal Chemistry Letters* 24 (16): 3690–99. https://doi.org/10.1016/j.bmcl.2014.06.038.

Balcombe, Jonathan, Hope Ferdowsian, and Lauren Briese. 2013. "Prolonged Pain Research in Mice: Trends in Reference to the 3Rs." *Journal of Applied Animal Welfare Science* 16 (1): 77–95. https://doi.org/10.1080/10888705.2013.741004.

Bentham, Jeremy. 1996. *The Collected Works of Jeremy Bentham: An Introduction to the Principles of Morals and Legislation.* Clarendon Press.

Bentham, Jeremy. 2001. *A Fragment on Government.* The Lawbook Exchange, Ltd.

Buchholz, F., P. O. Angrand, and A. F. Stewart. 1998. "Improved Properties of FLP Recombinase Evolved by Cycling Mutagenesis." *Nature Biotechnology* 16 (7): 657–62. https://doi.org/10.1038/nbt0798-657.

"C57BL/6." 2016. *Wikipedia.* https://en.wikipedia.org/w/index.php?title=C57BL/6&oldid=757154618.

Catterall, William A. 2000. "From Ionic Currents to Molecular Mechanisms: The Structure and Function of Voltage-Gated Sodium Channels." *Neuron* 26 (1): 13–25. https://doi.org/10.1016/S0896-6273(00)81133-2.

Chari, Raj, Nan Cher Yeo, Alejandro Chavez, and George M. Church. 2017. "sgRNA Scorer 2.0: A Species-Independent Model To Predict CRISPR/Cas9 Activity." *ACS Synthetic Biology* 6 (5): 902–4. https://doi.org/10.1021/acssynbio.6b00343.

Cosgrove, Ben. 2014. "'Concentration Camps for Dogs': Revisiting a Grisly LIFE Classic." Time. November 20, 2014. http://time.com/3589751/concentration-camps-for-dogs-revisiting-a-grisly-life-classic/.

Cottingham, John. 1978. "'A Brute to the Brutes?': Descartes' Treatment of Animals." *Philosophy* 53 (206): 551–59. https://doi.org/10.1017/S0031819100026371.

Crimmins, James E. 2018. "Jeremy Bentham." In *The Stanford Encyclopedia of Philosophy*, edited by Edward N. Zalta, Summer 2018. Metaphysics Research Lab, Stanford University. https://plato.stanford.edu/archives/sum2018/entries/bentham/.

Danielian, P. S., Y. Echelard, G. Vassileva, and A. P. McMahon. 1997. "A 5.5-Kb Enhancer Is Both Necessary and Sufficient for Regulation of Wnt-1

Transcription in Vivo." *Developmental Biology* 192 (2): 300–309. https://doi.org/10.1006/dbio.1997.8762.
Descartes, René. 1969. *Discourse on Method and the Meditations*. Vol. 3. Prabhat Prakashan.
Descartes, René. 1970. "Letter to Henry More, February 5, 1649." In *Descartes: Philosophical Letters*, 243–45.
Deuis, Jennifer R., Katharina Zimmermann, Andrej A. Romanovsky, Lourival D. Possani, Peter J. Cabot, Richard J. Lewis, and Irina Vetter. 2013. "An Animal Model of Oxaliplatin-Induced Cold Allodynia Reveals a Crucial Role for Nav1.6 in Peripheral Pain Pathways." *PAIN®* 154 (9): 1749–57. https://doi.org/10.1016/j.pain.2013.05.032.
Doench, John G., Ella Hartenian, Daniel B. Graham, Zuzana Tothova, Mudra Hegde, Ian Smith, Meagan Sullender, Benjamin L. Ebert, Ramnik J. Xavier, and David E. Root. 2014. "Rational Design of Highly Active sgRNAs for CRISPR-Cas9–mediated Gene Inactivation." *Nature Biotechnology* 32 (12): 1262–67. https://doi.org/10.1038/nbt.3026.
Drenth, Joost P. H., and Stephen G. Waxman. 2007. "Mutations in Sodium-Channel Gene *SCN9A* Cause a Spectrum of Human Genetic Pain Disorders." *The Journal of Clinical Investigation* 117 (12): 3603–9. https://doi.org/10.1172/JCI33297.
Ericsson, Aaron C., Marcus J. Crim, and Craig L. Franklin. 2013. "A Brief History of Animal Modeling." *Missouri Medicine* 110 (3): 201–5.
Fenwick, Nicole, Gilly Griffin, and Clément Gauthier. 2009. "The Welfare of Animals Used in Science: How the 'Three Rs' Ethic Guides Improvements." *The Canadian Veterinary Journal* 50 (5): 523–30.
Franklin, Benjamin. 1790. *The Way to Wealth*. London: Leavitt, Trow & Co.
Gallinger, Jacob Harold. 1900. *56th Congress, 1st Session. S. 3424 ... March 2, 1900 /*. [Providence, R.I. : http://hdl.handle.net/2027/uc1.31378008356399.
García-Villegas, Refugio, Luz E. López-Álvarez, Stephan Arni, Tamara Rosenbaum, and Marco A. Morales. 2009. "Identification and Functional Characterization of the Promoter of the Mouse Sodium-Activated Sodium Channel Nax Gene (Scn7a)." *Journal of Neuroscience Research* 87 (11): 2509–19. https://doi.org/10.1002/jnr.22069.
Gingras, Jacinthe, Sarah Smith, David J. Matson, Danielle Johnson, Kim Nye, Lauren Couture, Elma Feric, et al. 2014. "Global Nav1.7 Knockout Mice Recapitulate the Phenotype of Human Congenital Indifference to Pain." *PLOS ONE* 9 (9): e105895. https://doi.org/10.1371/journal.pone.0105895.
Gorter, Jan A., Emanuele Zurolo, Anand Iyer, Kees Fluiter, Erwin A. Van Vliet, Johannes C. Baayen, and Eleonora Aronica. 2010. "Induction of

Sodium Channel Nax (SCN7A) Expression in Rat and Human Hippocampus in Temporal Lobe Epilepsy." *Epilepsia* 51 (9): 1791–1800. https://doi.org/10.1111/j.1528-1167.2010.02678.x.

Hansson, Per. 2003. "Difficulties in Stratifying Neuropathic Pain by Mechanisms." *European Journal of Pain* 7 (4): 353–57. https://doi.org/10.1016/S1090-3801(03)00051-X.

Harrison, Peter. 1992. "Descartes on Animals." *The Philosophical Quarterly (1950-)* 42 (167): 219–27. https://doi.org/10.2307/2220217.

Hasegawa, Hiroshi, Sara Abbott, Bao-Xia Han, Yi Qi, and Fan Wang. 2007. "Analyzing Somatosensory Axon Projections with the Sensory Neuron-Specific Advillin Gene." *Journal of Neuroscience* 27 (52): 14404–14. https://doi.org/10.1523/JNEUROSCI.4908-07.2007.

Hodgkin, A. L., and A. F. Huxley. 1952. "A Quantitative Description of Membrane Current and Its Application to Conduction and Excitation in Nerve." *The Journal of Physiology* 117 (4): 500–544.

Horii, Takuro, Yuji Arai, Miho Yamazaki, Sumiyo Morita, Mika Kimura, Masahiro Itoh, Yumiko Abe, and Izuho Hatada. 2014. "Validation of Microinjection Methods for Generating Knockout Mice by CRISPR/Cas-Mediated Genome Engineering." *Scientific Reports* 4 (March): 4513. https://doi.org/10.1038/srep04513.

Hume, David. 2003. *A Treatise of Human Nature*. Courier Corporation.

Ingraham, Paul. 2018. "Pain Is Weird." www.PainScience.com. January 15, 2018. https://www.painscience.com/articles/pain-is-weird.php.

Jose L. Ochoa, M. D. 2018. "Neuropathic Pain: Redefinition and a Grading System for Clinical and Research Purposes." *Neurology*, August. http://n.neurology.org/content/neuropathic-pain-redefinition-and-grading-system-clinical-and-research-purposes.

Ke, C. B., W. S. He, C. J. Li, D. Shi, F. Gao, and Y. K. Tian. 2012. "Enhanced SCN7A/Nax Expression Contributes to Bone Cancer Pain by Increasing Excitability of Neurons in Dorsal Root Ganglion." *Neuroscience* 227 (December): 80–89. https://doi.org/10.1016/j.neuroscience.2012.09.046.

Lau, Joanne, Michael S. Minett, Jing Zhao, Ulla Dennehy, Fan Wang, John N. Wood, and Yury D. Bogdanov. 2011. "Temporal Control of Gene Deletion in Sensory Ganglia Using a Tamoxifen-Inducible Advillin-Cre-ERT2 Recombinase Mouse." *Molecular Pain* 7 (1): 100. https://doi.org/10.1186/1744-8069-7-100.

Levin, Stephen I., Zayd M. Khaliq, Teresa K. Aman, Tina M. Grieco, Jennifer A. Kearney, Indira M. Raman, and Miriam H. Meisler. 2006. "Impaired Motor Function in Mice With Cell-Specific Knockout of Sodium Channel Scn8a (NaV1.6) in Cerebellar Purkinje Neurons and Granule

Cells." *Journal of Neurophysiology* 96 (2): 785–93. https://doi.org/10.1152/jn.01193.2005.
Levin, Stephen I., and Miriam H. Meisler. 2004. "Floxed Allele for Conditional Inactivation of the Voltage-Gated Sodium Channel Scn8a (Nav1.6)." *Genesis* 39 (4): 234–39. https://doi.org/10.1002/gene.20050.
Lewis, Ace E., Harish N. Vasudevan, Audrey K. O'Neill, Philippe Soriano, and Jeffrey O. Bush. 2013. "The Widely Used Wnt1-Cre Transgene Causes Developmental Phenotypes by Ectopic Activation of Wnt Signaling." *Developmental Biology* 379 (2): 229–34. https://doi.org/10.1016/j.ydbio.2013.04.026.
Marks, P. W., M. Arai, J. L. Bandura, and D. J. Kwiatkowski. 1998. "Advillin (p92): A New Member of the Gelsolin/Villin Family of Actin Regulatory Proteins." *Journal of Cell Science* 111 (Pt 15) (August): 2129–36.
Meisler, Miriam H., Jennifer Kearney, Andrew Escayg, Bryan T. Macdonald, and Leslie K. Sprunger. 2001. "Sodium Channels and Neurological Disease: Insights from Scn8a Mutations in the Mouse." *The Neuroscientist* 7 (2): 136–45. https://doi.org/10.1177/107385840100700208.
"Mice and Rats in Laboratories." n.d. *PETA* (blog). Accessed April 25, 2017. http://www.peta.org/issues/animals-used-for-experimentation/animals-laboratories/mice-rats-laboratories/.
Minett, Michael S., Mohammed A. Nassar, Anna K. Clark, Gayle Passmore, Anthony H. Dickenson, Fan Wang, Marzia Malcangio, and John N. Wood. 2012. "Distinct Nav1.7-Dependent Pain Sensations Require Different Sets of Sensory and Sympathetic Neurons." *Nature Communications* 3 (April): 791. https://doi.org/10.1038/ncomms1795.
Minett, Michael S., Vanessa Pereira, Shafaq Sikandar, Ayako Matsuyama, Stéphane Lolignier, Alexandros H. Kanellopoulos, Flavia Mancini, et al. 2015. "Endogenous Opioids Contribute to Insensitivity to Pain in Humans and Mice Lacking Sodium Channel Nav1.7." *Nature Communications* 6 (December): 8967. https://doi.org/10.1038/ncomms9967.
Minett, Michael S., Sarah Falk, Sonia Santana-Varela, Yury D. Bogdanov, Mohammed A. Nassar, Anne-Marie Heegaard, and John N. Wood. 2014. "Pain without Nociceptors? Nav1.7-Independent Pain Mechanisms." *Cell Reports* 6 (2): 301–12. https://doi.org/10.1016/j.celrep.2013.12.033.
Mogil, J. S., S. G. Wilson, K. Bon, S. E. Lee, K. Chung, P. Raber, J. O. Pieper, et al. 1999. "Heritability of Nociception I: Responses of 11 Inbred Mouse Strains on 12 Measures of Nociception." *Pain* 80 (1–2): 67–82.
Nassar, Mohammed A., L. Caroline Stirling, Greta Forlani, Mark D. Baker, Elizabeth A. Matthews, Anthony H. Dickenson, and John N. Wood.

2004. "Nociceptor-Specific Gene Deletion Reveals a Major Role for Nav1.7 (PN1) in Acute and Inflammatory Pain." *Proceedings of the National Academy of Sciences of the United States of America* 101 (34): 12706–11. https://doi.org/10.1073/pnas.0404915101.

National Research Council (US) Committee to Update Science, Medicine. 2004. *Continuing Efforts to More Efficiently Use Laboratory Animals*. National Academies Press (US). https://www.ncbi.nlm.nih.gov/books/NBK24662/.

Nelis, H. J. C. F., and A. P. De Leenheer. 1981. "Evidence for Metabolic Inertness of Doxycycline." *Journal of Pharmaceutical Sciences* 70 (2): 226–28. https://doi.org/10.1002/jps.2600700230.

"Office of Laboratory Animal Welfare - Public Health Service Policy on Humane Care and Use of Laboratory Animals | Grants.nih.gov." n.d. Accessed April 25, 2017. https://grants.nih.gov/grants/olaw/references/phspol.htm.

Oliva, Carolina A., Carla Montecinos-Oliva, and Nibaldo C. Inestrosa. 2018. "Wnt Signaling in the Central Nervous System: New Insights in Health and Disease." In *Progress in Molecular Biology and Translational Science*, 153:81–130. Elsevier.

Oliveros, Juan C., Mònica Franch, Daniel Tabas-Madrid, David San-León, Lluis Montoliu, Pilar Cubas, and Florencio Pazos. 2016. "Breaking-Cas—interactive Design of Guide RNAs for CRISPR-Cas Experiments for ENSEMBL Genomes." *Nucleic Acids Research* 44 (W1): W267–71. https://doi.org/10.1093/nar/gkw407.

"PHS Policy on Humane Care and Use of Laboratory Animals." n.d. Accessed August 27, 2018. https://olaw.nih.gov/policies-laws/phs-policy.htm#Introduction.

Qin, Jane Yuxia, Li Zhang, Kayla L. Clift, Imge Hulur, Andy Peng Xiang, Bing-Zhong Ren, and Bruce T. Lahn. 2010. "Systematic Comparison of Constitutive Promoters and the Doxycycline-Inducible Promoter." *PLOS ONE* 5 (5): e10611. https://doi.org/10.1371/journal.pone.0010611.

Rideout, William M., Teruhiko Wakayama, Anton Wutz, Kevin Eggan, Laurie Jackson-Grusby, Jessica Dausman, Ryuzo Yanagimachi, and Rudolf Jaenisch. 2000. "Generation of Mice from Wild-Type and Targeted ES Cells by Nuclear Cloning." *Nature Genetics* 24 (2): 109–10. https://doi.org/10.1038/72753.

Russel, William Moy Stratton, Rex Leonard Burch, and Charles Westley Hume. 1959. *The Principles of Humane Experimental Technique*. Vol. 238. London: Methuen.

Ryder, Richard D. 1998. "The Political Animal: The Conquest of Speciesism." eweb:185131. 1998.

https://repository.library.georgetown.edu/handle/10822/909275?show=full.

Singer, Peter, Louis P. Pojman, Paul Pojman, and Katie McShane. 1998. "A Utilitarian Defense of Animal Liberation." In *Environmental Ethics: Readings in Theory and Application*, 96–105. Cengage Learning.

Sittl, Ruth, Angelika Lampert, Tobias Huth, E. Theresa Schuy, Andrea S. Link, Johannes Fleckenstein, Christian Alzheimer, Peter Grafe, and Richard W. Carr. 2012. "Anticancer Drug Oxaliplatin Induces Acute Cooling-Aggravated Neuropathy via Sodium Channel Subtype NaV1.6-Resurgent and Persistent Current." *Proceedings of the National Academy of Sciences* 109 (17): 6704–9. https://doi.org/10.1073/pnas.1118058109.

Stokes, E L, P A Flecknell, and C A Richardson. 2009. "Reported Analgesic and Anaesthetic Administration to Rodents Undergoing Experimental Surgical Procedures." *Laboratory Animals* 43 (2): 149–54. https://doi.org/10.1258/la.2008.008020.

Tannenbaum, Jerrold, and B. Taylor Bennett. 2015. "Russell and Burch's 3Rs Then and Now: The Need for Clarity in Definition and Purpose." *Journal of the American Association for Laboratory Animal Science: JAALAS* 54 (2): 120–32.

Twilley, Nicola. 2018. "The Neuroscience of Pain." *The New Yorker*, June 25, 2018. https://www.newyorker.com/magazine/2018/07/02/the-neuroscience-of-pain.

Wallace, David Foster. 2005. *Consider the Lobster: And Other Essays*. Little, Brown.

Wang, Yuanming, Kaiwen Ivy Liu, Norfala-Aliah Binte Sutrisnoh, Harini Srinivasan, Junyi Zhang, Jia Li, Fan Zhang, et al. 2018. "Systematic Evaluation of CRISPR-Cas Systems Reveals Design Principles for Genome Editing in Human Cells." *Genome Biology* 19 (1): 62. https://doi.org/10.1186/s13059-018-1445-x.

Watanabe, Eiji, Takeshi Y. Hiyama, Ryuji Kodama, and Masaharu Noda. 2002. "NaX Sodium Channel Is Expressed in Non-Myelinating Schwann Cells and Alveolar Type II Cells in Mice." *Neuroscience Letters* 330 (1): 109–13.

Weiss, Jan, Martina Pyrski, Eric Jacobi, Bernd Bufe, Vivienne Willnecker, Bernhard Schick, Philippe Zizzari, et al. 2011. "Loss-of-Function Mutations in Sodium Channel Nav1.7 Cause Anosmia." *Nature* 472 (7342): 186–90. https://doi.org/10.1038/nature09975.

"Wiley Press Images, 2012." n.d. Accessed August 15, 2018. http://media.wiley.com/mrw_images/cp/cpcb/articles/cb1911/image_n/ncb191107.gif.

Zetsche, Bernd, Sara E. Volz, and Feng Zhang. 2015. "A Split-Cas9 Architecture for Inducible Genome Editing and Transcription Modulation." *Nature Biotechnology* 33 (2): 139–42. https://doi.org/10.1038/nbt.3149.

Appendix I

DM Analgesia

Full sequence can be found at (https://benchling.com/s/seq-kXJcjWCKCMaPqfoEkGOQ)

Below is the list of primers used for DM Analgesia, the plasmid that was designed to add ERT2 control to the Wnt1 Cre recombinase mouse.

#	Primer	Tm	Info
DN354	ACCTCACACCATGCUctcgagccatctgctg gag	68.7	FWD USER primer ERT2
DN355	gctgatcagcgagctctagaagctttgtgactatcaag ctgtggcaggga	69.9	REV OEPCR for ERT2
DN356	tagtcacaaagcttctagagctcgctgatcagctcgac tgtgccttctagttgc	69.4	FWD OEPCR 3' with ERT2 hom new
DN357	AGGCTTTTGACTTGGCUgagggctttctgc tcttttcttcttccct	69	REV USER wnt 3' homology new
DN364	cggAGGCTTTTGACTTGGCTgagggctttc tgctcttttcttcttccc	69.6	REV OEPCR for wnt 3' homology new
DN365	tcatagggaagaagaaaagagcagaaagccctcAG CCAAGTCAAAAGCCTc	68.6	FWD OEPCR for DM BB with 3' hom
DN366	tggcaggatgaaAGTTCCTCCTGGGCTgac ttcag	67.4	REV primer for DM BB OEPCR (DN365)
DN381	AGCCCAGGAGGAACUgacggttagcctgtc agc	69.2	FWD USER primer wnt_cre_5_2
DN382	AGCATGGTGTGAGGUcgagtaattggtaga attcacgcg	66.3	REV USER primer wnt_cre_5_2 gblock
DN383	AGCCAAGTCAAAAGCCUccgaccggaggc tttt	68.5	FWD USER DM BB primer DM analgesia

Below are the PCR settings for the DNA fragments amplified for DM Analgesia assembly. All PCRs were carried out using Phusion U Hot Start Mastermix.

PCR	Fragment	FWD Primer	REV Primer	Template	Temp 1	Temp 2	Extension	Expected Length
1	DM BB with OEPCR hom	DN365	DN366	DM anything	58	68	1 min	1709
2	Wnt1-Cre 5' homology	DN381	DN382	Wnt1-Cre 5' homology gblock	55	67.5	1 min	1695
3	ERT2 with OEPCR	DN354	DN355	pCAG Cre ERT2	56	68	1 min	1023
4	Wnt1-Cre 3' OEPCR hom	DN356	DN364	Wnt1-Cre 3' homology gblock	57	69	1 min	1094
5	DM BB + 5'	DN383	DN382	PCR 1+ PCR 2	52	67	2 min	3361
6	ERT2 + 3'	DN354	DN357	PCR 3+PCR 4	53	69	1 min 15 sec	2063

All of these PCRs were two temperature PCRs, where the first five cycles are at the first temperature and the last twenty-five cycles at the second temperature. Below is the thermocycler program:

Stage 1	Stage 2			Stage 3			Stage 4	
98 C	98 C	T1	72 C	98 C	T2	72 C	72 C	12 C
30 sec	10 sec	15 sec	Ext	10 sec	15 sec	Ext	7 min	hold

Below are the g-block ordered from IDT for 5' and 3' homology arm to the B6 Wnt1-Cre2 mouse (Jax #022501) with slight modifications made to destroy the Cas9 PAM sites used to integrate the construct:

B6 Wnt1-Cre 5' homology gblock:

GGAACTGACGGTTAGCCTGTCAGCTCTTTGCTCAGACCGGCAAGA
GCCACAGCTTCGCTCGCCACTCATTGTCTGTGGCCCTGACCAGTG
CGCCCTGGTGCTTTTAGTGCCGCCCGGGCCCGGAGGGGCAGCCT
CTTCTCACTGCAGTCAGCGCCGCAACTATAAGAGGCCTATAAGAG
GCGGTGCCTCCCGCAGTGGCTGCTTCAGCCCAGCAGCCAGGACA
GCGAACCATGCTGCCTGCGGCCCGCCTCCAGACTTATTAGAGCCA
GCCTGGGAACTCGCATCACTGCCCTCACCGCTGTGTCCAGTCCCA
CCGTCGCGGACAGCAACCACAGTCGTCAGAACCGCAGCACAGAAC
CAGCAAGGCCAGGCAGGCGATATCCCTATTAATATTCCGGAGTAT
ACGTAGCCGGCTAACGTTAACAACCGGTACCCCATTGTATGGGAT
CTGATCTGGGGCCTCCGTGCACATGCTTTACATGTGTTTAGTCGA
GGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTT
CCTTTGAAAAACACGATGATAATATGGCCACAACCATGCCCAAGA
AGAAGAGGAAGGTGTCCAATTTACTGACCGTACACCAAAATTTGC
CTGCATTACCGGTCGATGCAACGAGTGATGAGGTTCGCAAGAACC
TGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGCATACCT
GGAAAATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCA
AGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTC
GCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAA
CTATCCAGCAACATTTGGGCCAGCTAAACATGCTTCATCGTCGGT
CCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTTA
TGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAA
AACAGGCTCTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTT
CACTCATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGG
CATTTCTGGGGATTGCTTATAACACCCTGTTACGTATAGCCGAAAT
TGCCAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAG
AATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGCACCGC
AGGTGTAGAGAAGGCACTTAGCCTGGGGGTAACTAAACTGGTCGA
GCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTA
CCTGTTTTGCAATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGT
GTTGCCGCGCCATCTGCCACCAGCCAGCTATCAACTCGCGCCCTG
GAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAG
GATGACTCTGGTCAGAGATACCTGGCCTGGTCTGGACACAGTGCC
CGTGTCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATA
CCGGAGATCATGCAAGCTGGTGGCTGGACCAATGTAAATATTGTC
ATGAACTATATCGTAACCTGGATAGTGAAACAGGGGCAATGGTGC

GCGTGCTGGAAGATGGCGATTACGCATCGCGTGAATTCTACCAAT
TACTCG

B6 Wnt1-Cre 3' homology gblock:

CTCACACCATGGCTCACAAAGCTTCTAGAGCTCGCTGATCAGCCT
CGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCC
CGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTC
CTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCA
TTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGG
ATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCT
ATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCGGTACCTCAACT
ATAGCTAGCATGCGCAAATTTAAAGCGCTGATATCGATCGCGCGC
AGATCTCTTTTCCAGGGCCTGAGCAAGGACCCTGAGATCCTGACC
CTTGGATGACCCTAAATGAGACCAACTAGGGATCCAGGTCTCCTG
AGAGAAAAGCCTGCTCAAAAGCTCCTGCAGGGCTCTTCCTCCTGT
TCTGGCAGCAGCTGGTTAAGTGATGAGGACAAGTGTGTCACCTGA
TGGGGACTCAGCACACCAACAGTCCAGCTCTCCTAGTGCAAGAGC
TGGGGAGGTAGCAGCAATGAAAGGAAAGGATAGCCATGACTACAT
CCTGAGAGGGGGAGGGGAAGCTCAAGCAGAAAGGGTGTCATTGC
CCCTGGGAGGCTATCAACCTTTTCTGCAGTCTGAGCTTTGAGCAG
TGGAAGGCCCACCTCTTACACTCTTCCTGGAAATTCTTTATTAGCA
ACCCAACCCTAACCTACAGGTAAGGGACATCCAGGAACAAGCCAT
GGGTTAGAGCTGGAAAGATGGCTCAGAGGTTAAGAGCACTGGCT
GCTCTTCTAGAAGTCCTGAGTTCAATTCCCAGCAACCACATGGTG
ACTCACAACCATCTGTTAATGGGATCTGGTGCACTCTTCTGGTGT
GTCTGAAGACAGCTATGGTGTGCTCATATAAATAATAATAAAGAA
ATGATATTAAAGAAAAGCCATGGGTTAACAGTGGGCCCTAAACTT
GAACTAGAAAACTTAAAGATGCTCATAGGGAAGAAGAAAAGAGCA
GAAAGC

Below are the Cas9 target sites used for the DM Analgesia integration into the B6 Wnt1-Cre2 mouse (PAM is in parenthesis, not used in guide design):

Guide name	Guide sequence
DM_Analgesia_1	ggtaatcgccatcttccagc(agg)

DM_Analgesia_2	aattactcgacctcacacca(tgg)
DM_Analgesia_3	ggtagaattcacgcgttaat(ggg)
DM_Analgesia_4	gcaactagaaggcacagtcg(agg)

Below are scores from various guide analysis software used to analyze for more effective guides (Oliveros et al. 2016)(Chari et al. 2017).

Guides	Breaking Cas	sgRNA Scorer 2.0
DM_Analgesia_1	91	0.131603259
DM_Analgesia_2	91.4	-0.090439974
DM_Analgesia_3	98.1	-0.771734077
DM_Analgesia_4	86.1	1.409282225

Below is the analysis from the Broad Designer tool (Doench et al. 2014).

Guide	Orientation	sgRNA Cut Position (1-based)	Target Cut Length	Target Total Length	Target Cut %	On-Target Ruleset	On-Target Efficacy Score	On-Target Rank	Off-Target Rank	Combined Rank
DM_Analgesia_1	antisense	37	36	445	8.1	Azimuth_2.0	0.5647	21	40	27
DM_Analgesia_2	sense	92	91	445	20.4	Azimuth_2.0	0.7127	1	4	1
DM_Analgesia_3	antisense	58	57	445	12.8	Azimuth_2.0	0.3004	64	2	34
DM_Analgesia_4	antisense	134	133	445	29.9	Azimuth_2.0	0.667	4	11	4

UC floxed SCN9a

Full sequence can be found at (https://benchling.com/s/seq-f4OCv9txNVCj4kHU4wPm).

Below is the list of primers used for UC floxed SCN9a, the plasmid that was designed to add lox around key exons of the *SCN9a* gene encoding the Nav1.7 sodium ion channel.

Primer	Sequence	Tm	Info
DN141	agacttagggacccUttgacttgatcggcacgtaaga	65.9	FWD USER primer UC Gaia for floxed
DN204	aagaggacatccggUATACATTTCAGTTTGAAAAACAGAAAACTGCATGTG	65.2	FWD USER primer 5' homology with J4
DN206	AGAGTTGCACCAUCGATAACTTCGTATAATGTATGCTATACGAAGTTATTCACTTCTTTGAGATGGAGACAGTC	67.2	FWD USER primer floxed 1 with lox site and recoding
DN207	CATGGGGCCAATAGAGGAAAATATAACTTCGTATAGCATACATTATACGAAGTTATCTTCAGTGTCAACCCACAAGTG	67.7	REV primer for flox 1 with lo and homology to flox 2
DN208	GGTTGACACTGAAGATAACTTCGTATAATGTATGCTATACGAAGTTATATTTTCCTCTATTGGCCCCATGTAAT	65.4	FWD primer for flox 2 with XhoI
DN209	agggtccctaagtcUCTCGAGTTTCTTCAAGGTCATAACTAATAGGAGACTAA	65.7	REV USER primer for flox 2 with XhoI and J5 for UC BB attachment
DN369	accggatgtcctctUggtagaaaatcaaaggatcttcttgaga	64.8	REV USER primer for UC BB
DN373	ATGGTGCAACTCUAAAAGTGAcGGCATGCAGGAATATGAACAGAGGCACACCAcGCATATTCATCTTTAAGTGAAAATGCTTCATTT	70.7	REV USER primer for new PAM edit SCN9a
DN374	ATGCTATACGAAGTTATTATTCAGAGATTTCTGCATTAGAATTTGTTC	60.7	FWD primer 1 for SCN9a 2kb
DN375	AGTTATGACCTTGAAGAAACTCGAGATAACTTCGTATAATGTATGCTATACGAAGTTATTATTCAGAGATTTCTG	65.4	FWD primer 2 SCN9a 2kb

	DN376	CTCGAGATTGAGGATTCTTA TCACAACTTATTTGCA	59.6	REV primer 1 SCN9a 2kb
	DN377	agggtccctaagtctCTCGAGATTG AGGATTCTTATCACAAC	64.5	REV primer 2 SCN9a 2kb

Below are the PCR settings for the DNA fragments amplified for UC floxed SCN9a assembly.

PCR	Fragment	FWD Primer	REV Primer	Template	T1	T2	Extension	Expected Length
1	UC Backbone	DN141	DN369	UC Rope	55	65	1 min	1872
2	Floxed exon 14	DN206	DN207	mouse gDNA	56	67	45 sec	1239
3	Floxed exon 15	DN208	DN209	mouse gDNA	55	65	45 sec	1266
4	Flox exon 14+15	DN206	DN209	PCR 2+PCR 3	63	66	1 min 30 sec	2435
5	SCN9a 5' homology	DN204	DN373	mouse gDNA	55	67.5	45 sec	1038
6	SCN9a 3' homology	DN374	DN376	mouse gDNA	54	60	1 min 30 sec	2017
7	SCN9a 3' homology for gibson	DN375	DN377	PCR 6	60	65	1 min 30 sec	2082

All of these PCRs were two temperature PCRs, where the first five cycles are at the first temperature and the last twenty-five cycles at the second temperature. Below is the thermocycler program:

Stage 1	Stage 2			Stage 3			Stage 4	
98 C	98 C	T1	72 C	98 C	T2	72 C	72 C	12 C
30 sec	10 sec	15 sec	Ext	10 sec	15 sec	Ext	7 min	hold

Below are the Cas9 target sites used for the UC floxed SCN9a integration into the standard C57BL6/J mice (Taconic B6) (PAM is in parenthesis, not used in guide design

Guides	Guide Sequence
SCN9a_1	ATGGTGCAACTCTAAAAGTG(AGG)
SCN9a_2	TCACTTTTAGAGTTGCACCA(TGG)
SCN9a_3	AATATGAACAGAGGCACACC(AGG)
SCN9a_4	CCATCTCAAAGAAGTGACCA(TGG)

Below are scores from various guide analysis software used to analyze for more effective guides (Oliveros et al. 2016)(Chari et al. 2017).

Guides	Breaking Cas	sgRNA Scorer 2.0
SCN9a_1	84.3	0.291880669658
SCN9a_2	81.8	0.00213112153575
SCN9a_3	73.2	0.0995941647656
SCN9a_4	61	0.622321078208

Below is the analysis from the Broad Designer tool (Doench et al. 2014).

Guide	Orientation	sgRNA Cut Position (1-based)	Target Cut Length	Target Total Length	Target Cut %	On-Target Ruleset	On-Target Efficacy Score	On-Target Rank	Off-Target Rank	Combined Rank
SCN9a_1	antisense	268	267	513	52	Azimuth_2.0	0.6398	5	8	2
SCN9a_2	sense	281	280	513	54.6	Azimuth_2.0	0.68	2	12	4
SCN9a_3	antisense	236	235	513	45.8	Azimuth_2.0	0.5665	11	2	3
SCN9a_4	antisense	287	286	513	55.8	Azimuth_2.0	0.6254	6	13	6

UC floxed SCN7a

Full sequence can be found at(https://benchling.com/s/seq-zGglVvCaHbADu3ZbKiAM).

Below is the list of primers used for UC floxed SCN7a, the plasmid that was designed to add lox around a key exon of the *SCN7a* gene encoding the Na_x sodium-like ion channel.

Primer	Sequence	Tm	Info
DN141	agacttagggacccUttgacttgatcgg cacgtaaga	65.9	FWD USER primer UC Gaia for floxed
DN214	AGCATACATTATACGAAGU TATTGGAGCCAGGTTTCCT TAAAGT	63.2	Rev USER primer 5' homology with part of lox site to disrupt cut sites
DN215	ACTTCGTATAATGTATGCU ATACGAAGTTATCGAGGAA GCAAGATTTTGGCAT	64.8	Fwd USER primer floxed exon with part of lox site
DN216	acgccatgggcaUTCTCCAGAAT ATCACTTTACTCTAGGC	65.8	Rev USER primer floxed exon with J2
DN260	aACCGGTgctttUgacttgatcggc ac	63.4	Fwd USER primer for UC BB
DN350	atgcccatggcgUATAACTTCGT ATAATGTATGCTATACGAA GTTATTATAAGATGGTATA ATTGTGGTGATGAAAATCA TTTTT	67.2	FWD USER J2 primer for SCN7a 3' homology 2kb
DN351	agggtccctaagtcUAAATTTCAT TGTAACTGCTTAATCCAAT CCAGATAGTT	64.7	REV USER J5 primer for SCN7a 3' homology 2kb
DN352	CTTCCATATACCTGTATGG AATTGGAAACTATAGGTggt agaaaatcaaaggatcttcttgaga	65.5	REV OEPCR for UC BB for SCN7a
DN368	TGGAGCCAGGTTTCCTTAA AGTGAGAGAACAAACACTA ACTTAGAAACAGGTAGTAT TACAAGCcGCAGACAAGCA TTTTACTAACAAATGCCTAT GGCAAGCgCATTTTCAGTT GAGCCCCGTC	71.6	REV ultramer with the extra PAM changes for 4 guides SCN7a
DN370	caagcagcagattacgcgcagaaaaaa aggatctcaagaagatcctttgattttct accaagaggacatccggtACCTATA GTTTCCAATTCCATACAGG T	70.5	FWD primer for 5' SCN7a (to go with DN368)
DN371	TGTATGCTATACGAAGTTA TTATAAGATGGTATAATTG TGGTGATGAAAATCATTTT TAAAGC	63	FWD primer 1 for 3' homology 2kb (half the lox site)

Below are the PCR settings for the DNA fragments amplified for UC floxed SCN9a assembly.

PCR	Fragment	FWD Primer	REV Primer	Template	T1	T2	Extension	Expected Length
1	SCN7a floxed exon	DN215	DN216	mouse gDNA	55	64	30 sec	1133
2	3' homology 2kb pt.I	DN371	DN351	mouse gDNA	58	63	1 min	2000
3	3' homology 2kb pt.II	DN350	DN351	PCR 2	62	66	1 min	2000
4	OEPCR for UC BB and 5' hom for SCN7a	DN260	DN352	UC Gaia	55	63	1 min	1850
5	floxed SCN7a 5' homology with PAMs	DN370	DN368	mouse gDNA	55.5	71	1 min	1000
6	UC BB +SCN7a 5' hom	DN141	DN214	PCR 4+PCR 5	70	65	2 mins	2800

All of these PCRs were two temperature PCRs, where the first five cycles are at the first temperature and the last twenty-five cycles at the second temperature. Below is the thermocycler program:

Stage 1		Stage 2			Stage 3		Stage 4	
98 C	98 C	T1	72 C	98 C	T2	72 C	72 C	12 C
30 sec	10 sec	15 sec	Ext	10 sec	15 sec	Ext	7 min	hold

Appendix II

IACUC Survey

Below is the exact language and logic if the survey distributed to the IACUC members through the AALAS list-serv. The survey was designed and distributed using Qualtrics.

IACUC Survey

Start of Block: Consent

Q1 Thank you for taking the time to complete this 5 minute survey!

Participation is voluntary and you may decline to answer any questions or stop at any point. This survey is confidential and your personal information will not be stored.

I am a researcher working in the Esvelt Lab at MIT on a project aiming to reduce animal suffering and improve scientific outcomes by engineering several strains of "inducible pain-free" mice. When fed a small molecule inducer, these mice will permanently lose the ability to feel a certain type of pain. In particular, mechanosensory, inflammatory, and classes of neuropathic pain are being targeted. Investigators would induce the appropriate engineered mouse strain before performing a procedure, thus abating perception of that form of pain from the animal during the duration of the experiment and hopefully causing less suffering than would occur with the standard analgesics.

While developing these strains of mice, I am interested in determining what

type of information or experimental demonstrations are needed for IACUC committees to be comfortable with recommending their researchers consider these mice as a further method of refinement in experimentation. People outside the scientific community may argue that this project will lead to additional experiments that might not otherwise receive approval. This survey aims to ensure that no such outcomes occur through gaining understanding of IACUC members' opinions and recommendations for the project. It should be noted that mice are being developed on a nonprofit basis to enable swift and widespread adoption if appropriate; we are doing this to reduce animal suffering and benefit science.

This survey has received an IRB exemption because it is anonymous and poses minimal risk. The survey results will be written up as part of my Master's Thesis along with the design and preliminary results for the inducible pain-free mice. With sufficient data from this survey, I plan to publish the results in a peer-reviewed article in a scientific journal or otherwise make the results available to the IACUC community.

Feel free to contact devora@mit.edu with any question or comments.

Page Break

Q2 I understand the procedures described above and I agree to participate in this study.

- ○ I consent (1)
- ○ I do not consent (2)

End of Block: Consent

Start of Block: IACUC info

Q3 Which member of the IACUC committee do you represent?

- ○ Veterinarian (1)
- ○ Non-scientist (2)
- ○ Non-affiliated member (representing community) (3)
- ○ Practicing scientist experienced in animal research (4)
- ○ Chairperson (5)
- ○ Administrator (6)
- ○ Other (7) _____

Q4 How many members are there on your IACUC committee?

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Q5 How does your IACUC typically review protocols?

- ○ Designated Member Review (mainly or only) (4)
- ○ Full Committee Review (mainly or only) (5)
- ○ Both depending on circumstances (6)

End of Block: IACUC info

Start of Block: Category E

Q6 What percentage of protocols submitted monthly for review to your IACUC involve Category D animals? (Category D is classified as animals subjected to potentially painful or stressful procedures for which they receive appropriate anesthetics, analgesics and/or tranquilizer drugs.)

- ○ 0-20% (1)
- ○ 21-40% (2)
- ○ 41-60% (3)
- ○ 61-80% (4)
- ○ >80% (5)
- ○ Other (6) _____

Q7 What percentage of protocols submitted monthly for review to your IACUC involve Category E animals? (Category E is classified as animal use activities that involve accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, tranquilizing drugs or other

methods for relieving pain or distress are not used or delayed for scientific purposes. Scientific justification has to be provided.)

- ○ 0-20% (1)
- ○ 21-40% (2)
- ○ 41-60% (3)
- ○ 61-80% (4)
- ○ >80% (5)
- ○ Other (6) _____

Q8 In your opinion, is there is a need for further refinement strategies for research involving Category D and Category E animals?

- ○ Yes (1)
- ○ No (2)
- ○ Other (3) _____

End of Block: Category E

Start of Block: Refinement

Q9 In your opinion, how would you rate the problem of animal pain in the Category D studies performed at your institution?

○ Low (Either procedures are not painful or the pain is well controlled.) (1)

○ Moderate (Painful procedures are limited, and pain is controlled the best we can, but animals do sometimes experience some pain.) (2)

○ High (Procedures are very painful and controlling pain is a challenge.) (3)

Q10 If specific pain pathways in an animal model could be lastingly shut down prior to experimentation, could this be considered to be a "refinement" strategy for experimentation?

○ Yes (1)

○ No (2)

Q11 If mechanosensory and inflammatory pain perception could be lastingly shut down prior to experimentation, should such an animal be classified in a similar way to an animal that receives anesthesia or an analgesic?

○ Yes (1)

○ No (2)

Q12 Would you be willing to approve the use of additional animals in Category D experiments in order to determine the extent to which the new method reduces suffering without interfering with experimental outcomes?

- ○ Yes (1)
- ○ No (2)

Q13 Would you be willing to approve the use of additional animals in Category E experiments in order to determine the extent to which the new method reduces suffering without interfering with experimental outcomes?

- ○ Yes (1)
- ○ No (2)

Q14 How many peer-reviewed studies in a relevant area of research would you find necessary to be comfortable with an investigator using this mouse model in their research in place of traditional analgesics or anesthesia?

- ○ 1 (1)
- ○ 2 (2)
- ○ 3 (3)
- ○ 4 (4)
- ○ 5+ (5)

Q15 Assuming sufficient peer-reviewed studies show that this mouse model reduces suffering without interfering with the pathways being studied by researchers, would you be willing to require its use by investigators?

- ○ Yes (1)
- ○ Yes, if the investigators cannot use traditional analgesics or anesthesia (2)
- ○ No, but I could recommend it as a further refinement strategy (3)
- ○ No (4)

End of Block: Refinement

Start of Block: Further Comments

Q16 If you have any final comments or recommendations regarding the survey, please feel free to write below.

_____

_____

IACUC Survey Results

Default Report
IACUC Survey
August 20th 2018, 10:33 am MDT

Q2 - I understand the procedures described above and I agree to participate in this study.

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	1.00	1.00	0.00	0.00	23

#	Question	Unknown		Total
1	I consent	100.00%	23	23
2	I do not consent	0.00%	0	0

Q3 - Which member of the IACUC committee do you represent?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	7.00	4.52	2.18	4.77	23

#	Question	Unknown	
1	Veterinarian	21.74%	5

2	Non-scientist	4.35%	1
3	Non-affiliated member (representing community)	0.00%	0
4	Practicing scientist experienced in animal research	13.04%	3
5	Chairperson	13.04%	3
6	Administrator	30.43%	7
7	Other	17.39%	4
	Total	Total	23

Question	Unknown		Total
and chair	100.00%	1	1
Compliance	100.00%	1	1
Coordinator	100.00%	1	1
Statistician	100.00%	1	1

Q4 - How many members are there on your IACUC committee?

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Q5 - How does your IACUC typically review protocols?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	4.00	6.00	5.26	0.74	0.54	23

#	Question	Unknown		Total
4	Designated Member Review (mainly or only)	100.00%	4	4
5	Full Committee Review (mainly or only)	100.00%	9	9
6	Both depending on circumstances	100.00%	10	10

Q6 - What percentage of protocols submitted monthly for review to your IACUC involve Category D animals? (Category D is classified as animals subjected to potentially painful or stressful procedures for which they receive appropriate anesthetics, analgesics and/or tranquilizer drugs.)

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	6.00	3.41	1.40	1.97	22

#	Question	Unknown		Total
1	0-20%	100.00%	2	2
2	21-40%	100.00%	4	4
3	41-60%	100.00%	6	6
4	61-80%	100.00%	5	5
5	>80%	100.00%	3	3
6	Other	100.00%	2	2

Question	Unknown		Total
I don't know for sure, but think it would be >80%.	0.00%	0	0
varies, and I can't quickly access numbers now	0.00%	0	0
i dont know	100.00%	1	1
in many of our studies anesthesia is used for restraint not specifically for pain management	100.00%	1	1

Q7 - What percentage of protocols submitted monthly for review to your IACUC involve Category E animals? (Category E is classified as animal use

activities that involve accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used or delayed for scientific purposes. Scientific justification has to be provided.)

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	6.00	1.77	1.51	2.27	22

#	Question	Unknown		Total
1	0-20%	100.00%	15	15
2	21-40%	100.00%	4	4
3	41-60%	0.00%	0	0
4	61-80%	100.00%	1	1
5	>80%	0.00%	0	0
6	Other	100.00%	2	2

Question	Unknown		Total
varies, and I can't quickly access numbers now	0.00%	0	0
i dont know	100.00%	1	1
o	100.00%	1	1

Q8 - In your opinion, is there is a need for further refinement strategies for research involving Category D and Category E animals?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	3.00	1.36	0.64	0.41	22

#	Question	Unknown		Total
1	Yes	100.00%	16	16
2	No	100.00%	4	4
3	Other	100.00%	2	2

Question	Unknown		Total
I'm not sure it is a 'need' but it would be highly desirable	0.00%	0	0
Category D I think is managed well. Category E could be further evaluated	100.00%	1	1
This is a regulatory requirement	100.00%	1	1

Q9 - In your opinion, how would you rate the problem of animal pain in the Category D studies performed at your institution?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	3.00	1.59	0.58	0.33	22

#	Question	Unknown		Total
1	Low (Either procedures are not painful or the pain is well controlled.)	100.00%	10	10

2	Moderate (Painful procedures are limited, and pain is controlled the best we can, but animals do sometimes experience some pain.)	100.00%	11	11
3	High (Procedures are very painful and controlling pain is a challenge.)	100.00%	1	1

Q10 - If specific pain pathways in an animal model could be lastingly shut down prior to experimentation, could this be considered to be a "refinement" strategy for experimentation?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	2.00	1.14	0.34	0.12	22

#	Question	Unknown		Total
1	Yes	100.00%	19	19
2	No	100.00%	3	3

Q11 - If mechanosensory and inflammatory pain perception could be lastingly shut down prior to experimentation, should such an animal be classified in a similar way to an animal that receives anesthesia or an analgesic?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	2.00	1.35	0.48	0.23	20

#	Question	Unknown		Total
1	Yes	100.00%	13	13
2	No	100.00%	7	7

Q12 - Would you be willing to approve the use of additional animals in Category D experiments in order to determine the extent to which the new method reduces suffering without interfering with experimental outcomes?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	1.00	1.00	0.00	0.00	22

#	Question	Unknown		Total
1	Yes	100.00%	22	22
2	No	0.00%	0	0

Q13 - Would you be willing to approve the use of additional animals in Category E experiments in order to determine the extent to which the new method reduces suffering without interfering with experimental outcomes?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	2.00	1.19	0.39	0.15	21

#	Question	Unknown		Total
1	Yes	100.00%	17	17
2	No	100.00%	4	4

Q14 - How many peer-reviewed studies in a relevant area of research would you find necessary to be comfortable with an investigator using this mouse model in their research in place of traditional analgesics or anesthesia?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	2.00	5.00	4.00	1.02	1.05	21

#	Question	Unknown		Total
1	1	0.00%	0	0
2	2	100.00%	1	1
3	3	100.00%	8	8
4	4	100.00%	2	2
5	5+	100.00%	10	10

Q15 - Assuming sufficient peer-reviewed studies show that this mouse model reduces suffering without interfering with the pathways being studied by researchers, would you be willing to require its use by investigators?

#	Field	Minimum	Maximum	Mean	Std Deviation	Variance	Count
1	Unknown	1.00	3.00	2.45	0.78	0.61	22

#	Question	Unknown		Total
1	Yes	100.00%	4	4
2	Yes, if the investigators cannot use traditional analgesics or anesthesia	100.00%	4	4
3	No, but I could recommend it as a further refinement strategy	100.00%	14	14
4	No	0.00%	0	0

Q16 - If you have any final comments or recommendations regarding the survey, please feel free to write below.

I am concerned this survey is too broad and does not address the numerous strain specific, sex specific responses to pain and recognition of pain. This technology would in my mind require validation of proper pain control similar to that used to validate proper management and control when paralytic agents are employed until such measurements and validations are confirmed

For this question: "If mechanosensory and inflammatory pain perception could be lastingly shut down prior to experimentation, should such an animal be classified in a similar way to an animal that receives anesthesia or an analgesic?" my answer would have changed to "yes" if only analgesics were referenced. I would not feel comfortable foregoing anesthetics for surgery as there are other components to consider (such as animals remaining motionless)

If mechanosensory and inflammatory pain perception could be lastingly shut down prior to experimentation, should such an animal be classified in a similar way to an animal that receives anesthesia or an analgesic? This question was hard for me to definitively answer. I answered "No", but there may be cases where I would not agree - for instance if the animal was treated to develop a physiological response (illness) that would cause the animal to loose some ability, I may want it to be still classified as cat. "D")possibly "E"???) Also, I am NOT in favor of the IACUC mandating which animal a PI uses, it is the PI's job to justify the use of the animal it chooses. The IACUC can ask questions about the justification, but to blanket require an animal model like this I would be very hesitant to support

I found this project worth a Nobel Price! Wonderful! The main concern in IACUC committees is basically making sure that whoever is working with the animals are doing their best to guarantee their well being. Having an animal that cannot experience pain is the best that you could ask for, IACUC wise. However, even though I did say I would approve more animals for a study with this characteristics, is still challenging to maintain a balance when as IACUC, you also want to have fewer animals being used. Wonderful project! Please keep the IACUC ListServe updated of your outcomes, posters or any other progress made towards this goal, I can't wait to hear that this mouse strain is available to be able to recommend it to every other friend I have in the scientific community. Best luck!

Hi, your survey does not allow for additional qualification of the yes or no answers. Those comments might be useful to your discussion section.

There are several things that are taken into account when reviewing an animal use proposal, pain management through pharmacological means (general anesthesia, NSAIDs, opioids, local anesthesia, etc) as well as non-pharmacological techniques (Heat/cold application, physical therapy, socialization, human contact, enrichment, etc) are one aspect of the review.

The other component to take into consideration is distress to the animal and research staff. Developing a model without pain receiving pathways is a refinement that potentially addresses pain, but I would require scientific justification for withholding analgesia and a thorough description on how distress would be appropriately managed (example: could the animal be conditioned or acclimated to the procedure(s)?). There would need to be strong scientific justification and a complete literary search. It is difficult to make a determination without having a full understanding of the entire proposal.

This is a worthy goal, but the basic premise of pain categorization in US-based research is that if we would feel pain, we should infer that the animal will feel pain. Modifiable pathways that demonstrably minimize or eliminate pain would be a great refinement to add to our options, but that's not going to change the categorization criteria, particularly since most of us use procedure-based categorization for the most part, rather than individual animal-based assessments.

I answered the D and E questions for the highest category. I just went back through two months of protocols and counted -- I don't have a long term percentage on that. I suspect that answers to these specific knowledge questions will be unreliable. // I have no idea how many studies I would need to be comfortable -- one or two thorough and good ones might be enough! // The biggest issue I see with this interesting idea is that many researchers are using genetically-modified strains already and may be averse to adding modifications. They even resist using male mice if previous studies have used females -- changes like that can affect the outcome. // Some researchers do studies in which the development of an inflammatory condition is the outcome. They resist analgesics, because they could undermine the inflammation. But perhaps a pain-free mouse model would be OK there, if the lack of pain did not affect the inflammatory process.

pain perception would only be one criteria, IACUC must still weigh tissue damage, impact on normal behaviors, etc. when determining if a particular model is appropriate.

the pain system especially peripheral nerves interacts with all body systems and tissues. if the pain system is shut down via genetic approaches from birth then I would expect their body systems to not be normal and as such data from these animals may not approximate to normal animals. this situation would not be comparable to short term use of a analgesic or anesthetic in a mature animal which i would suggest is much less likely to impact experimental results.

Good luck with your survey - pain free mouse strains sounds very intriguing!

This would be great!