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Referred pain: Connections between injured bladder afferents and their uninjured neighbors in dorsal root ganglia

By

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CHAPTER 9

WISL

Emily L Tran

SUMMARY: The science we conduct only matters if we can communicate about it to the world. If we do not put any effort into making the complex and sometimes convoluted interpretations of our research into understandable, digestible pieces, we lose our ability to reach the most important audience – our communities. Science need not be inaccessible, and in fact immensely benefits when other voices are involved. I am grateful to the Wisconsin Initiative for Science Literacy program, specifically Bassam Shakhashiri, Cayce Osborne, and Elizabeth Reynolds for encouraging productive science communication and help editing my writing. This chapter is an addition to my thesis that walks through my project for a non-science audience. All of the information has been described in depth in previous chapters.

INTRODUCTION

Startling news from the CDC reported that, as of 2023, one out of every five U.S. adults experience chronic pain (Rikard et al., 2023). Think of your five closest friends or family members. Imagine that at least one of them is living with pain that has persisted for months, and may be seriously lowering their quality of life. Sometimes chronic pain arises from a disease, other times an injury, but often a lack of obvious or visible cause makes it difficult to diagnose, let alone properly treat. In the pain world, doctors' toolkits are limited in their ability to help patients because (1) it can be hard to identify the cause of the pain, (2) it can be difficult for patients to describe their pain, and (3) pain research tends to be under-funded, limiting our basic scientific understanding of pain. Pain is something the majority of us experience to some degree, and yet we remain ill-equipped to explain it, especially for complex chronic pain conditions.

In more mild cases, the pain can be managed with over-the-counter drugs and rest, and perhaps is not constant but occurs as "flare ups." In more severe cases, prescribed drugs may be required along with regular doctor visits, physical therapy, or periods of immobility. These are often not perfect remedies and provide minimal relief, even if the condition is characterized by inconstant pain or "flare ups." In all cases, doctors struggle to find the ideal treatment and typically only reduce pain, not completely get rid of it. Tragically, in some cases, attempted treatment may worsen or prolong pain. Imagine the life of one of your close friends or family members again. They may worry about getting through their day in pain, accommodate their day around the pain, and are faced with the reality that it may never quite improve or go away.

People with chronic pain experience high rates of depression and anxiety (Aaron et al., 2025), substance misuse (Ripon & Maleki, 2025), and risk of suicide (Racine, 2018). These circumstances are not hard to imagine given the challenges these individuals must overcome or live with. Now let's complicate things further. A proportion of people with chronic pain suffer from

pain in multiple locations throughout their body. These pains can affect many areas; the head, which results in migraines, other limbs, which result in skin sensitivities, and internal organs, which are described as internal pains. In some cases, the pain is caused by systemic issues that tie them together. In other cases it appears as secondary, or referred, from the more discernable pain condition.

Think of the symptoms of a heart attack. What is one of the most commonly reported signs, aside from chest pain, described when one is experiencing a heart attack? Arm pain. Arm pain that arises from a heart attack is not because something is wrong with the arm – the heart is the problem – yet there is no denying its pain. This is considered secondary, or referred, pain. There are many more examples of these afflictions, such as a stomachache that radiates to your back, or a stinging in the nipple upon receiving a neck tattoo. Individuals with chronic pain, however, are quite familiar with secondary pain that is not as easy to treat. The variability of *where* pain occurs in the body, the *number* of locations it can occur, and the *degree of severity* make pain treatment challenging, and we are not even going to dive into inequalities in U.S. healthcare regarding minoritized individuals, including women .

A high proportion of chronic pain arises in the pelvic region, which impacts upwards of 16% of women (Dydyk, Singh, & Gupta, 2025). One out of six women you know may have chronic pelvic pain of some kind. Chronic pelvic pain can be a tremendous burden on women; reporting symptoms of pain or discomfort that can make even simple, necessary activities like urination challenging or outright agonizing. Symptoms affecting the bladder in this case are often diagnosed as “cystitis,” which is commonly characterized by bladder pain with no obvious cause. Along with sometimes painful urination, women will report feelings of urgency that cannot be ignored, or having to use the bathroom often at night, disrupting their sleep.

Imagine one of the women in your life experiencing these struggles, and how hard it must be to plan their day around having to use the bathroom, canceling plans to avoid

challenges using the bathroom, and the bitter pain that is felt when she finally gets the courage to use the bathroom (or is forced to because of her biological needs). Additionally, imagine the isolation or embarrassment she might feel if she keeps such experiences to herself. The negative psychological impacts of cystitis are not surprising. But what does this have to do with referred pain?

Unfortunately, many women with cystitis experience chronic body-wide pain as well (Chelimsky, 2021), where some of these patients even demonstrate nerve damage in their lower leg (Matthews et al., 2019). Why do patients that have nerve damage and pain in the skin of their leg also have pelvic or bladder pain when the bladder itself is otherwise okay? Why do people who have heart attacks experience pain in their arm when the arm itself is otherwise okay? Well, we don't fully know.

We have some good ideas about how pain is sensed from spinal cord neurons to neurons in the brain that have led to helpful treatments. However, while we know that problems in the spinal cord can contribute to referred pain, it cannot fully explain why some cystitis patients also have nerve damage in their leg. Where else can we look, if not the skin or the bladder itself? Many people do not realize that we actually have neurons *outside* of the spinal cord and brain. These neurons are responsible for sensation of the outside world and internal organs. They are fittingly called "sensory neurons." Sensory neurons have cell bodies that sit in bundles right outside the spinal cord. These bundles, technically called sensory ganglia, have 3 major jobs: (1) gather sensory information from your external environment (the world around you) and internal environment (the world inside you, i.e. your organs); (2) interpret and enhance or tone down the sensation; and (3) send that sensory information into the spinal cord so it can travel up to the brain and be perceived.

The location of the bundle in which a sensory neuron nests determines where it collects sensory information. The "information gathering" part of the neuron actually extends beyond the

bundle, coursing through the body (sometimes at long lengths) and turns into “nerve endings” in whichever body part it is responsible for. Each bundle, and each sensory neuron within it, corresponds to a different level of the spinal cord. For example, sensory neurons that gather information from the legs sit next to lower levels of the spinal cord (towards your butt), and sensory neurons responsible for the arms are higher in the body (towards your chest) **(Figure 1).** The geography of the human body is nicely laid out so the nerve endings of the legs do not need to travel too high up in the spinal cord, and vice versa for the arms (and every other part of the body).

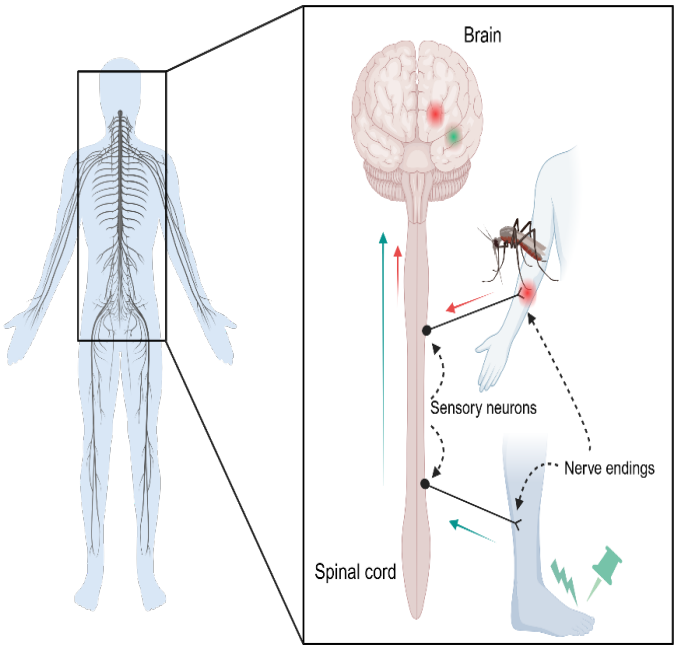

Figure 1. Example of the geography of the human body. Sensory information, gathered from nerve bundles in the arm or leg, is sent into the spinal cord and up to the brain. A mosquito bite in your arm or pain in your foot from stepping on a thumbtack are felt by different sets of nerve endings (and their attached sensory neuron) but eventually all lead back to the spinal cord and into the brain (Images created with BioRender).

Bundles are a mix of sensory neurons from different parts of the body. This makes it possible for sensory neurons from the bladder to communicate with sensory neurons of the feet – both sit in lower bundles. However, not only do sensory neurons create a bundle with a mix of body parts, the neurons themselves are responsible for different sensations. For example, your skin can feel so many different sensations – hot, cold, light touch, pain – and so can many of your internal organs. So within these many bundles, you have a mix of skin neurons responsible for touch, bladder neurons responsible for heat, and likely even colon neurons responsible for pain...and everything in between **(Figure 2)**.

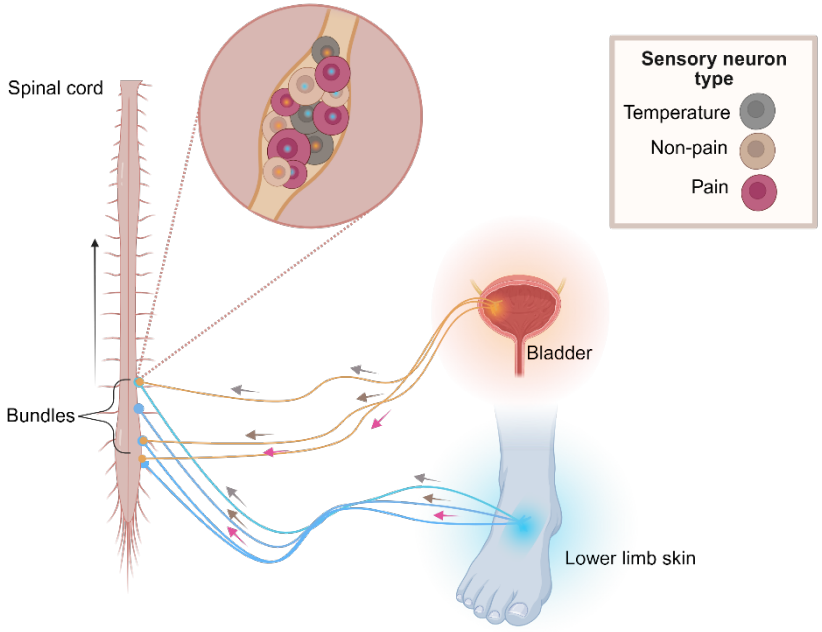

Figure 2. Mixing of sensory neurons in bundles. All organ systems, including the skin, have a variety of neurons that are responsible for different sensations. These various sensory neurons of different organs mix with each other in the same bundles. (Images created with BioRender).

You can imagine then how sensory information, pain in particular, traveling up to the brain can get muddled. My research focus is how pain information in these bundles become crossed after someone experiences cystitis, leading to lower limb pain. The “someone” in my case are lab mice, and the lower limb we examine is their hind paw.

A NOTE ON LAB MICE

Although some people argue rodents are imperfect models of human disease, a better alternative has yet to be discovered. While we cannot directly translate mice biology to humans, the biology of mice is the same or similar to humans. We can make educated assumptions about how humans function based on testing mice biology. This does not necessarily mean that a drug that treats referred pain in mice can be used in humans, but it does tell us *what* makes referred pain feel better in the mice. This knowledge helps scientists find the same "feel better" machinery for humans (think of it this way, bike tires and car tires do the same thing – they transport you from A to B – but you can't switch a car tire for a bike tire).

Mice are great starting points, and without their involuntary involvement in science we would not have the amazing medicines we do now. From smaller achievements like rash creams for eczema to life-changing advancements like the polio vaccine, rodents have been essential to improving human health. And if you care less about humans and more about your pet, rodents have been the stars of veterinary medicine as well. All of our experiments were done with intention and immense effort to minimize the suffering of our mice, working alongside veterinarians to ensure they are properly cared for. As I proceed with describing my research, remember that it was made possible by the mice. Take a moment in your head to thank them as I have for each experiment, each data point, and each interesting new piece of knowledge. Their contributions should not be taken lightly or for granted. Thank them for the medical advancements we have today that you benefit from even if you don't realize it.

MY PROJECT

Section 1: Overview

There are so many ways to test a hypothesis and so many questions to ask when designing an experiment to test that hypothesis (too many for one person, certainly). I spent much of my first two years in graduate school building not only my technical skills for doing

experiments, but also the background knowledge necessary to form a hypothesis. Without getting into the weeds, a small list of some of the many things I had to deeply grasp included basic neuron function, how to test basic neuron function (there are so many ways), mouse expressions of pain, how to test mouse expressions of pain, how do neurons express pain ("what do neurons in pain say to neurons that are not in pain" is the start to a great joke if only I had time to think of one), how do you make a graph out of the things neurons say to each other, how do you even see neurons when they are so tiny, which bundles hold bladder and paw neurons... and so on and so forth. A small list.

Through trials and tribulations, blood, sweat, and tears, I came up with my central hypothesis: cystitis causes hyper-activity of "uninjured" paw neurons in the same bundles that also have "injured" bladder neurons. The hyper-activity of paw neurons is what is causing the referred pain in the hind paw skin of these mice. To break my hypothesis down, I am stating that mice with cystitis and referred pain in their hind paws have sensory neurons that are *sensing more* than they should be (being over-dramatic, one could argue).

The idea behind my hypothesis comes from patients with severe nerve damage who experience a loss of nerve endings (the "information gathering" part of your neuron) but retain physical or phantom pain in the area (ever heard of phantom limb syndrome?). Interestingly, this is something almost anyone can relate to, on a much smaller scale of course. If you have ever burned yourself, the area over or around the burn hurts – it is sensitive to the touch. Like nerve injury patients, the injury causes some loss of nerve endings. It is very logical to think that, if you are *losing* the "sensing" part of the neuron - the nerve ending - wouldn't this cause someone to become *numb*? Unfortunately for us, our biology does not always seem to work based on logic.

Injured skin can become more sensitive after losing nerve endings because of the other sensory nerves around it (other, uninjured cell bodies of nerves in those bundles way up near the spinal cord). A peculiar thing happens to these uninjured neurons. In some cases uninjured

sensory neurons *switch* their function when they are near injured neurons (Tran & Crawford, 2020). It is as if those injured, sometimes dying neurons are crying out in pain and the uninjured neurons panic and also cry out in pain. In cases of just a small burn, this might be helpful in recruiting immune cells to heal the damaged skin. However, in cases where the pain persists, the cries from uninjured neurons are quite burdensome and can even be debilitating. My hypothesis is essentially saying that these uninjured hind paw neurons are crying out in pain after seeing their fallen brethren, the bladder neurons, become injured (via cystitis).

There were many different levels to consider when we thought about exploring the biology of animals and how it changes after we would experiment on them. Firstly, we wanted to know if our treatment (cystitis) does in fact cause mice to have more sensitive hind paws. The biology we were testing in this case was their behavior. Next, we wanted to dive deeper and see what was happening in the neurons of the paws. I decided to look at protein expression, which I will explain in a moment. Finally, we wanted to look at the neurons' behavior. For this, I decided to look at their *neurophysiology,* which is a fancy word for examining neuron behavior at the *molecular level* (in other words, their activity level, which is pretty much invisible to the naked eye - molecular). I'll walk through each biology level briefly and then move on to the exciting parts – the results and implications for humans.

Section 2: Mouse behavior

To be honest, I enjoy talking about methods most, so saying that results are the most exciting part of science is not all true (to me at least). Good methods (theoretically) provide good results, and your interpretation of results is only as good as the experiments they came from. Therefore, it is important to be "rigorous," as academics like to say. Really, this just means employing well thought-out experiments, with honed techniques from well-trained hands.

However, there are trade-offs for doing the best science ever and *realistic* science with the tools and resources you have, often requiring a balance of both. In humans, cystitis affects both men and women, and spans all ages of adulthood and older adulthood (ignoring pediatric patients). A rigorous experimental design might then include both male and female mice, and several age groups. We instead opted for only female mice of average adult ages. We did this because cystitis in humans tends to affect women more than men (Patnaik, et al., 2017), and aging out mice to be “elderly” can be a pretty significant time commitment. Our trade-off was targeting a specific mouse population that is quite relevant to the human population, and being efficient with our time (as grad students, the time really flies). Later on for other projects we used male mice, but I will not be discussing those studies. Before I explain how we tested paw sensitivity, it would be helpful to know how the mice got cystitis.

Earlier, I mentioned that cystitis is often diagnosed when a patient describes bladder pain but does not show an obvious cause. This definition may be a bit misleading, because cystitis many times is a result of *something*. For instance, patients with cystitis may have previously had a urinary tract infection (UTI), undergone chemotherapy, or been afflicted with some other condition causing bladder inflammation (or irritation). The remarkable part of cystitis is that *even when the other problems are resolved* (the UTI goes away, chemotherapy has ended, or bladder irritation is healed), the pain is still there. Furthermore, in some of these patients, it is not just the bladder that has persisting pain, it might also be their lower limb, like their leg or foot. The way we chose to reproduce cystitis symptoms in our mice was to give them a form of temporary bladder inflammation. To do this, we (or rather, Sara Stuedemann, the wonderful other grad student in the lab) used a very small catheter, fit for a mouse, to slowly inject a small amount of an irritating chemical – acrolein. The mice were anesthetized during the process, and the acrolein sat in their bladders for a short amount of time until they woke up and urinated naturally. Over the course of a few days, we then studied their behavior.

Because we care about referred pain in the skin of the mouse's paw, we focused on tests of paw sensitivity. Mice move around a lot, so unfortunately for them we had to remove them from their cage separately for testing and place them in a smaller box atop a mesh-like surface. The smaller box still allows them to move freely, just in a more enclosed space, and the mesh-like surface allows us to access their paws from underneath. We used several "filaments" (similar to toothbrush bristles) to poke the mouse's paws **(Figure 3)**. Filaments are different thicknesses, corresponding to a "force" (think the pressure of someone's finger poking you), which mice respond to at varying levels (soft force = no pain, strong force = pain). We applied each filament to the underside of their hind paws (like the pads of your feet), soft to strong, and wrote down if a mouse moved its paw or not.

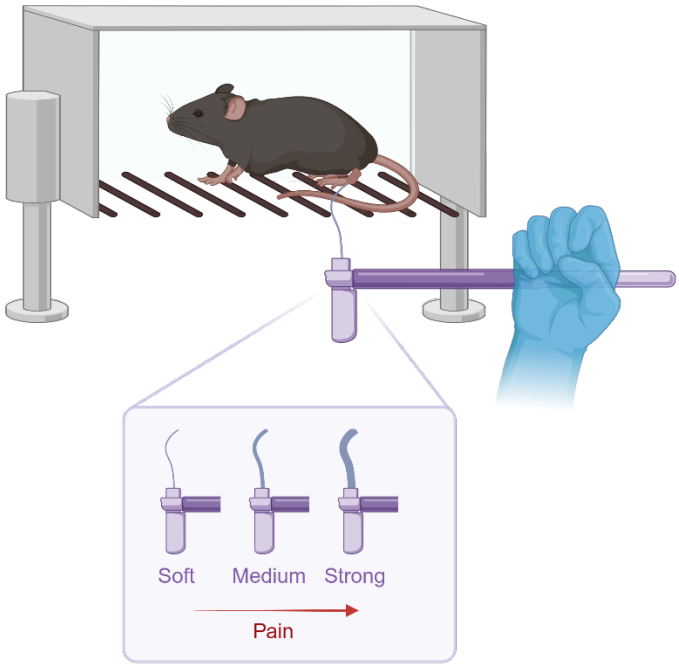

Figure 3. Testing of paw sensitivity with filaments. Mice are held in small boxes atop a mesh flooring. The holes in the mesh allow small filaments through, which we use to poke the bottoms of the paws. Filaments come in different sizes, which correspond to different forces. Small filaments are a softer force, and large filaments are a stronger force. The larger the filament (stronger force), the more painful the poke (Images created with BioRender).

How does this test pain? A mouse that is not in pain will likely not respond to a soft force, and may or may not respond to a strong force. However, a mouse *in pain* may respond to the soft force, and certainly to the strong force. We tested mice before and after reproducing cystitis, as well as mice that were "controls." Controls received no bladder irritant, but still got

catheterized. We then compared the pain levels of mice before and after treatment, and to control mice that received no treatment. Controls are important because without them, we would have no frame of reference for what a “painful response” looked like. We decided to “increase our rigor” in our experiments by using two types of controls: (1) responses of the same mouse when they were in pain (with cystitis) to when they were not (before cystitis) and (2) to separate mice not in pain at any point (controls, with catheter only).

So what did we find? As expected, the mice with cystitis had much higher pain responses compared to before they got cystitis, and also compared to control mice that never got cystitis at all. They demonstrated *more* paw sensitivity, telling us that our mice were experiencing referred pain in their paw skin. Remember, like in the human patients, the skin of their lower limbs were otherwise healthy and so the sensitivity must be due to some other cause...could it be the cystitis?

In these same mice, we also tested whether or not they even had cystitis. The tricky thing about animals is that they are as individual as humans, meaning they respond to treatments in their own individual way. For instance, some mice that have bladder irritation do not experience cystitis pain, but in other mice the irritation seems to really impair them. This is very similar to humans actually, where some patients with cystitis mostly go about their day, but some with severe cases would rather avoid moving at all. We needed to make sure that our mice with high paw sensitivity also had cystitis.

One of the ways to measure cystitis is to run what we call a “Void Spot Assay” (VSA), which is a fancy name for a urine test (but probably not the one you’re thinking of). We took the mice out of their home cage again and placed them into another cage (about the size of a shoebox), lined with filter paper on the bottom (you know the filter paper used to make coffee? That one). The mice roamed around for about three hours, and during this time they urinated (or “voided”) on this paper. When we removed them and looked at the filter paper, we could

measure how many “voids” they made in three hours, and how large the “void spots” were. Mice with cystitis tend to have much smaller “voids” that are all around the paper, whereas mice without cystitis have large “voids” usually in the corners of the cage. If we think about it, it makes sense for a mouse with bladder pain to “dribble” and have less control of their voiding (because they are holding it in to avoid pain upon voiding, or because sometimes cystitis also causes incontinence). When we looked at our mice who had paw sensitivity, they also showed clear signs of cystitis. Great! Then what? It was time to test their neurons.

Section 3: Protein expression and RNA exploration

The hardest part about working with neurons is not just that they are small or fickle, it also requires euthanasia of the animal. However, experiments that work directly with neurons are essential for understanding how they function. Once a mouse is euthanized (humanely, and with great care taken to minimize suffering), two things can happen: (1) we “fix” their tissue (think embalming) or (2) we keep the tissue alive and test neurons *outside* of the animal. “Fixing” tissue (in our case, neurons), preserves everything about them that would otherwise degrade once an animal has died. The second strategy, which preserves the life of the neuron after an animal has died, will be discussed later. The first strategy is what we use to assess how neurons could be changing based on their characteristics (think of it like changing an article of clothing). Neurons can be characterized in several ways, one of them is by the proteins they express and another is which RNA they express (and how much of it). I won’t go into detail about protein and RNA, but it is helpful to know why we care.

Proteins have many different roles; they provide structure to neurons that prevent them from falling apart, help neurons talk to one another, and help keep them healthy. Some of these proteins are responsible for pain sensing. Similar to proteins, RNAs are important for neuron function and carry the instruction manual for our DNA, which dictates our genes. RNA helps to produce proteins, so it is basically another way to test similar questions about neuron

characteristics. The proteins a neuron has (or "expresses") and how much of a certain RNA it contains can tell us what that neuron is responsible for. Is it a pain sensory neuron or a sensory neuron that gathers information about light touch? We can use the protein and RNA expression to decode this. When we consider that, after injury, some uninjured neurons *switch* to feel pain, we imagine that they also *switch* to have more pain-related proteins. Think about this switching like what we do when the weather switches from warm to cold. Instead of a short-sleeve shirt, we switch to a sweater to account for the new conditions we are in – the neurons do this too. We wanted to test if cystitis causes neurons to have more pain-related proteins (or RNAs), and if these neurons come from the hind paw.

But hold on, let's back up a moment. How do we know which neurons come from the hind paw? How do we know which *bundles* to even look in for hind paw neurons? Those answers came from a set of experiments where we harnessed the power of fancy fluorescent microscopes that see different color "probes." Probes were attached to either bladder or hind paw neurons of our mice. Basically, we "dyed" certain neurons different colors (probes) so that when we took a close look at each bundle, we could see where the colors were, and how many of them existed in a given bundle.

The probes work in a pretty neat way. We chose a blue probe for the hind paw neurons and green for bladder neurons. Each probe is in liquid form, which allows us to use a very small syringe with a very small needle to inject the liquid into the skin of the hind paw and the outer layers of the bladder. The mice of course were anesthetized, and when they awoke had sore paws and bellies, but otherwise continued to go about their lives as normal. These probes are called "retrograde tracers," where "retro" means backward and "tracer" is their action. The way the probes move from the paw or bladder all the way back into a bundle of neurons is by moving (tracing) in the backward direction, up through the nerve ending and into the cell body. Think of the way we sense things; information travels through our nerve endings from the paw or bladder

in the *backward* direction towards the bundles as well. The main difference is that, instead of a feeling that is traveling, a colorful probe is.

Without getting into all the details of why what we found was cool, I will emphasize that the new information may be somewhat controversial in the field. Other studies have used similar methods to identify neurons using probes. Remember when I explained that certain bundles have neurons responsible for body parts that they are nearest to (the leg has bundles lower toward your butt, arms toward your chest)? It has been mostly assumed that bladder neurons exist in the very lowest bundles, and hind paw neurons are mid-low range. To our surprise, we consistently found our green bladder probe in mid-range bundles as well. In fact, we found *both* probes together in the same bundles that we will call “L3”, “L5”, and “L6,” where our in-between “L4” bundles only had paw probes (i.e. paw neurons) **(Figure 4)**. The “L” corresponds to the level of the spinal cord these bundles are nearest, in this case the “lumbar” region. There were two highlights of this small study: (1) the real possibility that bladder and paw neurons were in contact, providing us rationale for referred pain, and (2) that we could use probes to look at specific neurons (paw or bladder) to understand how cystitis was affecting them.

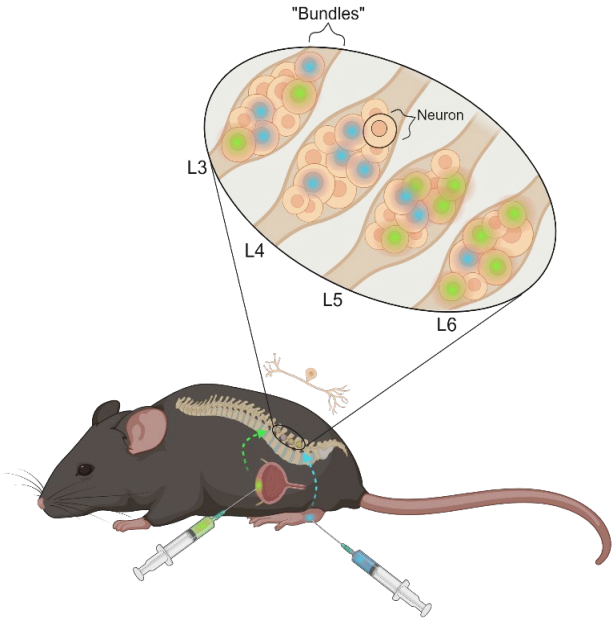

Figure 4. Using probes to identify bundles with bladder and paw sensory neurons. Green probes injected into the bladder were found in lower bundles *and* up in a mid-level bundle (L3), which was surprising. Blue probes injected into the hind paws were found in all mid- and low-level bundles. There was overlap between probes, meaning that certain bundles had both bladder and paw neurons (Images created with BioRender).

Back to the main test - protein and RNA expression. We tested protein and RNA at different times, and our RNA expression test was *exploratory* more than it was really experimental, so I will focus on protein. When looking for certain proteins in a neuron, we used a different type of probe that does not need to travel in any direction except onto the neuron. It directly “binds” or attaches itself. To tell different proteins apart, we simply used different color probes again. We also incorporated the same blue probe from earlier so that, when looking at a bundle, we could tell which neurons came from the paw and which had each protein depending on the mix of colors (probes) we could see. For example, if we attached a red probe to Protein A, and we saw a neuron with purple coloring, we might conclude that the paw neuron (blue probe from the paw mixed with the red probe from Protein A), had Protein A. If we see a neuron that only shows up as blue, perhaps that paw neuron does not have Protein A **(Figure 5)**. We had a few specific pain-related proteins in mind, and while their names are not important, it is helpful to at least know that we chose them because other studies have found them in their experiments of cystitis or nerve injury pain. If their expression is important for the two problems independently, could they also be related to referred pain?

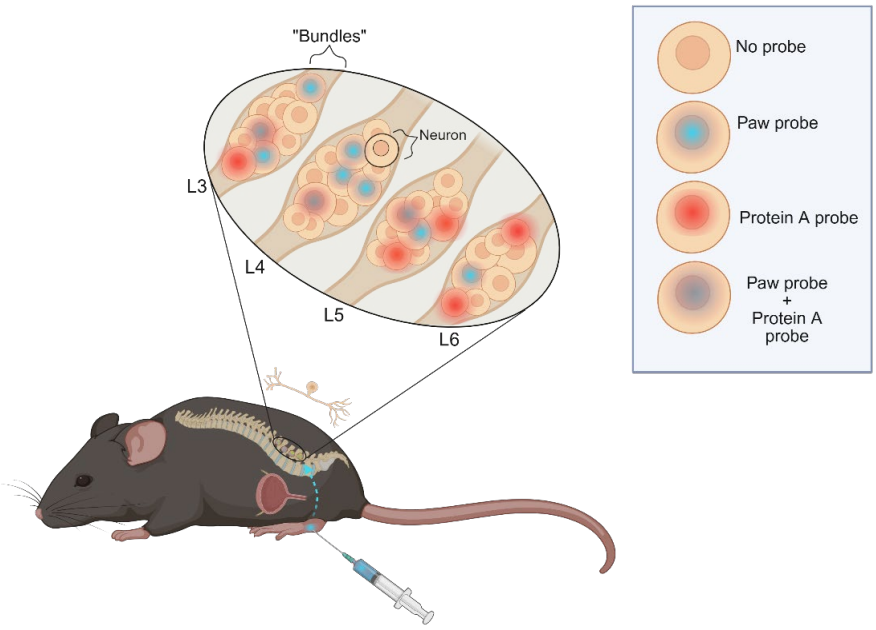

Figure 5. Probes identified in neurons tell us which proteins are expressed. Paw neurons (labeled with the blue probe) were studied to determine if they expressed a certain protein. Different proteins will have different color probes. In this example, Protein A is in red, and when expressed in non-paw (non-blue) neurons shows up as red. When Protein A (red) is expressed in paw neurons (blue), it shows up at purple, as if we were mixing paint colors together.

Using the same mice that had cystitis and paw sensitivity earlier, we collected their bundles and attached our probes. When we looked at our L3, L4, L5, and L6 bundles that had blue paw neurons, we saw pain-related proteins expressed all over (each probe was present in the neurons), but at least one of the probes showed up very brightly in paw neurons specifically. Remember, the paw neurons were uninjured and it was only the bladder that received any sort of injury. Very interestingly, our L3 bundle that had mostly paw neurons and only a few bladder neurons had the greatest amount of that pain-related protein. Seeing a ton of this one probe (this particular pain-related protein) in paw neurons in the *same* bundle that had injured bladder neurons brought us closer to confirming our hypothesis. Perhaps this pain-related protein was contributing to the sensitivity of the hind paws in our mice with cystitis.

In an early attempt to see the landscape below the protein expression and mouse behavior, we conducted a small study exploring the RNA content of our bundles. Because we were using an inflammatory strategy to produce cystitis in our mice, we sought to measure the levels of neuro-inflammatory ("neuro" = neuron + "inflammatory" = causing inflammation) RNA in bundles from mice with and without cystitis. We hypothesized that there would be high levels of neuro-inflammatory RNA after cystitis. However, to our surprise we mostly saw *low* levels after cystitis, except in L3 bundles. Because this was mainly an exploratory study, we could not make concrete conclusions about why this was the case. However, we noted that L3 bundles followed the pattern we saw with protein expression, in that they were uniquely changed compared to the other bundles. This will become relevant in the next series of studies I discuss.

Section 4: Neurophysiology ("neuron" + "physiology" aka neuron behavior)

The final test of our hypothesis was on neuron behavior. We determined that cystitis affects mouse behavior (causing paw sensitivity) and paw neurons (expressing pain-related proteins). We then needed to study how *living* neurons of the mouse act and react. Successfully completing these studies took the rest of my five years in grad school, and it still feels like there is much left to finish.

My first endeavor for starting these experiments was finding a way to keep neurons alive after they leave the mouse. I had to think critically about the needs of a neuron; what they consume, what they waste, where they exist in space (i.e. their environment). Then, I had to figure out a way to recreate all of these things artificially. I also needed to learn how to *remove* the bundles from the mice so that the neurons do not separate from each other.

Overcoming the first task of recreating a neuron's environment was accomplished primarily by tapping into the research that had already been done with similar goals. From here, I was able to gather information about the fluid environment containing each bundle (quite

literally called “cerebral,” meaning brain, “spinal,” meaning spinal cord, “fluid”). I learned that it was pretty easy to make this fluid on my own, so I did. I also became deeply informed about the other important cells in each bundle that help keep neurons healthy and assist in their communication – satellite glia. If I had more time I would dive into what satellite glia are and all the cool things they do, but for now we can just be aware that they are vital to neurons. It would be hard to create satellite glia on my own, so I was pleased to learn that if I was just careful enough removing each bundle, I could not only preserve the neurons but the satellite glia they were enwrapped in.

With the guidance of Dr. Richard Lennertz and my P.I., Dr. LaTasha K. Crawford, I became an expert bundle remover (it only took three years...at least). The key to keeping neurons alive during the removal process was by ensuring they were always bathed in the artificial cerebral spinal fluid I reproduced, providing them with oxygen (yes, our neurons need it too) and, at least in the first steps, keeping them ice cold. The cold helps reduce any potential decay and keeps the neurons “quiet” so they produce less waste that would otherwise become toxic to them (imagine sitting in a pool of your own waste – not super pleasant). I also had to be very quick. Cells begin to die and decay right after the animal’s death. So, immediately after a mouse was euthanized I needed to remove the bundles (I will spare you the dirty details of this process). Now what was the purpose of this again?

We wanted to be able to study neuron activity close-up, and to do this we needed a way to *see* the activity. We harnessed the power of genetics and used mice that had green probes *built in* to their neurons. The probe only shows up when a neuron is active. So, under a powerful microscope, we could record when they were active (when green probes became visible) and how active they were (how bright the probe became). Isn’t this remarkable?

Once the bundles were out of the body, they were transferred to another type of artificial cerebral spinal fluid, out of the cold, and eventually into a small “chamber” (like a penny-sized

bowl) that was heated to the temperature of a living mouse. This chamber sits right under that powerful microscope. In real-time, I could watch neurons become active when I provided them with some sort of stimulation (think, a starter gun to kick off a race). My hypothesis was that neurons that came from mice with cystitis were more active (they ran faster after the starter gun went off) than neurons that came from mice with no cystitis

Data analysis for these experiments was an achievement on its own. It involved the tedious task of identifying individual neurons in an entire bundle, which can be in the hundreds, and measuring how much of the green probe lights up over time in each neuron. And I did this for dozens of bundles, over dozens of mice. To my great satisfaction, we discovered that neuron bundles from mice with cystitis did in fact have brighter probes, telling us that they were more active. However, this was only true of bundles that had both bladder and paw neurons – bundles L3, L5, and L6. The L4 bundle, which only had paw neurons, did not demonstrate more activity after cystitis. This suggested that neurons must be in close proximity (i.e. the same bundle) to have an effect on each other, at least in our case. Remarkably, in line with our protein expression experiments, L3 bundles had the *most* activity despite having the fewest bladder neurons (next to L4, without any at all). Perhaps the specific population of neurons within L3 bundles make them more susceptible to the effects of cystitis. A classic line in scientific papers would say “further research is required to interpret these findings.” For instance, we could narrow our focus on neurons in L3 bundles and run experiments on only these neurons.

One common way to test if something has an effect on something else is to change that thing and examine the outcome. In science, we can change, or manipulate, the environment L3 neurons are in and see if cystitis still has the same impact. For example, we could introduce some sort of pain-relieving drug and see if cystitis still caused (1) mouse paw sensitivity, (2) changed pain-related protein expression, or (3) increased activity of the L3 neurons. If all of these things still happened, even with the pain-relieving drug, we could infer that L3 neurons

were not largely causative of changes we saw after cystitis. If any of the outcomes were changed, however, we could say that L3 neurons *were* critical to the changes we saw after cystitis. I left these experiments in the hands of future students.

Section 5: Conclusions

My experiments targeting three different levels in biology; animal behavior, protein expression, and neuron behavior, have shown how cystitis could lead to referred hind paw pain. Changes in protein expression and neuron activity lent credibility to the idea that cystitis affects not just bladder neurons, but their neighbors as well. Though this is a tough comparison to make with humans, it is reasonable to think that human neurons affected by cystitis are more active, and likely express some pain-related proteins. Maybe these changes occur in or around lower limb neurons and contribute to referred pain in cystitis patients. The saying that “more research needs to be done...” is always true. Indeed, a deeper understanding of what causes neuron activity to change in specific bundles needs to be examined.

In my final years, I have only started digging into what could be happening to uninjured paw neurons after cystitis. Two separate experiments were aimed at this. The first was studying the activity of neurons when harnessing their own systems of controlling activity. Neurons have built-in ways of either enhancing or lowering activity. By taking advantage of these systems already in place (for instance, how they lower activity) we ca test how the system might change after cystitis. What if cystitis removes a neuron’s ability to lower activity (what if you remove the brake pedal from a car), is this what makes it more active? The second set of experiments used our familiar friend, the blue retrograde tracer, to see if changes in activity occur specifically in blue paw neurons. What we did before was simply look at what happened in bundles *with* paw neurons. Taking it a step further and seeing what happens *in* paw neurons is important for confirming if the referred paw pain we see in live animals has anything to do with behavior of their neurons.

Another important part of any project is acknowledging and understanding its limitations. Each level of experiments, from animal behavior to protein to neurons, had its own challenges. Generally, for all experiments it is hard to describe our techniques as closely relating to reality. In other words, if we think about the way we tested paw sensitivity, in the real world who is going around poking the bottoms of our feet? Our idea was that our feet feel a sort of pressure when we take steps, like the force of several pokes, but it certainly is not an exact measure.

Additionally, protein experiments can only come so close to representing what neurons actually express when an animal is alive. Several things happen before we can even see the protein (mouse euthanasia, tissue fixation, probe binding, etc.). On top of that, we are limited to only one point in time. We cannot, for instance, ask questions about cystitis symptoms that go on for weeks, months, or years with just a single study. We instead chose a couple timepoints (days or weeks) and conducted several different studies with the same endpoints. The hope is that our findings still shed light on the way our neurons change, even if in the short-term.

And finally, when considering neuron behavior, a very obvious limitation is that we are studying neurons when they are in an artificial environment. By its nature, bundle removal causes a degree of neuron damage since it requires physical separation from the rest of the body. However, we still saw effects of cystitis on certain neurons, appearing to overcome the damage from removal. This strengthens our results.

That aside, because we have an appreciation for recreating the neuron environment as closely as possible, we have also tested the possibility of removing more than just the bundle from the animal. Instead, we have kept intact each part of the sensory system. The hind paw skin, sciatic and branching nerves that lead from the skin to the bundles, and the spinal cord remained *all connected* **(Figure 6)**. The purpose was to address the “physiologic relevancy” issue, an attempt to more realistically test activity of neurons. The goal is to eventually study

more directly how neurons activate when poking the hind paw skin, in a way combining animal and neuron behavior experiments.

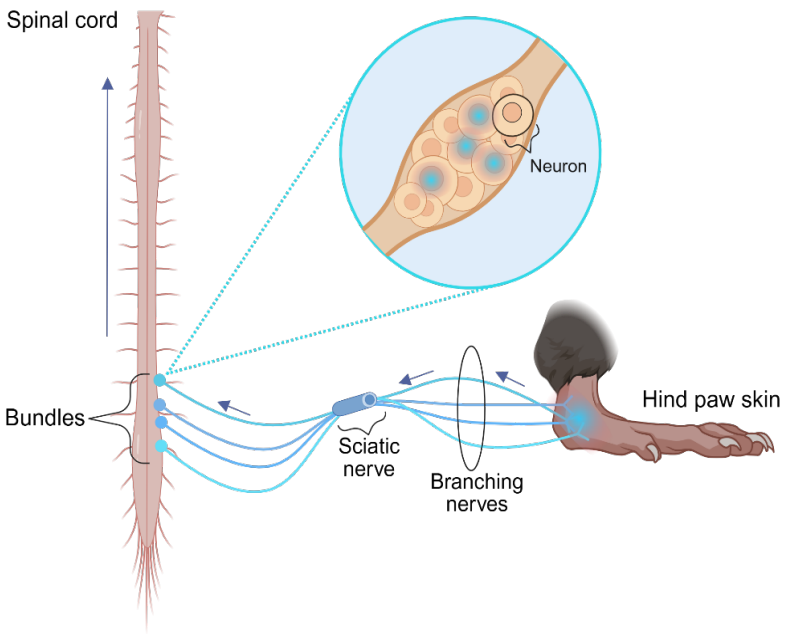

Figure 6. Diagram of sensory system kept intact. Mouse hind paw skin with nerve endings, connected nerve branches, and the main sciatic nerve are carefully preserved. The sciatic nerve splits and connects to each L3-L6 bundles, which has the connected hind paw sensory neurons within them. These bundles sit next to the spinal column and, through other connecting nerves, send sensory information into the spinal cord.

Why not just test on live animals? It is true that the most realistic study of neuron activity is when they are not removed from the mouse at all. These experiments are possible and well-utilized in other labs. However, like with any of our methods, they have their own limitations and challenges.

Testing in live animals sometimes requires use of anesthesia during the experiment. When studying neurons, anesthesia is not ideal as it can affect activity levels. Even testing in animals that are awake can be challenging since you need to be able to record what the neurons are doing while the animal is freely moving (anyone who has been around rodents

knows they move a lot). These experiments require some sort of implanted device, technology which must be small and reasonably accurate in collecting neuron activity. Such tests also in some ways can be more demanding on researchers since they must keep animals healthy and free of infection after surgical implantation of the device (mice like to pick at their injuries, often resulting in more harm to themselves and/or removal of the device). We opted to avoid live animal studies for the time being because of the equipment and skillsets we had immediately available, and because we felt our hypothesis could still properly be tested.

FINAL THOUGHTS

While we are not much closer to fully relieving referred pain conditions, my findings have improved our understanding of what could cause them. Discovering that L3 bundles experience remarkable changes after cystitis is an unexpected and exciting finding that may point to these neurons as targets for treatments.

Already, complex chronic pain relief in more severe cases relies on electrical stimulation treatment of certain bundles or levels of the spinal cord. We propose that more mid-level bundles (like L3) could be targeted to treat bladder/pelvic and referred lower limb pains. Additionally, in thinking about diagnostic challenges, physicians may benefit from exploring multiple body regions and more careful screening of patient symptoms when considering chronic pain treatments. Perhaps cystitis or other bladder pain conditions should not be viewed as independent of other skin sensitivities, and therapeutic strategies could aim to treat both.

On the other hand, certain patients may respond better to treatments focusing on a single ailment. For instance, if cystitis is severe enough doctors might prioritize treating the bladder symptoms with the idea that lower limb sensitivity is also relieved (especially in cases where no nerve damage is present in the skin). In the other direction, cystitis patients with lower limb sensitivities should be screened for nerve damage as that could point to a more serious

disease and therefore more involved pain treatment. In all of these cases, understanding that hyper-active neurons in specific bundles can result in bladder pain *and* referred pain in the skin gives us more opportunity to improve pain relief strategies.

Think once more of that one woman you may know (out of your six family members or friends) who could be suffering from chronic pain. Imagine the relief she might feel when the doctor tells her that they have a great treatment to prevent her pain from lasting forever, and will likely improve in all regions of her body. Imagine she no longer has to schedule her day around using the bathroom or worry about the pain in her legs or feet that lessen her mobility. Pain research is a necessary field that moves closer and closer to improving the quality of life for so many people. My drive to complete this project and pride in my work comes from the hope that we are a few days closer to this reality.

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