Blocking the Thrombin Receptor Promotes Repair of Demyelinated Lesions in the Adult Brain

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Reports by Alexa Roemmich and Haley Masters

The Healing Process: Researchers seek to treat MS by harnessing cells' ability to regrow, repair
By Alexa Roemmich, Science Correspondent; **Lay Audience**

Do you know someone with Multiple Sclerosis? If you do, you are not alone. The autoimmune and neurodegenerative disease, also referred to as MS, affects one million people in the United States and 2.5 million people worldwide. In healthy bodies, cells of the nervous system are coated in a fatty layer called myelin that helps speed up the electrical messages sent from the brain to the body. In MS, the immune system attacks this layer, interrupting the messages and causing numbness, weakness, vision problems, and other symptoms. MS patients and their families are eager for a cure, but current treatments focus on decreasing inflammation and symptoms of MS attacks – not the underlying cause. However, that could one day change thanks to researchers at the Mayo Clinic who recently found a way to regrow myelin in the lab, one big step on the path to stop MS in its tracks.

The team, led by Dr. Isobel A. Scarisbrick, focused on the cells that make up the myelin sheath, called oligodendrocytes. These are the cells that are damaged in MS, but recent studies have found that oligodendrocytes can regrow and begin to repair that myelin layer. The key is unlocking this ability in MS, even as the oligodendrocytes are under attack. To do this, immature cells called oligodendrocyte precursor cells (OPCs) need to do two things – get to the right places, and mature into oligodendrocytes. Strategies to get working oligodendrocytes to damaged areas in MS would help protect and regenerate myelin, getting to the heart of the disease.

However, the body's own defense mechanism gets in the way. At places of damage, the body activates an immune response which also leads to inflammation. Part of the inflammatory process involves a protein called Protease Activated Receptor 1 (PAR1), which recruits immune cells but also suppresses the production and maturation of those important OPCs. If PAR1 could be turned off, could these OPCs repopulate in damaged areas and rebuild the myelin? Scarisbrick and her team believed they could, and created a mouse lacking PAR1 to test out their hypothesis.

First, researchers mimicked local demyelination by injecting a drug called lysolecithin into the spinal cords of adult mice. After 2 weeks of recovery they saw that mice lacking PAR1 had 1.8x the amount of remyelinated axons in the spinal cord compared to normal mice, and 1.5x the signs of oligodendrocytes. Additionally, after one month of recovery, mice lacking PAR1 showed increases in an oligodendrocyte repair molecule. These were all signs of myelin regeneration, and suggested that PAR1 indeed plays a role in stopping OPCs from repairing myelin and protecting nerve cells.

Next, it was important to see what would happen in a broader model of demyelination, as happens during MS. A toxin called cuprizone kills oligodendrocytes in major brain regions and leads to demyelination that resembles the demyelination during MS. Normal mice and mice lacking PAR1 were fed cuprizone for 6 weeks. At the end of the six weeks, both normal mice and mice lacking PAR1 lost oligodendrocytes as expected. However, mice without PAR1 did retain more signs of nerve cell health, including more of a protein called neurofilament. In addition, scientists assessed the rodents' physical abilities using a test called the rotarod. During the test, a rodent is placed on a rotating bar much like a

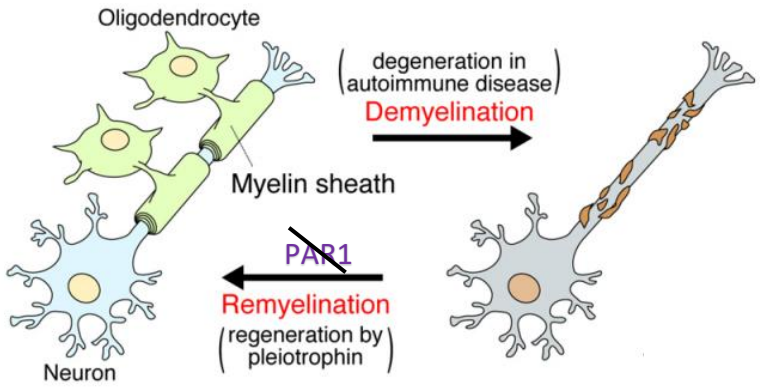

Image. Oligodendrocytes make up the myelin layer that protects nerve cells, and are damaged during MS. Scientists protected oligodendrocytes from damage by turning off a protein called PAR1, an important finding on the way to a cure for MS! *Adapted from multiplesclerosisnewstoday.com*

human log roller. Scientists found that after six weeks of cuprizone feeding, mice without PAR1 could stay on the rotarod at faster speeds, showing that even with demyelination, turning PAR1 off led to preserved balance and physical condition!

Next, the research team wanted to see how the mice would recover from demyelination. When mice eat normal food after being on cuprizone, myelin repairs itself. Would there be any differences in the amount of repair between normal mice and those without PAR1? After three weeks of recovery, both types of mice had recovered oligodendrocytes, but mice without PAR1 had 20% more. PAR1-lacking mice also maintained 20% more neurofilament protein indicating nerve cell health, and once again, performed better on the rotarod. Decreasing PAR1 in mice not only leads to protection of myelin and neurons during damage, but also promotes repair when the threat is over.

While these were exciting and important findings on their own, oligodendrocytes are not the only cells that support the nerves – there are two other kinds of helper cells, called astrocytes and microglia, that are involved with the troublesome inflammation that comes with demyelination and nerve damage in MS. What was happening to these cells? Following cuprizone treatment, mice without PAR1 showed fewer signs of astrocyte and microglia presence than normal mice, a difference which remained 3 weeks into recovery. Additionally, mice lacking PAR1 showed almost no inflammatory response, unlike normal mice which had a strong inflammatory response to cuprizone that took almost six weeks to recover. Therefore, deleting PAR1 may decrease inflammation by eliminating some astrocytes and their associated inflammatory response.

Of course, for scientists, it's never enough to know the *what*, they always want to know the *how*. What was happening within the cells when PAR1 was deleted to lead to such beneficial changes? To figure this out, Scarisbrick and her team studied oligodendrocytes *in vitro*, in a dish. In this up-close look, they saw that, compared to normal OPCs, OPCs lacking PAR1 had the shapes of mature oligodendrocytes, and an increase in mature oligodendrocyte protein levels. Curiously, this increase was similar to the response of OPCs to the classic growth factor BDNF, a protein well-known for supporting growth and health of cells in the brain. Next the researchers looked at a dish containing both oligodendrocytes and astrocytes. When astrocytes had PAR1 blocked, the oligodendrocytes with them had increased markers of function and maturation again, telling researchers that astrocytes without PAR1 can help oligodendrocytes mature, even if the oligodendrocytes themselves still have PAR1. How could this happen, and could BDNF be involved?

The final step for the Mayo Clinic team was to look at the detailed characteristics of astrocytes with and without PAR1. Astrocytes lacking PAR1 had more pro-repair characteristics versus astrocytes with PAR1 that had more inflammatory characteristics. One of the characteristic changes was again in BDNF, with PAR1-less astrocytes having more BDNF than those with PAR1. To see if BDNF truly was the major player behind astrocytic differences with and without PAR1, scientists blocked BDNF in astrocytes without PAR1 and saw that the oligodendrocytes in the dish with these astrocytes had lower signs of function and maturity, matching oligodendrocytes associated with astrocytes with PAR1 intact. Therefore, taking away PAR1 in astrocytes increases BDNF production by those astrocytes that can then help oligodendrocytes grow, mature, and regenerate myelin.

Overall, Dr. Scarisbrick and her team found that eliminating PAR1 in the nervous system of mice undergoing demyelination led to the regrowth of functional oligodendrocytes that could repair the myelin layer damaged in MS, and that this change was due to astrocyte helper cells providing nutrients instead of inflammation. There are two important caveats to this work, namely that more behavioral studies are needed to show that PAR1 elimination improves the condition of MS mice, and that only male mice were used in this study even though MS affects women 3 to 1. However, this research suggests PAR1 is an important target to improve myelin regeneration and decrease inflammation, and therefore provides an exciting new drug target in the fight against MS.

Suppression of PAR1 leads to remyelination of axons following demyelination suggesting a novel potential treatment for MS
By Haley Masters; **Scientific Report**

Multiple sclerosis (MS) is a neurodegenerative disease that effects about 2.5 million people around the world. This disease occurs when the immune system begins to attack the myelin sheaths that are wrapped around neurons. The presence of the myelin sheaths insulates the axons of neurons and in doing so facilitate the action potential traveling down the axon allowing the signal to be sent quickly and efficiently. The absence of the myelin sheaths prevents the efficient communication between neurons and the rest of the body leading to impaired motor abilities and organ dysfunctions. Currently, there is no cure for MS but there are treatments that mainly focus on suppressing the immune response. A major setback in the current treatments is the lack of focus on replenishing the myelin population that was degraded, leaving the state of MS the same. Given that most treatments for MS focus on suppressing the immune response, this leaves a lot of potential for developing treatments to replenishing the lost myelin populations. It is observed that in normal circumstances upon injury there is a population of oligodendrocytes precursors that replenish the myelin population, and this process is severely impaired in MS. The Scarisbrick lab took on this challenge to develop a method to replace the lost myelin by encouraging the precursor population to replenish the myelin population.

The Scarisbrick lab began to ask this question by looking into factors that are dysregulated in demyelinated diseases, such as MS, with the hopes of finding candidates that might act as a switch to kickstart the remyelination process. This search showed that a major difference in MS and control cases are with regards to proteolytic enzymes. Preliminary in vitro studies by this group showed that upregulated proteases led to suppression of oligodendrocyte differentiation. This is particular interesting since myelin is made from oligodendrocytes extending their processes around axons, thus heavily relying on oligodendrocytes to be differentiated from oligodendrocyte precursor cells (OPC). Further investigation showed that these proteases worked by cleavage activation of the protein PAR1. PAR1 has been sited to play a role in myelin development due to the absence of PAR1 leading to an increase in myelin abundance. Due to their previous findings they hypothesized that PAR1 acts as a regulator for OPC production and oligodendrocyte differentiation. They propose that when PAR1 is down regulated, the OPC population increases as well as efficiency differentiates into mature oligodendrocytes that then replenish the degraded myelin sheaths around neurons.

To efficiently investigate the role and potential for PAR1 in restoring the myelin population in vivo, the Scarisbrick lab used two mouse models where they looked at the effect of the absence of PAR1 in response to demyelination. The two modes differed in their method of demyelination. The first model induced demyelination locally by microinjection of lysolecithin in the spinal cord while the second model induced demyelination globally in the corpus callosum through the oligotoxin cuprizone that was introduced in the mouse food. The Scarisbrick lab utilized a PAR1 knockout and wildtype mouse line and used these two methods of demyelination in investigate the role that PAR1 is playing in oligodendrocyte precursor abundance and their ability to differentiate. Along with mouse models, they investigated PAR1s effect on OPC in vitro by culturing purified OPC and astrocytes to more directly look at the role of PAR1 is playing on OPCs and the cells they interact with, astrocyte, by blocking PAR1 through small molecule inhibitor, vorapaxar sulfate.

The first question the group asked was how local demyelination in the presence and absence of PAR1 would affect OPC proliferation and differentiation. They predicted that in the PAR1 knockout mouse, demyelination would trigger the remaining OPC population to proliferate and differentiate into oligodendrocytes which then wrap around neurons to replace the lost myelin and that this process would not occur as much in the wildtype mouse. Post demyelination quantification showed that the PAR1 knockout mouse had a greater abundance of remyelinated axons 4 weeks post injection of lysolecithin. This finding suggested a greater number of oligodendrocytes in the knockout and quantification did show a greater abundance of oligodendrocyte lineage marker Olig2 positive cells and mature oligodendrocyte marker CC-1 at the site of the injection, suggest a greater population of oligodendrocytes are being made

in the absence of PAR1 at the site of demyelination. The results of these studies led the group to conclude that PAR1, when suppressed, leads to an increase in OPC and their differentiation into oligodendrocytes. To understand the whole picture of how PAR1 is affecting the other cells in the CNS, the group also looked into the phenotype of astrocytes and microglia. Astrocytes and microglia are involved in the immune response in the CNS and are recruited to damaged sites. Certain phenotypes of these cells have been associated with whether they are in a pro-inflammatory or pro-repair state, with the ladder providing the environment necessary for efficient remyelination. The PAR1 knockout mouse showed higher number of astrocytes based on GFAP positive staining as well as astrocytes that were positive for S100A10, which is a marker that represents a pro-repair state. They did not notice a difference between the microglia of the PAR1 knockout and wildtype mouse in response to demyelination. Not only did the PAR1 knockout show an increase in OPC and their differentiation but also an increase in astrocytes, which are promoting a microenvironment that facilitates repair over inflammation.

To further understand the role that PAR1 was playing in myelin regeneration, the group utilized a mouse model where global demyelination was induced by oral consumption of the toxin cuprizone. After feeding the mice the cuprizone treated food for 6 weeks, they measured the levels of markers that represent OPC and differentiated oligodendrocytes. The advantage of using the cuprizone method of demyelination, is that when the cuprizone is removed remyelination can be observed. This method provides a positive control for the process they are hoping to induce. When cuprizone was removed the PAR1 knockout and wildtype both lead to replenishment of Olig2+ cells however the difference is that the PAR1 knockout mouse replenished their Olgi2+ cells faster than the wildtype mouse. Along with a faster rate of remyelination in the PAR1 knockout mouse, they observed more mature markers for oligodendrocytes. The PAR1 knockout likely triggered the intrinsic remyelination pathway that the tissue has, suggesting that the use of PAR1 inhibition for remyelination purposes is promising for therapy purposes where the intrinsic remyelination pathway is not being triggered.

Using the cuprizone model, they also looked at the role that other cell types are playing in remyelination. This model allows them to see if the response by the astrocytes and microglia seen in the PAR1 knockout is similar to the natural remyelination pathway observed once cuprizone is removed in the wildtype. In PAR1 knockout mice, astrocytes and microglia showed less proinflammatory properties and more pro-repair phenotypes after treatment of cuprizone. From these studies the group concluded that part of the regeneration process may involve the astrocytes providing a pro-repair environment instead of proinflammatory state. Microglia, which act as the immune cell of the CNS and play a role in the targeting cells for destruction, was showed to be higher in wild type and absent in the PAR1 knockout line. This further supported their conclusion that the reduction of microglial in the PAR1 knockout is due to a shift towards a pro-repair state.

While the immunostaining of the PAR1 knockout in varying demyelination mouse models suggest that the phenotype of MS is resolved, the question remains of if this leads to functional restoration of the remyelinated neurons in the CNS. The Scarisbrick lab wanted to better understand the clinical readout of the PAR1 knockout by measuring the motor coordination and strength using accelerated rotarod paradigm across the lines and treatment. MS is noted for impaired neuron firing and communication with the rest of the body. The angular speed that mice were able to maintain their balance on the rotarod improved in the PAR1 knockout during two phases of the treatment, first being during the cuprizone treatment and second being after the cuprizone treatment. The read out of the clinical measurements show that PAR1 loss has the potential to preserve neurological function. A major benefit of this study is their insight into the functionality of the potential treatment and not just stopping at the immunostaining to show that cell types are present but that they are working together to carry out the functions of a tissue.

While the studies in the mouse models were great to understand the in vivo effect of PAR1, it limits the ability to directly study the role that PAR1 is playing on remyelination. To understand the direct effect that PAR1 is having on the OPC and astrocytes populations, these cell types were purified from wild type mice and cultured. PAR1 was blocked using an FDA approved small molecule inhibitor, called Vorapaxar. When PAR1 was blocked, the OPC population increased as well as the PLP+ cells population, suggesting that not only are the OPC population increasing but they are differentiating well into mature

oligodendrocytes. The advantage of this method is the use of an FDA approved drug, making it more likely that the application of these findings can be put towards a potential treatment for MS or other demyelinating diseases. The culture system also allowed them to look at the effect that PAR1 knockout has on astrocyte cultures and specifically how the astrocytes are regulating the OPC population. When the PAR1 knockout OPC cultures were treated with PAR1 knockout astrocytes compared to wild type astrocytes, there was an increase in the Olig2 population. These findings support the in vivo studies where the astrocytes were adopting markers and morphology that suggested a pro repair state. The Scarisbrick lab dived deeper into the question of how the PAR1 knockout and wildtype differ by measuring RNA, levels through RT-qPCR, of various factors that have been associated with repair or inflammation. The results showed that PAR1 knockout astrocytes had lower levels of pro inflammatory transcripts and increase in some promyelinating growth factors, supporting the notion that the PAR1 knockout astrocytes are promoting a pro repair environment.

Overall the approach and the experiments carried out by the Scarisbrick lab highlighted an important regulator for myelination that can be used in cases where re-myelination is needed. Their studies were done using in vivo and in vitro models and these direct and indirect studies gave strength to their conclusions that PAR1 acts as a switch for whether or not myelination will occur. They further concluded that PAR1 suppresses the progenitor pool from increasing and differentiating into oligodendrocytes. Another strength this paper had, was the insight into how other cell types may be involved in the process making for a much better understanding of the expected outcomes of a potential therapy for humans. The final advantage that this study had is their use of clinical measurements to see if the replenished myelin population was resulting in improved neuron communication to the rest of the body. While the Scarisbrick lab showed great progress for potential therapies for replenishing myelin in MS patients, I think there are a few more questions to ask, many related to the safety of the patient when PAR1 is inhibited. Once such question is the effect of long-term effect of knocking out or inhibiting PAR1 and how this may be affecting the rest of the body. Is the effect they see on the immune system harmful to maintaining homeostasis in the long run by suppressing a pro-inflammatory state? It is possible that inhibition of PAR1 for a long time may lead to an overgrowth of the POC population and eventually adopt a tumor like state. While this paper showed that when mice are treated with a drug demyelination occurs, but MS is due to the immune system attacking the myelin, so how would the PAR1 knockout play out in mice that are a better representation for the cause of MS and would the immune system continue to attack any re-myelinated axons? Another step that needs to be taken before PAR1 can be used in a therapy for MS, is to understand how the treatment will work in human cells. IPSC from patients with and without MS should be taken and differentiated into OPC and treated with an inhibitor for PAR1 to see if the same effect is observed in human cells as it is in mice cells. Overall, the paper was able to shed light on a part of the MS treatment that has gone unnoticed, which is re-myelination not just suppressing the immune attack. The experiments showed the promise of PAR1 in understanding how re-myelination may be possible in different models of demyelination and that the myelin that is restored upon PAR1 knockout leads to restoration of neural communication with the rest of the body.

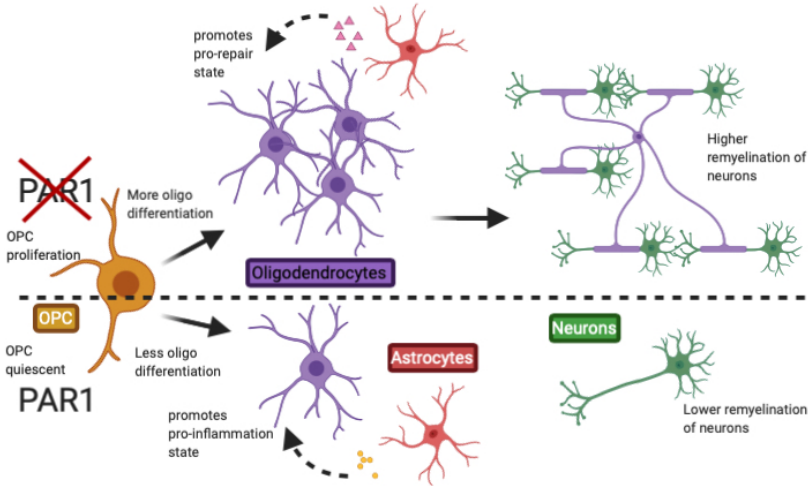

Image 1: When PAR1 is turned on, pro-inflammatory astrocytes are present which leads to suppression of OPC proliferation and genes involved in myelination. When PAR1 is turned off, the astrocytes switch to a pro repair state and secrete OPC growth factors leading to OPC proliferation and differentiation into myelin which wrap around the axons of neurons.