AMAZING PAPERS IN NEUROSCIENCE: Remyelination and Ageing: Ethical Considerations of Using Surgically Joined Animals in Research

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Remyelination is a key repair process that ensures neurons remain protected following injury. This process is mediated by remyelinating oligodendrocytes in vertebrates, however, similarly to other neurobiological processes, the rate and efficiency of remyelination decreases across age and under pathological conditions. This has largely been attributed to two main contributors: 1) decreased exogenous signals supporting remyelination; and 2) aging of precursor cells that no longer differentiate into remyelinating oligodendrocytes. Here we discuss a key paper by Ruckh et al. (2012) who presented novel evidence that exposure to soluble bloodstream factors of young mice significantly rescues remyelination in old mice following a demyelinating insult. In this paper, a parabiosis approach was used where young and old mice were surgically joined for three weeks before and then left as a pair throughout the experiment. Ruckh and colleagues also offer novel insight into the role played by immune system cells, specifically macrophages, in clearance of myelin debris, a further contributor to remyelination. This paper is a good tool to expose undergraduate neuroscience students to basic molecular processes underlying conduction and transmission, helping them link cellular and network components. It also offers a platform for introducing the practicalities of *in vivo* research and debating ethical controversies that arise in animal research.

Key words: demyelination; remyelination; oligodendrocytes; macrophages; central nervous system (CNS); Multiple Sclerosis (MS); parabiosis; green fluorescent protein (GFP) molecular physiology; ethics; animal research

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The myelin sheath insulates neurons and helps conduct electrical activity (Morell and Quarles, 1999). In response to injury or ageing, the myelin sheath may sustain damage. This loss of myelin is termed 'demyelination' and can have a detrimental impact to both the central nervous system (CNS) and peripheral nervous system (PNS). Due to the debilitating impact of demyelination, key repair mechanisms allow continued function of the nervous system and essential processes (Dubois-Dalcq et al., 2008). Oligodendrocytes are myelinating cells in the CNS of vertebrates that can restore myelin sheaths following a demyelinating insult, a repair process termed 'remyelination' (Duncan et al., 2018; Franklin, 2008). How the myelin sheath is produced and rehabilitated is a core part of any neuroscience education.

Multiple Sclerosis (MS) is characterized by loss of myelin within the CNS (Kuhlmann et al., 2008; Rodriguez, 1992). This disorder is a classic example of how excessive demyelination in the absence of efficient repair mechanisms leads to neuronal death (Goldenberg, 2012; Ferguson, 1997). Current therapeutic developments to alleviate pathological features of MS are targeting either decreased or inefficient remyelination to restore physiological function (Chang, 2002). As previously mentioned, an imbalance between demyelination and remyelination is not only characteristic of different disorders, but also a feature of ageing (Tang et al., 1997; Sim et al., 2002). Therefore, demyelination could also be an important contributor to age-related neurodegenerative disorders. Demyelinated axons become vulnerable to toxins and pathological insults, which could account for the high prevalence of pathologies such as Alzheimer's disease (AD) or Parkinson's disease (PD) in elderly populations (Bartzokis, 2004; Maghzi et al., 2016). Understanding the mechanisms underlying remyelination of demyelinated axons thus could have a wide array of implications for the development of future therapies.

The Ruckh et al. (2012) paper 'Rejuvenation of remyelination in the aging central nervous system' is an excellent example of remyelination research and provides a key account of cellular contributors to this repair process in aged animals. Ruckh et al. (2012) also provides evidence of the importance of immune cells in remyelination, supporting subsequent research that showed increased myelin debriefs could overwhelm the CNS intrinsic defenses and contribute to dysfunction both in aged brains and pathology (Safaiyan et al., 2016; Zuo et al., 2022). These researchers used a parabiosis model to investigate age-related changes in remyelination. Parabiosis involves surgical joining of two living organisms and has been widely used for biomedical research, specifically in understanding age-dependent mechanisms (Yang et al., 2021). Not only is it an unusual research method, but it also raises several ethical questions about the need and legitimacy of using such a method. This study is very well described which makes it easy to follow even when not a specialist in the field, whilst promoting critical thinking and evaluation of the research, making it ideal for undergraduates.

RESEARCH ARTICLE

The researchers surgically joined two mice, one old mouse and one young mouse, to form heterochronic parabiosis pairs (Ruckh et al., 2012), which allows the animals to have

shared blood circulation and exposure to soluble factors and cells from the other animal (Conese et al., 2017). Lysolecithin was then used to induce demyelination, in the spinal cord, in the old mouse, 3 weeks after the parabiosis pairs were formed. Lysolecithin injections was the selected methodology as it causes acute demyelination, making it better suited for studying remyelination. Authors suggested this was a more robust model than chronic demyelination, which could directly affect an organism's ability to remyelinate and thus may have off-target effects. By isolating the demyelinating insult, researchers are able to investigate the effect of endogenous remyelination systems of young mice in old mice. The young mouse was marked with green fluorescent protein (GFP) and the mixing of GFP positive (GFP+) and negative (GFP-) cells in both animals was used to check parabiosis and to investigate if there were specific cells that were recruited to the demyelinated site from the young animals. Young and old isochronic (same age) pairs were used as controls, for the heterochronic pairs, where one would be marked with GFP and one would have the lysolecithin injection but the order, in these control pairs, was irrelevant (Ruckh et al., 2012).

Histological analysis showed that by day 21 after demyelination, when the young animals have completed remyelination, the heterochronic parabiosis pair had significantly higher remyelination compared to the old isochronic controls. Remyelination was not completely restored to isochronic pairs control levels but remyelination in the old mice was partially rescued due to the attachment to the young animal (Ruckh et al., 2012). Ruckh and colleagues also analyzed the importance of oligodendrocytes, such as oligodendrocyte precursor cells (OPC's) and mature oligodendrocytes, as these form the myelin sheath of neurons within the CNS. Oligodendrocytes do not proliferate in the adults CNS and therefore, when there is damage to the myelin, OPC's are needed to produce more oligodendrocytes to allow remyelination (Qiao et al., 2016). In both cases they found there were significantly higher levels of these cells in the heterochronic pairs as compared to old isochronic controls. Although there were increased OPC's and oligodendrocytes, they found no co-localization of these cells with the GFP+ stain and therefore concluded that these cells originated in the old mouse. Apparently, these cells still can conduct efficient remyelination and that it was something else within the circulatory, from the young mouse, that was improving the remyelination (Ruckh et al., 2012).

Ruckh et al. (2012) also found GFP+ macrophages, an immune cell, at the spinal cord where demyelination had occurred. There were other immune cells present at the site that were also GFP+, however they were in very small quantities. The macrophages from young animals were then tested against macrophages from old animals to assess if this cell type could be causing the more efficient remyelination in the heterochronic pair. They could not find any differences in growth factors or cytokine production in these populations. However, they found that clearing of the damaged myelin by these cells seemed to be ineffective in old isochronic controls as there was a large amount of myelin debris in these animals. Conversely, heterochronic pairs were much more similar to young isochronic controls with significantly less myelin debris than old isochronic controls. Myelin has been shown to inhibit OPC's differentiation into mature oligodendrocytes and therefore more effective clearance of the damaged myelin may be the reason for the more efficient remyelination in the heterochronic pairs (Ruckh et al., 2012).

Macrophages are recruited to areas with inflammation by a receptor called CCR2 (Ruckh et al., 2012). Due to this receptor being needed for recruitment, the researchers also conducted an experiment where they made the young mouse in the heterochronic pair to be both GFP+ and CCR2 deficient to investigate the outcome if the young macrophages were unable to be recruited. They found that the recruitment of GFP+ macrophages was impaired however these heterochronic and CCR2 deficient pairs still had significantly more mature oligodendrocytes compared to old isochronic controls. Therefore, the macrophages are not aiding in the progression from OPC's to mature oligodendrocytes and it is more likely that the clearing of myelin debris is how the macrophages are rejuvenating remyelination (Ruckh et al., 2012). Although this paper is focused on the macrophages themselves, which is what they chose to investigate further, this specific experiment highlights that there are other cells and/or soluble factors at work that must be aiding in the development of more oligodendrocytes.

TEACHING VALUE

The paper is a relatively short paper, with each experiment, as well as the justification for their methods and sampling, well explained, making it easily accessible to undergraduate students. The use of distinct cell-type markers and use of the lysolecithin injection model are clearly explained. The paper allows a less experienced reader to familiarize themselves with a variety of important and diverse techniques.

Most undergraduate neuroscience programs will have courses that include lectures on myelin sheaths, demyelination, remyelination, and their contribution to disease. Moreover, some modules may likely focus on neuropathologies such as MS. This paper would be an ideal example of how physiological processes can easily transition into pathologies and it illustrates how therapies are developed to restore physiological function. The paper also gives an example of a cellular component (macrophages) that can aid or hinder remyelination depending on the demands of the system. They also highlight how age can affect the remyelinating ability of a cell and that cell function is heterogeneous across the lifespan.

Although the experiments and techniques are good for teaching, another important aspect of this paper is the use of animals within research. In the UK, for example, the Home Office regulates animal testing. To conduct animal research, three different licenses are needed: a personal license for the person conducting the experiments, a project license for the project/study, and an establishment license for the location where the experiments are conducted (Animals in Science Regulation Unit, 2015). Many neuroscience undergraduates will go onto careers within

research and therefore knowledge about the practical aspects of research is useful and introduces the idea of laws and ethical consideration as important.

On the other hand, the animal research aspect of this paradigm opens an important ethical discussion for undergraduates. The method is a particularly controversial since it involves sewing two animals together (parabiosis) and allowing them to live joined together. This paper opens the discussion of whether individuals believe the experiments in this paper are justifiable. The 3Rs, replacement, reduction, and refinement, are used to guide animal research and help to make it as humane as possible. These principles are present across the UK, US, and EU, with many international authorities being committed to ensure the implementation of the 3Rs in animal research (European Parliament, 2010; National Research Council, 2011).When conducting animal research, the aim is to replace animals with the closest alternative, reduce the number of animals used as much as possible, and refine the experiments to minimize the pain and distress to the animals, all while keeping your study experimentally and statistically sound (Animals in Science Regulation Unit, 2015). These principles could be taught and discussed when using this paper with undergraduates to get them thinking about what could be changed in this experiment to reduce animal suffering.

TARGET AUDIENCE AND USE

The Ruckh et al. (2012) paper is a valuable paper for undergraduate teaching and can be utilized for many different course levels and degrees. The inclusion of GFP methods and the ethical consideration raised could be used in entry-level undergraduate classes to spark interest in neuroscience. The authors' focus around remyelination and experiments to better understand and treat MS lends itself to upper level courses when they are studying research papers in more depth. By the time they reach upper level courses, students will have basic knowledge of most of the processes and cells discussed in the paper. Demyelination and remyelination could be first explained with details of how and when these processes occur. This paper could then be used as an example for how these processes can go wrong with age and within disease as well as for teaching and discussing MS itself and the debate about whether MS is a neurodegenerative disease or an autoimmune disorder (Trapp and Nave, 2008).

The ethical considerations raised by this study could make this paper useful within any biomedical or biomedical-related degree program. These wider biomedical audiences could benefit from discussing the controversial techniques in the paper. An ethics debate could be carried out alongside a neuroscience lecture where this paper is used as an example. It could also be used as the main paper in a group discussion with a small group of students, for example in a tutorial/facilitated group discussion. With the paper's short length and accessible language, students could be asked to read the paper in advance along with ethical guidelines followed by a class discussion of their own opinions of the paper and animal research in general.

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