////Title: Strategies and Tools for Studying Microglial-Mediated Synapse Elimination and Refinement

////Stand-first: Synapse elimination is a tightly regulated process, and an aberrant number of synapses both during the early stages of development or later in aging, has been observed in several neurological conditions, including Alzheimer's disease, schizophrenia or autism. Effective methodological approaches for investigating how supernumerary synapses and apoptotic material are removed from the brain are vital for understanding the underlying mechanisms, and this, in turn, may provide exciting avenues for the development of new treatments. Dr Morini, Dr Filipello and colleagues at Humanitas University and at Washington University in St Louis have recently published a review about the different strategies and tools available to study the role of microglia [mai·krow·**glee**·uh], the phagocytes of the brain, in neuronal and synaptic substrates phagocytosis.

////Body text:

A newborn infant's brain contains billions of nervous cells, known as neurons. Neurons 'communicate' by passing along electrical or chemical signals. Synapses are highly specialized structures that act as gateways, allowing neuron to neuron communication. In the early phases of human development, namely at birth and during the first two years of life, an excess of neuronal connections is formed.

Neurons are organised into circuits. When a child grows up, the experiences strengthen relevant circuits, while those connections that are surplus to requirements become weaker. The redundant or weak neurons and synapses are eliminated, and resources are reallocated to those remaining, allowing them to grow stronger. This selective removal of synapses, which is known as synaptic 'pruning', in humans begins a few years after birth, continues for all the adolescence and in some brain regions doesn't end until the early 20s. Synapse elimination can also be incorrectly reactivated later in life, and this may be a factor in synapse loss associated with neurodegenerative diseases.

Phagocytosis [faguh-sie-tOH-sis] is a fundamental process by which specialized cells engulf other cells or large particles, thus removing unwanted material from the surrounding environment. Microglia are immune cells which continuously monitor the brain microenvironment, and are considered the resident phagocytes of the central nervous system. Recently microglia have been identified as key players in the process of synapse formation and elimination. Thus, understanding how this process works and is regulated may help to identify therapies for neurodevelopmental and neurodegenerative diseases. The researchers at Humanitas University and Washington University in St Louis, recently reviewed the currently available experimental strategies for investigating neuronal and synaptic substrates phagocytosis by microglia.

Experimental research conditions consist of *in vivo* settings (within the living organism – usually a mouse), *ex vivo* (freshly isolated organs) or *in vitro* (outside the living organism, conducted in a Petri dish). Despite the *in vitro* conditions provide an over-simplified scenario and have important limitations, microglial cultures *in vitro* are broadly considered a useful tool to study the phagocytic properties of microglia because most of the experimental variables can be controlled.

Immortalised microglia cell lines, grow and divide indefinitely *in vitro* and are robust and readily available but may not be directly comparable to primary or *in vivo* cells. Primary microglial cultures represent an important advancement toward the use of immortalized cell lines, they are directly isolated from the tissue of mouse embryos and new-born pups, and are therefore more comparable to the microglia in the living organism.

Microglia phagocytic capacity can be assessed *in vitro* by adding specific substrates directly to microglia cultures, or by co-culturing microglia and neurons in order to investigate the molecular

pathways and the functional consequences of synapses elimination. Many experiments compare mutant or genetically modified mice to normal or wild type mice. Co-cultures are also used to test experimental drug treatments since neurons and microglia are grown separately before the co-culture which means the two cell types can be subjected to individual treatments before the co-culture.

Dr Morini and colleagues reiterate the importance of additional *ex vivo* and *in vivo* experiments to verify that the results derived from the *in vitro* analyses are indeed representative of the *in vivo* situation .

Because of the clear evidence of the central role played by microglia either in physiological or in pathological conditions, the possibility of isolating intact microglia from the adult brain has been investigated by many groups. Despite the challenge of preserving microglia gene signature and preventing microglia activation during the isolation process, several protocols have been published optimizing this procedure. These protocols are based on an initial step of brain tissue homogenization, followed by subsequent separation steps to untangle myelin debris and unwanted brain material from the microglia.

Once the tissue has been homogenized, microglia are separated using one of the following processes: fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting, or immunopanning. These three methods exploit immune cell markers exposed on microglia cells surface to separate them from other major central nervous system cell types. Once microglia have been isolated, they can be tested immediately for their phagocytic capacity *ex vivo,* or can be cultured for several days or weeks *in vitro*. In this latter approach, microglia phagocytic properties can be investigated by adding specific substrates such as liposomes, synaptosomes, fluorescent microbeads or apoptotic neurons in a defined quantity and for a specific time window.

Multiple signals are required to identify a synapse that needs to be eliminated, and conversely, protective molecules are exposed by synapses that should not be removed. Multiple specific substrates can be used to investigate the nature of the 'eat-me' signals which need to be exposed on the target surface membrane to trigger microglial phagocytosis. Due to their composition, liposomes have been used to both characterize the phagocytic properties of microglial cells and to assess their responsiveness to stimuli. Apoptotic cells display specific 'find-me' and 'eat-me' signals which may or may not be displayed at synaptic sites, whereas synaptosomes maintain the molecular and biochemical features of a synapse. Fluorescent microbeads enable fast and quantitative analysis of basic phagocytosis and represent a useful tool to demonstrate that the phagocytic machinery of microglia is properly functional.

Broadly used methods for assessing the outcomes of phagocytosis assays are flow cytometry and microscopy-based approaches, such as confocal imaging, electron microscopy, two-photon microscopy and lightsheet microscopy. More recently, mass cytometry (CyTOF) has also been used to assess the phagocytic properties of myeloid cells. This cutting-edge technology, which combines flow cytometry and mass spectrometry, has enabled a high-dimensional analysis of cell surface markers, signalling molecules, cytokines and the engulfed material at the single-cell level.

Several groups have identified differences between mouse and human microglia in terms of gene signature and fundamental properties. Therefore, in recent years the scientific community has taken advantage of human embryonic stem cells or the reprogramming of adult cells to generate induced pluripotent stem cells (iPSCs). Subsequently, human microglia-like cells, highly resembling the cells from a specific patient, are generated from iPSCs though specific protocols.

A 2019, a study described an *in vitro* approach to analyse the synaptic pruning process by human microglia-like cells derived from schizophrenic patients and healthy individuals. Patient-derived

synaptosomes were added to microglia derived from healthy or schizophrenic patients and their engulfment capacity was measured. Notably, microglia cells derived from patients, phagocytosed an increased amount of synaptosomes, thus suggesting that abnormal pruning is a typical feature of schizophrenia.

The Ionized Calcium-Binding Adapter 1 (IBA-1) is expressed by microglia and anti-IBA-1 antibodies have been traditionally used to label microglia and quantify their amount, morphology, and distribution. Anti-IBA1 antibodies are not specific for microglia only and also recognise other cells such as border-associated macrophages (BAMs) as well as subsets of peripheral myeloid cells. Therefore, more recent studies have focused on identifying microglia-specific markers that can reliably distinguish microglia from other leukocytes.

Thanks to the advancement of high resolution live microscopy techniques over the past 20 years, we now know that microglia are highly motile cells, able to extend and retract their processes. It has been shown that microglia are able to make contacts with pre- and post-synaptic neuronal spines and also interact with peri-synaptic astrocytes and the synaptic cleft. Advanced microscopy techniques have shown more and more clearly that the close proximity of microglia to synaptic structures appeared to result in phagocytosis of synaptic materials leading to re-wiring of neuronal circuits.

Moreover, the phagocytic process has been further investigated by using the combination of microscopy and three-dimensional (3D) modelling to demonstrate the presence of synaptic material inside microglial intracellular structures.

Recently, another group adopted time-lapse imaging technique to visualize microglia and neurons in organotypic tissue culture, an *ex vivo* model that preserves tissue architecture. The authors showed that microglia do not engulf the whole synapse, but selectively prune neuronal presynaptic structures through a partial phagocytosis termed 'nibbling'. Dr Morini and collaborators note that further lines of investigation are needed to continue elucidating the precise mechanisms underlying microglia-mediated synaptic elimination.

Although modern microscopy is a powerful tool for a quantitative analysis of synaptic material inside microglia, it has technical limitations, and there are drawbacks when fast and unbiased quantification is required. Therefore, the development of alternative approaches is currently in high demand. In this regard, holographic microscopy and multi-isotope imaging mass spectrometry are two promising candidates for improvements in microscopy.

When considering recent advances, such as the ability to create human microglia-like cells, 3D visualisation, high-resolution microscopy and mass cytometry, Dr Morini, Dr Filipello and colleagues are hopeful that future research will bring a better understanding and a more and more clear picture of microglia functions in neurological diseases.

This SciPod is a summary of the paper 'Strategies and Tools for Studying Microglial-Mediated Synapse Elimination and Refinement' published in the open access journal *Frontiers in Immunology.*

This research was supported by CARIPLO grant 2018 to Dr Filipello and by PRIN, and Ministero della Salute 2016 to Professor Matteoli. For further information, you can connect with Dr. Fabia Filipello and Professor Michela Matteoli.

Contacts:

Dr Fabia Filipello filipellof@wustl.edu;

Prof. Michela Matteoli michela.matteoli@hunimed.eu