Diversity of microglia

Their contribution to multiple sclerosis lesion formation

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Publication date
2020
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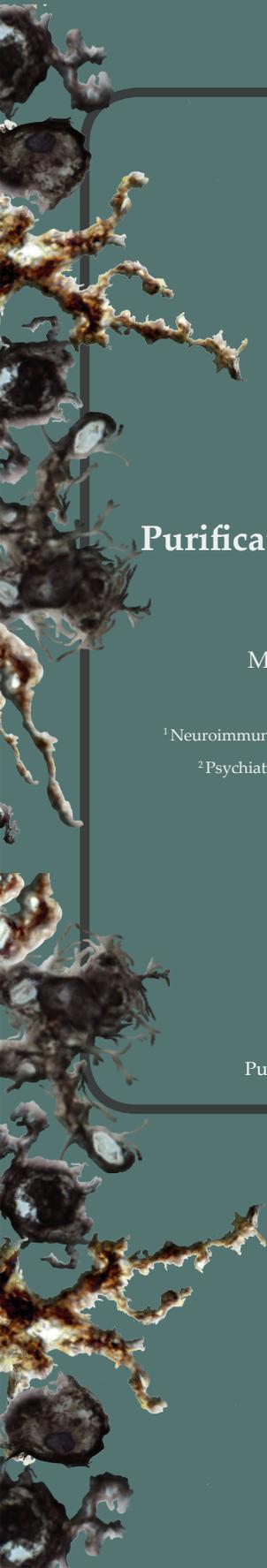**Citation for published version (APA):**
van der Poel, M. (2020). *Diversity of microglia: Their contribution to multiple sclerosis lesion formation*. [Thesis, externally prepared, Universiteit van Amsterdam].

Chapter 3

Purification of cells from fresh human brain tissue: primary human glial cells

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Abstract

In order to translate the findings obtained from post-mortem brain tissue samples to functional biologic mechanisms of central nervous system disease, it will be necessary to understand how these findings affect the different cell populations in the brain. The acute isolation and analysis of pure glial cell populations are common practice in animal models for neurologic diseases, but are not yet regularly applied to human post-mortem brain material. The development of novel cell isolation techniques and methods for transcriptomic and proteomic analysis have made it possible to isolate and phenotype primary human cell populations from the central nervous system. The psychiatric program of the Netherlands Brain Bank has considerable experience with the purification of glial cells. This chapter will review the rapid isolation and phenotyping procedures for two major glia cell populations in the human brain, microglia and astrocytes, and will also discuss the potential for biobanking these cells, as well as the possible alternatives to cell isolations. The acute isolation of glial cells without culture-based adherence steps allows the analysis of glial alterations that underlie, or are the result of, disease neuropathology of the donor.

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Introduction

Pathophysiologic investigations into neurologic or psychiatric disorders of the central nervous system (CNS) will eventually converge into research questions that focus on the individual functional units of the CNS, i.e., the individual cells. Traditionally research into CNS disorders has focused on genetic studies, non-invasive imaging studies, post-mortem CNS tissue analysis, and animal and cellular models, and only more recently has the use of primary human brain cells been developed successfully for neuroscience research. This is not surprising, since the techniques required to isolate intact, viable cells from the human CNS have only emerged in the last decade. In addition, the availability of fresh human CNS tissue, derived from either rapid autopsy or surgical resection, is limited or unobtainable for most researchers. Fortunately, the current global rise in brain banking initiatives is enabling more researchers to work with this valuable material[1]. Although the use of intact, frozen or fixed, post-mortem human tissue remains an invaluable tool to study human CNS disorders, the possibility of studying isolated populations of primary human CNS cells provides a new way to directly study changes that are associated with CNS disorders, leading to disease-relevant cellular mechanisms. Current research methods now allow the investigation of gene expression profiles in heterogeneous populations of single cells or in specific combined subpopulations of cells[2]. Cellular biobanking will be necessary in order to more widely distribute primary human CNS cells and to make this valuable resource available to more researchers. The purification, storage, and dissemination of glial cells were key points in the development of the psychiatric program of the Netherlands Brain Bank (NBB-Psy).

In this chapter we will provide an overview of current methods to isolate and characterize human primary glial cells. We will discuss the inherent limitations, as well as possible alternatives to study specific CNS cell populations from human post-mortem tissue.

Glial cells in neurological and psychiatric diseases

The use of primary human cells is considered standard practice in fields where access to human tissue or fluid to derive primary cells from is easily obtained. For example, in immunology, oncology, and hematology, human cells are used extensively to further our understanding of the disease process. However, in neuroscience research, only a small number of publications exist in which primary human CNS cells are isolated and characterized. This limitation is inherent to the field of human neuroscience, due to the nature of the human brain, which is inaccessible without highly invasive methods (brain surgery and biopsies). The inaccessible nature of the brain also leads to an extended period of time between death and sampling, or post-mortem delay (PMD). Moreover, historically primary CNS cell biology has been neglected because it was reasoned that neurons should only be studied as networks, and not as single cells. This reasoning also affected primary glial cell research. The development of novel ways to extract information from post-mortem brain tissue is therefore a highly relevant area of neuroscience research, which will be crucial in forming new insights into human CNS disorders.

The use of human brain tissue provides the most direct strategy to develop and test new hypotheses about the molecular and cellular basis of neurologic and psychiatric disorders. Recent years have seen great advances in our understanding of the molecular alterations underlying many CNS disorders; however these insights have not led to effective new therapeutic approaches to prevent or reverse brain diseases. A possible reason for this is the focus on neuronal functioning in identifying affected pathways underlying CNS disorders. While the focus on neuronal dysfunction to explain neurologic symptoms seems a logical one, the molecular substrate for dysfunction might also lie in glial cells. It is becoming increasingly clear that glia, astrocytes, microglia, and oligodendrocytes influence all major aspects of normal neuronal processes, including synaptic transmission and neuronal plasticity, under both homeostatic and pathologic circumstances, and in both neurologic and psychiatric disorders[3–5]. It is therefore important to combine research efforts on both neurons and glia in order to understand the neuropathologic processes underlying brain disease. The search for altered gene expression pathways and disturbances in neuronal communication and function should therefore expand to include glial cell populations as well. A recent example illustrates the importance of this expanded view, by focusing on the biologic mechanism underlying a genetic risk factor for schizophrenia through microglia-mediated effects on synapse elimination[6]. Only by combining genomic, transcriptomic, immunohistochemical, and cell biologic data derived from all CNS cells will we come to novel insights into the development and pathophysiology of neurologic and psychiatric disorders, and subsequently novel approaches to counteract or prevent these changes.

Microglia

Microglia are brain-resident phagocytic cells, with their ontogeny traced back to a population of myeloid progenitors from the yolk sac during embryonic development[7]. Under homeostatic conditions the microglia population in the adult CNS is maintained through self-renewal without influx of peripheral cells[9,10]. Microglia are key players in normal CNS function, fulfilling essential roles in neurodevelopment, adult synaptic plasticity, and brain immunity[11,12]. In the adult brain, microglia act as surveyors of the local environment to sustain homeostasis and are therefore highly sensitive to changes associated with damage, inflammation, or infection within and outside the CNS. It is therefore not surprising that changes in microglia function have been described in or proposed to underlie changes in most known neurologic disorders like Alzheimer's disease[13], Parkinson disease[14], and multiple sclerosis (MS)[15]. While neuropathologic alterations can be clearly identified in the post-mortem brain tissue of these neurologic disorders, the neuropathology is less obvious in most neuropsychiatric disorders. But even in the absence of clear neuropathologic alterations, microglia have been implicated in disorders like major depressive disorder, schizophrenia, and bipolar disorder[3,16].

Most research on the function of microglia and on their role in health and disease has been conducted on *in vivo* murine models, where a wide range of genetic models have greatly facilitated our understanding of microglia characteristics in health and disease (reviewed by Crotti and Ransohoff, 2016[17]). Studies into human microglia function have highlighted similarities but also crucial differences between mice and humans[18]. However, regardless of these similarities and differences, the disease mechanism of many human CNS disorders without a known underlying cause will never be replicated in animal models. To investigate the role of microglia in the context of human CNS

disorders, it is therefore crucial to also study human primary microglia.

Astrocytes

In the adult brain, astrocytes are the most abundant cell type, constituting about 40% of all cells, although a recent publication provides proof of a more conservative number, ranging from 10% to 20%[19]. In contrast to microglia, astrocytes derive from the same neuronal precursor cells as neurons and oligodendrocytes during early CNS embryogenesis. Long considered to be mere passive support cells for neurons, the view on astrocyte function has changed tremendously from their initial classification. Astrocytes, like microglia, have been shown to fulfil a plethora of functions crucial for normal developmental and adult CNS functions. Genetic and disease animal models have implicated astrocyte involvement in all aspects of synaptic regulation[3], including synapse elimination[20]. Astrocytes have long been known to play a crucial role in buffering the extracellular space to regulate ion[21] and neurotransmitter[22] concentrations to ensure normal neuronal function. Furthermore, the regulation of CNS blood flow and blood–brain barrier homeostasis are functions that have long been attributed to astrocytes[23].

The central role astrocytes play in normal CNS physiology, perhaps unsurprisingly, implicates astrocytes in most neurologic disorders: from developmental disorders[24] and psychiatric disorders[3] to neurologic disorders where neuroinflammation plays a large role[25]. The morphology of human astrocytes is considerably different from their murine counterparts[26] and likely reflects a profound functional difference. Interestingly, transplanting human glial progenitors into a mouse brain is reported to be able to improve the cognitive function of the mouse[27]. In addition, in animal models of different CNS disorders, activated microglia can induce a subtype of reactive astrocyte to drive neuronal dysfunction and neurodegeneration[28] and highlights the need to study separate CNS cellular populations from within the same brain regions. By dissecting the contributions of different CNS cells and cellular subtypes to certain pathologic states, we can unravel the specific processes involved and increase our potential to intervene in these processes. To fully understand astrocyte function and dysfunction in the human brain, a method is needed that allows the direct purification and analysis of these cells from diseased and control brain tissue.

Oligodendrocytes

Oligodendrocytes are the myelinating cells of the CNS that allow the fast and efficient transfer of neuronal communication through the myelination of axons. By ensheathing axons with laminated, lipid-rich membranes, oligodendrocytes produce the electric insulation needed for fast saltatory axonal conduction. Besides providing these segments of insulation, oligodendrocyte myelin is important for axonal integrity and provides direct metabolic support. This is illustrated by the observation that small changes in oligodendrocyte metabolism lead to neurodegeneration[29]. Oligodendrocytes derive from oligodendrocyte precursor cells (OPCs) both during development and in the adult brain[30]. Many aspects of human oligodendrocyte biology are poorly understood, in part due to being hampered by the limitations of studying primary human cells. The importance of oligodendrocyte functions and the need for insights into the process of (re)myelination are ex-

emplified by the chronic demyelinating disease MS, where inflammatory demyelination results in lesions that eventually fail to be remyelinated, leaving axons without proper insulation and support, and resulting in various severe neurologic symptoms[15]. Changes in oligodendrocyte function, as evidenced by expression of myelin and myelin regulating genes, have also been linked to psychiatric disorders like schizophrenia and bipolar disorder[31]. Understanding the dynamics of myelination, axonal support, and how these processes relate to neurologic and psychiatric disorders will require the specific purification of OPCs and oligodendrocytes from the human brain.

Obtaining pure glial cell populations

To obtain purified populations of cells, fresh, unfixed brain tissue needs to undergo dissociation in order to loosen intact cells from the extracellular matrix and to produce a (single) cell suspension. Most published methods rely on a combination of mechanic and enzymatic tissue dissociation, although a method using only mechanic dissociation has been described for microglia as well. An overview of the different studies and their methods used to obtain primary glia is given in **Table 19.1**. After tissue dissociation, most methods incorporate a density based method to exclude the bulk of the myelin debris. Since myelin consists of tightly wrapped oligodendrocyte membranes, it is unclear whether this step already excludes the presence of most mature oligodendrocytes in the final suspension. One study indirectly describes the presence of mature oligodendrocytes in the resulting cell suspension[32]. The most widely used methods rely on density gradient separation using either Percoll or glucose gradients, yielding a mixed glial cell population. This suspension likely contains most CNS cell types, including astrocytes, microglia, oligodendrocytes, and OPCs, to be further purified in downstream steps. For isolations from gray-matter tissue, this fraction also includes soma of disrupted neurons. The resulting cell suspension can then be used in downstream methods to purify specific cell types through culture or capture techniques. As many studies use different enzymatic digestions for the various CNS cell types, it is unclear if certain enzymatic digestion favors the presence of specific cell types, or even subpopulations of cells. One has to consider the fact that an over- or underrepresentation of a certain cell type in the mixed suspension can arise as a result of the chosen dissociation strategy. A schematic overview of the different methods and downstream analyses, described below, is depicted in **Figure 19.1.**

Adherence-based methods

A number of methods make use of the differential adherent properties of glia to enrich for microglia, astrocytes, or oligodendrocytes through culture. These differential adherent properties can rely on adhesion to culture plastics or specific substrates, like poly-L-lysine. An added advantage of this approach is that the nonadhering cells can be collected and used to purify other cells like neuronal precursor cells[41] or oligodendrocytes[34] as byproducts. Since human primary microglia do not readily proliferate in culture, some protocols rely on the addition of mitogens like macrophage colony-stimulating factor and granulocyte–macrophage colony-stimulating factor to expand microglial cultures *in vitro*. These methods usually have high cellular yields and ideally only have *in vitro* applications, such as functional assays for phagocytosis or chemotaxis. While these methods are highly suited to

study *in vitro* responses, microglia in culture have been shown to deviate greatly from noncultured primary microglia under basal culture conditions and show a fast decline in expression levels of well-described microglia markers[42,49]. Especially when mitogens are added in culture, it is highly probable that these cells no longer reflect *in situ* changes and are therefore unsuited to relate findings to the neuropathologic state of the donor material.

Table 19.1 | Overview of studies using various isolation methods to obtain primary human glia

Reference	CNS cell type	Tissue source and dissociation	Purification method	Downstream applications
Dick *et al.* (1997)[33]	Microglia	Biopsy, collagenase	Density-based gradient centrifugation	Flow cytometry
de Groot *et al.* (2000)[34]	Microglia, oligodendrocytes	Autopsy, trypsin	Adherence in culture	*In vitro* culture and response to stimuli
Olah *et al.* (2012)[35]	Microglia	Autopsy, no enzyme, trypsin, collagenase	Density-based separation, CD11b capture	Flow cytometry, *in vitro* culture and response to stimuli, functional assays (chemotaxis, phagocytosis)
Melief *et al.* (2012, 2013, 2016)[36–38]	Microglia	Autopsy, collagenase	Density-based separation, CD11b capture	Flow cytometry, *in vitro* culture and response to stimuli
Durafourt *et al.* (2013)[39]	Microglia	Biopsy, trypsin	Adherence in culture	*In vitro* culture, stimuli-induced polarization
Hendrickx *et al.* (2014)[40]	Microglia	Autopsy, collagenase	Density-based separation, CD11b capture	*In vitro* culture, functional assay (phagocytosis)
Rustenhoven *et al.* (2016)[41]	Microglia, neural precursor cells	Biopsy, autopsy, papain	Adherence in culture	*In vitro* culture, response to stimuli, functional assay (phagocytosis)
Mizee *et al.* (2017)[42]	Microglia	Autopsy, collagenase, trypsin	Density-based separation, CD11b capture	Flow cytometry, *in vitro* culture
de Groot *et al.* (1997)[43]	Astrocytes	Autopsy, mechanical	Adherence in culture (PLL coating)	*In vitro* culture
Kooij *et al.* (2011)[44]	Astrocytes	Autopsy, mechanical	Adherence in culture (PLL coating)	*In vitro* culture, response to stimuli, functional assays (efflux)
Sun *et al.* (2013)[45]	Astrocytes	Biopsy, papain	GLT1-positive cell sorting	Gene expression analysis (microarray)
Zhang *et al.* (2016)[46]	Astrocytes	Biopsy, papain	Sequential immuno-panning, hepacam capture	RNA-sequencing, *in vitro* culture, functional assays (synapse engulfment, trophic support)
Windrem *et al.* (2004)[47]	OPCs	Biopsy, papain	Antibody-mediated capture	Grafted myelination assays
Othman *et al.* (2011)[32]	Oligodendrocytes, OPCs	Biopsy, papain	Adherence in mixed glial culture	*In vitro* culture, immunocytochemistry
Medina-Rodríguez *et al.* (2013)[48]	OPCs	Biopsy, papain	Adherence in mixed glial culture	*In vitro* culture, immunocytochemistry, differentiation assay

CNS = central nervous system; OPCs = oligodendrocyte precursor cells; PLL = poly-L-lysine.

Antibody-based sorting methods

In order to specifically study multiple aspects of human glia, it is essential to obtain pure glia populations from post-mortem human brain samples. The main reason to use an acute and direct purification of glial cells from post-mortem CNS samples is to exclude phenotypical changes induced in these cells by prolonged adherence steps used in other isolation protocols[34,39,41]. Any phenotypical change detected in acutely isolated populations should therefore be relevant to the neuropathologic status or CNS location of the samples from which the cells were extracted. Depending on the specificity of the purification strategy, immediate isolation of glial cells will also reduce the number of contaminating cells from undesired cell types. This increased specificity can come with the disadvantage of having lower cell yields, thereby limiting the possibility of large-scale culturing applications. The basis for the methods to directly isolate microglia lies in the method developed to isolate and phenotype rat microglia[50], which was further adopted to work with human brain tissue[33]. To enhance the purification of human microglia, we have developed a procedure based on cell density separation and antigen capture using magnetic beads coupled to anti-CD11b antibodies[36]. A major advantage of this isolation procedure in comparison with culture-based methods is that it allows for direct analysis of isolated microglia. Using this technique, based on the membrane expression of CD45 and CD11b, microglia can be distinguished from autologous peripheral macrophages[36,42]. The enzymatic digestion of post-mortem brain tissue and subsequent isolation of cell populations based on extracellular markers have been used by other groups as well[35,46] and currently provide the best means to study CNS disease-related changes in a pure population of human microglia. With the increasing number of RNA-sequencing studies performed on acutely isolated mouse microglia[17], many new and specific extracellular markers for microglia are being described. A recent study shows that transmembrane protein 119 can be used to specifically purify mouse microglia using a cell-sorting approach[51], although this has not been confirmed using human tissue.

In contrast to studies on microglia, there are only a few publications to date that describe methods to directly purify human astrocytes. Recently, Zhang and colleagues (2016)[46] published a novel way to directly purify adult primary human astrocytes from CNS tissue, based on the membrane presence of hepacam. An earlier study described the purification of adult astrocytes by sorting GLT1-positive cells from three CNS samples[45]. In both studies, only samples resected from the adult CNS during neurologic surgery were used, and therefore the question remains whether these cells can be purified from post-mortem tissue as well. Both studies highlighted the uniqueness of the human astrocyte gene expression profile, compared to murine astrocytes. Furthermore, clearly defined gene expression profiles could be seen in astrocytes isolated from glioblastoma tumors. The same study also describes the acute purification and analysis of mature oligodendrocytes, whereas other studies only use isolated OPCs to generate oligodendrocytes in culture (**Table 19.1**).

Biobanking potential of purified cell populations

The ongoing increase in the number and size of brain banking initiatives worldwide has tremendous promise for all neuroscientists who wish to expand their research into post-mortem CNS samples[1]. Although this will increase the availability of fixed and/or frozen samples, it remains a challenge

to implement a widespread distribution of fresh post-mortem or surgically resected CNS tissue for multiple research purposes. To enable researchers worldwide without local access to fresh autopsy and biopsy material to address research questions using purified primary cell populations, professional biobank facilities are needed to process, characterize, store, and dessiminate samples. Within NBB-Psy, described in Chapter 1 of this volume, microglia isolations have been implemented in the brain bank procedures in a standardized way and can then be applied for through the regular application procedures of the NBB. The NBB purifies microglia in a standardized way from subcortical white matter and occipital cortex from all nonpsychiatric control and psychiatric donors who come to autopsy. Microglia from each donor are assessed for viability and percentage of cells that are CD45$^+$CD11b$^+$, of which the mean expression can also be used as a measure for microglial activation[37]. From this population a proportion of cells is immediately lysed in Trisure for RNA extraction, and a proportion of cells is cryogenically stored in freezing medium containing 10% dimethyl sulfoxide.
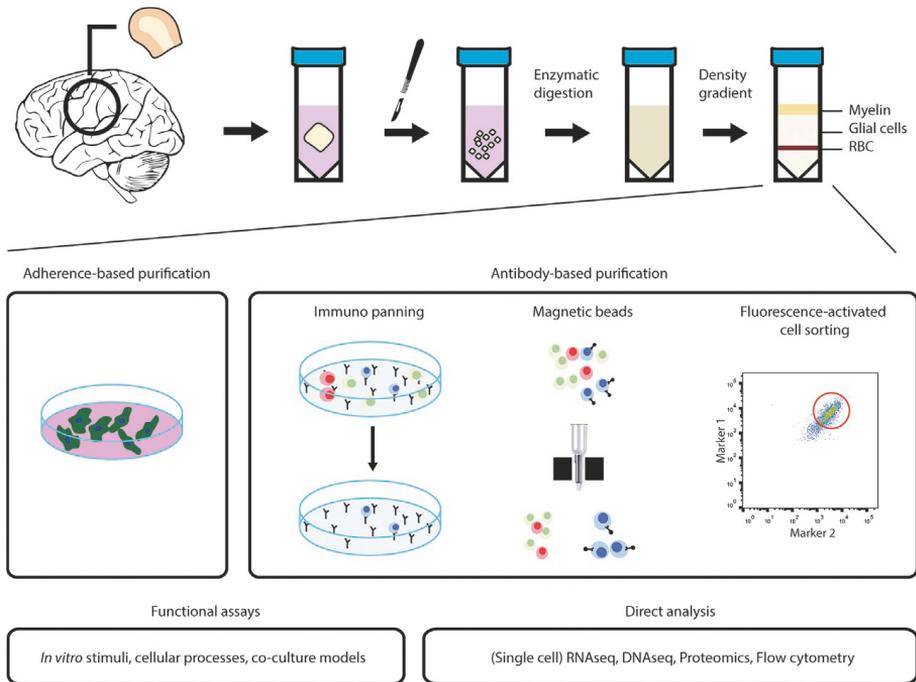
Figure 19.1. | A schematic overview of the isolation of glial cells from human brain samples. Brain tissue derived from biopsy or autopsy is collected in medium and kept cold until processing. The tissue is then mechanically and enzymatically treated to dissociate the cells. An enriched glial population can be obtained by subjecting the dissociated cell suspension to a density-based separation (Percoll or glucose gradient). The resulting (glial) cellular fraction is mostly free from myelin debris and red blood cells (RBC), and can be subjected to downstream purifications. For single-cell RNA sequencing, the whole glial fraction can be used. To enrich for a specific cell type, both adherence-based and antibody-based methods are described. The choice of isolation strategy depends on the desired cell type and downstream application (direct analysis or functional assays).

Microglia from each donor are assessed for viability and percentage of cells that are CD45$^+$CD11b$^+$, of which the mean expression can also be used as a measure for microglial activation[37]. This allows

researchers to process the viable cells as desired upon reconstitution. We have performed multiple pilot experiments to assess the feasibility of cryogenic storage and recovery of freshly isolated microglia. Recovery rates of cryogenically stored microglia are highly variable, averaging around 30%[42]. The usability of these cells depends on the desired downstream applications. Culturing microglia after cryogenic recovery is not advisable based on our observations, in which we rarely observed viable cultures upon reconstitution after thawing. However, recovery of cells with the purpose of extracting RNA, DNA, or protein samples is feasible. When kept cold during the thawing and washing process, expressional changes are minimal, but should be taken into account nonetheless. Since we have observed an increase in CD45 expression after cryogenic storage, acutely analyzed microglia and cryogenically stored microglia should not be compared directly. As we did not observe an effect on CD11b expression, cellular markers might be differentially affected. We expect the same will hold true for astrocyte isolations. Primary human astrocytes isolated through culture expansion do have the potential for cryogenic storage and recovery[43]. However, the recently published, and currently only reliable, method for acute purification of primary astrocytes[46] is very costly and therefore we have not yet standardized the isolation and storage of astrocytes from post-mortem samples.

Downstream applications of pure glia cell populations

Once a purified population of cells is obtained, various opportunities are generated to analyze the glial phenotype in relation to disease. Genomic, epigenetic, transcriptomic, and proteomic approaches can be applied and even combined to generate cell-specific signatures of various neurologic and psychiatric disorders. As the largest body of work on the various applications of primary glia concerns primary microglia, we have focused primarily on this cell type in this section.

Multi-omics analysis of purified primary glial cells

The advent of next-generation sequencing allows the use of small samples of DNA or RNA as input, while generating a large amount of data on transcript or gene count levels. This level of sensitivity is therefore ideally suited to follow the purification of the oftentimes relatively low number of viable cells from post-mortem CNS tissue. Transcriptomic analyses can be used to generate cell specific gene expression signatures and disease-specific gene expression patterns, as described for human healthy and tumor-associated astrocytes[46] and mouse microglia[17], and with the analyses of the human microglia gene expression profile coming shortly. Similarly, microRNA expression profiles can be assessed to provide insights into the fine-tuning of disease-related gene expression changes, as well as provide possible ways for therapeutic intervention. DNA sequencing can be used to generate cell-specific epigenomic data using DNA methylation profiling, which can then shed light on the importance of disease-associated genotypes found in genome wide association studies by profiling active sites of transcription.

Epigenetic profiles can also provide a link between long-lasting changes induced by early-life events, and susceptibility to psychiatric disorders in later life[52]. This is an area in which cell type-specific information is currently lacking. An exciting new addition to the neuroscientist's toolbox is analysis of transcriptomics or epigenomics on a single-cell level[2]. Using a single-cell approach

allows the identification of different subtypes of cells within a population of cells. This can lead to ground-breaking new insights in heterogeneity of cellular responses in certain pathologic states, or to the discovery of novel subtypes within existing populations of cell types. Furthermore, single-cell analysis may at some point eliminate the need to purify specific cell types, as unbiased clustering of transcriptomic or epigenomic data will allow the subsequent separation of data into different cell (sub)type clusters[53]. Purified populations of cells can also be used to directly study proteomic changes that underlie various CNS disorders. Isolating intact cells enables the investigation of the proteomic changes that occur in different cellular compartments, or the specific changes in lysosomal protein content that may reflect recent phagocytosis. Studying the different cellular compartments also enables the investigation of the nuclear versus cytoplasmic presence of transcription factors. The study of proteomic changes can either be performed on protein lysates, for protein-wide approaches, or directly on individual cells using flow cytometry in more directed approaches, as described below for human microglia.

Flow cytometry characterization of primary glial cells

Phenotyping acutely isolated microglia based on flow cytometric analysis is a fast technique to quantify expression of multiple proteins on a single-cell level. Flow cytometry is a powerful tool to define subpopulations of cells within a heterogeneous cell suspension and to characterize these subpopulations based on a panel of antibodies, which is not feasible with transcriptomics when not using a single-cell approach. Only a small number of cells are required to study an extensive panel of proteins of interest by flow cytometry. After excluding nonviable cells using a viability dye, CD11b purified cells are defined as CD45-positive and CD15-negative cells to separate them from any other cell types. Additional markers will provide information about the activation status of microglia, such as the pattern recognition receptor CD14, Fc-gamma receptors, and CD80 and CD86 for T-cell interaction. Our group has previously shown that white-matter microglia isolated from donors with peripheral inflammation[36] and donors diagnosed with MS[37,42] display increased size, granularity, Fc-gamma receptor and CD45 expression when compared with microglia derived from control donors, indicating an alerted or primed state in the patient groups. Similar findings exist for glioblastoma-derived microglia[35]. These findings clearly demonstrate the potential for flow cytometry of purified microglia to shed light on the neurologic disease processes as they manifest shortly before death. In our ongoing study, a broad panel of cell surface markers, including HLA-DR and CD40 to define the activation status of microglia, CD200R and SIRPA as suppressive immune markers, scavenger receptors as markers for phagocytic activity and complement receptors to define the humoral response of microglia, will all expand the microglial phenotyping in normal-appearing MS tissue. Although not part of the glial repertoire of CNS cell types, lymphocytes play a sizable role in the pathogenesis of MS, so post-mortem tissue can also be used to purify and characterize CNS exogenous cells[54]. Besides the characterization of cell populations, flow cytometry is an indispensable technique to sort specific cell populations from a heterogeneous population for downstream applications like RNA-sequencing[51,55].

Functional assays using primary glial cells

The immediate analysis of the proteome or transcriptome of acutely isolated microglia will continue to be the most accurate reflection of microglial phenotype *in situ*. However, functional assays using primary human microglia could provide a unique tool to study functional glial responses to various stimuli *in vitro*, related to either neurologic disease mechanisms or therapeutic interventions. Functional assays can also help to study and understand specific human aspects of cellular processes like myelination of axons by oligodendrocytes[27], human myelin-phagocytosis by microglia[40,56], and to gain mechanistic insight into the isolated responses of glial cells to various inflammatory stimuli (**Table 19.1**). Although cultured microglia deviate from uncultured microglia, they seem to be preferable over the use of immortalized cell lines or a monocyte-derived microglia model[38] in terms of certain cellular markers. For primary human astrocytes, these functional assays lead to a new understanding of the role of astrocytes in both synapse formation and synapse elimination[46]. Cellular responses of cultured cells can in part still reflect the neuropathologic state of the donor material, as was shown for astrocytes cultured from MS lesion tissue or from adjacent normal-appearing white-matter tissue[44]. This may result from the persistent, long-lasting epigenetic cellular changes. However, the changes that cells undergo while in culture are confounding factors when relating the data to the *in situ* characteristics of the donor tissue and therefore should not be used as such. In addition, for cell types like the different subtypes of neurons it will not be possible to isolate intact and specific populations of cells. Possible alternatives to study neurons and other cell types for which purification is not feasible are described in the final part of this chapter.

Discussion

The use of fresh CNS tissue to purify glial populations is not yet a widely applied technique in human neuroscience studies. However, both the increasing availability of donor material to researchers, and the greater knowledge regarding the purification of glial cells, will result in a rapid increase in the number of studies that apply these methods. Considering the continuously developing and increasing sensitivity of the methods that can be used to generate data from isolated populations of cells, it is essential that we understand the issues that arise from using post-mortem tissue, such as donor-to-donor variability and disease-related factors that are difficult to control for.

Donor variables affecting glial cell yield

Inherent to working with post-mortem human CNS tissue is the absence of control over donor parameters other than neurologic diagnosis, both antemortem and post-mortem, that have the potential to induce changes in the tissue. This not only holds true for the isolation of viable cells, but also affects post-mortem fixed and frozen CNS samples, albeit to a lesser extent. The average PMD for NBB donors is 6 hours, counted from the time of death until the end of the autopsy. Although it seems likely that increasing PMD affects the physiology of cells residing in the brain tissue after death, surprisingly we and others found no correlation between viable microglia yield and PMD, nor with time until tissue processing[35,42]. This finding is in line with a study that showed no loss of RNA integrity, as a measure of tissue integrity, with increasing PMD of the brain donor[57]. The same study also showed that the presence of antemortem events like artificial ventilation, coma, and respiratory

illness were negatively associated with RNA integrity. Furthermore, tissue pH was a good indicator of tissue/RNA integrity. In our own microglia isolation cohort cerebrospinal fluid (CSF) acidity at the time of autopsy was a strong indicator of viable microglia yield, as was also shown in a smaller study[35]. As CNS acidity has been shown to relate to agonal state, we showed in our cohort that the cause of death relates to CSF pH. Brain donors who suffered from cachexia or pneumonia before death showed lower CSF pH than donors who underwent euthanasia and who did not suffer from cachexia or pneumonia. Thus, it seems that total viable microglia yield is not affected by variations in PMD or by the total time elapsed after death and before the tissue was processed, nor is the age of the donor of predictive value[42]. Furthermore, we did not find any indication in our cohort that PMD, age, or CSF pH was correlated with flow cytometric analysis of CD45 and CD11b expression. All considered, we recommend carefully matching the CSF or tissue pH of brain donors as a precaution for unwanted noise in the final data set, either before or after tissue processing.

Alternatives to intact cell isolations

In this chapter we have focused on the isolation of primary human glial cells from fresh post-mortem brain tissue. The reason to exclude neurons from this overview is that intact, viable terminally differentiated neurons are not considered to be present in cell suspensions, as described in this chapter. Although the isolation and culture of human primary neurons have been described[58], it seems more likely that these cultures arise from neuronal precursor cells in culture, rather than represent acutely isolated viable neurons. Recently, a publication described the use of isolated nuclei from human post-mortem frozen tissue as a source for cell-specific transcriptomic information[59]. By making use of the small amounts of mRNA present in cell nuclei[53,60], and a single nucleus sequencing approach, the authors were able to generate neuronal subtype specific transcriptomic profiles from post-mortem tissue. If these methods can be adapted to purify specific nuclei from any cell type from high-quality (frozen) CNS tissue samples, they will provide major advantages over the isolation of intact cells and will unlock precious frozen and well-characterized human brain tissue collections for cell-specific analyses. We can select donor material retrospectively, gather information on multiple cell types in a given tissue block, and reduce the influence on gene expression by PMD to a minimum. This method might also be combined with laser dissection microscopy to select specific regions of interest using morphology or even immunohistochemistry. The use of single-cell RNA sequencing will provide an even greater advantage, since no nuclear cell-specific markers need to be used to purify specific nuclei. Statistical methods using unbiased clustering will provide the means to extract expression profiles of specific populations. Disadvantages lie in the fact that only epigenomic and transcriptomic analyses are possible, and there are currently high costs associated with single cell/nucleus sequencing.

Another alternative to the isolation of primary human cells is the generation of primary cells from donor-derived induced pluripotent stem cells (iPSCs). Primary human fibroblasts can be obtained from skin samples acquired through biopsy or post-mortem. Fibroblasts are then reprogrammed into a pluripotent stem cell phenotype and can be transdifferentiated into the desired cell type[61]. This allows the study of patient-specific, physiologically active CNS cells, which has proven to be useful in many fields of neuroscience, including molecular psychiatry[62]. The generation of iPSC

biobanks can aid in generating sample sets large enough to perform high throughput analysis in pharmacologic screening studies. Within the NBB-Psy program, post-mortem skin samples from all psychiatric and control donors are routinely processed into iPSC lines and stored. This allows researchers worldwide who have the capacity to differentiate these cells to work with primary cellular models in neuropsychiatric research. Although the neurons and glia generated this way do not reflect *in situ* CNS changes from brain donors, these models can help to generate working models of pathophysiology for disorders with a clearly defined genetic component.

Future perspectives

The use of post-mortem human brain tissue to generate cell type-specific information in neurologic and psychiatric disorders has come a long way in recent years. Human brain banking initiatives have made these advances possible. Although modern analyses of fixed and frozen sections have provided a wealth of cell-specific information regarding cellular processes in neuropathologic circumstances, the use of animal models and *in vitro* assays has provided most of the mechanistic insights we possess today. Although extensive, these mechanistic insights still lack the human elements that are desperately needed for translation purposes. Especially in the case of glial cells, acute purification of cells from relevant human post-mortem samples will add a new layer of understanding and translation potential to the available hypotheses.

A pitfall of using human brain tissue can be the relatively low availability of samples when compared to the high degree of variation between donor parameters. In order to transcend the high level of noise, the number of samples will need to be increased. Standardization of isolation strategies between research groups can help to build complementary sample sets both for validation and to increase the analysis power. In addition, the biobanking initiatives that wish to purify cells can play a role by standardizing procedures. While the isolation of tissue nuclei seems like a promising strategy to replace the isolation of intact cells, it is not yet clear exactly how nuclear RNA sequencing relates to whole-cell RNA sequencing. Furthermore, functional assays using primary human CNS cells will still rely on fresh tissue isolates. An interesting future application is the possible combination of human CNS cells derived from patient-derived iPSCs and primary human cells. It will be interesting to see which direction human cellular neuroscience takes. However, the use of fresh CNS tissue as a source for primary cells, especially while progressing into the single-cell analysis age, is likely to remain a highly relevant field of research directed at finding novel explanations for neurologic and psychiatric pathophysiology.

Acknowledgements

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