Emerging technologies to study glial cells

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Abstract
Development, physiological functions, and pathologies of the brain depend on tight interactions between neurons and different types of glial cells, such as astrocytes, microglia, oligodendrocytes, and oligodendrocyte precursor cells. Assessing the relative contribution of different glial cell types is required for the full understanding of brain function and dysfunction. Over the recent years, several technological breakthroughs were achieved, allowing "glio-scientists" to address new challenging biological questions. These technical developments make it possible to study the roles of specific cell types with medium or high-content workflows and perform fine analysis of their mutual interactions in a preserved environment. This review illustrates the potency of several cutting-edge experimental approaches (advanced cell cultures, induced pluripotent stem cell (iPSC)-derived human glial cells, viral vectors, in situ glia imaging, opto- and chemogenetic approaches, and high-content molecular analysis) to unravel the role of glial cells in specific brain functions or diseases. It also illustrates the translation of some techniques to the clinics, to monitor glial cells in patients, through specific brain imaging methods. The advantages, pitfalls, and future developments are discussed for each technique, and selected examples are provided to illustrate how specific "gliobiological" questions can now be tackled.
1 | INTRODUCTION

Neurons and glial cells were first described at the same period, in the early 19th century, using a simple microscope and the mollusk as animal model (Dutrochet, 1824; reviewed in Fan and Agid 2018). Virchow coined the term "neuroglia" ("Nervenkitt") in 1856 and referred it as to a type of "connective tissue" that held nervous elements together. This established the concept of "glue," which dominated the history of glial cells for a long period. About a decade later, the introduction of fixative and staining techniques allowed for a finer description of both neuronal and glial cell morphologies (Deiters, 1865; Golgi, 1873). By improving the "black reaction" technique implemented by Golgi, Ramon y Cajal provided the first histological evidence of the structural individuality of neurons and glial cells (Ramon y Cajal, 1888). During this period, thanks to the initial development of electrophysiology, the physiological study of nerves progressed rapidly, whereas the study of glial cells remained purely morphological. Nevertheless, careful microscopic observations suggested remarkable functions for glial cells, in particular, transport of nutrients from blood to neurons (Golgi, 1875), and axon guidance in the developing brain (His, 1888). During the three following decades (1889–1921), the three main classes of glial cells of the central nervous system (CNS), namely astrocytes, oligodendrocytes, and microglia, were described and the first hypotheses regarding their roles in neurotransmission, brain function and behavior, were put forward (reviewed in Fan and Agid 2018).

Pathologists also described morphological changes of glial cells under pathological conditions, and the phagocytic properties of glial cells were suggested.

In the next 30 years (1922–1950), glial research came to a standstill while the study of neurons made spectacular progress, thanks to developments in cellular electrophysiology and biochemistry. Indeed, as glial cells do not generate action potentials that could be detected by the first recording techniques, and because glial cells do not directly control macroscopic body responses, such as muscle contraction, determination of their functions relied on morphological observations. Moreover, the hypotheses driven from glial cell observations could hardly be tested experimentally. In addition, the concept of "glue," inherited from the previous century, also reinforced the general disinterest in the physiology of glial cells. Finally, in the 1950s, new developments in intracellular recording methods enabled the detection of resting potential and depolarization in glial cells (Coombs, Eccles, & Fatt, 1955; Hild, Chang, & Tasaki, 1958). At the same time, development of histochemical techniques opened the way to biochemical studies in glial cells (Koelle, 1955).

From that point, glial research has progressed at a regular pace and changed our conceptual views of the roles of these cell types in brain function. Nowadays, glial cells are recognized to be not only support cells for neurons but also active players in all important CNS processes. Briefly, glial cells have a dual origin: (a) ectodermal for oligodendrocytes, oligodendrocyte progenitor cells (OPCs), and astrocytes (Allen & Lyons, 2018), and (b) mesodermal for microglia (Prinz, Jung, & Priller, 2019; Thion, Ginhoux, & Garel, 2018), but all are long-lived cells in the CNS. Microglia cells derived from the yolk sack invade the brain parenchyma when neural progenitors actively divide and generate the first neurons. They are the main brain immune cells, are tightly adapted to their local environment, and play key roles in the surveillance and maintenance of brain homeostasis (Colonna & Butovsky, 2017). Oligodendrocyte precursors and astrocytes are generated from neural progenitors at a later phase of fetal brain development (Bergles & Richardson, 2015; Verkhratsky & Nedergaard, 2018). Oligodendrocytes mature from their precursors and myelinate axons, whereas astrocytes provide trophic and metabolic support to neurons. However, over the past 20 years, it became clear that the functional role of each of the glial cells is much richer and complex, and that glial cells engage in many interactions not only with neurons but also among themselves. Several reviews provide a comprehensive view of how the diverse and dynamic functions of glial cells actively participate in essentially all aspects of the nervous system formation, function, and dysfunction (Allen & Barres, 2009; Allen & Lyons, 2018; Barres, 2008). Among those, several key discoveries drastically transformed our view of glial cell roles under physiological conditions: (a) the demonstration that raising intracellular calcium (Ca2+) concentration in astrocytes affects synaptic transmission in adjacent neurons, which led to the concept of the tripartite synapse and established astrocytes as key active players in the integration of synaptic information (Araque, Parpura, Sanzgiri, & Haydon, 1999); (b) by combining patch-clamp recording in OPC and electron microscopy, Bergles, Roberts, Somogyi, and Jahn (2000) established the existence of glutamatergic synapses between neurons and OPCs. Although the physiological significance of this highly specialized form of communication is not yet clearly established, it revealed that OPCs are not solely precursors of oligodendrocytes; (c) in vivo imaging of GFP expressing microglia revealed that they are never-resting cells that continuously move their processes, inferred a surveillance role for microglia in the brain parenchyma, and opened the way to decipher microglial roles in brain physiology and pathology (Davalos et al., 2005; Nimmerjahn, Kirchhoff, & Helmcen, 2005); (d) a lentiviral approach to knockdown the MCT1 transporter from mature myelinating oligodendrocytes revealed a key role of mature oligos in the maintenance of axonal integrity and neuronal survival (Lee et al., 2012); (e) the diversity of glial cell types and the existence of specialized cell types (such as radial glia, Schwann cells, peri-synaptic Schwann cells) was also highlighted. In addition to their physiological roles, glial cells are key drivers of neuroinflammation, which are arguably the central tenet connecting all neurodegenerative diseases (Ising & Heneka, 2018). In many, acute or neurodegenerative pathological conditions, glial cells undergo massive molecular and functional changes that might directly influence disease progression. Later, genetic association studies revealed that glial cells likely play important roles in the initiation and/or progression of some common neurodegenerative conditions such as Alzheimer disease (Verheijen & Sleezers, 2018).

Application of various technologies to glial cells (i.e., electrophysiology, calcium imaging, in vivo imaging, conditional knockout) has been instrumental to further understand their roles in physiology and pathology. The latest technological improvements
involve high-resolution and/or high-content techniques and multi-parametric analyses to capture the complexity of their biological responses. In this review, we present a selection of in vitro, ex vivo, and in vivo cutting-edge approaches that have been recently developed to study the role of glial cells in brain (dys)functions. Examples of key discoveries achieved with these approaches are presented. We also discuss the advantages, pitfalls, limitations, and future developments for each of these approaches. In the near future, we believe these emerging technologies will further emphasize the importance of glial cells in brain (dys)functions. This review mainly focuses on CNS astrocytes, oligodendrocytes, OPCs, and microglia, the most studied glial cells. However, some of the techniques highlighted lean on other nonneuronal cells of the brain (see Verkhratsky, Ho, Zorec, & Parpura, 2019) because they play important roles for brain functions.

2 | NEW IN VITRO MODELS: OPPORTUNITIES AND CHALLENGES

Historically, glia cells were only grown to provide trophic support for cultured neurons, and therefore their isolation and culture were most certainly an after-thought. The twin realizations that glia are more than just brain-glue and that astrocytes and microglia are highly reactive cells that change their phenotype significantly when cultured have led to improved culture methods.

2.1 | A brief history of neural cell culture

The development of tissue culture methods to grow neural and glial cells and their precursors was intimately linked to early work in developmental biology (Hamburger & Levi-Montalcini, 1949). Several seminal advances allowed for the selection, purification, and maintenance of primary neural and glial cultures, as well as growth and directed differentiation of tumor lines of neural and glial origin (Alliot & Pessac, 1984; Bottenstein & Sato, 1979; Raff, Abney, Cohen, Lindsay, & Noble, 1983; Raff, Brookes, Fields, & Mirsky, 1979). Enriched mixed glial cultures were initially extracted from embryonic rodent brains, by protocols relying on their staged emergence in the brain, along with selective adhesion properties (McCartey & de Vellis, 1980). These “McCartey-De Vellis” glial cultures allow for the progressive selection of highly purified OPCs and astrocytes. Later, it was determined that microglia can be harvested from the same astrocyte cultures (Giulian & Baker, 1986).

In these cultures, serum was used as a supplement to a balanced salt and nutrient solution (Eagle, 1959) to provide an extensive cocktail of growth factors that are still difficult and expensive to generate in purified forms. As the blood–brain barrier does not allow serum entry into the brain and because serum has drastic effects on cell morphologies and functions (Figure 1a), there has been a strong effort to develop serum-free media. Minimum supplements have been defined to grow neural and glial cells without serum, allowing reproducibility and customization (Barres, 2008; Bottenstein & Sato, 1979). In parallel, combinations of amino acid homopolymers and extracellular matrix proteins (e.g., laminins, fibronectin, collagen) were used for cell attachment (Baron-Van Evercooren et al., 1982).

The more directed method of immunopanning, which relies on the recognition of cell-surface antigens by specific antibodies, was initially developed to capture lymphocytes (Wysocki & Sato, 1978) and was quickly adapted to efficiently select neurons and glia (Barres et al., 1992). Immunopanning was then further adapted and became a precursor for successful sorting strategies such as magnetic cell sorting (Schachner, 1982; Wright, Fitzgerald, & Colello, 1997). Later on, pure, serum-free immunopanned cultures of retinal ganglion cells and OPCs were generated from neonatal rodents (Barres et al., 1992; Barres, Jacobson, Schmid, Sendtner, & Raff, 1993; Meyer-Franke et al., 1998; Meyer-Franke, Kaplan, Pfrieger, & Barres, 1995). Purified OPCs underwent apoptotic cell death in culture, highlighting their need for exogenous trophic factors, and it was then discovered that astrocytes and microglia rely on exogenous factors for survival. Identification of the crucial growth factors to culture each glial cell type in a nonreactive state without serum has been key to refine the study of glia (Barres, Jacobson, et al., 1993; Barres, Schmid, Sendtner, & Raff, 1993; Bohlen et al., 2017; Foo et al., 2011). These studies uncovered novel interactions between brain cells, and molecular regulation of their reactive state (Bohlen et al., 2017; Foo et al., 2011; Liddelow et al., 2017; Rothhammer et al., 2018; Scholze, Foo, Mulinyawe, & Barres, 2014; Wheeler et al., 2019). Immunopanning is a gentle process compared with other cytometry-based methods and can be implemented in any laboratory, without the need for a dedicated sorter or transgenic animals with cell type-specific fluorescent transgenes. In general, immunopanning affords a good yield of purified cells.

The availability of murine embryonic stem cells (ESCs Evans & Kaufman, 1981; Martin, 1981) soon provided another means to obtain neural and glial cells through a deterministic in vitro process. Amphibian, avian, and eventually rodent models revealed the need for BMP antagonism to give rise to neural precursor cells (Ang, Conlon, Jin, & Rossant, 1994; Graff, Thies, Song, Celeste, & Melton, 1994; Smith & Harland, 1992). The effects of retinoic acid (Durston et al., 1989; Sive, Draper, Harland, & Weintraub, 1990) and sonic hedgehog (Echelard et al., 1993; Riddle, Johnson, Laufer, & Tabin, 1993) in the spatial patterning of the differentiating neural axis were also demonstrated. This led to the development of several methods to generate neural and glial precursor cells, originally identified in rodent fetal and adult brains, and thereby neurons, astrocytes, and oligodendrocytes (Fraichard et al., 1995; Mufti et al., 1999; Okabe, Forsberg-Nilsson, Spiro, Segal, & McKay, 1996).

2.2 | From rodent to human cell cultures

Both “McCartey-De Vellis” and immunopanned-based glial rodent protocols have been applied to fetal human cells. They can even be applied to adult human brain cells (Nunes et al., 2003; Sim et al., 2011; Windrem et al., 2004). However, the use of human fetal
samples is naturally limited by ethical concerns and regulatory constraints, while available material from adult humans is largely restricted to abnormal tissue and pathological states.

When human ESCs and later human-induced pluripotent stem cells (hiPSCs) became available, protocols similar to those implemented for rodent ESC cultures allowed the generation of human neural precursor cells (Chambers et al., 2009; Reubinoff et al., 2001; Takata et al., 2017; Zhang, Wernig, Duncan, Brustle, & Thomson, 2001), oligodendrocytes (Douvaras et al., 2014; Hu, Du, Li, Ayala, & Zhang, 2009), astrocytes (Brennand, 2017; Krencik & Zhang, 2017), and microglial precursors (Takata et al., 2017). These cells can be used in a variety of applications, including transplantation into neonatal mouse brains, to study disease models and develop treatments.

**Figure 1**
Methods to culture glial cells. (a) Left, Phase contrast images of immunopanned astrocytes (IP astrocytes) cultured in heparin-binding EGF-like growth factor in the absence of serum, display many processes. Center, IP astrocytes cultured in the presence of 10% serum convert into flat and fibroblast-like cells. Right, Astrocytes are prepared by the traditional shake-off method and cultured in 10% serum, as described by McCarthy & de Vellis (1980). MD astrocytes. Serum cultured IP-astrocytes resemble MD-astrocytes. (b) Human PSCs can be driven exclusively towards neuroectoderm (TGFβ inhibition with dual SMAD inhibition, ≈15 days) into rosette-forming neural stem cells (RNSCs). RNSCs depend on bFGF and insulin for proliferation. Upon removal of these growth factors, multipotent RNSCs differentiate into postmitotic neurons (>30 days) or can be steered toward glial lineages including astrocytes (in the presence of CNTF and BMP4, >45 days), or oligodendrocytes (in the presence of PDGF and T3, >60 days). Alternatively, PSCs can be driven toward the mesodermal lineage (in the presence of BMP4 and activation. CSF1R-dependent primitive macrophages/microglial precursors proliferate in response to CSF1/IL34 (>15 days). Factors such as IL34, CD200, TGFβ1, or CX3CL1 can favor their maturation in microglia-like cells. (c) Immature and proliferative microglial precursors can be added to various organotypic culture systems to expose them to a parenchymal neural environment and complete their maturation to a homeostatic state. These include aggregated cultures of PSC-derived cells as in (b), addition to PSC-derived cerebral organoids, and mouse brain slices. They may also be transplanted into neonatal mouse brains, where they will engraft. Future efforts include the introduction of microglial precursors at a relevant mouse developmental stage (e.g., ~E8–E10). Figure (b) and (c) was created with Biorender
and eventually microglia (Abud et al., 2017; Douvaras et al., 2017; Muffat et al., 2016; Pandya et al., 2017). Cells of neuroectodermal origins are obtained through inhibition of TGFβ signaling, giving rise to fibroblast growth factor (FGF)-responsive neural progenitors. In the absence of maintenance growth factors, these progenitors differentiate toward multiple neuronal and glial lineages (Figure 1b). Many of the important developmental origins of brain cells were known by the time ESCs became a model system, except for microglia whose origins were still elusive. Seminal work in the mouse showed that most, if not all, microglia derive from primitive yolk sac macrophages, migrating to the brain before closure of the blood–brain barrier (Alliot, Godin, & Pessac, 1999; Ginhoux et al., 2010; Vitry, Bertrand, Cuman, & Dubois-Dalcq, 2003). This knowledge supported original protocols to generate microglia from hiPSCs (Abud et al., 2017; Douvaras et al., 2017; Muffat et al., 2016; Pandya et al., 2017; Takata et al., 2017). Our evolving understanding of microglial development will continue to allow differentiation protocols to improve (Lee, Kozaki, & Ginhoux, 2018) as ontogeny and cell identity become better defined (see the following section and Figure 1c).

Generating brain cells from iPSCs requires defining the cell type(s) of interest, an issue which is the object of emerging technologies discussed further in this review (see Section 4). A cell type was historically defined by ontogeny, cytoarchitecture, and physiological outputs or by a few key expression markers identified by immunostaining or qPCR. Today, a combination of higher-resolution mapping of ontogeny and high-content multiomics profiles, including epigenetic and full-genome expression, is available to compare cells (Arendt et al., 2016; Gokce et al., 2016; Gosselin et al., 2017; Lavin et al., 2014; Macosko et al., 2015; Tabula Muris et al., 2018; Zeng & Sanes, 2017). We are increasingly aware of the distance separating some in vitro models from their in vivo reference counterparts. For example, one of the first thorough transcriptome-level descriptions of astrocytes in a disease-relevant context pointed out similarities between “McCarthy-De Vellis” cultured astrocytes and poststroke astrocytes (Cahoy et al., 2008; Zamanian et al., 2012), while immunopanned astrocytes appeared to better match the in vivo astrocyte transcriptome (Foo et al., 2011). Choice of culture methods must therefore carefully balance practical experimental needs (e.g., working with human neurons to study Alzheimer disease) with the necessary degree of relevance of the model (e.g., a tumor line grown under diabetic conditions may be sufficient to study the fundamental aspects of cell division).

Lessons learnt from rodent cell cultures, for example, the discovery of factors required for glia survival and oligodendrocyte differentiation, have been integral in the development of hiPSC-derived glia (Sloan et al., 2017). The use of PSCs as the source material provides a unique opportunity to work with patient cells and to engineer the genome of the future glial cells (see Section 3). Thanks to nuclease-based gene targeting, such as CRISPR/Cas9 technology, PSCs can be targeted with fate reporters, physiological sensors, or disease-related genetic variants to perform isogenic comparisons (Li et al., 2013; Omer Javed et al., 2018; Soldner et al., 2009). However, the decision to work with human cells comes with cost: glial cell differentiation from human PSCs scales to human developmental timing, thus often requiring higher costs than similar work performed with mouse cells. Human cells should primarily be used when therapeutic translation is key, or for the study of human-specific biology such as the emergence of novel cell types during development, interactions with unique pathogens, or age-dependent selective neuronal vulnerability.

2.3 2D pure cultures for drug screening and high-content analysis

Most of the culture systems described earlier are 2D culture systems that have been integral in advancing our understanding of the CNS at the molecular and cellular levels. Complex questions such as identification of key regulators of synaptic connectivity have been answered in these simplified systems (Araque et al., 1999). Primary rodent cultures as well as neuronal and glial immortalized cell lines are used by academia and industry. They are particularly compatible with medium- and high-throughput analysis with simple readouts for drug screening and profiling. Such tests enable proof-of-concept in vitro studies before more extensive in vivo studies of candidate drugs in small animals or nonhuman primates.

The information value of these cultures can be further magnified by high-content imaging with unbiased automated imaging platforms. Coupled to powerful software and machine-learning algorithms, the images can be analyzed automatically, providing multiple parameters for hundreds to thousands of cells per well or condition. Automatic population analysis provides the scale necessary to obtain reliable drug dose–response curves, for example. Additional sophisticated live high-content imaging machines enable the longitudinal examination of complex and important cellular behaviors such as cell division, migration, process extension, and cell death. Therefore, defined, simplified 2D cell culture systems provide a high-throughput platform to obtain mechanistic information in a cost and time-effective way. They allow quick hypothesis testing and provide the backbone of more complex culture systems that better recapitulate CNS complexity.

2.4 Combination of cell types and organoids

Although the reductionist approach has been long attempted to fragment the nervous system into its individual components, new culture platforms for glial cells try to reverse-engineer the complex cell–cell interactions found in vivo.

Well-defined monocultures can be refined by combining them to create more complex multicellular culture systems to understand important questions such as the molecular mechanisms regulating reactive gliosis, myelination, or synapse formation (Allen et al., 2012; Christopherson et al., 2005; Rothhammer et al., 2018; Watkins, Emery, Mulinyawe, & Barres, 2008; Wheeler et al., 2019). Indeed, there is a growing recognition that cells cannot be what they are in isolation. Microglia are an extreme example of this problem where the tissue microenvironment adds an important layer to their identity (Gosselin et al., 2017). Although microglia ontogeny has now been reasonably replicated in the dish (Abud et al., 2017; Ditadi et al., 2015; Douvaras et al., 2017).
Some of these culture platforms are self-assembling, such as brain organoids or neural spheroids (Lancaster et al., 2013; Li et al., 2017; Sloan et al., 2017). They allowed the study of developmental processes (Omer Javed et al., 2018) or disease progression. Other systems to study brain cells combine predifferentiated cells in culture devices that can be observed and manipulated longitudinally (Choi et al., 2014). For example, microglia precursors derived from hiPSCs can be embedded in cylindrical 3D cultures containing neurons, astrocytes, and oligodendrocytes (Muffat et al., 2016) or in cerebral organoids (Abud et al., 2017) (Figure 1c). In these cultures, microglia adopt the ramified morphology and surveying behavior that match their in vivo counterparts more faithfully than isolated iPSC-derived microglia. These systems are not only relevant for human cells, but applicable to the mouse, where neuronal cocultures also help PSC-derived macrophages acquire microglial features (Takata et al., 2017). Interestingly, astrocytes grown in spheroid cultures mature to advanced developmental stages more rapidly than they would have on their own (Sloan et al., 2017). In the same line, oligodendrocytes fated inside cerebral organoids develop into myelinating cells (Madhavan et al., 2018; Marton et al., 2019; Pasca et al., 2015). In vitro and in vivo models can intersect: mouse microglial precursors and embryonic macrophages (Bennett et al., 2018), as well as human iPSC-derived microglial precursors (Abud et al., 2017), have been successfully transplanted to the neonatal mouse brain. Such techniques, along with organotypic brain slice cultures, complement the 3D culture modalities available to experimentalists looking to take advantage of the plethora of mouse lines carrying relevant gene variants (Figure 1c). In these complex culture systems, we can now embed lineage tracers and physiological actuators or sensors at the pluripotent stage. Virtually all the transgenic tools described in the following sections can be delivered in vitro using viral vectors (Kiskinis et al., 2018; Weick et al., 2010), and yet better integrated in specific loci through CRISPR/Cas9 gene targeting in pluripotent cells (Roberts et al., 2017), to be propagated to the relevant lineages. In the process, we gain access to human neural tissue mimics that are simply not available otherwise and will contribute to the comparative study of glial biology with other species.

Although great progress has been made in glia cell culture, there are still many opportunities to improve growth conditions. Indeed, the cell culture environment is flawed: often hyperoxic or hypoxic, rarely physiological, never homeostatic. We must move past our historical reliance on media formulations designed for convenience and cost efficiency (Barry, Coogan, & Commins, 2016; Brewer et al., 2001; Cantor et al., 2017; Muffat et al., 2016). For example, albumin supplementation remains a convenient staple of many defined media, but is a serious source of unpredictability (Chen et al., 2008) and is aberrant, considering its virtual absence in brain interstitial fluids. Glucose concentrations in generic media are essential to highly proliferative tumor lines, but are often too high for neurons and glia, placing them under extreme diabetic stress. Osmotic pressures developed to enhance viability of embryonic rodent neurons at low density have to be reevaluated when our goal is to study human glial cultures approaching adult maturity. Improper culture conditions could easily mask some of the more important phenotypes or doom rescue strategies to fail. Individual components of the media, normally provided by the complex tissue microenvironment, can be catalogued in vivo, screened, and provided in vitro, to better replicate the baseline status of individual cell types (Bohlen et al., 2017) and to understand normal biology as well as disease mechanisms.

### 3 | NEW TOOLS TO STUDY GLIA CELLS AT CELLULAR RESOLUTION

There are now a multitude of tools to study glial cells in vivo: many transgenic mouse lines expressing fluorescent proteins, biosensors, opto/chemogenetic proteins (see Section 3.3), Cre recombinase, Cas9, or suicide genes under glial cell-type-specific, Cre-dependent, or tetracycline-dependent promoters (Guttenplan & Liddelow, 2019; Slezk et al., 2007; see also http://networkglia.eu/en/animal_models). For each approach involving overexpression, activation, recombination, knockdown, or alation, there are a number of options in terms of activity and specificity of promoters, and the timing of control of their expression. There are a fewer options to target some understudied glial cells such as perisynaptic Schwann cells or satellite glial cells. However, by combining existing transgenic lines, or by a more efficient genome manipulation with the CRISPR-Cas9 system, new tools are expected to be available in the near future, as recently illustrated for pericytes (Berthiaume & Shih, 2019). Most of these tools have been reviewed and discussed earlier (Guttenplan & Liddelow, 2019; Wieghofer, Knobeloch, & Prinz, 2015), and we will therefore focus here on emerging technologies to monitor and manipulate glial cells in vivo.

#### 3.1 | Viral approaches to manipulate glia genome or modulate gene expression in glia

Viral vectors, which are highly versatile and easy to generate, offer an interesting option to modulate gene expression in mice or other species, and to evaluate potential therapeutic strategies (Deverman, Ravina, Bankiewicz, Paul, & Sah, 2018). Furthermore, viral vectors can be used to transduce glial cells in primary cultures, hiPSCs (Chen, McCarty, Bruce, Suzuki, & Suzuki, 1998; Jiang et al., 2018; Li et al., 2010; Merienne et al., 2017; Rosario et al., 2016) or human brain organoids (Amin & Pasca, 2018). The potential and safety of viral vectors is highlighted by the ongoing CNS clinical trials (Cartier et al., 2009; Hudry & Vandenbergh, 2019; Palfi et al., 2018) and the recent approval of Zolgensma (AAV9-SMN) for the treatment of spinal muscular atrophy (Mendell et al., 2017).
Most gene transfer experiments in the CNS of rodents or large animals rely on two delivery systems: the lentiviral (LV) and recombinant adeno-associated viral (rAAV) vectors. These replication-deficient viral vectors have been engineered to eliminate their pathogenic genes and to lead to long-term expression in large brain areas (Sakuma, Barry, & Ikeda, 2012; D. Wang, Tai, & Gao, 2019). Each system has specific features in terms of cloning capacity, immune response, genome integration, tropism, and diffusion (Table 1). A detailed description of these viral vectors is beyond the scope of this publication (for reviews see Berry & Asokan, 2016; Lundberg et al., 2008; Murlidharan, Samulski, & Asokan, 2014; Sakuma et al., 2012; Salganik, Hirsch, & Samulski, 2015; D. Wang et al., 2019). Viral gene transfer applications are extremely diverse, ranging from expression of reporter genes, biological sensors (Grienberger & Konnerth, 2012), optogenetic probes (Adesnik, 2018; Almad & Maragakis, 2018), transfer applications are extremely diverse, ranging from expression of reporter genes, biological sensors (Grienberger & Konnerth, 2012), optogenetic probes (Adesnik, 2018; Almad & Maragakis, 2018), expression of coding or noncoding genes, and endogenous gene replacement for loss-of-function disorders (Grienberger & Konnerth, 2012; Mendell et al., 2017), endogenous gene activation (CRISPRa), endogenous gene inactivation (CRISPR/Cas9 and CRISPRi (Powell, Gregory, Akbarian, & Rabinowitz & Samulski, 1998). In recent years, more than 100 rAAV serotypes have been discovered and a substantial number of variants have been created (Asokan, Schauffer, & Samulski, 2012; Hammond, Leek, Richman, & Tjalkens, 2017; Wu, Asokan, & Samulski, 2006). The cellular entry of AAV is mediated by interaction with specific surface glycans and receptor/coreceptor(s). Identified glycan moieties include heparan sulfate proteoglycans (rAAV2, rAAV3, and rAAV6), N-terminal galactose (rAAV9), and specific N- or O-linked sialic acid moieties (rAAV1, rAAV4, rAAV5, and rAAV6) (Murlidharan et al., 2014; Shen, Bryant, Brown, Randell, & Asokan, 2011). The preferential neuronal tropism of rAAV2 was shown to correlate with the higher heparan sulfate proteoglycan availability on the membrane of neurons compared with that on glia (Hsueh et al., 1998; Hsueh & Sheng, 1999). AAV receptor (KIAA0319L) is an essential cellular receptor required for the transduction of vectors derived from multiple AAV serotypes, including the evolutionarily distant serotypes rAAV2 and rAAV5 (Pillay et al., 2016). Fibroblast growth factor receptor 1 was identified as a coreceptor for rAAV2 (Qing et al., 1993). An improved astrocyte tropism was reported with the glycoprotein derived from the Mokola virus (MOK-G; rhabdoviruses) in rat hippocampus (Pertusa et al., 2008). Finally, LV pseudotyped with Chikungunya envelope (CHIKV; alphaviruses) target astrocytes effectively in vivo (60% of transduced cells are astrocytes) and present strong astrocyte tropism in vitro (Eleftheriadou et al., 2017). It is important to note that these vectors can suffer from limited transduction and low viral titers and that they can also have inflammatory/toxic effects.

The rAAV2 was the first serotype used for CNS gene transfer and its injection in the rat hippocampus leads to a largely neuronal transduction pattern (Bartlett, Samulski, & McCown, 1998; Rabinowitz & Samulski, 1998). In recent years, more than 100 rAAV serotypes have been discovered and a substantial number of variants have been created (Asokan, Schauffer, & Samulski, 2012; Hammond, Leek, Richman, & Tjalkens, 2017; Wu, Asokan, & Samulski, 2006). The cellular entry of AAV is mediated by interaction with specific surface glycans and receptor/coreceptor(s). Identified glycan moieties include heparan sulfate proteoglycans (rAAV2, rAAV3, and rAAV6), N-terminal galactose (rAAV9), and specific N- or O-linked sialic acid moieties (rAAV1, rAAV4, rAAV5, and rAAV6) (Murlidharan et al., 2014; Shen, Bryant, Brown, Randell, & Asokan, 2011). The preferential neuronal tropism of rAAV2 was shown to correlate with the higher heparan sulfate proteoglycan availability on the membrane of neurons compared with that on glia (Hsueh et al., 1998; Hsueh & Sheng, 1999). AAV receptor (KIAA0319L) is an essential cellular receptor required for the transduction of vectors derived from multiple AAV serotypes, including the evolutionarily distant serotypes rAAV2 and rAAV5 (Pillay et al., 2016). Fibroblast growth factor receptor 1 was identified as a coreceptor for rAAV2 (Qing et al., 1993) and platelet-derived growth factor receptor alpha for rAAV5 (Pasquale et al., 2003).

In 2009, rAAV6 was the first serotype shown to cross the blood–brain barrier and to efficiently target CNS cells following intravenous injection (Foust et al., 2009). Both neuronal and glial cells can be transduced via different injection routes in both small and large animal models (Foust et al., 2009; Gray et al., 2011; Samaran et al., 2013). In 2016, Schober and collaborators showed that injection in the barrel cortex of rAAV6 preferentially targets astrocytes (90% of transduced astrocytes in the targeted areas; (VSV-G) was used for this (Naldini et al., 1996). It was shown that LV/VSV-G mainly transduces terminally differentiated neurons, an observation confirmed by other groups (Björklund et al., 2000; Deglon et al., 2000; Mitrophanous et al., 1999). To alter the tropism of LV, envelopes from the Retroviridae, Paramyxoviruses, Rhabdoviruses, Alphaviruses, and Orthomyxoviruses families were used (Joglekar & Sandoval, 2017).

With regard to astrocyte targeting, glycoproteins derived from the lymphocytic choriomeningitis virus (rhabdoviruses) or amphotropic envelope of the Moloney murine leukemia virus (retroviridae) resulted in an astrocytic tropism in the substantia nigra of rats (Cannon et al., 2011). However, because integration of the Moloney murine leukemia virus genome is closely dependent on mitosis, only a small proportion of astrocytes are infected (Roe, Reynolds, Yu, & Brown, 1993). An improved astrocyte tropism was reported with the glycoprotein derived from the Mokola virus (MOK-G; rhabdoviruses) in rat hippocampus (Pertusa et al., 2008). Finally, LV pseudotyped with Chikungunya envelope (CHIKV; alphaviruses) target astrocytes effectively in vivo (60% of transduced cells are astrocytes) and present strong astrocyte tropism in vitro (Eleftheriadou et al., 2017). It is important to note that these vectors can suffer from limited transduction and low viral titers and that they can also have inflammatory/toxic effects.

### TABLE 1

<table>
<thead>
<tr>
<th>Features of lentiviral and adeno-associated vectors</th>
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<tr>
<td><strong>Lentiviral vectors</strong></td>
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<tr>
<td>Infect both dividing and nondividing cells</td>
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<tr>
<td>Integrating virus integration-deficient LV</td>
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<tr>
<td>Single-stranded RNA viruses</td>
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<tr>
<td>8 kb of capacity</td>
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<td>2.2 kb of capacity for self-complementary AAV</td>
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<td>Dependovirus genus of the Paroviridae</td>
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<tr>
<td>Not enveloped</td>
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<td>Preexisting neutralizing antibodies</td>
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<tr>
<td>High diffusion</td>
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However, the tropism of rAAV is altered depending on the species, targeted brain regions, developmental stages, and promoter activity (Hadaczek et al., 2016; Lawlor, Bland, Mouravlev, Young, & During, 2009; Liu, Martins, Chiorini, & David-son, 2005). In addition, the degree of purity of viral vectors affects the profile of expression (Kleinet al., 2008). Three years ago, the first rAAV variant with preferential tropism for oligodendrocytes was described (Powell et al., 2016). This Olig001 serotype is a mixture of rAAV1-2-6-8-9 and is functional both in vitro and in vivo, showing a low affinity for peripheral organs. Finally, targeting microglial cells has been challenging, probably because these cells are involved in the destruction of pathogens by phagocytosis including viruses (Maes, Colomb, Schulz, & Siegert, 2019; Rosario et al., 2016). One study showed a selective but low in vitro and in vivo transduction of microglial cells with microglia-specific promoters (F4/80 or CD68) combined with triple mutation in rAAV6 capsid reducing proteasome degradation (Rosario et al., 2016).

### 3.1.2 Defining glial cell type-specific promoters

The second strategy to modify viral vector tropism relies on cell-type-specific promoters. Unfortunately, the number of fully characterized glial promoters that efficiently regulate transgene expression is still extremely low. The promoter of the excitatory amino acid transporter 1 (GLAST, human Scl1a3; 2.0 kb) was used in MOK-LV, but the level of transgene expression was low (Colin et al., 2009). Brenner et al. made a great contribution with the characterization of the glial fibrillary acid protein (GFAP) promoter (Brenner, Kisseberth, Su, Besnard, & Messing, 1994). However, its large size (2.2 kb for Gfa2) limits its use to small transgenes (the packaging capacity is 4.5 kb for rAAV and 8 kb for LV, see Table 1). The intranigral or striatal injection of an AAV2/5-Gfa2-GFP demonstrated very high astrocyte transduction in rats and mice (Drinkut, Tereshchenko, Schulz, Bahr, & Kugler, 2012; Meunier, Merienne, Jolle, Deglon, & Pellerin, 2016). A shorter version, the gfaABC1D (600 bp) promoter (Lee, Messing, Su, &
Brenner, 2008), was generated to allow inclusion of larger transgenes. Finally, three copies of an enhancer (B3 element) were later inserted to increase transgene expression and generate the gfa2(B3) and GfaABC1D(B)3 promoters (de Leeuw et al., 2006; Lee et al., 2008). The injection of an rAAV2/5-GfaABC1D(B)3-GFP in the mouse striatum showed a strong expression of GFP in glutamate synthetase-positive astrocytes and no colocalization with NeuN-positive neurons (Merienne et al., 2017).

The most commonly used oligodendrocyte-specific promoter is that of the myelin basic protein (Chen et al., 1998). Recently, different fragments of human myelin-associated glycoprotein (MAG, 2.2, 1.5, and 0.3 kb) promoter have been characterized. The corresponding rAAV1/2-MAG-GFP vectors reached around 90% transduction of oligodendrocytes after intraparenchymal delivery in adult mice. Of note, all these MAG promoter fragments were inactive in vitro (von Jonquieres et al., 2016), which probably reflects functional differences between embryonic cultures and adult cells. Additional regulatory regions might be needed to promote transcription in vitro.

As mentioned in the earlier section, the F4/80, CD68, and also the CD11 promoters restrict transgene expression to microglial cells in primary cultures and in neonatal/adult brains, when injected locally (Jiang et al., 2016; Rosario et al., 2016). However, microglia are resistant to robust AAV or lentivirus infection in vivo and the available approaches are very limited.

### 3.1.3 Manipulating posttranscriptional regulation

The final target used to enhance viral selectivity toward specific glial cells such as astrocytes is posttranscriptional gene expression. This “de-targeting” strategy was initially proposed by Naldini (Brown & Naldini, 2009; Gentner & Naldini, 2012). It takes advantage of cell-type-specific microRNAs (Bartel & Chen, 2004) that block transgene expression in nontargeted cells (Eulalio, Huntzinger, & Izaurralde, 2008). To prevent residual expression in neurons and improve astrocyte tropism, four copies of the target sequence of the neuron-specific miR124 (miR124T) were inserted in the 3’UTR region of the transgene (Colin et al., 2009). Combining miR124T with MOK-LV increases transgene targeting in astrocytes in several mouse brain regions (striatum, hippocampus, and cerebellum) (Colin et al., 2009; Pannasch et al., 2014; Petrelli et al., 2018; Taschenberger, Tereshchenko, & Kugler, 2017). To further improve astrocyte-specific expression, the Déglon laboratory incorporated a second miRNA target sequence, the miR9-T (Ko et al., 2008) and showed that it further decreases residual activity in neurons (Merienne et al., 2017). As microglial cells also lack microRNA-9 activity, a similar strategy was used to restrict transgene expression in these cells: four target sequences of the miR-9 were inserted in an LV (Akerblom et al., 2013). After brain injection, the GFP reporter gene was only expressed in the cells of neocortical origin, including microglia but not in astrocytes or neurons.

### 3.1.4 Glia silencing or gene inactivation

The abovementioned examples all pertain to the overexpression of a gene of interest in glial cells, but gene silencing in glia may be achieved as well. Gene silencing with small hairpin RNA (shRNA) relies on polymerase III promoters that are active in both neurons and glial cells (Drouet et al., 2009; Mazuel et al., 2017; Toro Cabrera & Mueller, 2016). To achieve astrocyte silencing, a detargeting strategy is not suitable because miRT sequences are cleaved during shRNA processing and maturation. To overcome this limitation, the Déglon laboratory indirectly regulated the expression of shRNA with a tetracycline-regulated (Tet) system in LV (Merienne et al., 2015). As an alternative to both LV and rAAV vectors, miR-embedded shRNAs located in the 3’ untranslated region (3’UTR) have been developed (Fellmann et al., 2013). The optimized miR30E backbone ensures an efficient maturation of the shRNA and increases the knockdown efficiency of LV (Fellmann et al., 2013).

CRISPR technology could also be used to inactivate or modulate genes in glial cells (Figure 2a). A nonviral approach called CRISPR-Gold was used to deliver the RNA-guided endonucleases Cas9 and Cpf1 into the brains of adult mice (B. Lee, K. Lee, et al., 2018). Two weeks after stereotaxic injection, approximately 10–15% of the cells around the injection site (1–2 mm) were edited. In the hippocampus, half of the edited cells were astrocytes, 40% were IBA1-positive and 10% NeuN-positive (corresponding to 33% editing efficiency in astrocytes, 19% in microglial cells, and 3% in neurons). Using LV delivery and the Kamicas9 self-inactivating system, Merienne et al. (2017) reached high editing efficiency of the Huntingtin gene in primary and iPSC-derived astrocytes. In vitro studies in an immortalized microglial cell line showed that a double LV approach to provide Cas9 and the sgRNA in different vectors induced complete deletion of glia maturational factor exon 3 (Raikwar et al., 2019). Finally, a new AAV with a synthetic surface peptide was shown to target terminally differentiated human astrocytes much more efficiently than neurons (Kunze et al., 2018). This rAAV encoding both Cas9 and sgRNA was used to deliver HIV-inhibitory genes to astrocytes and inactivate persisting HIV-1 proviruses in this reservoir.

### 3.1.5 Pitfalls and future directions for glial cell tools

As illustrated earlier, this first generation of cell-type specific vectors provides a large panel of tools to investigate and facilitate the analysis of glial functions. If viral vectors are very popular, they come with some intrinsic limits (e.g., transgene size, mostly local effects, risk of inflammation, and biosafety requirements), but they are extremely versatile and should further reveal the contribution of glial cells in CNS disorders and promote the development of targeted therapies. Researchers should be aware that the experimental design is essential to avoid toxicity, such as massive transgene overexpression (Klein et al., 2006) or immune/inflammatory responses (Ortinski et al., 2010). To ensure purity, potency, and safety, appropriate purification
methods and quality assessment of virus batches should be considered (Schnodt & Bunings, 2017). Finally, the doses administered and also the in vivo injection procedures (volume, injection rate, cannula) are critical to avoid functional impacts and result misinterpretation (Kohro et al., 2015). Under optimal conditions, long-term expression is obtained with reactive astrocytes and microglia detected exclusively around the injection site. Beside intraparenchymal delivery, noninvasive systemic administration has been used to promote a widespread vector distribution in the CNS (Chan et al., 2017; Saraiva, Nobre, & Pereira de Almeida, 2016).

The second major limitations of gene transfer for the CNS are the number of glial-specific promoters, which are still extremely limited to study and target cell subpopulations. Microglial cells are difficult to transduce with viral vectors (Maes et al., 2019; Rosario et al., 2016), and few promoters of the macrophage lineage (F4/80, CD68, and CD11b) have been used to restrict transgene expression in primary microglial cultures and in neonatal/adult brains (Jiang et al., 2016; Rosario et al., 2016).

Single-cell mRNA sequencing studies have shown that the Zika virus entry receptor AXL is enriched in radial glia, microglia, and astrocytes in the developing human cortex (Nowakowski et al., 2016). However, the use of the Zika virus as an efficient delivery system has not been yet described. A better understanding of the intracellular mechanisms is needed to define strategies to overcome this bottleneck and design viral vectors efficiently targeting microglia. Of note, viral vectors are used extensively and efficiently in vitro, where they can display different properties in terms of transduction efficiency or tropism.

3.2 Live imaging to monitor glial cell behavior

The study of glial cells has traditionally lagged behind that of neurons in part due to a dearth of methodologies to elucidate their precise functions in vivo. However, advances in optical in vivo imaging and development of genetically encoded sensors have dramatically improved capabilities for glial studies in the intact brain. A few examples of these studies include time lapse intravital imaging of microglia that revealed their rapid dynamics and response to injuries (Davalos et al., 2005; Nimmerjahn et al., 2005); visualization of OPCs showing their self-repulsive behavior during adult proliferation (Hughes, Kang, Fukaya, & Bergles, 2013); and imaging of astrocyte processes with 

\[ \text{Ca}^{2+} \] sensors demonstrating time-correlated \n
\[ \text{Ca}^{2+} \] responses to neuronal activation (Bindocci et al., 2017). The palette of tools has continued to blossom with refined and potent techniques such as targeted two-photon holographic activation of channelrhodopsins in individual cells (Papagiakoumou et al., 2010) and genetically encoded optical voltage and neurotransmitter sensors (Abdelfattah et al., 2019; Marvin et al., 2013; Patriarchi et al., 2019), which in the future will allow a more sophisticated in vivo dissection of the role of glial cells within complex neural networks. Overall, in vivo imaging techniques expand capabilities for elucidating the interactions between neuronal and nonneuronal cells in health and disease. Rather than a comprehensive review on all imaging techniques, here, we will focus on three additional examples of simple, yet powerful methodologies for in vivo imaging and experimental manipulation of glial cells.

3.2.1 In vivo imaging to disentangle the cellular mechanisms of neurovascular coupling regulation

The brain has very large energetic requirements and has thus evolved into a sophisticated system to precisely match regional cerebral blood flow to the constantly fluctuating activity-dependent local energetic demands. This mechanism, otherwise known as neurovascular coupling, is of great interest, not only because its disruption may be involved in a variety of cerebrovascular and neurodegenerative conditions (Iadecola, 2017), but also because functional magnetic resonance imaging, a widely used technique for neuroscience research, detects increases in paramagnetic oxygen due to changes in cerebral blood flow during neurovascular coupling (Ogawa et al., 1993; see also Section 5). Several recent reviews have comprehensively covered this topic (Iadecola, 2017). In this methodological review, we chose to focus on recent neurovascular studies from the Grutzendler laboratory that involve new imaging approaches, even if some findings remain a topic of controversy (Attwell, Mishra, Hall, O’Farrell, & Dalkara, 2016).

Although the mechanisms controlling neurovascular coupling are not well understood, previous work demonstrates the involvement of complex interactions between neurons, glia, and vascular mural cells (Iadecola & Nedergaard, 2007), which include arteriolar smooth muscle cells and capillary pericytes (Rouget, 1874). A major challenge is to study the roles of the different elements of the neurovascular unit in intact physiological states.

The precise anatomical patterns of mural cell distribution throughout the cerebral vascular tree were established using transgenic mice expressing a red fluorescent protein under the smooth muscle actin (\(\alpha\)-SMA) promoter (Armstrong, Larina, Dickinson, Zimmer, & Hirschi, 2010) and one expressing GFP under the mural cell-specific PDGFRβ promoter. There was a sharp demarcation between \(\alpha\)-SMA-expressing ring-like cells and \(\alpha\)-SMA-negative mural cells with long processes that surrounded ~80% of intraparenchymal small vessels (Figure 3a). The expression of \(\alpha\)SMA in ring-like cells suggested that these cells are contractile, while fusiform pericytes are not. The properties of these cells were unambiguously determined by single-cell in vivo two-photon optogenetic stimulation of mural cells and intravital imaging of the microvasculature during neural activation tasks (Hill et al., 2015). These experiments showed that increases in microvascular diameter correlated well with neuronal activation, only in vessels expressing \(\alpha\)SMA, while capillary pericytes did not dilate as a result of different activation tasks. Expression of the genetically encoded \(\text{Ca}^{2+}\) sensor GaCaMP6 in mural cells showed that fluctuation of \(\text{Ca}^{2+}\) levels correlate with changes in vessel diameter only in \(\alpha\)-SMA-expressing ring-like mural cells. This is consistent with \(\text{Ca}^{2+}\) involvement in relaxation and contraction of smooth muscle cells, but not in pericytes, where \(\text{Ca}^{2+}\) may have different functions. The
Grutzendler laboratory then implemented a single cell optogenetic approach by combining vascular imaging during two-photon optical activation of channelrhodopsins in individual mural cells (Figure 3a and Section 3.3). This allowed to unambiguously determine the precise location and nature of individual contractile cells along the vascular tree. This confirmed that only αSMA-expressing cells with ring-like morphology exert contractile radial forces sufficient to alter microvascular diameter or flow. Such high-resolution mural cell imaging with fluorescent reporters, Ca²⁺ sensors, and optogenetic single cell stimulation has also been applied to a variety of studies of neurovascular coupling and cerebral blood flow regulation under physiological conditions, ischemia, spreading depolarization and neurodegenerative conditions (Hill et al., 2015).

### Intravital label-free myelin imaging

Myelin is a critical structure of the nervous system that increases the speed of propagation of action potentials and plays a role in axonal metabolic support (Simons & Nave, 2015). The biology of myelin has been extensively studied at the molecular and ultrastructural levels. However, intravital myelin imaging has been generally restricted to early development in zebrafish (Kirby et al., 2006) primarily due to limited availability of tools for high-resolution imaging of myelin in the live mammalian brain. As a result, little is known about the rates of myelin internode formation, the stability of internodes, and the longevity of oligodendrocytes at various stages of life and in pathological processes.

Several methodologies for label-free myelin imaging have become available over the past decade, including optical coherence microscopy (OCM), coherent anti-Stokes Raman scattering (CARS) microscopy, and harmonic generation (THG) microscopy (Farrar, Wise, Fetcho, & Schaffer, 2011; Wang, Fu, Zickmund, Shi, & Cheng, 2005). These techniques have unique advantages, such as chemical sensitivity for myelin lipids with CARS (Wang et al., 2005) and deep tissue imaging capability, which can reach subcortical white matter, with THG (Farrar et al., 2011). However, these techniques are not easy to implement and require sophisticated equipment and setups not readily available in most laboratories (reviewed in Hill & Grutzendler, 2019). In contrast, spectral confocal reflectance microscopy (SCoRe) is a recently developed, simple, but yet powerful method for high-resolution intravital label-free imaging of myelinated axons in the mammalian brain (Schain, Hill, & Grutzendler, 2014). This method uses a conventional laser scanning confocal microscope to obtain reflection images of individual myelinated internodes. Contrast is generated by taking advantage of the large refractive index mismatch between highly lipidic (and thus reflective) myelin and the surrounding aqueous medium in other cells and in the interstitial space. Images are generated by using multiple lasers in the visible wavelength range, as each laser generates a patchy and incomplete reflection pattern, but when all wavelengths are combined, a full axonal reconstruction can be created. Using SCoRe, it is possible to visualize axons in the cortex and to track them for hundreds of microns (Figure 3b), thus allowing the reconstruction of the internodes of individual axons and their longitudinal visualization over extended intervals of time. Because SCoRe is label-free, it is a very practical method for in vivo studies of mutant and aging mice which otherwise would require prohibitive long-term cross-breeding with fluorescent reporter mice for visualization. SCoRe can also be combined with fluorescence imaging of transgenic or virally labeled reporters in axons, oligodendrocytes or other cells, thus providing a powerful toolset for studying the biology of myelin in vivo and its interactions with neurons and glial cells. Furthermore, because the SCoRe signal is very sensitive to changes in myelin compaction, it is uniquely able to detect areas of myelin wrapping that do not form a compact layered structure, such as the paranodal region and under pathological dysmyelinating conditions.

Using SCoRe in combination with fluorescence optical imaging, the Grutzendler laboratory recently conducted one of the first intravital longitudinal studies of myelin plasticity at single axon resolution in the mammalian brain (Hill, Li, & Grutzendler, 2018; Hughes, Orthmann-Murphy, Langseth, & Bergles, 2018). These studies showed that individual axons in cortex are partially myelinated with large...
patches that remain unmyelinated in early adulthood, consistent with recent optical imaging (Hughes et al., 2018) and serial electron microscopy studies (Tomassy et al., 2014). However, these unmyelinated regions continue to fill in across the lifespan such that by 2 years of age in mice, axons were nearly fully myelinated. Modest remodeling of existing myelin internodes across the lifespan was observed, suggesting that the addition of new myelin internodes is the main mode of myelin plasticity. The precise reason for this protracted cortical myelination process is unclear, but the progressive addition of myelin internodes to partially myelinated axons could have significant effects on axonal conduction and neuronal spike timing-dependent plasticity, which could be critical for memory encoding (Caporale & Dan, 2008). Furthermore, examining how these processes are altered during development, aging, neurendogenesis, and other disorders are now possible by using SCoRe imaging alone or in combination with fluorescence microscopy (Jafari et al., 2019; Olmos-Serrano et al., 2016). In the near future, a combination of label-free techniques such as SCoRe, CARS, and THG microscopy may provide additional complementary in vivo data about myelin biology.

3.2.3 Two-photon chemical apoptotic-targeted ablation of cells in the live brain

Investigating the mechanisms and consequences of cell death and interactions with glial cells in the mammalian brain is of great importance for understanding brain development and neuropathology. However, research has been limited by the lack of methods to induce and visualize this process with cellular specificity as well as spatial and temporal precision in vivo. Several methods for targeted ablation of cells have become available over the years such as genetically encoded constructs for conditional expression of death-inducing signaling such as with diphtheria toxin receptor (Saito et al., 2001), Caspase 3 (Mallet et al., 2002), or the herpes simplex thymidine kinase/ganciclovir system (Moolten, 1986). These methods and others (Roberts, Rosen, & Casciola-Rosen, 2004) are very effective at inducing cell death but require introduction into specific cell types by viral vectors, transgenesis, or in utero electroporation, and it is difficult to control the location and degree of cell death. The Grutzendler laboratory recently developed an easily adoptable and robust method for targeted induction of death of single cells or small clusters of cells in living animals. This method, called 2Phatal, combines in vivo nuclear labeling by topical application of a DNA-binding dye (Hoechst 33342) followed by gentle photobleaching of a small area on labeled nuclei by brief (10–20 s) focal illumination with a femtosecond-pulsed laser during two-photon microscopy leading to local production of reactive oxygen species (ROS), without causing thermal injury. This likely triggers ROS-mediated DNA damage and the subsequent induction of apoptosis (Figure 3c), but without acute cellular disruption of adjacent cells as would occur with thermal laser ablation methods (Davalos et al., 2005).

By implementing 2Phatal in mice that express fluorescent reporters in microglia and astrocytes, it is possible to visualize their reactions to adjacent dying cells at high spatiotemporal resolution. This allows precise quantification of the timing of engagement of glial processes with the dying cells; the efficiency of engulfment; and the ultimate degradation of cell bodies, processes, and nuclei. Using this technique on mutant mice, it is now feasible to precisely quantify in vivo the effects of candidate molecules on the death, engulfment, and degradation processes, providing for the first-time dynamic data in vivo. Additional experiments that are feasible with 2Phatal include the selective elimination of cell populations of interest to assess their functional effects on neural circuits. No such study has been performed with glial cells so far, but sparse elimination of interneurons was shown to cause adjacent excitatory neurons to increase their firing frequency (Hill, Damisah, Chen, Kwan, & Grutzendler, 2017).

Overall, the field of glial neurobiology is greatly benefiting from exciting neuroscience tool developments that promise to revolutionize our understanding of the diversity of nonneuronal cells in the nervous system and their interaction with neurons during development, aging, and disease. Among the future developments that may further enhance glial research are superresolution microscopy techniques that can be applied to imaging the live mammalian brain with subcellular resolution such as stimulated emission depletion microscopy (Badeley & Bewersdorf, 2018). Methods for deep tissue imaging such as two- and three-photon imaging (Horton et al., 2013), fiber optic microscopy, and miniaturized implantable scan-heads (Ghosh et al., 2011) are revolutionizing the investigation of deep brain structures in live and behaving animals. Emerging genetically encoded neurotransmitter sensors will open the possibility for investigating the functions of astrocytes within networks (Haustein et al., 2014). Further refinement of techniques for single cell manipulation will be critical for investigating the molecular and cellular mechanisms of neuroglial interactions with greater precision. Advances in optogenomics for two-photon-inducible expression of genes in single cells (Yao et al., 2019) promise to revolutionize the analysis of glial pathophysiology in their intact in vivo microenvironments.

3.3 Chemogenetic and optogenetic tools to manipulate glia cell functions

Classical glia research relied on two kinds of manipulations: either pharmacological or chronic genetic ones, both of which have limitations. Pharmacological manipulations provide limited temporal precision and are rarely completely cell type-specific. Chronic genetic manipulations, on the other hand, are specific to a type of glial cell, but provide low temporal precision (at the scale of days or weeks) and often no spatial specificity either. Modern optogenetic and pharmacogenetic tools developed for neuronal research can be adapted to glial cells and provide temporal and spatial precision, while avoiding side effects on neighboring cells (Figure 4a).

Importantly, both methodologies can be combined with many other investigation tools, like electrophysiology (from the single cells or cell populations in brain sections, to in vivo) and behavior, or combined with each other. Based on these clear advantages, these techniques have already revolutionized neuronal studies and are starting
to transform astrocyte research as well. This section will describe both methods, their advantages and caveats, and discuss general issues one should consider when manipulating astrocytic activity with these tools.

### 3.3.1 Chemogenetic modulation of astrocytes

Chemogenetic (also known as pharmacogenetic) tools allow the inducible and reversible control of selected intracellular signaling pathways in genetically defined cell populations. This goal is achieved by the expression of designer receptors engineered to be unresponsive to any innate ligands, but respond only to an otherwise inert designer drug (Roth, 2016). These receptors are known as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). DREADD expression can be achieved either by the injection of viral vectors encoding its DNA under an astrocytic promoter (see Section 3.1) or by generating transgenic animals expressing a receptor in all astrocytes. The major disadvantage of intracranial viral infection is the surgery and its associated local immune activation. However, this drawback is compensated by significant advantages: spatial specificity (determined by the injection location), versatility (new tools can be implemented immediately), time-restricted burden of overexpression, and the possibility of combinatorial expression (e.g., receptor and indicator or two receptors targeting different populations).

Transgenic mice-expressing DREADDs in astrocytes do not require intracranial injections, but their clear disadvantage is that the widespread receptor expression provides no spatial specificity and
can lead to systemic effects upon designer drug administration (see later). The designer drug is usually administered by intraperitoneal injection, and can also be delivered directly into the brain, or given in the drinking water. Several chemogenetic receptors are now available, they recruit different intracellular pathways and respond to different ligands (Figure 4b).

Chemogenetic tools have been used on astrocytes for over a decade, with the first studies relying on transgenic mice, followed by a transition to virus-mediated expression in mice. The most commonly used tools are presently Gq-coupled DREADDs (Figure 4b). Transgenic mice expressing the Gq-coupled DREADD MrGAR1R have major systemic effects when this designer receptor drug is administered, like changes in the autonomic nervous system and in motor activity globally, whereas the drug has no effect on synaptic plasticity (Agulhon et al., 2013; Agulhon, Fiacco, & McCarthy, 2010). Local expression of a different Gq DREADD hM4Di in dorsal CA1 induced de novo plasticity and improved memory acquisition (Adamsky et al., 2018). Expression of this DREADD in centromedial amygdala astrocytes selectively increased inhibitory synaptic transmission, depressed excitatory transmission, and reduced fear perception (Martín-Fernandez et al., 2017). When the DREADD was targeted to astrocytes in the nucleus accumbens core, it inhibited the motivation for ethanol and cocaine seeking (Bull et al., 2014; Scofield et al., 2015). However, it should be noted that the exact doses of the DREADD’s ligand is crucial and different doses can even lead to opposite results (Chen et al., 2016; Yang, Qi, & Yang, 2015). Furthermore, chemogenetic experiments should include control groups both for the intended effect of CNO (in DREADD-expressing animals) and also for a possible influence of CNO per se (without DREADDs).

Transgenic mice overexpressing the Gs-coupled DREADD Rs1 were shown to have constitutive ligand-independent Gs-coupled activity, accompanied by impaired memory after prolonged expression. Ligand application dramatically impaired memory consolidation and recall (Orr et al., 2015). The Gi DREADD hM4Di induces Ca2+ elevations in astrocytes (Durkee & Araque, 2019; Nagai et al., 2019). When it was expressed in striatal astrocytes, hM4Di activation increased local excitatory synaptic transmission and induced hyperactive behavior (Nagai et al., 2019).

The effects of the different chemogenetic tools on neurons are straightforward, whereas astrocytic modulation is harder to construe. For example, Gq-coupled receptors depolarize DREADD-expressing neurons and increase their spiking activity in response to ligand application, whereas inhibition is achieved by Gi-coupled receptors, which reduce spiking upon ligand application. The effects of astrocyte manipulations (using intracellular Ca2+ levels as a proxy for their activity) are harder to interpret, mainly because there is no accepted definition of astrocytic activation or inhibition. It is becoming clear that Ca2+ dynamics in different astrocyte compartments (soma vs. proximal processes) have different speed, duration, and spatial spread, are mediated by different intracellular mechanisms (Bazargani & Attwell, 2016), and may represent different kinds of information processing within astrocytes. This complexity of Ca2+ signals is exemplified by the following findings: (a) the same chemogenetic tools can differentially affect astrocytic Ca2+ dynamics in different brain regions (Chai et al., 2017); (b) when used in astrocytes, both Gq- and Gi-coupled receptors induce Ca2+ elevations in these cells, despite the fact that they recruit different pathways, by mimicking different neurotransmitters (Durkee & Araque, 2019; Nagai et al., 2019). As it is unlikely that astrocytes respond similarly to different neurotransmitters, activating different receptors, it is similarly unlikely they are identically modulated by different DREADDs. Advanced analysis methods may soon provide insight into the intricacy of Ca2+ dynamics activated by DREADDs in astrocytes (Y. Wang, DelRosso, et al., 2019).

### 3.3.2 Optogenetic modulation of astrocytes

Optogenetics is a technique allowing the control of membrane potential or intracellular signaling in genetically defined cell populations by expressing opsins (light-sensitive proteins) and stimulating them by light with high temporal accuracy (Fenno, Yizhar, & Deisseroth, 2011; Figure 4c). As in chemogenetics, opsins (pumps, channels, or receptors) can be either virally induced or transgenetically expressed. Light is usually delivered either ex vivo via the microscope objective or in vivo through the tethering to an optical fiber connected to a light source right before the experiment, which can be stressful, and thus requires appropriate control groups (Goshen, 2014). The most commonly used optogenetic tools in neuroscience are ion pumps or ion channels, directly affecting membrane potential. This kind of manipulation is intuitively fitting in neurons, whereas the inducible changes in ionic concentration and membrane potential do not mimic any known physiological process in astrocytes. The significance of the light stimulation frequency is even more obscure when considered in the context of astrocyte manipulation.

Until recently, optogenetic manipulation in astrocytes relied mainly on channelrhodopsin variants. Channelrhodopsin optogenetics modifies neuronal firing rate; affects respiratory responses, pupil dilatation, sleep disturbance; and suppresses feeding (Gourine et al., 2010; Gradinaru, Mogri, Thompson, Henderson, & Deisseroth, 2009; Okada et al., 2012; Pelluru, Konadhide, Bhat, & Shiromani, 2016; Perea, Yang, Boydien, & Sur, 2014; Sasaki et al., 2012; Sweeney, Qi, Xu, & Yang, 2016; Yamashita et al., 2014). The outward proton-pump archaerhodopsin was also used in astrocytes to shift the oscillatory state in cortex (Poskanzer & Yuste, 2016). It should be noted that optogenetic effects on neuronal activity are induced with millisecond precision, whereas the change in astrocytic activity develops very slowly on a timescale of minutes (Gourine et al., 2010; Gradinaru et al., 2009; Okada et al., 2012; Poskanzer & Yuste, 2016). The delayed effect and the uncertainty regarding the physiological nature of the manipulation raises the possibility that the resulting phenotype (of neuronal activity or behavior) is not mediated by a precise effect mimicking astrocytic activity, but rather from some overall environmental factors. Indeed, a recent study showed that stimulation of the commonly used cation-channel channelrhodopsin causes an increase in extracellular potassium concentration and induces a nonspecific excitatory effect on neuronal activity (Octeau et al., 2019). More
recent studies used light-sensitive G-coupled receptors to manipulate astrocytic activity, which may better mimic physiology. For example, the Gq-coupled optoGq and melanopsin in CA1 astrocytes induce de novo plasticity in neighboring neurons and improve cognitive performance (Adamsky et al., 2018; Mederos et al., 2019).

### 3.3.3 Future directions for opto/chemogenic tools in glia cells

Although chemogenetics and optogenetics are highly prevalent in neuronal research, these are early days for their use on glia. Furthermore, within the glia field, these techniques have been used almost exclusively in astrocytes, probably because of the ease of genetic access to this cell type using viral vectors (see Section 3.1). In the future, with the development of effective targeting strategies of these tools to oligodendrocytes and microglia, their power could be harnessed to promote research in these cell types as well.

Optogenetics and chemogenetics have numerous advantages, but they should be used in a careful and controlled way. Especially, major differences between glia and neurons should be kept in mind when using tools originally developed for neurons. For example, optogenetic cation channels are perfect for neuronal activation, but not for astrocytic modulation. Similarly, G-coupled receptors will recruit the same intracellular pathway, but induce very different effects in neurons and astrocytes and can even diverge in their effects between astrocytes in different brain regions. To sum up, the use of these new tools in gli research, especially in combination with conventional tools like electrophysiology and behavior, and employing careful controls, has the potential to transform the field.

### 4 HIGH-CONTENT APPROACHES TO STUDY GLIAL CELLS

#### 4.1 Sequencing approaches

At the beginning of the 2000s, the emergence of cell isolation and high-content sequencing empowered the unsupervised analysis of the repertoire of genes expressed by specific CNS cell types. This allowed the study of changes in gene expression profiles across physiopathological conditions, and was proved instrumental to better understand the roles of glial cells. However, it is important to note that these methods rely on preconceived ideas of what constitutes a cell type: through cell markers for fluorescence-activated cell sorting (FACS) or cell epitopes for immunopanning. Only single cell analysis (without sorting) does not rely on prior classification of cells (see below).

#### 4.1.1 Bulk genome-wide analysis of glial cells

It really started with the pioneering work from the Barres and Wu laboratories, which expanded the initial microarray-based study (Cahoy et al., 2008) and uncovered the expression profiles of different postnatal cell types of the CNS including astrocytes, microglia, oligodendrocytes, newly formed oligodendrocytes, and OPCs (Zhang et al., 2014). In several glial cell types, bulk sequencing approaches revealed that glia cells express specific patterns of genes across developmental stages (microglia: Matcovitch-Natan et al., 2016), brain regions (astrocytes: [Chai et al., 2017; John Lin et al., 2017; microglia: [Grabert et al., 2016]], sex (microglia: Guneykaya et al., 2018), and aging (astrocytes: [Boisvert, Erikson, Shokhirev, & Allen, 2018; Clarke et al., 2018]; microglia: [Hickman et al., 2013]). Some of these factors were also studied in combination, as for development and sex (microglia: Thion et al., 2018).

In vivo changes in gene expression profiles of glial cells have also been investigated across various pathological conditions. In general, these studies highlighted the versatility of glial cell reaction to disease conditions. These sequencing studies further characterize the loss of homeostatic functions and acquisition of new functions of reactive glial cells. In mouse microglia, the transcriptomic reaction has been thoroughly compared under various pathological conditions, leading to the identification a core reaction signature and of disease-specific profiles (for reviews Dubbelaar, Kracht, Eggen, & Boddeke, 2018; Hirbec, Noristani, & Perrin, 2017). The pathological response of astrocytes and OPCs/oligodendrocytes, on the other hand, has been less well characterized although astrocytes do develop disease-specific molecular responses (Escartin, Guillemaud, & Carrillo-de Sauvage, 2019; Liddelow & Barres, 2017; Zamanian et al., 2012). Interestingly, Srinivasan et al. (2015) determined the concomitant remodeling of the neuronal, astrocytic, and microglial transcriptome in both acute inflammation and neurodegenerative models. Under endotoxemia conditions, they showed that astrocytic but not microglial transcriptomic remodeling was TNF-receptor dependent. Transcriptome remodeling of OPCs was investigated under demyelination and remyelination conditions (Moyon et al., 2015) and morphine treatment (Avey et al., 2018).

The extreme plasticity of glial cells requires changes in the composition and structure of chromatin through the action of epigenetic modulators. Their importance in controlling glial cell phenotypes is just starting to be unraveled, and can be assessed by studying histone modifications, DNA methylation, as well as microRNA and long non-coding RNA expression. Epigenome modifications can be studied in acutely isolated glial cells either in bulk or at the single-cell level. Several high-throughput sequencing approaches such as methyl-seq, chromatin immunoprecipitation and assay for transposase-accessible chromatin sequencing (e.g., ChIP-Seq and ATAC-Seq), which respectively measure DNA methylation, patterns of transcription factor DNA binding, and DNA accessibility to transposase can be used. Through these approaches, it was demonstrated that epigenetic regulation is important for the differentiation of OPCs in mature oligodendrocytes (Moyon, Liang, & Casaccia, 2016). In microglia as well, epigenetic changes play key roles for the acquisition of specific phenotypes (reviewed in Cheray & Joseph, 2018). In particular, alterations in microglial gene enhancers were implicated in chronic pain (Denk, Crow, Didangelos, Lopes, & McMahon, 2016). Epigenetic mechanisms...
are also likely to define the immune memory of microglia, and possibly other glial cell types, a process that is thought to be involved in the differential responses of individuals to neurodegenerative conditions (Wendeln et al., 2018).

Glia cell sequencing studies have mostly been performed in mice, although some were performed in humans (astrocytes: [Zhang et al., 2016]; microglia: [Galatro et al., 2017; Gosselin et al., 2017; Olah et al., 2018]). Of note, human oligodendrocytes/OPCs transcriptome were explored either using microarrays or single-cell RNA-seq (scRNA-seq) (see Table 2). Globally, these studies highlighted that although mouse and human glial cells present largely overlapping gene expression profiles, human cells exhibit unique characteristics that should be taken into consideration for therapy design and justify the use of human models for experimental manipulation.

From these genome-wide data, we learned that each glial cell types display heterogeneous phenotypes. Such heterogeneity may reflect the tight adaptation of these cells to their local environment to best support neuronal function. The identification of such diversity also offers a great opportunity for therapeutic intervention; indeed, the existence of distinctive glial subpopulations will allow specific treatments that either promote the beneficial subpopulations and/or of inhibit the deleterious ones.

4.1.2 | From bulk to single cell analysis

scRNA-seq is a powerful method to disentangle the heterogeneity of specific glial cell populations and to investigate the diversity of individual cellular responses (for review Hedlund & Deng, 2018 and also Table 2 and Table S1). Microglial heterogeneity is more pronounced in the embryonic and early-postnatal periods than in the adult (Hammond et al., 2019; Li et al., 2019; Matcovitch-Natan et al., 2016; see Table 2). Interestingly, by increasing the sequencing depth (Hammond et al., 2019) or the number of analyzed cells (Li et al., 2019), the two most recent scRNA-seq studies identified a yet uncharacterized subpopulation of microglia in the developing white matter. scRNA-seq also allowed the characterization of the mouse oligodendrocyte cell lineage in unprecedented detail. Despite being generated using different sequencing technologies, the available data sets show remarkable agreement and overlap in terms of expressed genes (for review see van Bruggen, Agirre, & Castelo-Branco, 2017). The most comprehensive study identified 12 oligodendrocyte-related cell clusters, including six distinct mature oligodendrocyte cell states, with heterogeneity observed between ages and brain regions; it also revealed a continuous process of oligodendrocyte differentiation across age and brain regions (Jakel et al., 2019; Marques et al., 2016).

scRNA-seq has also been used to identify cell subpopulations under pathological conditions. Disease Associated Microglia (DAM) or DAM-like microglia were identified in many different neurodegenerative conditions including Alzheimer, amyotrophic lateral sclerosis, frontotemporal dementia, and severe neurodegeneration models (Keren-Shaul et al., 2017; Mathys et al., 2017). In a different mouse model of Alzheimer disease, Sala Frigerio et al. (2019) recently identified activated- and interferon response microglia. The activated response microglia (ARMs) partly overlap with DAMs, but by applying cell trajectory inference methods (also referred as pseudotime analyses) these authors demonstrate that ARMs were not solely present in disease conditions and are part of the normal brain-aging process. A different category of microglia was also identified only under acute inflammatory conditions as a heterogeneous microglia subpopulation with distinct transcriptional profile compared to DAMs (Sousa et al., 2018).

4.1.3 | Advantages, pitfalls, and future developments for glial cell sequencing

High-content sequencing approaches are unsupervised methods that highlight the complexity of glial cell responses to their environment and allow generation of new hypothesis. Furthermore, advanced bioinformatics tools allow combining analyses from different laboratories, models, and species in meta-analyses to identify common and specific features of different cell (sub)populations. scRNA-seq is still in its infancy and bioinformatics tools are regularly developed to enable the thorough analysis of the huge amount of data generated by this approach. As an example, cell trajectory inference methods can be used to study the transition of cells from one subtype to another and assess the relevance of transient subpopulations during disease progression and recovery (Masuda et al., 2019; Tay et al., 2018). Bioinformatics packages have also been developed to study the crosstalk between different cell types isolated from the same brain samples.

Although sequencing methods are remarkable approaches to study glial cells, there are some important pitfalls. A major issue in transcriptional profiling of dissociated cells, including scRNA-seq, is that transcriptional perturbations can occur during tissue dissociation to mask the true conditions-induced transcriptional changes (Haimon et al., 2018). Mechanical cell dissociation also breaks glial cell processes and the RNA contained in these distant processes or domains will be lost. Promising approaches based on the use of proteases working at low temperature (Adam, Potter, & Potter, 2017) or transcription inhibitors (Wu et al., 2017) have emerged to reduce experimental alteration of the transcriptome. Transgenic mice in which ribosomal complexes are tagged by HA or GFP (i.e., Ribotag mice) offer an alternative approach to isolate cell-specific translantome from complex homogenates including interesting subcellular domains or intricate processes, but this approach also has its weaknesses. Indeed, only RNAs being actively translated are analyzed. Furthermore, this technique may be more prone to contamination by RNA from other cells and it cannot be used at the single cell level (Sanz et al., 2009). Laser-capture microdissection prevents artefactual transcriptional changes and preserves some spatial information but is more subject to contamination, especially for small glial cells (Merienne et al., 2019; Okaty, Sugino, & Nelson, 2011). Cell extraction protocols also influence which cell populations are being analyzed, and myelin removal
<table>
<thead>
<tr>
<th>Reference</th>
<th>CNS cells analyzed</th>
<th>Species</th>
<th>Tissue</th>
<th>Condition</th>
<th>Stage</th>
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<th>Accession number</th>
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<td>Neurodegeneration</td>
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<td>GSE103334</td>
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<td>Tay, Sagar Dautzenberg, Grun, and Prinz (2018)</td>
<td>Micro</td>
<td>Mouse</td>
<td>FN</td>
<td>FNX</td>
<td>2mo, 0/7/30 d postaxotomy</td>
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<td>Mouse</td>
<td>WB</td>
<td>LPS</td>
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<td>Hammond et al. (2019)</td>
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<td>Mouse</td>
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<td>Physio and demyelination</td>
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<td>Li et al. (2019)</td>
<td>Micro/monocytes</td>
<td>Mouse</td>
<td>WB—Ctx/CB/Hpp/St/ OB/CP</td>
<td>Control</td>
<td>E14.5—P7/8 mo</td>
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<td>Mouse</td>
<td>Retina</td>
<td>Retinal degeneration</td>
<td>48 hr after light exposure</td>
<td>NS</td>
<td>GSE121081</td>
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<td>Masuda et al. (2019)</td>
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<td>10 brain regions</td>
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<td>E16.5—P21/4 mo</td>
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<td>Surgical resections</td>
<td>Control and MS</td>
<td>NS</td>
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<td>Mouse</td>
<td>10 brain regions</td>
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<td>P21-30/2 mo</td>
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<td>Mouse</td>
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<td>E13.5/P7</td>
<td>1,514 (+ cells from Marques et al. (2016))</td>
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<td>Mouse</td>
<td>SC</td>
<td>MS (EAE model)</td>
<td>15–20 d post-induction</td>
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<td><strong>All brain cells but significant findings on glial cells</strong></td>
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<td>Zeisel et al. (2015)</td>
<td>All</td>
<td>Mouse</td>
<td>Hpp (CA1) and sCtx</td>
<td>Control</td>
<td>P20-30</td>
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<td>La Manno et al. (2016)</td>
<td>All</td>
<td>Mouse</td>
<td>vMB</td>
<td>Development</td>
<td>E11.5-18.5/P19-27/ P28-57</td>
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<td>Human</td>
<td>vMB</td>
<td>Development</td>
<td>6 w gestation</td>
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<td>Chen, Wu, Jiang, and Zhang (2017)</td>
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<td>Hyp</td>
<td>Control</td>
<td>8–10 wo</td>
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<td>Mouse</td>
<td>mAmy</td>
<td>Physio &amp; seizure</td>
<td>8–10 wo</td>
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<td>6–10 wo</td>
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<td>PFC, V1 Ctx and MGE</td>
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<td>6–36 w gestation</td>
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<td>Morphine treatment</td>
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<td>Visual Ctx</td>
<td>Visual stim.</td>
<td>7–8 wo</td>
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<td>Control</td>
<td>8–10 wo</td>
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<td>Zeisel et al. (2018)</td>
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<td>19 regions (incl. SC, sensory, sympathetic, enteric nervous system)</td>
<td>Control</td>
<td>P12-30/6-8 wo</td>
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<td>NS</td>
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<td>Retina and Ctx</td>
<td>Control and hypoxia</td>
<td>Adult</td>
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<td>Clark et al. (2019)</td>
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<td>Retina</td>
<td>Control</td>
<td>10 stages (E11-&gt;P14)</td>
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<td>Mouse</td>
<td>Retina</td>
<td>NMDA injection</td>
<td>6–14 wo</td>
<td>49,860</td>
<td>Available on request</td>
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<td>Gunner et al. (2019)</td>
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<td>Mouse</td>
<td>sCtx</td>
<td>Whisker deprivation</td>
<td>P5, 24 hr post-deprivation</td>
<td>48,000</td>
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<td>Ximerakis et al. (2019)</td>
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<td>Hindbrain</td>
<td>Aging</td>
<td>2-3 mo/21–23 mo</td>
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<td>AD</td>
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(Continues)
### Table 2

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<th>Condition</th>
<th>Stage</th>
<th>CNS cells analyzed</th>
<th>Accession number</th>
<th>Number of cells</th>
<th>Reference CNS cells analyzed Species Tissue Condition Stage Number of cells Accession number</th>
</tr>
</thead>
</table>

We performed a PubMed search in September 2019, with key words: "(single-cell sequencing or scrna-seq or single-cell rna-seq) and (glia or glial cell or microglia or astrocyte or oligodendrocyte)" over the last 10 years. We retrieved 160 references. We only included studies performed on mammalian models, which provided substantial insight into glial cells. We did not include studies on glioma. Single-nuclei based studies were searched independently. This table shows that single-cell/single-nuclei approaches have brought key new insights into glial cell diversity in development, health, and disease (see column "Key findings" in Table S1). The number of cells indicated is the number of cells that passed quality control and were analyzed. Note the preponderance of single-cell/transcriptomics on microglia or oligodendrocytes compared with other glial cells. Interestingly, some findings are validated in several studies (e.g., higher microglia heterogeneity at early developmental stages), but the exact number of cell clusters’ states or classes is quite variable across studies, depending on the sensitivity of the study, sequencing depths or region studied.

Abbreviations: Cells: Micro, microglia; NSC, neuronal stem cells; Oligo, oligodendrocytes. Brain regions: ACC, nucleus accumbens; Cb, cerebellum; CC, corpus callosum; CP, choroid plexus; Ctx, cortex; DG, dentate gyrus; FN, facial nucleus; Gap, gap junctions; GC, granule cell; GC, granule cell; GC, granule cell; GCL, granule cell layer; HC, hippocampus; Hyp, hypothalamus; LGN, lateral geniculate nucleus; mAmy, medial amygdala; MGE, medial ganglionic eminence; OB, olfactory bulb; PFC, prefrontal cortex; SC, spinal cord; sCtx, somatosensory cortex; St, striatum; vMB, ventral midbrain; WB, whole brain. Others: AD, Alzheimer disease; FNX, unilateral facial nerve axotomy; MS, multiple sclerosis; NS, not specified; stim, stimulation; TBI, traumatic brain injury; w, weeks/months/years old.

Technological developments in the field are arising at an astounding rate, offering many new opportunities. Among them single-nucleus RNA-seq (snRNA-seq) and spatial transcriptomics (Stahl et al., 2016; Strell et al., 2019) are worth mentioning. In snRNA-seq, the cell suspension is gently lysed, nuclei are isolated by centrifugation on gradients, and nuclear RNA is sequenced. The major interest of the approach is that nuclei are more resistant to mechanical assaults and can be isolated from frozen or fixed samples such as those archived in human biobanks. Due to the low abundance of nuclear RNA, snRNA-seq detects only half of the transcripts detected by scRNA-seq. Still, high-depth snRNA-seq has proved sufficient to discriminate between closely related neuronal cell types (Bakken et al., 2018). snRNA-seq was recently used to analyze no less than 80,000 nuclei isolated from the prefrontal cortex of 48 patients at different stages of Alzheimer pathology (Mathys et al., 2019). This study highlighted the complexity of neuron–glia interactions and revealed that myelination-related processes were disturbed in Alzheimer disease. A similar approach was used to demonstrate that upper-layer excitatory neurons and microglia are the preferentially affected cells in autism patients (Velmeshev et al., 2019) (see Table 2).

Another pitfall of scRNA-seq is that potentially important spatial information is lost during cell dissociation. To overcome this issue, different transcriptomic approaches have been developed to explore gene expression profiles with high spatial resolution (for review see Strell et al., 2019). Until recently, spatial cellular resolution was only achieved at the cost of detection efficiency. However, the recently developed STARmap (spatially resolved transcript amplicon readout mapping; Wang et al., 2018) and seqFISH+ (sequential fluorescence in
situ hybridization; Eng et al., 2019) methods allow the simultaneous
detection of hundreds to thousand genes in mouse brain sections at
single-cell spatial resolution with high efficiency, accuracy, and repro-
ducibility. This technology opens the way to study glial cells in their
native physiopathological context, while preserving important spatial
information. This tool seems particularly adapted to study the diver-
sity of reactive glial cells in pathological contexts in which discrete
lesions are observed (e.g., amyloid plaques; dystrophic neurites; stroke
infarcts; demyelinating lesions).

4.2  |  Profiling glial cells at the protein level

Although transcriptional profiling provides useful information on the
molecular status of a given cell population or subpopulation, genes
and transcripts are not the cell’s ultimate effectors and major discrep-
ancies are reported between gene and protein expression (Liu,
Beyer, & Aebersold, 2016; Sharma et al., 2015). Protein levels are con-
stantly adapted to environment changes and proteomic-based studies
may provide the most accurate understanding of glial cell (dys)func-
tions (Altelaar, Munoz, & Heck, 2013). Cell-type specific proteins can
be studied by mass spectrometry-based approaches or by using spe-
cific antibodies. The first methods allow the exploration of hundreds
to thousands of proteins at once, while the other approaches are
targeted and focus on pre-established epitopes, but they directly pro-
vide (sub)cellular resolution (see Levy & Slavov, 2018).

4.2.1  |  Mass spectrometry

Proteomics (large-scale study of proteins) has lagged behind genomics
and transcriptomics because of technical limitations imposed by our
inability to amplify proteins or amino acids, contrary to nucleic acids.
Protein yields required for mass spectrometry (typically tens of thou-
sands of mammalian cells) are difficult to obtain from a pure cell-type
population of nondividing cells. With recent technical advances
achieved in both mass spectroscopy accuracy and sensitivity (Altelaar
et al., 2013), proteomics is now used more commonly to complement
high-content genomic and transcriptomic analysis. Beyond the identi-
fication and quantification of proteins, proteomics may also support
the analysis of many important proteins features, including exact
sequence, state of modification, structure, and interaction partners
Several approaches exist for proteomics analysis and quantification,
but it is beyond the scope of this review to discuss them (Aebersold &
Mann, 2016; Altelaar et al., 2013). Among available proteomics
methods, liquid chromatography with tandem mass-spectrometry is
the most popular and widely used technology for quantitative high-
throughput cell-type specific proteomics (Wilson & Nairn, 2018;

Complex morphologies and heterogeneity of glial cells make
“glioproteomics” very challenging. Most analyses are performed on
primary cultures of glial cells or acutely isolated cells, which are
prepared with methods that damage to fine subcellular structures and
generate low protein yields. Sharma et al. (2015) recently performed
an analysis of both acutely isolated and primary cultured astrocytes,
oligodendrocytes, and microglia in 10 major brain regions. In-depth
analysis identified more than 11,500 proteins in each glial cell type, of
which 10,529 proteins were detected in all cell types. Parallel RNA-
seq analysis demonstrated a great depth of coverage of the
glioproteome. Results generated by this study constitute the largest
proteomic dataset of the adult mouse brain thus far. Another recent
study assessed striatal and hippocampal proteomes from acutely iso-
lated astrocytes. Among the 3,509 identified proteins, the authors
found 18 proteins enriched in striatal astrocytes and 12 in hippocam-
pal astrocytes (Chai et al., 2017). These results are interesting because
each of these proteins is a potential region-specific astrocyte marker.
Proteomics is also crucial for therapeutic approaches, as it identifies
disease-related proteins (Rangaraju et al., 2018) and/or early alter-
ations that occur during prodromal stages of the disease (Boza-Serrano,
Yang, Paulus, & Deierborg, 2018).

Although glial cell proteomics has been applied in animal models,
it is challenging in human postmortem brain samples because of post-
mortem protein degradation and limited availability of human fresh
samples (Mizee, Poel, & Huitinga, 2018). The recent development of a
procedure to rapidly isolate pure human microglia from autopsy tissue
could improve this in the near future, but its usefulness will depend
on the possibility to implement it in regular clinical practice (Mizee
et al., 2018).

Advances in proteomics have also helped to characterize part of
the “gliosecretome” by quantifying the expression of secreted pro-
teins from conditioned media of cultured glial cells (Jha et al., 2018).
Proteomic profiles of secreted proteins from microglia, astrocytes,
and other glial cell types are now well documented (Jha et al., 2013).
Among the rare examples of gliosecretome analysis in human cells,
the profile of cytokines and chemokines produced from normal human
astrocytes derived from 15-week human fetus (Choi, Lee, Lim,
Sato, & Kim, 2014) and the secretome of human neural stem cells-
derived oligodendrocytes were established (Kim et al., 2014).

4.2.2  |  Mass cytometry and imaging mass
cytometry

Cytometry by time of flight (CyTOF) is a revolutionary technology
merging conventional flow cytometry and mass spectrometry (for
review see Brodin, 2019). The main difference with conventional flow
cytometry is that fluorophore-tagged antibodies used to label cells are
replaced by metal-tagged ones. Thus, after ionization of the isolated
labeled cell, the specific tags are identified by time of flight of discrete
masses. Low mass spectral overlap signal offers the advantage of
avoiding compensation. CyTOF has also low background signal, and it
is thus particularly suited for high-dimension functional and pheno-
typic analysis at the single cell level. CyTOF was used to (a) identify
different sub-populations of brain-resident macrophages under physi-
ological conditions and (b) study the remodeling of the entire brain.
immune landscape under pathological conditions including Alzheimer
disease and experimental autoimmune encephalomyelitis (Mrdjen
et al., 2018). As an example of the power of the CyTOF approach,
these authors identified a specific microglia subpopulation associated
with Alzheimer disease and aging, which is reminiscent of DAMs iden-
tified by Keren-Shaul et al. (2017). Furthermore, by analyzing 1.8 mil-
cion cells from seven experimental conditions with a panel of
39 antibodies, three different CNS-resident myeloid subpopulations
were identified, including a disease-specific one (Ajami et al., 2018).
Interestingly, these authors demonstrate that although defined as rela-
tively homogeneous by cell surface markers, under pathological con-
ditions, each subpopulation contains heterogeneous functional
subsets. More recently, CyTOF, together with sophisticated bioinfor-
matics analyses, was used for a comprehensive characterization of
postmortem human microglia (Bottcher et al., 2019). Of note, this
study confirmed that human microglia can be phenotypically distin-
guished from peripheral myeloid cells and supports the notion of
microglial heterogeneity in the human brain.

Also based on metal-tagged antibodies, imaging mass cytometry tech-
niques have been developed to produce images of formalin-fixed,
paraffin-embedded tissue sections (for review see Chang et al., 2017).
Two similar technologies have been developed: imaging mass cytometry
and multiplex ion beam imaging. In brief, stained tissue sections are
scanned by a 1-μm-diameter pulsed laser spot. Tissue vaporized on each
laser spot is then analyzed by using the mass cytometer. Reconstructed
images from the tissue sections have a resolution comparable to light
microscopy. Of note, another related approach based on the use of
photocleavable oligonucleotide-tagged antibodies instead of metal-tagged
antibodies has been developed. It can detect and quantify up to 40 differ-
ent proteins in each region of interest (Blank et al., 2018). A recent study
used imaging mass cytometry to explore heterogeneity of myeloid and
astrocytes populations around human MS lesions (Park et al., 2019). The
multiplexing possibilities offered by these methods will undoubtedly be
useful to study the diversity of glial cells at the protein level.

4.2.3 | Advantages, pitfalls, and future directions
for proteomics approaches

Mass spectrometry-based approaches are powerful unbiased methods
for biomarker discovery and therapeutic strategy development as mass
spectrometry allows identification of protein expression changes that
can be used as markers of pathogenesis or disease progression
(Bayes & Grant, 2009; Severino, Farina, & Chambry, 2013). Compari-
sion of the gliosecretome with proteins found in the cerebrospinal fluid
of patients provided new biomarkers for brain diseases (Suk, 2010).
The upcoming challenge will now be to reach single-cell resolution
while preserving wide proteome coverage (Doerr, 2019; Levy & Slavov,
2018). A new mass spectrometry-based approach was recently devel-
opled that quantified ~600 proteins in single differentiating mouse
embryonic stem cells (Budnik, Levy, Harmange, & Slavov, 2018).

However, as mentioned for scRNA-seq approaches (Section 4.1),
even if proteomics sensitivity improves to the single cell level, the
experimental challenge of isolating entire single glial cells remains.
Indeed, cell isolation is a key primary step for mass spectrometry but
also for CyTOF and can result in major artifacts and loss of important
cell domains. A solution to circumvent this problem is to target protein
labeling agents to specific cell types, as recently developed by the
Chin and Schuman groups. The strategies are based on the cell-type-specific
expression (through viral vectors or in transgenic mice) of an
exogenous tRNA transferase, which incorporates mass spectrometry-
detectable noncanonical amino acids administered orally (Alvarez-
Castelo et al., 2017; Krogager et al., 2018). After isolation of labeled
peptides, they were able to establish the cell-type specific proteome of
the targeted cells in vivo. The importance of specialized subcellular com-
partment is emerging in glial research (e.g., single myelinating segments,
perisynaptic or perivascular astrocyte processes, and membrane
nanodomains). Therefore, complementary methods providing subcellular
resolution, sometimes at the expense of throughput, can help illuminate
this important issue. They include methods based on intact brain sections
like imaging mass cytometry or proteomics-based methods of a specific
compartment isolated through subcellular fractionation or labeling. This
kind of subcellular mass spectrometry analysis was performed on axonal
growth cones (Poulopoulos et al., 2019), or mitochondria tagged in spe-
cific brain cell types (Fecher et al., 2019). Alternatively, in situ proximity
labeling with an exogenous biotin ligase can be used to identify protein
complexes and partners in specific subcellular compartments (Uezu et al.,
2016). However, these methods are still in their early days and require
more optimization (Wilson & Nain, 2018).

The great advantage of CyTOF and mass imaging cytometry over
conventional cytometry and immunohistochemistry approaches, respec-
tively, is to considerably improve the multiplexing potential. Indeed,
whereas conventional cytometry is limited to staining panels of ~25
fluorophores, CyTOF uses staining panels of 35–45 metal-tagged anti-
bodies, and the multiplexing possibilities are likely to improve in the
coming years. However, these approaches still require complex, expen-
sive instruments and analysis tools. In addition, as more markers are
included or higher resolution is needed, longer acquisition times are
required. For example, the rate of image acquisition with imaging mass
cytometry is slow (about 0.75 mm²/hr) and set the current limit of these
approaches. High-content analysis of these data is certainly one of the
greatest challenges to the effective implementation of these
approaches in research. Indeed, researchers need to visualize and ana-
lize these high-dimensional data to obtain a comprehensive picture of the
different cell subpopulations within a given physiopathological con-
text. New clustering and dimensionality-reduction algorithms are con-
stantly developed to resolve this issue. However, the choice among the
different tools is not always straightforward.

5 | TOWARD THE CLINIC: NONINVASIVE
BRAIN IMAGING TECHNIQUES TO MONITOR
GLIAL CELLS IN HUMANS

Several noninvasive brain-imaging methods linked to glial cells have
been developed in preclinical studies and some have been translated
to the clinic. They are mostly based on magnetic resonance imaging or magnetic resonance spectroscopy, which are basically sensitive to the water or proton-containing molecules present in brain tissue. They either provide images or maps or quantitative data in a voxel of interest. Other imaging techniques such as positron emission tomography are based on the intravenous injection of a radioactive molecule (radioligand) that will enter the brain, bind to a particular receptor, enzyme or cell, be detected by their emission of positrons, and used to assemble 3D functional images.

5.1 | Magnetic resonance imaging of myelin formed by oligodendrocytes

Many magnetic resonance imaging modalities are used to indirectly image myelin (see Filippi & Agosta, 2010; Heath, Hurley, Johansen-Berg, & Sampaio-Baptista, 2018; Figure 5b). Classic T2-weighted images show contrast between gray and white matter and are routinely used to detect demyelinated lesions in humans. Contrast agents like gadolinium are useful to detect focal myelin loss as found in multiple sclerosis patients. Additional techniques based on water diffusion along myelinated tracks, such as diffusion tensor imaging, reveal the directionality and size of myelinated axons (and change thereof). Magnetization transfer ratio imaging is sensitive to the amount of macromolecules present in myelin, such as lipoproteins, allowing an indirect assessment of myelin content in situ. However, comparison of magnetic resonance imaging data in postmortem samples reveals that axonal damage, water content, or inflammation with accumulation of microglia and macrophages may strongly influence the signals (Filippi et al., 2019; Heath et al., 2018; Vavasour, Laule, Li, Traboulsee, & MacKay, 2011). It will thus be essential to develop more specific imaging techniques to monitor myelin loss and recovery (see Section 5.3).

5.2 | Magnetic resonance spectroscopy

Magnetic resonance spectroscopy can measure the concentration of abundant glial metabolites like myo-inositol or glutamine or quantify glial oxidative pathways, with 13C-labeled metabolites such as acetate, which are metabolized preferentially in astrocytes (Escartin, Valette, Lebon, & Bonvento, 2006; Lebon et al., 2002). Changes in metabolite concentration or metabolic fluxes in specific CNS regions can reveal ongoing disease processes or changes in brain cell metabolism.

Recently, diffusion-weighted magnetic resonance spectroscopy of glial metabolites was used to infer morphological changes in astrocytes when they become reactive (Ligneul et al., 2019; Palombo et al., 2016). However, it is important to note that the relative distribution of several of these metabolites between glial cells and neurons was established in vitro, in conditions where cell metabolism may be very different from in vivo, due to the absence of complex cellular interactions.

FIGURE 5  Non-invasive imaging of glial cells in patients. (a) Translocator protein positron emission tomography imaging with [18F]-DPA-714 in prodromal Alzheimer disease patients and healthy control subjects. Sagittal, coronal, and axial views of standard uptake value ratio show significant radioligand binding in the temporal and parietal cortex of prodromal patients. (b) T1-weighted magnetic resonance imaging scan (i), T2-weighted scan (ii), standard uptake value image of a [11C]-PIB positron emission tomography scan (iii) from a multiple sclerosis patient. Arrows show two typical demyelinating lesions visible in all three modalities. (c) Myelin dynamics monitoring by longitudinal [11C]-PIB positron emission tomography imaging in a different multiple sclerosis patient. Demyelinating and remyelinating voxels appear in red and blue, respectively. Adapted with permission from (a) Hamelin et al. (2016) and (b,c) Bodini et al. (2016)
interactions, or vascularization and exposure to higher O$_2$ and glucose levels (see Section 2). For example, N-acetylaspartate, an abundant metabolite broadly used as a neuronal marker, is in fact deeply influenced by the reactive state of astrocytes (Carrillo-de Sauvage et al., 2015). N-Acetylaspartate is also a key intermediate in myelin production (Amaral, Meisingset, Kotter, & Sonnewald, 2013), challenging the dogma that N-acetylaspartate is a pure neuronal metabolite.

5.3 | Positron emission tomography

Several positron emission tomography imaging approaches are based on radioligands targeting specific glial cell molecular targets such as translocator protein (TSPO) and TSPO positron emission tomography imaging is widely used to track microglial activation, including in patients where positive signal can be visible at early disease stages, for example, in patients with Alzheimer or Huntington disease (Edison, Donat, & Sastre, 2018; Hamelin et al., 2016; Tai et al., 2007; Figure 5a). However, reactive astrocytes also express translocator protein and cannot be discriminated from reactive microglia with available tracers (Lavisse et al., 2012).

Efforts are ongoing to develop more specific ligands to individual cell types and ligands that recognize changes in cellular status in disease conditions (Aiello et al., 2019; Narayanaswami et al., 2018). For example, radioligands targeting monoamine oxidase B, which is induced in reactive astrocytes, may be of use, but this enzyme is also expressed in monoaminergic neurons (see Narayanaswami et al., 2018 for references). Other specific receptors induced in reactive glial cells could provide alternative targets for positron emission tomography. Recent database describing the “sensome” of microglial cells (for example Hickman et al., 2013), could help find molecular targets for positron emission tomography imaging or repurpose available radioligands. A good example of such repositioning is $[^{13}C]$-Pittsburg compound, which is classically used for the detection of amyloid deposits but was found to also bind myelin. The local rate of demyelination/remyelination was measured with this ligand in active lesions of relapsing-remitting multiple sclerosis patients (Bodini et al., 2016), revealing significant heterogeneity in myelin dynamics within and between patients (Figure 5c).

5.3.1 | Advantages, pitfalls, and future directions for noninvasive brain imaging

Brain imaging methods generally provide a rather coarse picture of glial cells throughout the CNS, with limited spatial resolution, well below that of the cellular imaging techniques presented in Section 3.2. However, by imaging glia molecular targets, it becomes possible to indirectly monitor the global behavior of specific glial cell populations. In addition, these techniques have the major advantage of allowing longitudinal subject monitoring. This provides invaluable insight into disease evolution and potential recovery with treatment in the same subject and reduces animal use in preclinical research. Interestingly, some techniques already used in preclinical and clinical studies are based on functions that are strongly regulated (if not totally) by glial cells. For example, positron emission tomography imaging of $[^{18}F]$-fluorodeoxyglucose uptake may be directly influenced by astrocytes regulating glucose uptake and lactate supply to neurons through the astrocyte-to-neuron lactate shuttle (Belanger, Allaman, & Magistretti, 2011). Doppler-based methods, or functional magnetic resonance imaging, measure blood flow and/or brain oxygen perfusion, and blood flow is regulated by astrocytes and other nonneuronal cells (Grutzendler, Kasthuri, & Gan, 2002; Mishra, 2017).

In conclusion, imaging of glial cells is already in place in the clinics either directly or indirectly. However, developments are needed to make it more specific for a single cell type, a specific state, more sensitive, and of broader applicability. Of importance, noninvasive brain-imaging methods in animal models in preclinical research and in humans in clinics use very similar equipment and techniques. These approaches are thus among the few that can really be translated to the clinic.

6 | CONCLUDING REMARKS AND FUTURE DIRECTIONS

As illustrated in the previous sections, the glia field is blooming with many tools and techniques that provide efficient, specific, and refined options to monitor and study glial cells in different experimental settings. It is important to note that glial cells encompass many different cell types with different ontogenies, molecular, and morphological features or functional roles in the CNS. Therefore, some techniques are only appropriate and relevant for a given cell type, and only few of them are truly applicable to all glial cells.

Alternative animal models like zebrafish, Caenorhabditis elegans, or Drosophila have their own glial cells, remarkably homologous to those found in mammals. They provide great experimental opportunities, including genetic screening, whole brain/body imaging and rich behavioral assessment (Coutinho-Budd & Freeman, 2013; Freeman, 2015; Lyons & Talbot, 2014; Shaham, 2015). On the other hand, it is important to translate results obtained in rodents or in these alternative models, to nonhuman primates or even humans. Human glial cells are quite different from their mouse counterparts (Geirsdottir et al., 2019; Hodge et al., 2019). They may have a different morphology, gene expression profile, and some of their functions may differ as well (Oberheim et al., 2009; Zeng & Sanes, 2017; Zhang et al., 2016). Analysis of glia in nonhuman primate models as well as human samples and cells (through iPSC cells or brain biopsies) is important. An alternative approach is to graft human glial cells in the brain of rodent models to “humanize” these mice in an attempt to get closer to human patients (Arranz & De Strooper, 2019), although this approach is inevitably associated with some bias (e.g., need for surgery inducing mechanical injury and inflammation as well as immunodeficiency of the receiver mice). Such human-mouse chimera were used with astrocytes in Huntington disease
Inclusion of glial cells in the experimental arsenal for disease modeling, drug and biomarker discovery, and therapeutic testing is crucial (Garden & Campbell, 2016). It is becoming more and more evident that glial cells are central players in many brain diseases such as neurodegenerative disease, demyelinating diseases, epilepsy, and brain cancer (Ben Haim et al., 2015; Priller & Prinz, 2019; Zuchero & Barres, 2015). It is thus important to study glial cell behaviors in these diseases as well as their response to different treatments. The development of glial cell-based therapies is in its infancy but it opens huge opportunities for the treatment of many brain diseases (Moller & Boddeke, 2016).

Another exciting prospect for glia research is to develop research projects on overlooked glial cells, such as tanicytes and satellite glial cells. There are fewer or less characterized tools and less knowledge, yet, these cells may play key roles in brain physiopathology, as it the case for tanicytes of the hypothalamus in metabolic regulation (Prevot et al., 2018), satellite glial cells in pain (Ohara et al., 2009), and enteric glial cells in gut–brain axis interactions (Grubisic & Gulbransen, 2017). There are certainly many discoveries to be made on these glial cells. This was recently illustrated with the description of a previously unknown cutaneous glial cell that ensheathes sensory nerve terminals and is involved in nocuous signal transduction (Abdo et al., 2019).

Clearly, one of the main challenges for glia research, and neuroscience in general, is to handle and make sense of the huge data sets being produced (e.g., by genomic, proteomic data, single cell profiling, electrophysiological recordings, behavioral video tracking). This calls for strong ties with other disciplines like bioinformatics, biostatistics, artificial intelligence, and systems biology (Kastanenka et al., 2019). Development of multidisciplinary approaches, combining mathematics (e.g., to model complex processes), physics (to develop new microscopes and detectors), chemistry (to generate new probes and sensors), and informatics (to compute large and complex data), will be key to reach a better understanding of glia cell function and dysfunction. In addition, glia researchers could seek inspiration from approaches developed by immunologists to study inflammation or cell heterogeneity and by developmental biologists to study cell specification or those implemented by endocrinologists to study cell–cell communications.

In conclusion, the glia field has developed significantly since the seminal research papers presented in the 1850s. Many important discoveries on these cells were facilitated by methodological developments or new tools, but above all, it is the strong, growing, and multidisciplinary community of researchers that makes the glia research field so productive and exciting.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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REFERENCES


Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutscher, I., ... Aubourg, P. (2009). Hematopoietic stem


Mooten, F. L. (1986). Tumor chemosensitivity conferred by inserted her-

pes thymidine kinase genes: Paradigm for a prospective cancer control

Moyon, S., Dubessy, A. L., Algrot, M. S., Trotter, M., Huang, J. K.,
Daiphinhot, L., ... Lubetzki, C. (2015). Demelination causes adult CNS
progenitors to revert to an immature state and express immune cues
that support their migration. *Journal of Neuroscience*, 35(1), 4–20.
https://doi.org/10.1523/JNEUROSCI.0849-14.2015

brainres.2015.06.009

Mrđjen, D., Pavlovic, A., Hartmann, F. J., Schreiner, B., Utz, S. G.,
Leung, B. P., ... Becher, B. (2018). High-dimensional single-cell mapping
of central nervous system immune cells reveals distinct myeloid sub-
doi.org/10.1016/j.immuni.2018.01.011

Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., ...
Jaenisch, R. (2016). Efficient derivation of microglia-like cells from
https://doi.org/10.1038/nm.4189

Mujtaba, T., Piper, D. R., Kalyani, A., Groves, A. K., Lucero, M. T., &
Murlidharan, G., Samulski, R. J., & Asokan, A. (2014). Biology of adeno-
associated viral vectors in the central nervous system. *Frontiers in
Molecular Neuroscience*, 7, 76. https://doi.org/10.3389/fnmol.2014.00076

Mukai, R., Rajabhanderi, A. K., Gangwani, M. R., Hachisuka, A., Coppola, G.,
Masmanidis, S. C., ... Khakh, B. S. (2019). Hyperactivity with disrupted
attention by activation of an astrocyte synaptogenic cue.
*Cell, 177(5), 1280–1292. https://doi.org/10.1016/j.cell.2019.03.019


Narayanaswami, V., Dahl, K., Bernard-Gauthier, V., Josephson, L.,
Cumming, P., & Vasdev, N. (2018). Emerging PET radiotracers and tar-
gets for imaging of neuroinflammation in neurodegenerative diseases:
1536011817782317

progenitors to revert to an immature state and express immune cues
that support their migration. *Journal of Neuroscience*, 35(1), 4–20.
https://doi.org/10.1523/JNEUROSCI.0849-14.2015

Nimesh, J., Gangwani, M. R., Allam, S. L., Tran, D., Huang, S., Hoang-
Trong, T. M., ... Khakh, B. S. (2019). Transient, consequential increases
in extracellular potassium ions accompany Channelrhodopsin2
celrep.2019.04.078

Ogawa, S., Menen, R. S., Tank, D. W., Kim, S. G., Merkle, H.,
blood oxygenation level-dependent contrast magnetic resonance
imaging. A comparison of signal characteristics with a biophysical model.
S0006-3495(93)81441-3

Ohara, P. T., Vit, J. P., Bhargava, A., Romero, M., Sundberg, C.,
1078358509363604

Okabe, S., Forsberg-Nilsson, K., Spiro, A. C., Segal, M., & McKay, R. D.
(1996). Development of neuronal precursor cells and functional
postmitotic neurons from embryonic stem cells in vitro. *Mechanisms of
Development*, 57(1), 89–102. https://doi.org/10.1016/0925-4773(96)
00572-2

Okada, Y., Sasaki, T., Oku, Y., Takahashi, N., Seki, M., Uijita, S., ...
Ikegaya, Y. (2012). Presynaptic calcium rise in putative pre-Botzinger complex
10.1113/jphysiol.2012.231464

Okaty, B. W., Sugino, K., & Nelson, S. B. (2011). Cell type-specific trans-
https://doi.org/10.1523/JNEUROSCI.0626-11.2011

Olah, M., Patrick, E., Villani, A. C., Xu, J., White, C. C., Ryan, K. J., ...
*Nature Communications*, 9(1). 539. https://doi.org/10.1038/s41467-
018-02926-5

Olmos-Serrano, J. L., Kang, H. J., Tyler, W. A., Silbereis, J. C., Cheng, F.,
neuron.2016.01.042

Omer Javed, A., Li, Y., Muffat, J., Su, K. C., Cohen, M. A., Lungjangwa, T., ...
doi.org/10.1016/j.celrep.2018.09.032

Onorati, M., Li, Z., Liu, F., Sousa, A. M. M., Nakagawa, N., Li, M., ...
Sestan, N. (2016). Zika virus disrupts phospho-TBK1 localization and
mitosis in human neuroepithelial stem cells and radial glia. *Cell Reports*,
16(10), 2576–2592. https://doi.org/10.1016/j.celrep.2016.08.038

https://doi.org/10.1038/nn.3930

Ortinski, P. I., Dong, J., Mungenast, A., Yue, C., Takano, H., Watson, D. J., ...

Palfi, S., Guruchaga, J. M., Lepetit, H., Howard, K., Ralph, G. S., Mason, S., ...
Mitrophanous, K. A. (2018). Long-term follow-up of a phase I/II study of
ProSavin, a Lentiviral vector gene therapy for Parkinson’s dis-
https://doi.org/10.1089/humc.2018.081

Palombo, M., Lagneul, C., Najac, C., Le Douce, J., Flament, J., Escartín, C., ...
Valette, J. (2016). New paradigm to assess brain cell morphology by
diffusion-weighted MR spectroscopy in vivo. *Proceedings of the
National Academy of Sciences of the United States of America*, 113(24),
6671–6676. https://doi.org/10.1073/pnas.1504327113

Pandya, H., Shen, M. J., Ichikawa, D. M., Sedlock, A. B., Choi, Y.,
Johnson, K. R., ... Park, J. K. (2017). Differentiation of human and
murine induced pluripotent stem cells to microglia-like cells. *Nature
Neuroscience*, 20(5), 753–759. https://doi.org/10.1038/nn.4534

Pannasch, U., Freche, D., Dallerac, G., Ghezali, G., Escartin, C., Ezan, P., ...
Rouach, N. (2014). Connexin 30 acts synaptic strength by controlling


congenitally dysmyelinated brain. Nature Medicine, 10(1), 93–97. https://doi.org/10.1038/nm974

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.