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REVIEW

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Towards PET imaging of the dynamic phenotypes of microglia

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Abstract

There is increasing evidence showing the heterogeneity of microglia activation in neuroinflammatory and neurodegenerative diseases. It has been hypothesized that pro-inflammatory microglia are detrimental and contribute to disease progression, while anti-inflammatory microglia play a role in damage repair and remission. The development of therapeutics targeting the deleterious glial activity and modulating it into a regenerative phenotype relies heavily upon a clearer understanding of the microglia dynamics during disease progression and the ability to monitor therapeutic outcome *in vivo*. To that end, molecular imaging techniques are required to assess microglia dynamics and study their role in disease progression as well as to evaluate the outcome of therapeutic interventions. Positron emission tomography (PET) is such a molecular imaging technique, and provides unique capabilities for non-invasive quantification of neuroinflammation and has the potential to discriminate between microglia phenotypes and define their role in the disease process. However, several obstacles limit the possibility for selective *in vivo* imaging of microglia phenotypes mainly related to the poor characterization of specific targets that distinguish the two ends of the microglia activation spectrum and lack of suitable tracers. PET tracers targeting translocator protein 18 kDa (TSPO) have been extensively explored, but despite the success in evaluating neuroinflammation they failed to discriminate

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between microglia activation statuses. In this review, we highlight the current knowledge on the microglia phenotypes in the major neuroinflammatory and neurodegenerative diseases. We also discuss the current and emerging PET imaging targets, the tracers and their potential in discriminating between the pro- and anti-inflammatory microglia activation states.

KEYWORDS
microglia phenotypes, neuroinflammation, PET imaging

INTRODUCTION

Microglia are the resident immune cells of the central nervous system (CNS). They originate from the yolk sac where progenitors, during development, migrate and populate the brain [1,2]. Following this migration, the microglia population is maintained during the lifespan by self-renewal through apoptosis and proliferative processes [3]. During development, microglia play a key function in shaping the neuronal network by supporting neural development and synapsis pruning [4,5], while in the adult CNS they carry surveillance functions to maintain homeostasis. Taking advantage of their ramified plastic morphology and their high mobility, they continuously sense their environment for pathological insults and harmful stimuli [6]. Microglia are the first line of defense in the brain. In response to injury and disease pathogens they undergo cellular activation characterized by morphological change (from ramified into amoeboid shape), gliosis and functional changes, including alteration of cell surface receptors expression and production of cytokines and chemokines. Depending on the nature of the stimuli, activated microglia have been largely classified in two phenotypes, namely pro-inflammatory and anti-inflammatory, sitting at opposite ends of the activation spectrum. Pro-inflammatory microglia are generally described as producers of inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, macrophage inflammatory protein (MIP)-1 α, nitric oxide synthase (NOS) and reactive oxygen species (ROS), via which they exert their detrimental effects [7]. Conversely, anti-inflammatory microglia are involved in dampening the inflammatory response and promoting the repair mechanisms. They are characterized by secretion of anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and transforming growth factor (TGF)-β and upregulation of markers such as arginase-1 (Arg-1), FIZZ-1, CD206 and YM-1 [7]. However, recent single-cell transcriptomics and proteomics studies disputed this pro-/anti-inflammatory dichotomy and identified a multitude of activated microglia phenotypes due to a variety of gene signatures associated with diseases and depending on their environmental context [8–10].

Despite this tremendous progress in identifying the different microglia phenotypes *in vitro* and *in vivo*, a clear understanding of these processes in the human brain *in vivo* is still lacking. A non-invasive imaging technique such as positron emission tomography (PET) is well suited for quantitative investigation of neuroinflammation and possesses the potential to discriminate between the different microglia phenotypes. Although it is now appreciated that the simple pro- and anti-inflammatory phenotype may not capture the whole diversity of microglia activation [11], this classification serves as a crucial guide for PET tracer development. In this context, we understand the pro-inflammatory response to be associated with disease progression and neurotoxicity, while the anti-inflammatory response is linked to dampening of harmful effects and recovery. Most of the development in PET imaging of neuroinflammation has focused upon targeting translocator protein 18 kDa (TSPO). Despite the large panel of tracers developed, TSPO appeared to be a less than ideal target due to, among others, its expression on different brain cells and the lack of ability to differentiate between pro- and anti-inflammatory microglia. These drawbacks encouraged the investigation of other targets that might be more selective for microglia and can differentiate between the activated phenotypes.

In the current review, we first summarize the different microglia activation phenotypes in the main chronic neuroinflammatory and neurodegenerative diseases, focusing on multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD). Then, we discuss the current and emerging PET imaging targets and their tracers and highlight, when characterized, their potential in discriminating between microglia activation phenotypes.

Microglia activation phenotypes in neuroinflammatory and neurodegenerative diseases

Neuroinflammation is an inflammatory process within the CNS and occurs as response to various pathological insults, such as infection, protein aggregation, trauma and ischemia. It is mainly driven by microglia and

astrocytes and in certain cases by infiltrated immune cells from the blood, especially when the blood–brain barrier (BBB) is inflamed and compromised. The secretion of pro-inflammatory cytokines and chemokines, nitric oxide (NO) and ROS usually mark this process. In an acute event, the mounted neuroinflammatory response will resolve when the triggering insults are eliminated, and microglia will transition into an anti-inflammatory and regenerative phenotype. However, in neurodegenerative diseases, neuroinflammation tends to become a chronic process that fails to resolve itself due to continuous stimulation. This chronic activation leads to a variety of microglia phenotypes that potentially contributes to neuronal damage and death and becomes a major driver of disease progression.

In this section we concisely highlight the activation phenotypes of microglia in neuroinflammatory and neurodegenerative diseases and how they contribute to the disease pathology, focusing upon MS, AD and PD (Table 1).

TABLE 1 Activated microglia phenotypes and the expressed cytokines and markers in neuroinflammatory and neurodegenerative diseases

Disease	Microglia phenotype	Expression of markers and cytokines	Reference
Multiple sclerosis			
Initial and early white matter active lesions	Pro-inflammatory	CD68, MHC-I, MHC-II, CD86	[14]
		ROS, p22phox	
Late active white matter lesions	Intermediate phenotype	MIP-1β (CCL4), OPN	[15–17]
		HLA-DR, CD11c, AXL, CD45, CD68, CD206, CD163	
Slowly expanding white matter lesions (active rim)	Pro-inflammatory	CD40, CD68, p22phox, iNOS, ferritin	[14,18]
		IL-1, IL-6	
Cortical lesions	MS 1	↓ $P2Y_{12}R$, ↑HLA-II, ↑CD68	[21]
	MS 2	↓ $P2Y_{12}R$, hyper-ramified morphology	[21]
EAE animal model (early phase)	Pro-inflammatory	CD86, CD40, MHC-II	[22]
		TNF-α, IFN-γ, IL-12, IL-6, iNOS, NO	
EAE animal model (recovery phase)	Anti-inflammatory	IL-4, IL-10 and IL-13	[22,23]
Alzheimer's disease			
AD (late stage)	Pro-inflammatory	IL-1β, TNF-α, p40, MHC-II, iNOS	[9,31]
	AD1 (associated with Aβ)	↓ $P2Y_{12}R$	[10]
		↑ TREM2, ↑ ApoE4, ↑ITGAX	
	AD2 (associated with pTau)	↑ GRID2, $P2Y_{12}R$, ↑CX3CR1	[10]
AD transgenic mice	Anti-inflammatory (early Aβ pathology)	YM1	[30]
	Dynamic shift towards pro-inflammatory (later stage)		
	DAM (phagocytic)	↓ P2y12, ↓ CX3CR1, ↓ TMEM119	[32]
		↑ ApoE4, ↑ Tyrobp, ↑TREM2	
	Dark microglia	CD11b, TREM2	[39]
Parkinson's disease			
PD	Pro-inflammatory	COX-2, iNOS	[43,44]
		IL-1β, IL-6, TNF-α	
PD animal models	Pro-inflammatory	IL-1β, IL-6, TNF-α, NOS2	[55]
		↓ IGF-1, ↓ MRC1	

Abbreviations: ↑, Over-expression; ↓, down-regulation; AD, Alzheimer's disease; ApoE4, apolipoprotein E-4; CX3CR1, CX3C chemokine receptor-1; EAE, experimental autoimmune encephalomyelitis; GRID2, glutamate receptor, ionotropic, delta 2; HLA-DR, human leukocyte antigen D-related; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; iNOS, inducible nitric oxide synthase; ITGAX, integrin subunit alpha X; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; MRC, mannose receptor C; NO, nitric oxide; OPN, osteopontin; $P2Y_{12}R$, purinergic 2Y receptor type 12; ROS, reactive oxygen species; TNF, tumor necrosis factor; TREM, triggering receptor expressed on myeloid cells; Tyrobp, transmembrane immune signaling adaptor.

Multiple sclerosis (MS)

MS is a chronic inflammatory disease of the CNS. The main pathological hallmark of MS is the presence of focal lesions in the white matter associated with extensive glia activation, demyelination and neurodegeneration [12,13]. Activated microglia are at the core of the neuroinflammatory response in MS pathology. Recent discoveries of specific markers and molecular signatures of microglia [transmembrane protein 119 (TMEM119) and purinergic 2Y receptor type 12 ($P2Y_{12}R$)] indicated their predominance over monocyte-derived macrophages in MS lesions [14,15]. Active demyelination and axonal injury are associated with the focal accumulation of activated microglia; however, the dynamics of microglia activation and their phenotypes in association with lesion types and disease progression is still not well understood. In white matter initial and early active lesion stages, microglia predominantly showed a pro-inflammatory phenotype with expression of phagocytic and antigen presentation markers [CD68, major histocompatibility complex (MHC) classes I and II and CD86] and production of ROS and enzymes that produce them (p22phox). In later stages of active lesions and inactive microglia-containing cores of active lesions, microglia adopted an intermediate phenotype and co-expressed anti-inflammatory markers (CD206 and CD163) together with pro-inflammatory markers [14,16,17]. Single-cell mass cytometry analysis on active lesions of progressive MS revealed enriched clusters of cells with higher expression of phagocytosis-related markers [human leucocyte antigen D-related (HLA-DR), CD11c, AXL, CD45, CD68] and inflammatory molecules such as inflammatory cytokines MIP-1β (CCL4) and osteopontin (OPN) [15]. In slowly expanding lesions, microglia present at the active rim adopted mainly a pro-inflammatory phenotype with expression of CD40, CD68, p22phox, inducible nitric oxide synthase (iNOS) markers and ferritin, while expression of anti-inflammatory markers was very low [14,18]. This phenotype was supported by data from RNA microarrays on micro-dissected tissues showing strong expression of complement factors and interleukins (IL-1 and IL-6), which play a role in pro-inflammatory processes [18]. It is evident that pro-inflammatory microglia contribute to the demyelination and axonal injury in the early active lesions and the active rim of chronic lesions [19]. The increased expression of antigen-presenting markers, inflammatory cytokines and ROS support their strong role in the development of MS pathology. Active remyelination in MS lesions is most prominent in active white matter lesions, which harbor a high content of microglia with intermediate phenotype. In contrast, no or minimal remyelination is seen in slowly expanding lesions [20] where mainly pro-inflammatory microglia are present. Cortical lesions are also present in MS; a recent study identified the presence of two distinct MS-specific microglia in these lesions named MS1, defined by a low expression of $P2Y_{12}R$, high HLA-II and CD68 and MS2 characterized by a hyper-ramified morphology with low $P2Y_{12}R$ expression [21]. Interestingly, only MS2 microglia were associated with neuronal loss in the cortical lesions [21].

Experimental autoimmune encephalomyelitis (EAE) is the most used model to mimic the pathology observed in MS. Pro-inflammatory microglia are predominantly present in the early phase of EAE and express high levels of CD86, CD40 and MHC-II on their surface. The prominent expression of pro-inflammatory cytokines such as TNF-α, interferon (IFN)-γ, IL-12, IL-6, iNOS, NO and proteases highlight their important role in the early phase of EAE development [22]. The level of anti-inflammatory microglia increases with EAE progression and peaks at the maximum of the clinical symptoms and in the recovery phase at the expense of pro-inflammatory microglia. This dynamic shift towards anti-inflammatory microglia dominance and the release of a variety of cytokines such as IL-4, IL-10 and IL-13 play an important role in the resolution of inflammation and repair mechanisms [22,23]. This pro- and anti-inflammatory polarization balance highly correlates with the demyelination/remyelination process in acute demyelination models, probably via modulation of the inflammatory niche and oligodendrocyte progenitor cell response [24,25].

Microglia activation in MS shows a very complex spatial and temporal pattern, and the exact role and contribution of microglia phenotypes to the evolution of MS lesions at different stages of the disease is still not totally clear.

Alzheimer's disease (AD)

AD is the most common type of neurodegenerative diseases of the CNS and is associated with progressive cognitive decline and memory loss. Pathologically, AD is characterized by extracellular deposition of amyloid beta (Aβ) plaques and intraneuronal accumulation of phosphorylated tau tangles [26]. Neuroinflammation is a prominent feature of AD. Microglia have been identified around Aβ plaques and shown to undergo dramatic morphological and electrophysiological changes in comparison to plaque-distant microglia, indicating an activated state [27,28]. In AD, microglia are able to bind soluble Aβ and Aβ fibrils via cell-surface and Toll-like receptors, and microglia phagocytic activity of Aβ has been identified *in vitro* and *in vivo*, exhibiting their role in Aβ clearing [29]. Microglia surrounding the plaques express YM-1 and are thus suggested to adopt an anti-inflammatory activation phenotype [30]. An age-related dynamic shift of microglia phenotype is reported in the $PS1^{M146Lx}APP^{751SL}$ double mutated mice. Anti-inflammatory microglia with phagocytic characteristics at the beginning of Aβ pathology could switch to a pro-inflammatory phenotype at the

advanced stage of disease in this model, which is induced in part by the age-dependent accumulation of soluble Aβ oligomers [30]. Activated microglia in a later AD stage showed an increased expression of pro-inflammatory markers such as IL-1β, TNF-α, p40, MHC-II and iNOS, but not the anti-inflammatory factors IL-4 or IL-10 [9,31].

Recent transcriptomics studies identified a microglia phenotype associated with neurodegenerative diseases, termed disease-associated microglia (DAM) [32]. DAM stained positively for intracellular Aβ particles and are spatially localized in the proximity of Aβ plaques in the 5XFAD mice, a transgenic animal model of AD, and in human post-mortem brain [32]. DAM demonstrated significant changes in gene expression, including down-regulation of homeostatic genes such as P2y12, CX3C chemokine receptor-1 (Cx3cr1) and TMEM119, and up-regulation of known AD risk factors such as apolipoprotein E-4 (ApoE4), transmembrane immune signaling adaptor (Tyrobp) and TREM2 [32,33]. Triggering receptor expressed on myeloid cells 2 (TREM2) is a microglia-specific cell surface receptor, and loss-of-function mutations in TREM2 are associated with increased risk of developing AD [34]. TREM2 binds Aβ and works as a sensor molecule for microglia activation [35]. TREM2-deficient microglia showed reduced uptake of Aβ-lipoprotein complexes *in vitro* and minimal evidence of Aβ internalization *in vivo* [36,37]. The stimulation of TREM2 initiates signal transduction pathways that promote microglial chemotaxis, phagocytosis, survival and proliferation, suggesting an anti-inflammatory and protective role of DAM in AD [34,38]. A new phenotype of microglia, referred to as 'dark microglia', was identified in the APP/PS1 AD mouse and demonstrated a highly activated state with strong expression of CD11b and TREM2 [39]. Microglia activation in AD is complex, and results in a large variety of phenotypes [9] due to the presence of different stimuli and variety of activation pathways. Microglia activation and phenotypes evolve as a function of disease progression. A loss of homeostatic signature and transition of microglia into DAM population is a function of disease progression and occurs in two phases with a TREM2-independent initial step and TREM2-dependent second step [9,32]. Recently, in a snRNAseq study on human AD tissues, two distinct AD-associated microglia profiles were identified that associated either with Aβ (termed 'AD1') or with hyper-phosphorylated tau (termed 'AD2') [10]. AD1 microglia correlated with phagocytic/activated markers including ITGAX, LPL, GPNMB, MYO1E and SPP1, while AD2 microglia were enriched with homeostasis genes such as CXCR1, $P2Y_{12}R$ and neuron-related genes such as GRID2, ADGRB3 and DPP10 [10]. Interestingly, AD1 microglia showed a similar gene expression profile as the DAM identified in the amyloid mice model [32] with increased expression of TREM2 and APOE and down-regulation of $P2Y_{12}R$ [10].

Current evidence suggests that at the early stages of AD, microglia have a protective role marked by phagocytosis and clearance of Aβ and secretion of anti-inflammatory cytokines. However, as AD progresses, chronic stimulation and activation cause phenotypical changes in microglia towards a pro-inflammatory phenotype. The pro-inflammatory environment can alter the phagocytic activity of microglia, which possibly leads to increased accumulation of Aβ [38], and can stimulate neurotoxic astrocytes via IL-1α and TNF [40]. In addition, complement-mediated (C3R) synapse phagocytosis and loss induced by pro-inflammatory microglia contribute to neurodegeneration and disease progression [38].

Parkinson's disease (PD)

Progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of intraneuronal Lewy body inclusions and neurites containing protein aggregates are the main pathological hallmarks of PD [41]. In PD patients, dopaminergic neuronal death results in progressive motor symptoms, including tremor at rest, bradykinesia, rigidity and postural instability. During the last decade it has become evident that neuroinflammation is associated with PD and activated microglia are present in the SNpc of PD patients [42]. In post-mortem PD tissues, activated microglia express high levels of inflammatory cytokines such as IL-1β, IL-6 and TNF-α, as well as iNOS and cyclooxygenase-2 (COX-2), suggesting a pro-inflammatory activated phenotype [43,44]. Analysis of cerebrospinal fluid (CSF) and peripheral blood plasma from PD patients also showed elevated levels of IL-1β, IL-6 and TNF-α [44–46]. These inflammatory cytokines were also increased in the 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD, while anti-inflammatory cytokines and markers such as TGF-β and CD206 were decreased [47,48]. In non-human primates, long-term glial activation and high levels of TNF-α and IFN-γ were detected several years following MPTP treatment [49]. Importantly, the amounts of IFN-γ and TNF-α in the SNpc correlated with the degree of neurodegeneration and motor impairment [49]. The exposure of dopaminergic neurons to inflammatory cytokines has been shown to promote neuronal death. Over-expression of TNF-α or IL-1β, pro-inflammatory microglia cytokines, in the SNpc using an adenovirus vector was sufficient to induce nigral neuronal degeneration [50,51]. However, neutralization of soluble TNF by intranigral administration of engineered dominant negative TNF compound XENP345 reduced the retrograde nigral degeneration in the 6-OHDA model by 50% [52]. This emphasizes the cardinal role of pro-inflammatory microglia and cytokines in dopaminergic neuronal degeneration. Another feature

of PD pathology is the presence of α-synuclein (α-syn) aggregates that constitute the main component of the Lewy bodies and neurites. Extracellular α-syn oligomers function as damage-associated molecular patterns. Signaling via Toll-like receptor (TLR)-2 and -4, α-syn activates microglia into a pro-inflammatory phenotype and induces the secretion of inflammatory cytokines and markers such as TNF, IL-1β, IL-6, COX-2, NOS and ROS [53,54]. Administration of preformed fibrils in mouse brains induced the expression of pro-inflammatory markers by microglia including NOS2, IL-6, TNF and IL-1β without affecting or decreasing anti-inflammatory markers such as IFN regulatory factor-4 (IRF-4), insulin-like growth factor-1 (IGF-1) and mannose receptor C-1 (MRC-1) [55]. Additionally, activated microglia play an active role in the process of α-syn transmission to neurons via exosomes that is further amplified by the presence of pro-inflammatory cytokines [56].

The role of pro-inflammatory microglia as initiator or secondary effector in PD is still not clear. Chronically activated microglia and the secretion of high levels of pro-inflammatory mediators that damage the neurons and induce the release of α-syn further activate microglia resulting in a feed-forward effect, promoting inflammation and neurodegeneration.

Translocator protein 18 kDa (TSPO) – gold standard for PET imaging of activated microglia

PET imaging research of microglial activation and neuroinflammation has been traditionally focused upon TSPO. TSPO is predominantly expressed on the outer mitochondrial membrane in multiple cell types in periphery and CNS and is linked to several critical cellular functions, including inflammatory pathways. TSPO PET, particularly using (*R*)-[^{11}C]PK11195, is often referred to as the gold standard for PET imaging of neuroinflammation. Limitations of (*R*)-[^{11}C]PK11195, including the low signal-to-noise ratio, have led to an extensive search for a more optimal TSPO radioligand, both taking into account an increase in affinity to reduce non-specific binding (second-generation TSPO ligands) and the difference in ligand binding affinity induced by single-nucleotide polymorphism *rs6971* (third-generation TSPO ligands), reviewed in detail recently [57,58]. Next to limitations of the PET ligands, targeting TSPO for neuroinflammation has challenges and limitations in itself. First, knowledge on levels and cellular localization of TSPO expression in healthy and diseased human brain largely stems from post-mortem studies using TSPO radioligands, but results are highly inconsistent [59,60]. Cellular sources of TSPO expression in healthy and diseased brain have recently been reviewed by Nutma *et al.* [61]. In short, immunohistochemical studies for TSPO in healthy human brain revealed that TSPO is expressed in glial cells (microglia and astrocytes), oligodendrocytes and vascular endothelial cells [62,63]. In post-mortem human AD brain samples, TSPO expression was demonstrated in microglia, macrophages and astrocytes but also in cells surrounding Aβ plaques [62] and only marginally significant differences in TSPO protein expression were found in temporal and frontal cortex between AD patients and healthy controls [63]. Furthermore, the TSPO burden did not correlate with neuropathology or with the glial response (microglia and astrocytes). In active and chronic active MS lesions, a strong correlation was shown between radioligand binding [(*R*)-[^{3}H]PK11195 and [^{3}H]PBR28], but astrocytic TSPO accounted for up to 65% of TSPO-positive cells in these lesions [64]. In PD rodent models, α-syn aggregates have been linked to microglial activation and correlated with expression of TSPO, but in PD patients TSPO PET has thus far yielded inconclusive results (recently reviewed by [65]).

Moreover, TSPO expression was also shown not to be phenotype-specific in microglia in active and chronic active MS lesions [64]. To directly correlate tracer binding to protein expression and distinguish cell types responsible for the radioligand uptake, Tournier *et al.* coupled fluorescence-activated cell sorting (FACS) to readout in radioligand-treated tissues (RTT) [66]. Using FACS-RTT, cellular origin of TSPO expression was shown to be highly dependent upon neuroinflammation type [66], and thus should be investigated for every pathology in order to attribute correct conclusions regarding TSPO PET imaging in human disease.

In line with these recent findings regarding TSPO protein expression, preclinical and clinical research results using TSPO PET in neurological diseases show highly variable outcomes. Nevertheless, some interesting recent imaging studies are outlined here. In a transgenic rat model of AD, using second-generation ligand [^{18}F]FEPPA, researchers were unable to detect activated microglia that were MHC-II-positive. Similarly, in a rat model of stroke, TSPO PET signal was only found in the insult region, but not in remotely present MHC-II-positive microglia [67].

In healthy humans, a significant correlation between volume of distribution (V_T) and age was found using second-generation ligand [^{11}C]PBR28 [59]. Interestingly, another study showed that the binding potential (BP_{ND}) values of second-generation ligand [^{11}C]DPA713 but not those of (*R*)-[^{11}C]PK11195 increased with age, although [^{11}C]DPA713 BP_{ND} values also did not reach significance [68]. Similarly, increased BP_{ND} values for [^{11}C]DPA713 were found in AD patients in all ROIs, but for (*R*)-[^{11}C]PK11195 BP_{ND} values were only elevated in precuneus [68], although patient cohorts for each tracer were different, so no direct comparison can be made.

(*R*)-[11C]PK11195 was used in a cohort of MS patients to follow-up on disease progress over 4 years [69]. Tracer uptake in normal-appearing white matter (NAWM) and thalamus of MS patients was significantly higher compared with healthy controls at the baseline scan, but no difference in cortical gray matter was observed. Interestingly, this is the first clinical study, to our knowledge, showing that higher TSPO binding predicts greater clinical disability, independent of relapsing status [69].

Taken together, being the 'gold standard' in PET imaging of neuroinflammation in the clinic, TSPO PET has had some success in revealing neuroinflammatory processes in human aging and disease. However, due to TSPO expression being non-specific for either pro- or anti-inflammatory status and even non-specific for a single cell type, there is still a need for the evaluation of other, more specific biological targets for PET imaging of neuroinflammation in human disease.

Emerging targets and PET tracers for imaging of activated microglia

To overcome the limitations of TSPO PET, other neuroinflammation and microglia imaging targets are being explored (recently reviewed in Narayanaswami *et al.* [70]). In the next part of this review, we describe the emerging targets for PET imaging of microglia and the most recent preclinical and clinical results. Focus lies in the most promising targets for which significant advances were made with regard to target characterization and for which PET tracer development includes initial biological evaluation (Table 2).

Purinergic 2X receptor type 7 ($P2X_7R$)

$P2X_7R$ is an ATP-gated ion channel expressed on cells of the myeloid lineage, lymphoid immune cells and mast cells. In CNS, the highest $P2X_7R$ density is found in microglia, but receptor expression is also found in astrocytes and oligodendrocytes, albeit to a lesser extent [71,72]. The involvement of $P2X_7R$ in the immune response is best described by its role in the mechanism of IL-1β secretion [71]. Following $P2X_7R$ activation by ATP, the efflux of large amounts of K^+ drive the assembly of NLRP3 inflammasome and subsequent caspase-1 activation [71,73,74]. High ATP concentrations (mM), generally an effect of cell damage or injury, are required to trigger receptor activation [73,74]. $P2X_7R$ is therefore regarded silent in normal physiological conditions; however, it is highly up-regulated during microglial activation. The increased microglial expression of $P2X_7R$ in neuroinflammatory disease is associated with a pro-inflammatory activation phenotype. *In vitro* in polarized adult human microglia, $P2X_7R$ is highly over-expressed in the pro-inflammatory but not the anti-inflammatory phenotype [25]. $P2X_7R$ was also over-expressed in inflammatory microglia in white matter of active and chronic active MS lesions [25]. Similarly, $P2X_7R$ expression was increased in activated microglia in the EAE rat model during the disease phase [25,75]. In AD mouse models, neuroinflammation induced by Aβ peptides increased the expression of $P2X_7R$ on microglia in senile plaques at advanced and late pathological stages [76], and $P2X_7R$ expression levels in plaque associated microglia progressively rose with age in line with the development of AD pathogenesis [77]. Altogether, $P2X_7R$ could serve as potential target for PET imaging of pro-inflammatory microglia.

The recent development of several PET tracers targeting $P2X_7R$ by different research groups (for overviews on purinergic receptor imaging see Janssen *et al.* and Zarrinmayeh and Territo [78,79]) highlights $P2X_7R$ as a promising target. *In vitro* autoradiography studies with [11C]GSK1482160 in EAE rats displayed increased tracer binding in lumbar spinal cord at 12–14 days post-immunization (peak of the disease) compared with spinal cord of healthy rats [80]. However, these results could not be replicated *in vivo* due to too-low affinity of [11C]GSK1482160 for rat $P2X_7R$. When inducing neuroinflammation in mice using lipopolysaccharide (LPS), [11C]GSK1482160 uptake was higher in brains of LPS-treated mice (72 h post-treatment) compared with saline-treated animals [81]. In a first-in-human study in healthy volunteers, brain uptake of [11C]GSK1482160 was demonstrated with a SUV of 1.0 at 20 min p.i. [82], deeming [11C]GSK1482160 suitable for further clinical evaluation.

Adamantyl benzamide [11C]SMW139 was used in a preclinical PET imaging study in EAE rats, displaying significantly elevated tracer uptake in spinal cord at the maximum of clinical symptoms in severe–relapsing EAE rats compared with recovery phase and control animals, but not in rats with mild–acute disease progression [75], probably due to milder inflammatory response. A first-in-human study showed good pharmacokinetics and quantifiable brain uptake of [11C]SMW139 in a small set of healthy volunteers and MS patients [83]. For every brain region investigated, V_T and BP_{ND} values were higher for MS patients compared with healthy subjects, but only V_T differed significantly.

To date, two Janssen R&D (Raritan, New Jersey, USA) compounds have been evaluated as a $P2X_7R$ PET tracer, both preclinically and clinically. In an acute rat model of PD (6-OHDA injection), [11C]JNJ-54173717 showed a time-dependent increase in tracer binding *in vitro*, co-localizing with Iba1 expression, but failed to show differences in tracer binding in a chronic A53T viral vector model of PD [84]. [11C]JNJ-54173717 showed desirable and quantifiable brain uptake in human, but no significant

TABLE 2 Activated microglia targets and their association with microglia phenotypes and potential tracers for PET imaging

Molecular target	Cellular localization	Pro-/anti-inflammatory expression	Tracers	*In vivo* (pre-)clinical research stage	References
$P2X_7R$	Microglia (mainly), to a lower extend on oligodendrocytes and astrocytes	Potentially specific to pro-inflammatory phenotype	[^{11}C]GSK1482160	First-in-human results in healthy volunteers warrant further clinical evaluation	[80–82]
			[^{11}C]SMW139	First-in-human results in healthy volunteers and MS patients warrant further clinical evaluation	[75,83]
			[^{11}C]JNJ-54173717	No differences in uptake between healthy volunteers and PD or ALS patients	[84–86,143]
			[^{18}F]JNJ-64413739	High inter-individual signal variability in non-human primates and human	[87–89]
$P2Y_{12}R$	Microglia	Potentially specific to anti-inflammatory phenotype	[^{11}C]5	No *in vivo* data	[25,94]
CB2R	Microglia, astrocytes, neurons, endothelial cells	No data on microglia phenotype expression	[^{11}C]NE40	Lower uptake in AD patients than healthy volunteers	[111,112]
			[^{11}C]RS-028	Unsuitable for clinic due to rapid washout of mouse spleen	[107]
			[^{18}F]11	No difference between ALS mouse model and wild-type	[110]
			[^{18}F]29	Rapidly metabolized in mice	[109]
COX-1	Microglia, neurons	No data on microglia phenotype expression	[^{11}C]KTP-Me	Unsuitable for further clinical evaluation due to rapid washout observed in healthy volunteers and MCI/AD patients	[125]
			[^{11}C]PS13	Specific uptake in non-human primates, no increase after LPS treatment, good test-retest reliability in healthy volunteers	[126,128,129,
			[^{18}F]PS13	Specific uptake in non-human primates	130
COX-2	Microglia, neurons	No data on microglia phenotype expression	[^{11}C]MC1	Increased uptake after LPS treatment in non-human primates, higher uptake in inflamed joints (and interestingly brain) of RA patients	[126,127]
			[^{11}C]TMI	Metabolically stable in non-human primates	[131]
			[^{11}C]MPbP	Metabolically stable in mice	[133]
			[^{18}F]1	Increased uptake in LPS-treated rats	[132]
CSF-1R	Microglia	No data on microglia phenotype expression	[^{11}C]AZ683	Low brain uptake in rats and non-human primates	[114]
			[^{11}C]CPPC	Increased uptake in LPS-treated, AD and EAE mice and LPS-treated non-human primates, *in vitro* data in human AD tissue warrants clinical evaluation	[115]
			[^{11}C]BLZ945	Efflux transporter substrate, unsuitable for clinical application	[122]
			[^{11}C]5	Does not cross BBB in rats	[123]

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BBB, blood–brain barrier; CB, cannabinoid receptor; COX, cyclooxygenase; CSF-1R, colony stimulating factor 1 receptor; EAE, experimental autoimmune encephalomyelitis; LPS, lipopolysaccharide; MS, multiple sclerosis; $P2X_7R$, purinergic 2X receptor type 7; $P2Y_{12}R$, purinergic 2Y receptor type 12; PD, Parkinson's disease; PET, positron emission tomography; RA, rheumatoid arthritis.

differences were detected between brain uptake in healthy volunteers and PD [85] or ALS patients [86].

In rats unilaterally treated with LPS, [^{18}F]JNJ-64413739 uptake was significantly higher in the ipsilateral hemisphere compared with the contralateral hemisphere, in *ex vivo* autoradiography and in *in vivo* PET imaging, but not in *in vitro* autoradiography, suggesting differences in binding mechanism between *in vitro* and *in vivo* experiments [87]. A 90-min dynamic PET acquisition in healthy humans showed limited bias, good test–retest variability and reliability [88], but no appropriate reference region could be determined both in human and non-human primates [88,89]. In addition, both [^{11}C]JNJ-54173717 and [^{18}F]JNJ-64413739 displayed high inter-individual signal variability, possibly due to a binding effect of a $P2X_7R$ polymorphism [85,88]; therefore, this needs to be further explored (Figure 1).

Purinergic 2Y receptor type 12 ($P2Y_{12}R$)

$P2Y_{12}R$ is a G-protein coupled receptor involved in chemotaxis. Following the increase in ATP and ADP concentrations post-brain injury, ADP acts as chemotactic signal that recruits microglia to sites of injury via activation of $P2Y_{12}R$ [90]. Receptor expression in the CNS is mainly restricted to microglia and $P2Y_{12}R$ is absent in peripheral macrophages, making it an ideal microglial marker [91]. $P2Y_{12}R$ is used as a homeostatic marker; however, *in vitro* studies also demonstrated significant up-regulation of $P2Y_{12}R$ expression in anti-inflammatory polarized microglia and down-regulation upon pro-inflammatory stimuli [25,92,93]. In rats, intracranial injection of recombinant IL-4 increased $P2Y_{12}R$ expression in microglia in the injected hemisphere compared to the contralateral side, and the expression co-localized with the anti-inflammatory marker CD206 [94]. In a model of parasitic brain infection known to induce a T helper type 2 (Th-2) response that promotes anti-inflammatory microglia polarization, $P2Y_{12}R$ expression was highly increased on CD163-positive microglia [92]. Conversely, $P2Y_{12}R$ expression is down-regulated in pro-inflammatory conditions. In MS, $P2Y_{12}R$ expression is absent in the active and chronic active lesions [14,91] and in AD a loss of $P2Y_{12}R$ expression is observed in microglia surrounding Aβ plaques and Tau tangles [95]. Thus, $P2Y_{12}R$ is a promising target for PET imaging of anti-inflammatory microglia. One prospective PET tracer ([^{11}C]5) has been reported, displaying decreased tracer binding in the affected brain regions in *in vitro* autoradiography experiments in post-mortem human and mouse brain tissue following stroke [94] and in brain tissue of MS patients and EAE rats [25]. In EAE rats, tracer binding increased in the recovery phase following the peak of clinical symptoms [25]. These results warrant the investigation of $P2Y_{12}R$ as a PET target for neuroprotective neuroinflammation, but unfortunately further preclinical evaluation of [^{11}C]5 was discontinued due to its inability to cross the BBB (Figure 2).

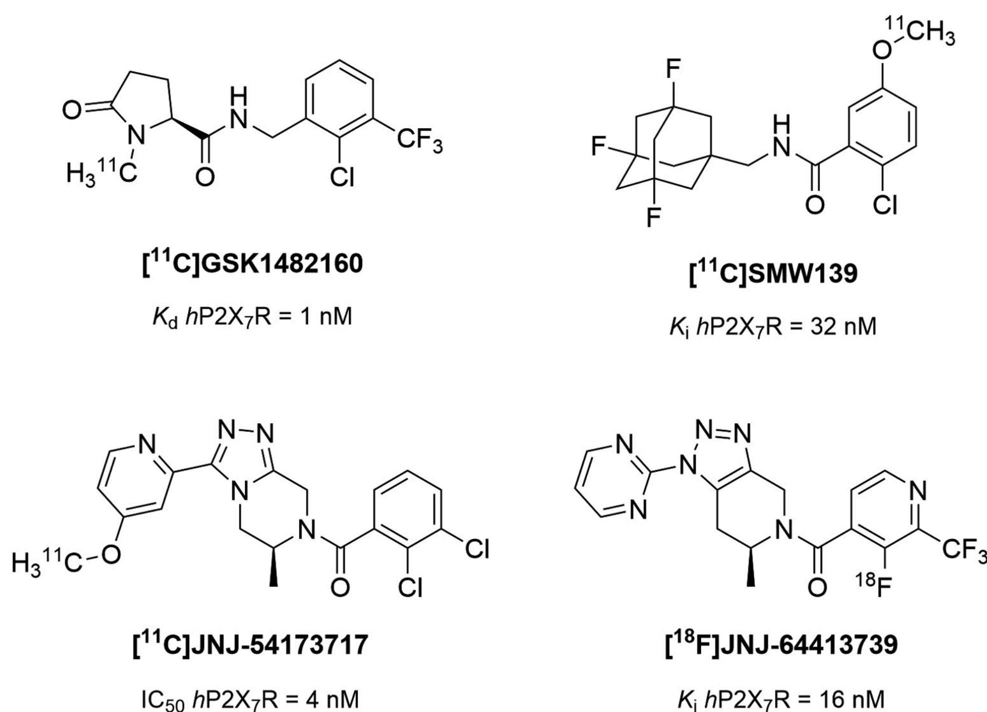

FIGURE 1 Positron emission tomography (PET) tracers for purinergic 2X receptor type 7 ($P2X_7R$)

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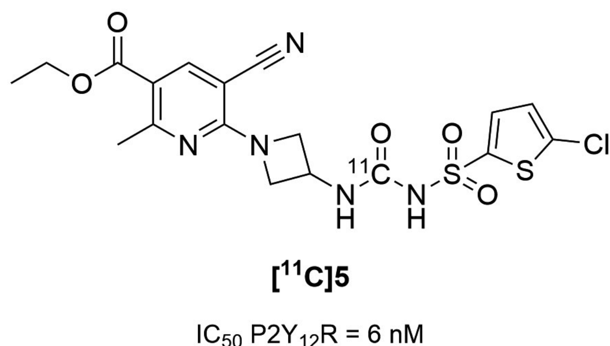

FIGURE 2 Prospective positron emission tomography (PET) tracer for purinergic 2Y receptor type 12 ($P2Y_{12}R$)

Cannabinoid receptor type 2 (CB2)

CB2 is mainly expressed in immune cells in the periphery, but in CNS under healthy conditions low expression is found in microglia and neurons [96,97]. Expression of CB2 on microglia significantly increases under neuroinflammatory conditions and in pathological conditions such as MS, AD and PD [98,99]. Limited data are available on the association of CB2 expression with microglia activation phenotypes. However, CB2 in microglia has been extensively investigated as a therapeutic target to help limit the inflammatory process. Activation of CB2 receptors by cannabinoids modulates microglial immune function by increasing their beneficial properties such as phagocytosis, as well as reducing their production of pro-inflammatory mediators such as TNF-α and free radicals (recently reviewed by Duffey *et al.* [100]).

As CB1 is constitutively expressed in CNS, PET tracers need to be highly selective for CB2 over CB1. The latest preclinical and clinical results in CB2 tracer development are reviewed here; for a complete overview the reader is referred to reviews by Ametamey and co-workers [101,102].

As a proof of principle for PET tracers dedicated to imaging of inflammation in CNS, LPS-induced neuroinflammation in rodents is a much-used tool. A number of CB2 targeting PET tracers have been evaluated in this model [103–108] with mixed results, the majority showing no significant increase in tracer uptake compared with control animals or non-specific binding that is too high to pick up on small differences in radioactivity read-out. A-836339 analogue [^{18}F]29 showed an increased uptake in brain of LPS-treated mice versus control mice, but its high metabolic rate (7% intact at 30 min p.i.) limits the applicability of this tracer [109].

[^{11}C]RS-028 showed significantly higher tracer binding in post-mortem spinal cord tissue of ALS patients versus healthy subjects, but was deemed unsuitable for *in vivo* purposes due to rapid washout in spleen (CB2-rich organ) of mice [107].

Diastereomeric mixture [^{18}F]11 was first tested *in vitro*, with similar levels of specific binding in control mouse brain (31%) compared with SOD1 mouse brain (ALS model), but the pattern of binding differed between the two [110]. *In vivo* PET imaging also showed no significant differences in time-activity curves between animals, unless using a Logan plot with the left heart ventricle as a reference region, showing slightly higher values for symptomatic versus asymptomatic mice only [110].

[^{11}C]NE40 is to date the only CB2 targeting tracer investigated in a clinical setting, showing rapid brain uptake and washout in healthy volunteers [111]. Brain uptake of [^{11}C]NE40 was higher in healthy volunteers than in AD patients [112], which can probably be attributed to neuronal loss in late-stage AD (Figure 3).

Colony stimulating factor 1 receptor (CSF-1R)

Colony stimulating factor 1 receptor (CSF-1R) is a cell surface, class III receptor tyrosine kinase mainly expressed by microglia in the brain, but expression has also been found on cells of the neuronal lineage [113]. Endogenous ligands of CSF-1R are cytokines CSF-1 and IL-34. CSF-1R is directly involved in controlling development, survival and maintenance of microglia [114,115]. In transgenic AD mice, high CSF-1R expression is found in close proximity to Aβ aggregates [116,117]. More recently, plaque-associated microglia were found to be more resistant to microglia depletion by CSF-1R inhibition than non-plaque-associated microglia in a mouse model displaying both Aβ and tau pathology [118]. In MS lesions, CSF-1R expression was lower than in NAWM and in EAE mice, CSF-1R inhibitors reduced disease severity [113]. Activation of microglia through CSF-1R was recently shown to induce demyelination [119]. In line with this, increased levels of CSF-1 in EAE mice correlated with microglial activation and neuronal loss [120]. In human brain tissue of MS patients, CSF-1R was only modestly increased in relapse–remitting MS but significantly elevated in progressive MS compared to NAWM from non-MS patients [121]. In addition, increased CSF-1 could be detected in cerebrospinal fluid of progressive MS patients [121].

Four PET tracers targeting CSF-1R have been evaluated *in vivo* to date (Figure 4). [^{11}C]BLZ945 was evaluated in healthy mice but showed a substantial level of non-specific binding in brain, both *in vitro* and *in vivo* [122]. In addition, low brain uptake due to strong efflux transporter substrate behavior was demonstrated, rendering [^{11}C]BLZ945 unsuitable for clinical application. [^{11}C]5 showed high specific binding *in vitro*, but selectivity for CSF-1R was limited [123]. In healthy rats, [^{11}C]5 did not cross the

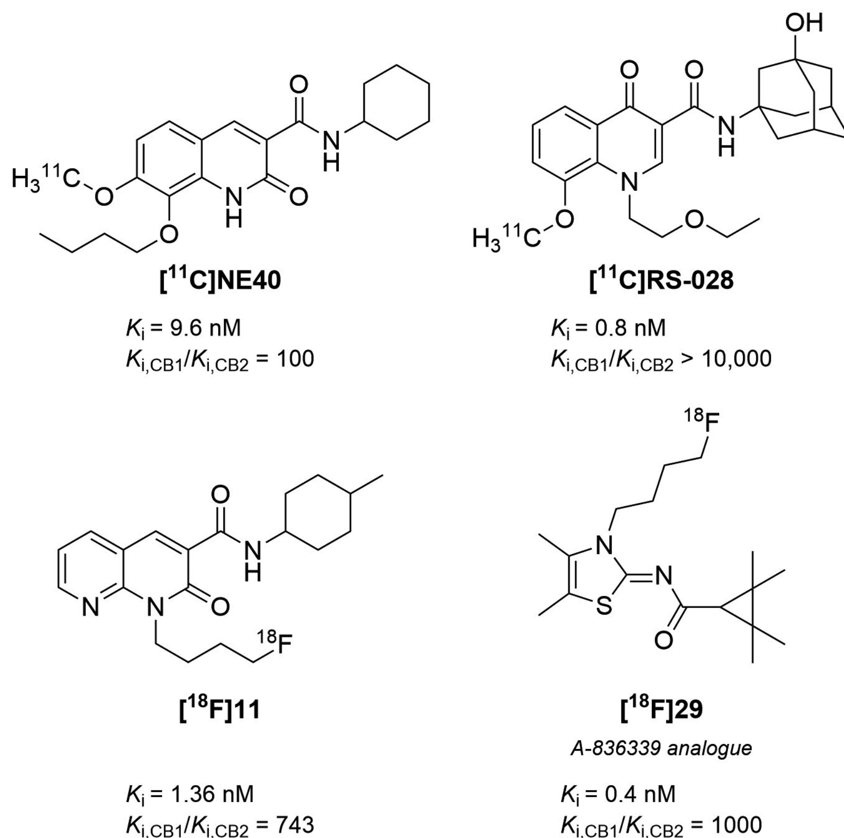

FIGURE 3 Positron emission tomography (PET) tracers for cannabinoid receptor type 2 (CB2) receptor

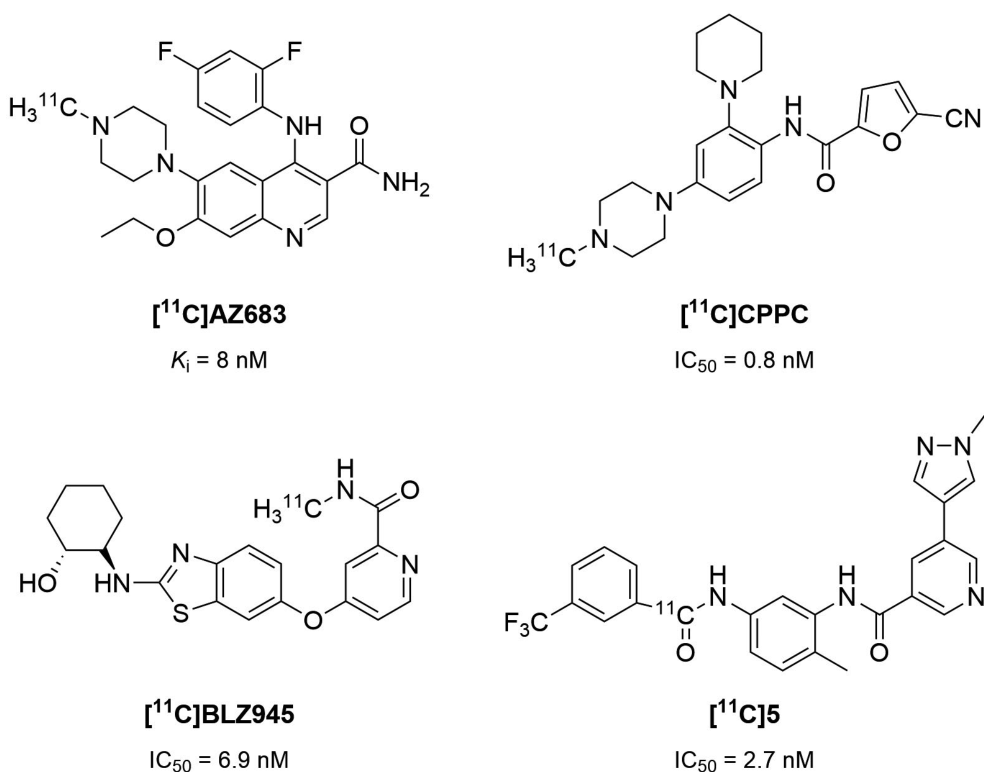

FIGURE 4 Positron emission tomography (PET) tracers for colony stimulating factor 1 receptor (CSF)-1R

BBB, even when efflux transporters were inhibited, and thus [^{11}C]5 is unfit for use in neuroimaging. [^{11}C]AZ683 showed low brain uptake and retention in both rats and non-human primates [114], but [^{11}C]CPPC showed increased tracer uptake in mouse and baboon brains after LPS-induced neuroinflammation [115]. Transgenic AD and EAE mice also showed an increase in PET signal, which correlated with disease severity in the EAE mice. [^{11}C]CPPC binding was highly increased (75–99%) in postmortem brain of AD patients compared with healthy control brain in *in vitro* autoradiography studies [115]. The increasingly evident role of CSF-1R in microglial activation and the first promising results in PET tracer development warrant further investigation of CSF-1R as a PET target for neuroinflammation imaging.

Cyclooxygenases

Cyclooxygenase (COX) enzymes are involved in activation of inflammatory pathways leading to prostaglandin production and chemokine, cytokine and ROS release [124]. Two known isoforms, COX-1 and COX-2, are both expressed in brain, although cell type localization and regulatory function differ. While COX-1 is constitutively expressed in CNS, COX-2 expression increases in inflammatory conditions. To accurately assess the role of both COX-1 and COX-2 in neuroinflammation by PET imaging, tracers targeting each isoform need to be highly selective over the other. In an attempt to develop a CNS-applicable radiotracer for intracellular target COX-1, a pro-drug of COX-1 inhibitor ketoprofen (ketoprofen methyl ester) was labeled with carbon-11 ([^{11}C]KTP-Me) and evaluated in healthy volunteers and MCI/AD patients [125]. Initial brain uptake was followed by a very rapid washout in all groups, probably due to hydrolysis of the ester moiety, and [^{11}C]KTP-Me is therefore rendered unsuitable as PET tracer. Recently, two isoform selective tracers, [^{11}C]PS13 (COX-1) and [^{11}C]MC1 (COX-2), were studied in a head-to-head comparison in which [^{11}C]PS13 showed high specific uptake in non-human primates, while uptake of [^{11}C]MC1 was mainly non-specific at baseline conditions [126–128]. However, scans acquired post-LPS treatment revealed significant up-regulation of COX-2 but not COX-1 [126,127]. Nevertheless, [^{11}C]PS13 was evaluated in healthy humans, showing good test–retest reliability, and thus it would be interesting to investigate binding differences between healthy subjects and, for example, AD patients [129]. To provide a radiotracer with a longer half-life, a [^{18}F]PS13 was developed, showing similar promising results in non-human primates, however, optimization of the radiolabeling procedure is required to obtain [^{18}F]PS13 with sufficient molar activity [130]. [^{11}C]MC1 was evaluated in two healthy volunteers and two patients with rheumatoid arthritis as a proof of principle, and notably higher binding in inflamed joints was observed in patients versus controls [127]. Unexpectedly, [^{11}C]MC1 uptake in brain of rheumatoid arthritis patients could be blocked by celecoxib, indicating a potential central response to peripheral inflammation [127]. As celecoxib is a well-known COX-2 inhibitor, but subject to rapid metabolism, its scaffold has been explored to improve metabolic stability, as well as selectivity over COX-1. Isoxazole derivative [^{11}C]TMI showed strongly enhanced metabolic stability in non-human primates, with a significant amount of radiometabolites (22% at 90 min p.i.) only observed in a blocking study using meloxicam [131]. Fluorinated derivative [^{18}F]1 showed high brain uptake and excellent metabolic stability in mice [132], but could only be obtained in low yields and low molar activity, hampering further evaluation of this tracer. Exploring a different scaffold, [^{11}C]MPbP showed increased uptake in brains of LPS-treated rats compared with non-treated rats; however, selectivity of this tracer over COX-1 is limited [133] (Figure 5).

Summarizing, COX-2 seems to be a legitimate target for PET imaging of neuroinflammation, whereas for COX-1 this remains unclear.

Therapeutic perspectives of modulating microglia phenotypes

The increased understanding of microglia phenotypes and their contribution to disease development and progression offers novel approaches for therapeutic intervention. As anti-inflammatory microglia promote regeneration and repair mechanisms, strategies aiming at modulating microglia into the anti-inflammatory phenotype has gained momentum in recent years. In animals, brain delivery of anti-inflammatory microglia inducers such as IL-4, IL-10 and IL-13 have been shown to be effective in reducing the clinical symptoms and the demyelination in the EAE model [134]. Similarly, intracranial delivery of IL-4 reduced the Aβ plaque load and improved cognition in the APP23 AD mice [134]. Although the results with these agents seem promising, the translation into human remains a challenge, as most of the cytokines do not cross the BBB. Extensive effort has been made to target other immunomodulators, such as the peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ is a transcription factor that has long been known to modulate microglia phenotype. PPARγ agonists are able to induce the anti-inflammatory microglia phenotype and have been shown to facilitate the clearance of Aβ in AD models and to exhibit a protective function against striatal dopamine depletion and restore cognitive function in the MPTP model of PD [135]. However, in a Phase III double-blind

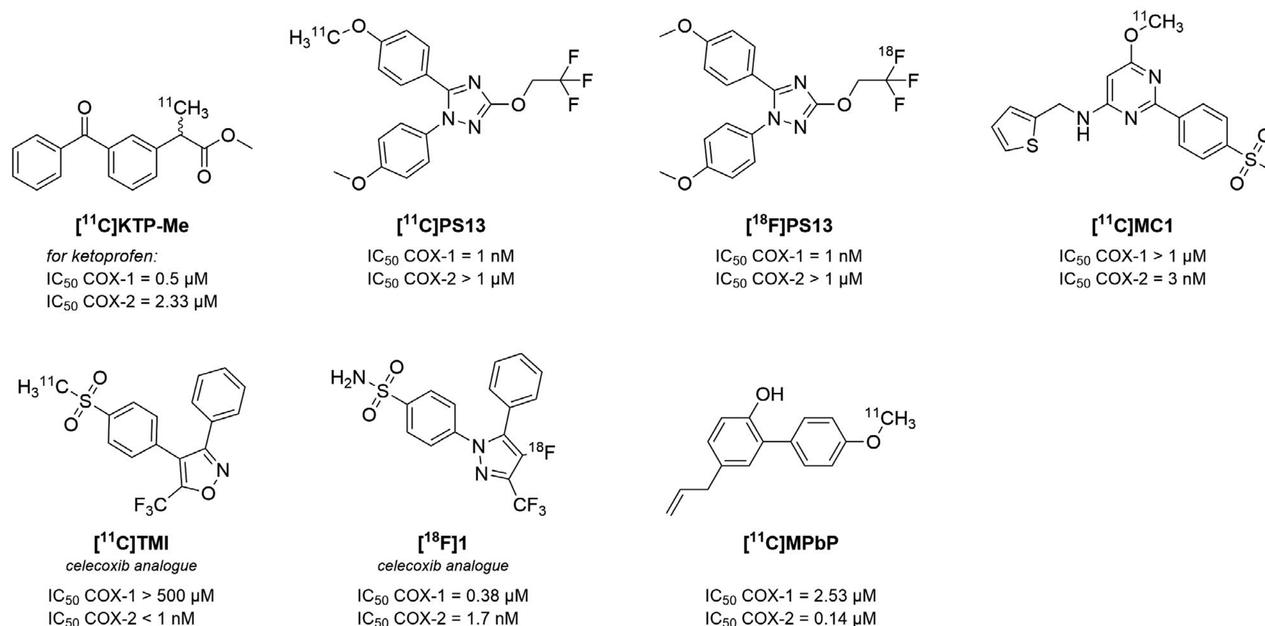

FIGURE 5 Positron emission tomography (PET) tracers for cyclooxygenase (COX)-1 and COX-2

randomized placebo-controlled study in AD patients, PPARγ agonist rosiglitazone showed no efficacy nor improvement in cognition [136]. This was probably due to the poor BBB permeability of the compound, which raises uncertainties as to whether target occupancy was sufficient. Recently, a Phase IIa study with a new PPARγ/δ agonist T3D-959 evaluated brain pharmacology of the drug by monitoring brain metabolism with [^{18}F]FDG-PET [137]. They were able to show that T3D-959 alters glucose metabolism in the brain in a dose-dependent manner. The study also provided an indication of improving cognition and higher executive function in mild-to-moderate AD supporting further clinical investigation [137]. Minocycline is a brain-penetrating antibiotic that was shown to have an anti-inflammatory effect *in vitro* and in animal models via reduction of inflammatory mediators such as IL-1β, IL-6, COX-2 and iNOS [138]. A randomized controlled trial in MS patients showed that the risk of conversion from a clinically isolated syndrome to MS was significantly lower with minocycline than with placebo over 6 months [139]. Conversely, in a clinical study with AD patients, minocycline at a dose of 200 and 400 mg failed to delay the progress of cognitive or functional impairment in people with mild AD during a 2-year period [Minocycline in Alzheimer's Disease Efficacy (MADE) trial] [140]. Recently, more attention has been directed to the immunomodulatory characteristics of the sphingosine-1-phosphate receptor modulator fingolimod (FTY720), an immunosuppressor that is clinically approved for MS. In AD and stroke animal models, fingolimod was able to shift microglia into an anti-inflammatory phenotype, which encourages further investigation [141,142].

Although the modulation of microglia is a promising approach, successful clinical translation has been challenging. Our understanding of the dynamics of microglia activation and resulting phenotypes in the different neuroinflammatory and neurodegenerative diseases is still incomplete. Translation from animal models is also not straightforward as doses used to effectively modulate neuroinflammation in animals may not be tolerated in humans, and lower doses would not provide the wanted neuroprotective effect. Furthermore, the timing for modulation of pro- and anti-inflammatory microglia is critical and needs to be tailored to the different diseases. Altogether, this stresses the need for reliable *in vivo* measurement methods that could enable the assessment of microglia phenotypes and their dynamics, define the therapeutic window and monitor the therapeutic efficacy to guide the clinical trial design.

DISCUSSION AND CONCLUSION

PET imaging is particularly well suited to quantitatively evaluate and discriminate the different microglia phenotypes, assuming that a selective target is identified and validated and proper radiotracers become available. Significant efforts have been made to go beyond TSPO PET and identify imaging biomarkers specific for microglia activation phenotypes. This proved not to be trivial, and only a couple of targets have been associated with pro- or anti-inflammatory microglia phenotypes. This can be attributed to several limitations; (i) the strategies used to identify differential phenotypical markers mainly rely on *in vitro* polarized microglia that may not capture the complexity of the stimulus in a pathological environment;

(ii) the lack of reliable antibody tools, which limits the validation of the identified biomarkers; and (iii) the limited availability and accessibility of human post-mortem tissues that are crucial for exploration and validation of the biomarkers in different pathologies.

Among the emerging targets, $P2X_7R$ and $P2Y_{12}R$ stand out as more specific for pro- and anti-inflammatory microglia, respectively. Several $P2X_7R$ tracers have been developed and evaluated in neuroinflammatory and neurodegenerative diseases but results were not consistent in showing clear differences between healthy control and disease state. In the case of $P2Y_{12}R$, the lack of a BBB penetrable tracer makes it difficult to assess its value. The remaining targets are less well characterized with respect to association with microglia phenotypes. Additionally, in the case of CB2, its neuronal expression may hamper imaging interpretation in neurodegenerative diseases. It is unlikely that a single imaging marker would be able to discern the inflammatory environment, and a combination of two or three markers would be needed. One could imagine combining $P2X_7R$ with $P2Y_{12}R$ and evaluating their relative dynamics as an indication of microglia activation status at different time-points during disease progression or treatment. This could be applied, for example, in the evaluation of disease-modifying therapies in longitudinal studies. A decrease of $P2X_7R$ tracer uptake that coincides with an increase of $P2Y_{12}R$ uptake could be indicative of a shift in microglia towards an anti-inflammatory phenotype. In addition to the targets described here, several other markers are being explored and more will emerge from mining microglia transcriptomic and snRNA sequencing studies. This stresses the importance to define basic characteristics for a PET target, which ideally should be (i) specifically expressed on microglia and minimal expression on other brain cells; (ii) localized on the cell membrane; (iii) differentially up-regulated on activated microglia in pro- or anti-inflammatory conditions; (iv) low homeostatic levels of expression; and (v) low level of polymorphism.

Additionally, radiotracer development for PET neuroimaging is a challenging task. An important criterion for a candidate PET tracer is its ability to cross the BBB. In the case of $P2Y_{12}R$, this has been a bottleneck for the development of a candidate PET tracer. While the developed tracer displayed favorable *in vitro* binding properties, it failed to show BBB permeability and brain uptake. To address this problem, new computational approaches can potentially be used to predict the BBB permeability of compound libraries and their affinity to efflux transporters. This will help in identifying the most suitable candidate compounds for translation into PET tracers and increase their chances of success in *in vivo* evaluation. Other downfalls that limit clinical translation of tracers are related to their non-specific uptake in the brain and the presence of radioactive metabolites that cross the BBB. This can present serious complications for accurate quantification of tracer uptake and the development of kinetic modeling approaches. It is also crucial for PET radiotracers to not be affected by the target polymorphism which was a hurdle for the TSPO PET tracer development.

It is now apparent that the recent advances in transcriptomic and snRNA sequencing have shone light upon the complexity of the microglia phenotypes in different pathologies and have taken it beyond the limits of the pro- and anti-inflammatory dichotomy. Conversely, they also provided a powerful tool to identify specific targets that can be used to image these subsets. Thus, there is a call to action to identify and validate biomarkers that better capture these complex and dynamic microglia activation phenotypes in order to accurately investigate their role in disease progression and monitor therapeutic response. It is also crucial that imaging biomarkers are extensively validated and shown to provide predictive value for clinical or therapeutic outcome before investing time and resources in developing radiotracers.

CONFLICTS OF INTEREST

A.D.W. is editor-in-chief of *Nuclear Medicine and Biology*; the other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

W.B. designed the manuscript and together with B.J. equally contributed to the writing. D.J.V., H.E.deV. and A.D.W. critically reviewed the manuscript.

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