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REVIEW

Applications of radiolabeled antibodies in neuroscience and neuro-oncology

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Positron emission tomography (PET) is a powerful tool in medicine and drug development, allowing for non-invasive imaging and quantitation of biological processes in live organisms. Targets are often probed with small molecules, but antibody-based PET is expanding because of many benefits, including ease of design of new antibodies toward targets, as well as the very strong affinities that can be expected. Application of antibodies to PET imaging of targets in the central nervous system (CNS) is a particularly nascent field, but one with tremendous potential. In this review, we discuss the growth of PET in imaging of CNS targets, present the promises and progress in antibody-based CNS PET, explore challenges faced by the field, and discuss questions that this promising approach will need to answer moving forward for imaging and perhaps even radiotherapy.

KEYWORDS
immunotherapy, molecular imaging, neuro-oncology, neuroscience, radiolabeled antibodies, radiotherapy

1 | INTRODUCTION

Brain positron emission tomography (PET) imaging has become an invaluable tool in both academic medicine[1] and drug discovery,[2] with notable applications in neuro-oncology[3,4] and neuroscience.[5,6] In academic medical centers, PET is widely used in clinical care and research applications, where it can aid in early diagnosis of disease, confirming eligibility for a given therapy, and monitoring a patient to ensure appropriate response to said therapy. The latter two concepts have taken on significant importance in oncology as they have been combined to create the concept of "theranostics," where the same drug can be labeled with both a diagnostic and a therapeutic radionuclide. The diagnostic (e.g., ^{18}F and ^{68}Ga) scan reveals target expression in a patient's tumor and confirms eligibility for subsequent treatment with the corresponding alpha (e.g., ^{225}Ac, ^{211}At, ^{213}Bi, and ^{212}Pb) or beta (e.g., ^{90}Y and ^{177}Lu) therapeutic. Although the most widespread application of theranostics to date has involved small molecules or peptides in the treatment of neuroendocrine tumors[7–9] and prostate cancer,[10–12] there is considerable interest from neuro-oncologists to also use them to treat brain tumors.[13,14]

Meanwhile, brain PET has played an integral part in the development of modern neuroscience. In the 1970s, [^{18}F]fluorodeoxyglucose (FDG) was originally employed to investigate glucose metabolism in the brain,[15] whereas its potential in oncology only began to emerge in the late 1980s and early 1990s.[16] Successful brain imaging with FDG in the late 1970s essentially began the field of brain PET, and extensive research aimed at developing radiopharmaceuticals for imaging central nervous system (CNS) disorders has been undertaken in the intervening

50 years.[17] Notable milestones include the first use of imaging to quantify a receptor in Eckelman's pioneering studies quantifying muscarinic acetylcholine receptors in Alzheimer's disease using [^{123}I]3-quinuclidinyl-4-iodobenzilate,[18] imaging of dopamine receptors using 3-*N*-[^{11}C]methylspiperone,[19] introduction of [^{11}C]deprenyl for imaging of monoamine oxidase,[20] determination of the distribution of dopamine in the basal ganglia using [^{18}F]6-fluoro-DOPA,[21] and measurement of cerebral blood flow with both [^{15}O]water and [^{11}C]butanol.[22] The 1980s and 1990s thus saw the introduction of radiotracers for imaging a whole host of neurological targets, including subtypes of receptors (e.g., opioid, dopamine, and both muscarinic and nicotinic acetylcholine), transporters (e.g., DAT, VMAT2, SERT, and VAChT), and enzymes (e.g., MAO and acetylcholinesterase). In 2004, Mathis and Klunk reported the use of [^{11}C]Pittsburgh compound B (PiB) for imaging amyloid plaques in dementia patients.[23] This work not only jumpstarted a search for imaging agents for other misfolded proteins implicated in neurodegenerative disorders, such as tau,[24] but also led to the widespread use of PET imaging as a tool in dementia research.

With all of these tools available, it is unsurprising that the utilization of PET to support drug discovery has grown considerably in the last 10–20 years (for a recent review, see Donnelly[2]). The 5R framework in the pharmaceutical industry represents an overarching goal in drug discovery. There are various iterations of the concept, but the overall idea is to get the "right drug" at the "right dose" via the "right route" to the "right patient" at the "right time," and molecular imaging has proven a critical tool in realizing the 5Rs. Hargreaves was a pioneer in this area, using [^{18}F]SPA-RQ to inform dosing of Emend (aprepitant), a neurokinin 1 receptor antagonist being developed by Merck for treatment of depression and chemotherapy-induced nausea, but also to make key go/no-go decisions about advancing the drug for both indications.[25] The establishment of amyloid plaques and tau aggregates as surrogate biomarkers of dementia and the maturation of amyloid and tau PET into sophisticated imaging procedures have built on this concept and, together, afforded a paradigm shift in the development of CNS drugs. Both amyloid and tau PET are now used routinely for dementia clinical trial enrichment, confirming target expression and eligibility for a given therapeutic trial (e.g., aducanumab) as well as to monitor subsequent patient response to the experimental therapy.[26] Lastly, drug hunters can also prepare radiolabeled versions of their therapeutic candidates to obtain information about the drug (e.g., target engagement, sites of off-target binding, receptor occupancy, and pharmacokinetics) that can be fed back/fed forward in the discovery pipeline so that the best candidates are being advanced.[2]

The fields of nuclear medicine and the radiopharmaceutical sciences therefore continue to push the boundaries of brain PET use in both neuro-oncology and neuroscience, both in terms of academic pursuits, such as elucidating disease mechanisms, and to support drug hunters who are using imaging to achieve the 5Rs for new therapeutics in development. Continuous innovation of this sort demands access to a cutting-edge pipeline of diagnostic (and therapeutic) radiopharmaceuticals, but at the frontline of radiopharmaceutical research, certain key challenges have emerged in the development of new small molecule agents:

- Newly discovered targets frequently have no known small molecule ligands to use as a starting point in radiopharmaceutical development (e.g., α-synuclein, transactive response DNA binding protein of 43 kDa [TDP-43]);
- High throughput screening (HTS) campaigns to identify potential hits for the development of theranostics around new targets are time consuming, costly, and often difficult to fund in an academic setting;
- It can be challenging to get selectivity between similar targets using small molecules (e.g., amyloid-β and α-synuclein);
- Some emerging therapeutic strategies inherently involve non-small molecule modalities (e.g., immunotherapies such as CAR T cell therapy and biologics);
- Developing theranostics is possible using small molecules (e.g., ^{18}F and ^{211}At) but is likely easier when using a larger molecule decorated with a chelating group compatible with both diagnostic and therapeutic radioactive metal ions.

For all of these reasons, in recent years, there has been considerable excitement around the introduction of radiolabeled antibodies as a platform for the rapid development of both diagnostic and therapeutic radiopharmaceuticals. Antibodies offer several key advantages to address the issues outlined above:

- Antibodies can be rapidly generated for newly discovered targets, faster than small molecules can be identified in HTS and optimized in medicinal chemistry structure–activity relationship (SAR) campaigns;
- The superior targeting specificity of monoclonal antibodies offers opportunity for gaining selectivity over structurally similar targets that have proven difficult to resolve with small molecules;
- Antibody imaging and therapy are inherently poised for translation into other antibody-based techniques/therapies;

- Antibodies are readily functionalized with chelating groups (e.g., DOTA and DFO), making them compatible with a large range of longer-lived diagnostic (e.g., ^{64}Cu, ^{89}Zr, and ^{203}Pb) and therapeutic (e.g., ^{225}Ac, ^{211}At, ^{213}Bi, ^{177}Lu, and ^{212}Pb) radionuclides[27] or, through pre-targeting approaches, with ^{18}F and ^{211}At.[28]

Given all of this, radiolabeled antibodies are being investigated for imaging and therapy[29–31] and, most recently, their use has also begun expanding to explicitly include theranostics.[32–34]

Uses of antibodies for PET imaging and immunotherapy have primarily focused on oncology, and numerous antibody-based radiopharmaceuticals have been successfully developed for a broad spectrum of targets expressed by tumors, metastases, and/or malignant cells (for recent reviews, see Wei et al.[35] and Manafi-Farid et al.[36]). There is also enormous interest in antibody-based radiopharmaceuticals in neuroscience and neuro-oncology applications.[37] However, CNS applications of radiolabeled antibodies present certain unique challenges that must be overcome:

- Antibodies do not typically penetrate into the CNS;
- Sufficient antibody concentrations must be reached in the brain in a timeframe compatible with radionuclide half-life for effective imaging and/or treatment.

In spite of these challenges, certain radiolabeled antibodies have been shown to enter the CNS,[38] and access to the CNS can be enhanced through a damaged blood-brain barrier (BBB) or via engineered approaches such as chemical opening of the BBB or receptor-mediated transport. In this review, we discuss the work of many groups that are actively developing antibody-based agents for brain imaging. We highlight strategies for engineering radiolabeled antibodies specifically for CNS applications, survey the current state of the art in use of radiolabeled antibodies for neuroimaging and CNS therapeutic applications, and consider remaining challenges, opportunities, and questions for PET with antibodies in neurological applications. This review article complements other recent reviews of the subject, such as a recent one from de Lucas et al. at the Turku PET Centre.[39]

2 | PROMISES AND PROGRESS

Antibodies present a powerful solution to many of the shortcomings of small molecule targeting agents. Perhaps the greatest advantage antibodies pose is the relative ease of attuning them to specific parts of a target antigen and/or specific forms/modifications of their antigens. This tunability is hugely advantageous for difficult protein targets, such as TDP-43, the aggregates of which have been implicated in a slew of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), the TDP-43 variant of frontotemporal lobar degeneration (FTLD-TDP), limbic-predominant age-related TDP-43 encephalopathy (LATE), and Alzheimer's disease.[40] Not only do many antibodies exist against the protein, but, more importantly, there are antibodies that purposely target different epitopes or modified versions of the protein,[41] including

- domains that are present in all forms of TDP-43;
- only the second RNA recognition motif (RRM2)[42];
- the N-terminus of the protein[43];
- the C-terminus of the protein[43];
- the C-terminus but only when phosphorylated at both serines 409 and 410.[44]

The specificity for particular domains of a protein and/or specifically modified forms of proteins is the critical feature that makes antibodies ideally suited for many CNS targets, especially protein aggregate targets. In the case of TDP-43, the ubiquitous, typically nuclear protein is too abundant in healthy cells for its imaging to likely be informative about specific disease states. At the same time, there is huge potential in imaging of TDP-43 that is mislocalized in the cytoplasm (where RRM2 is not bound to RNA)[45] and/or phosphorylated, as these forms of the protein are implicated in TDP-43 pathologies,[44,46,47] and selective binding only to these disease-relevant forms of the protein is likely necessary for useful TDP-43 imaging.

Another important target for brain PET imaging is amyloid. As noted above, PET imaging of amyloid plaques is well developed, and there are three FDA-approved small molecule radiotracers (Amyvid, Neraceq, and Vizamyl) as well as investigational agents in advanced clinical trials (e.g., [^{18}F]flutafuranol), all of which have been widely used in large multicenter studies (e.g., Alzheimer's Disease Neuroimaging Initiative) and drug trials. However, with clinical evidence that soluble amyloid-β protofibrils appear earlier in the development of Alzheimer's than plaques—perhaps early enough to allow for preventative or even restorative treatments—there is interest in selectively imaging these soluble plaque-precursors. To date, this has proven challenging with small molecules,[48] but antibodies against protofibrils have been generated, which show selectivity for this form over low-molecular weight polymers as well as fragments of the protein, monomeric amyloid-β, and also aggregate plaques of the protein.[49,50] Subsequently, these antibodies have been radiolabeled and used to probe

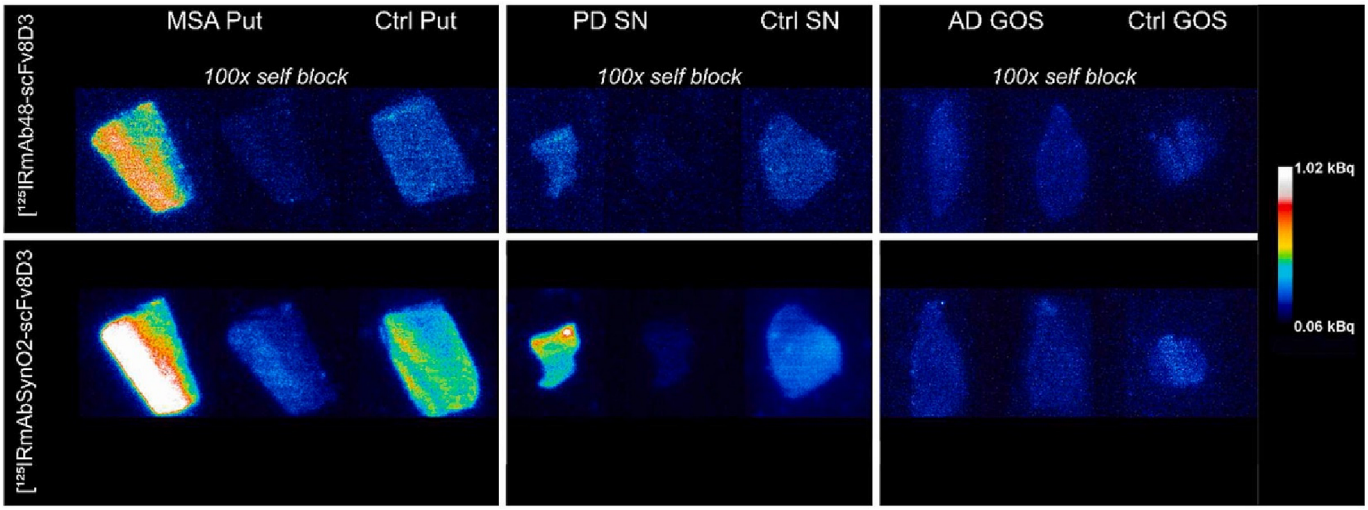

FIGURE 1 Autoradiography of two α-synuclein-targeting antibodies ([^{125}I]RmAb48-scFv8D3 and [^{125}I]RmAbSynO2-scFv8D3) on brain tissue samples of human multiple system atrophy (MSA) putamen (Put) and Parkinson's disease (PD) substantia nigra (SN), both of which contain aggregated α-synuclein, and Alzheimer's disease (AD) superior occipital gyrus (GOS), which contains amyloid-β pathology but not α-synuclein. In the cases of MSA and PD, signal was blocked by a 100-fold concentration application of unlabeled antibody, but the signal is unaffected in the AD tissue, demonstrating that the antibody does not bind to amyloid-β. Within each set of three images, the leftmost are the diseased tissues with radiolabeled antibody, the middle are the same tissues co-incubated with 100× self-blocking dose, and the rightmost are the corresponding tissues from non-diseased individuals. Reproduced from Roshanbin et al.[58] under the terms of a Creative Commons CC-BY license.

amyloid-β protofibrils in the brains of live mice.[50] In this way, many protein aggregate targets in the brain could benefit from (or may require) targeting of just one specific form of the protein. Moreover, if quantitation of pre-aggregate proteins in the CNS turns out to enable better diagnosis, stratification, and confirmation of target engagement for therapies, antibody frameworks may prove to be a necessary tool for the job.

Besides distinguishing modified forms of a protein from each other, in some instances, it has also proven very challenging to distinguish between structurally-related proteins using small molecule tracers, as with amyloid-β, tau, and α-synuclein.[51] A large part of this challenge stems from the small molecule agents' mode of binding: many of these agents bind to *structural motifs* such as β-pleated sheets[52] or paired helical filaments[53] rather than to a specific binding site (e.g., an amino acid-defined pocket in an enzyme/receptor). Thus, within a "motif recognition" model, large off-target binding components are inherent, and perhaps only moderate gains can be expected even with refinement. For example, there were early challenges in designing small molecule radiotracers for tau because of off-target binding to monoamine oxidase,[54,55] and efforts are ongoing to develop radiotracers that are selective for two of the six major isoforms: 3R and 4R tau.[56,57] Development of imaging agents that are selective for α-synuclein over other misfolded proteins is also an ongoing challenge, as noted above.[51] Because, to the best of our knowledge, there are no selective α-synuclein radioligands that do not also show affinity to amyloid, radiolabeled antibodies with this selectivity have been developed.[58] The radiolabeled antibodies are very promising in this case, selectively binding α-synuclein and penetrating into the CNS (Figure 1), but they cannot detect the protein in mouse models of Parkinson's disease, likely because of the intracellular location of the protein target.

Another benefit of antibody-based radiopharmaceuticals is their very tight binding to antigens, regularly in the tens of picomolar range.[59] In order to get sufficient signal, the K_d of a drug often needs to be less than 20% of the target's B_{max}. This poses a problem for imaging very low expression targets, but antibodies, with their tunability and high affinities, may be able to provide a solution. Taken together, all of these advantages have the potential to be of critical importance for non-aggregate targets in the CNS as well, such as transporters, receptors, and enzymes. For example, dopamine receptors of the D_2 and D_3 subtypes have high sequence similarity, but D_3 receptors possess a higher affinity for dopamine (~400×) and, unlike D_2 receptors, small changes in the number or function of D_3 can have dramatic effects on synaptic transmission.[60] Thus, there have been innumerable efforts into creating small molecules that are BBB penetrant and selective for one over the other. Current PET agents used clinically, such as [^{11}C]raclopride and [^{11}C]

PHNO, bind both receptors, hampering efforts to study levels of either individually.[61] However, antibodies selective for D_2 and D_3 have been known since at least the 1990s[62,63] and offer another potential approach for developing subtype selective imaging agents. To the best of our knowledge, however, radiolabeled antibodies have yet to be employed as dopamine receptor subtype-selective imaging agents.

Turning our attention to neuroinflammation, a similar problem exists in the imaging of the 18-kDa translocator protein (TSPO), an important target for understanding the role of neuroinflammation in various neurological conditions and diseases. The single nucleotide polymorphism in the gene for TSPO leads to differences in amino acid 147 of the protein and to a significant spread in binding affinities of TSPO ligands, resulting in to three classes of "binders" (high, medium, and low) and posing difficulties in translation of TSPO PET agents into routine clinical use. Here, too, could antibodies be utilized for a universal TSPO ligand by targeting an epitope distal from residue 147, leading to strong binding across the spectrum. This is the focus of some promising small molecule research as well[64]—to bind a disparate region from that which is affected by the single amino acid change—so it is not a unique avenue for antibody-based work; however, antibody generation against particular, pre-defined epitopes is routine and therefore a low-risk approach for alternate binding site/epitope radiopharmaceuticals. With TSPO, D_2/D_3, and other targets where intentional selection of binding sites could be a boon, antibodies pose a powerful tool for the rapid development of the desired agents. Translation of these antibodies into PET agents may be a more effective and expeditious path forward than efforts with small molecule agents.

In the growing field of theranostics, compounds labeled with radioactive metal ions ("radiometals") have proven to be quite advantageous because of the relative ease of switching radionuclides. With radiometals, many labeling procedures are similar between the diagnostic and therapeutic ions because of some generalizable coordination chemistry principles, and the speed and simplicity of adapting methods offer a huge advantage over some non-metal labeling strategies. There are theranostic pairs that provide for a seamless transition, like $^{64/67}$Cu, but even some pairs with quite dissimilar coordination chemistries have nonetheless been successfully partnered, such as diagnostic ^{68}Ga paired with therapeutic ^{177}Lu. An advantage of antibodies for CNS theranostics, then, is their amenability to labeling with radiometals. There is great diversity and flexibility in where, how, and with how many radiometals an antibody can be labeled, and antibodies' large sizes typically result in minimal disruption of binding properties or kinetics from the introduction of a chelating group and/or radiolabeling.

Part of using antibodies for CNS PET and/or radiotherapy is accounting for their typically slower pharmacokinetics (PK), though "bad" PK for therapeutics often equates to "good" PK for PET imaging and vice versa. Therapeutic uses of antibodies typically benefit from the long biological half-lives of antibodies, but the slow clearance from the blood pool is a problem for PET imaging as it is associated with high background signal; the slow uptake into the CNS may be acceptable for therapeutic uses, but PET scans with antibodies are hindered by this aspect of their pharmacokinetics because of the short-lived radionuclides typically employed for PET (Table 1). For example, in mice, brain levels of human IgG took about 24 h to peak,[71] and PET scans with radiolabeled antibodies are typically conducted around 72 h post-injection.[58] Given this slower uptake than most small molecules, some radioisotopes are inherently off-limits, such as ^{11}C with its 20 min half-life. It is important that the physical half-life of the radionuclide is matched with the biological half-life of the antibody as this ensures that the signal from radioactive decay is still at useful levels even after the time needed for delivery to and wash-out from the target tissue (Table 1). This typically means that, for antibodies ($t_{1/2}$ ~11–30 days for an IgG format[72,73]; shorter for a Fab format, e.g., 2–6 h[74]) that are radiolabeled prior to administration, longer-lived isotopes such as ^{124}I ($t_{1/2}$ = 4.2 days), ^{64}Cu ($t_{1/2}$ = 12.7 h), or ^{89}Zr ($t_{1/2}$ = 3.3 days) are employed for PET imaging.[75] ^{124}I poses some potential advantages, including rapid (<5 min reaction times) and direct radiolabeling of antibodies, without the need for prior modification to install a metal chelating group. To its disadvantage, though, ^{124}I produces relatively high energy positrons, leading to a lower resolution than those of ^{64}Cu and ^{89}Zr, and it also lacks the benefit of residualizing, wherein the metabolites of radiometal-labeled compounds are retained in cells, leading to higher sensitivity.[76–80]

Thus far, radiolabeled antibodies for the CNS have been explored with a number of radionuclides, including $^{124/125}$I, ^{18}F, ^{64}Cu,[81] ^{68}Ga,[41] and ^{89}Zr (Table 1). Interestingly, despite the low permeability of the BBB to antibodies, lesions in the brain have been detected using radiolabeled antibodies, likely because of disruption of the BBB proximal to brain metastases.[82] A list of radiolabeled antibodies that have imaged tumors in the CNS of humans is given in Table 2 (we are unaware of any successful attempts to image non-tumor targets in the human CNS using antibodies). An exhaustive list of preclinical studies in this avenue is beyond the scope of this review.

TABLE 1 Common radionuclides used to label antibodies.

Radionuclide	Labeling approach	Half-life	Decay mechanism	Application
^{18}F	Pre-targeting	110 min	β^+	PET
^{64}Cu	Chelation, with or without pre-targeting	12.7 h	β^+	PET
^{124}I	Direct	4.2 days	β^+	PET
^{89}Zr	Chelation	3.3 days	β^+	PET
^{67}Ga	Chelation	3.3 days	EC	SPECT
^{111}In	Chelation	2.8 days	EC	SPECT
^{123}I	Direct	13.2 h	EC	SPECT
^{125}I	Direct	59.4 days	EC	SPECT
^{131}I	Direct	8.0 days	β^-	SPECT/beta therapy
^{211}At	Direct	7.2 h	α/EC	Alpha therapy
^{225}Ac	Chelation	9.9 days	α	Alpha therapy
^{90}Y	Chelation	64.1 h	β^-	Beta therapy
^{177}Lu	Chelation	6.6 days	β^-	Beta therapy

Note: β^+, positron emission; EC, electron capture; β^-, beta/electron emission; α, alpha emission. Direct iodinations typically occur via electrophilic aromatic substitution meta to the hydroxy group on solvent-exposed tyrosine residues; the analogous astatine chemistry produces an unstable C–At bond,[65] so alternate methods have been developed for clinical use.[66–70]

Antibodies labeled with ^{89}Zr have been used to enable the identification of tumors that evaded detection by conventional imaging.[87,88] In terms of bispecific, shuttled antibodies (vide infra), most studies thus far have utilized $^{124/125}$I in translational and preclinical work. ^{18}F-Labeled antibodies are promising because of the radioisotope's widespread accessibility in Curie amounts from small medical cyclotrons, including for centers without an in-house cyclotron through established networks of commercial nuclear pharmacies. If fluorine-18 is to be used, then a pre-targeting approach should be considered, given the short half-life of the radionuclide (110 min). In pre-targeting, an unlabeled antibody bearing an appropriate handle is dosed first, allowed to accumulate at the target over several days, and then targeted with a small molecule labeled with a radioisotope that reacts with the antibody handle in vivo (Figure 2). This asynchronous targeting/labeling scheme enables the use of a pharmacokinetically slow antibody with a short-lived radioisotope, which benefits patients as they receive lower doses of radioactivity (cf. high dosimetry from ^{89}Zr circulating for days). Issues of kinetics are also greatly minimized using this approach because the antibody's entry into the CNS, binding, and efflux from the CNS are completely decoupled from the pharmacokinetics of the radiopharmaceutical and the half-life of the radioisotope. There are numerous ways pre-targeting has been accomplished, such as biorthogonal click reactions between ^{18}F-labeleled tetrazines and trans-cyclooctyne (TCO)-functionalized antibodies.[28,97–99] This approach has been used preclinically, including for PET imaging of amyloid-β in the brains of transgenic mice.[99,100] Interestingly, a related pre-targeting strategy has also recently been used with ^{64}Cu-labeled tetrazines and TCO-functionalized antibodies, even though copper-64 has a longer half-life (12.7 h).[101] However, to the best of our knowledge, the tetrazine/TCO pre-targeting approach has not yet been used clinically for CNS targets, and general questions remain about whether separate pharmacology/toxicology studies are going to be required for each component of a pre-targeting cocktail prior to clinical translation. Alternative to pre-targeted approaches, antibodies may be labeled with longer-lived radioisotopes like ^{64}Cu[102,103] and ^{89}Zr ($t_{1/2}$ = 3.3 days),[75] or radioiodine, with the advantage of simplifying procedures: just one dose is required rather than the two needed in pre-targeted methods. The various benefits of radiometals over radiohalogens in antibody-based PET have inspired our group to explore this combination for preclinical CNS PET,[41] and others have translated it for clinical use. For example, ^{89}Zr-labeled bevacizumab imaging has been used to investigate whether failed bevacizumab therapy could be attributed to inadequate uptake by the tumors in diffuse intrinsic pontine glioma (Figure 3).[39,85]

3 | CHALLENGES, LIMITATIONS, AND REMAINING QUESTIONS

Although antibody-based radiopharmaceuticals potentially offer many advantages, there are a number of noteworthy limitations and challenges. Probably the biggest

TABLE 2 Radiolabeled antibodies and antibody fragments used to clinically image or treat cancers in the CNS.

Monikers; formats tested	Applications and isotopes used	Representative publication
Peripheral administration (intravenous, intra-arterial, intra-carotid, etc.)		
Rituximab; chimeric mAb (human $IgG_1\kappa$ with mouse variable regions)	Imaging; I-123	[83]
Pertuzumab; humanized mAb	Imaging; Zr-89	[84]
Bevacizumab; humanized mAb	Imaging; Zr-89	[85]
IAB2M; minibody	Imaging; Zr-89	[86]
Fresolimumab; human IgG_4 mAb	Imaging; Zr-89	[87]
Trastuzumab; humanized IgG_1	Imaging; Zr-89, Cu-64	[88]
IMP288 peptide bound to pre-targeted bispecific antibody TF2; humanized mouse antibodies	Imaging; Ga-68	[89]
425; mouse IgG_{2a} mAb	Therapy; I-125	[90]
huJ591; humanized mouse antibody	Imaging; Zr-89	[91]
Ibritumomab; murine antibody[a]	Therapy, imaging; Y-90, In-111	[92]
Some peripheral, some direct brain/CNS administrations		
81C6, Mel-14; murine IgG_{2b}, chimeric human IgG_{2b}/mouse, $F(ab')_2$	Therapy, imaging; I-131, At-211, I-125	[93]
Administered directly to brain/CNS (into surgically created resection cavity, via Ommaya shunt, direct injection into brainstem, etc.)		
Omburtamab, 3F8, 8H9; mouse IgG_3 antibodies	Therapy, imaging; I-131, -124, -125	[94]
BC-4; mouse mAb	Therapy; Y-90, I-131	[95]
Nimotuzumab; humanized IgG_1 mAb from murine IgG_{2a}	Therapy; Re-188	[96]

Note: Each group has only one representative publication cited, but some agents have been the topic of numerous clinical trials and publications. Most involve access via a leaky BBB or direct injection into a surgically created resection cavity. We were not able to find any examples where antibodies accessed the CNS of a patient where administration was peripheral and the BBB was demonstrated to be intact. Antibodies studied together are listed together, along with alternate names. mAb = monoclonal antibody.

[a]Some studies include comparing administration with and without osmotic BBB opening.

challenge in the advancement of antibody-based CNS PET agents is the passage of antibodies across the BBB. Antibodies do not penetrate into the brain in significant amounts, with uptake values commonly claimed at 0.1% of injected dose (alternatively, a detailed pharmacokinetic study in mice found that 24 h after an intraperitoneally administered dose, 0.009%, and 0.0017% was present in the cortex and hippocampus, respectively).[71] Despite this, there are some methods by which antibodies inadvertently gain access to the CNS, and a number of reports exist of PET imaging of CNS targets using antibody platforms. In some cases, radiolabeled antibodies can enter the brain by attachment to (immune) cells that become privy to the CNS.[104] Alternatively, CNS targets can sometimes be imaged with antibodies via a disrupted or "leaky" BBB, as with some cancers[82,105] (Table 2), neuroinflammation,[104] and multiple sclerosis.[81] However, to expand the use of antibodies in diagnosis and treatment of CNS conditions, reliable engineered methods of delivering antibodies across an *intact* BBB are critical. One promising approach involves utilization of antibodies or antibody fragments that target receptors on the brain endothelium. These BBB-targeting antibodies are attached to drug or imaging molecules (e.g., a radiolabeled antibody against amyloid-β), and the BBB-targeting unit can bind to receptors/transporters on the brain endothelium and be internalized, pulling the drug or diagnostic unit with it. Many transporters have been targeted for transport-mediated transcytosis,[106] including transferrin receptor (TfR), insulin receptor, CD98hc/LAT1 heterodimer, and lipid transporters, with the transferrin receptor receiving by far the most attention to date.

Once a strategy has been decided upon, a bispecific antibody can be created by chemically ligating the BBB-targeting antibody and the imaging- or therapeutic-targeting antibody via complementary chemical units attached to each part, often using lysine or cysteine residues. For example, an azide may be attached to an anti-transferrin receptor antibody and an alkyne group to an antibody against a protein aggregate, allowing the two

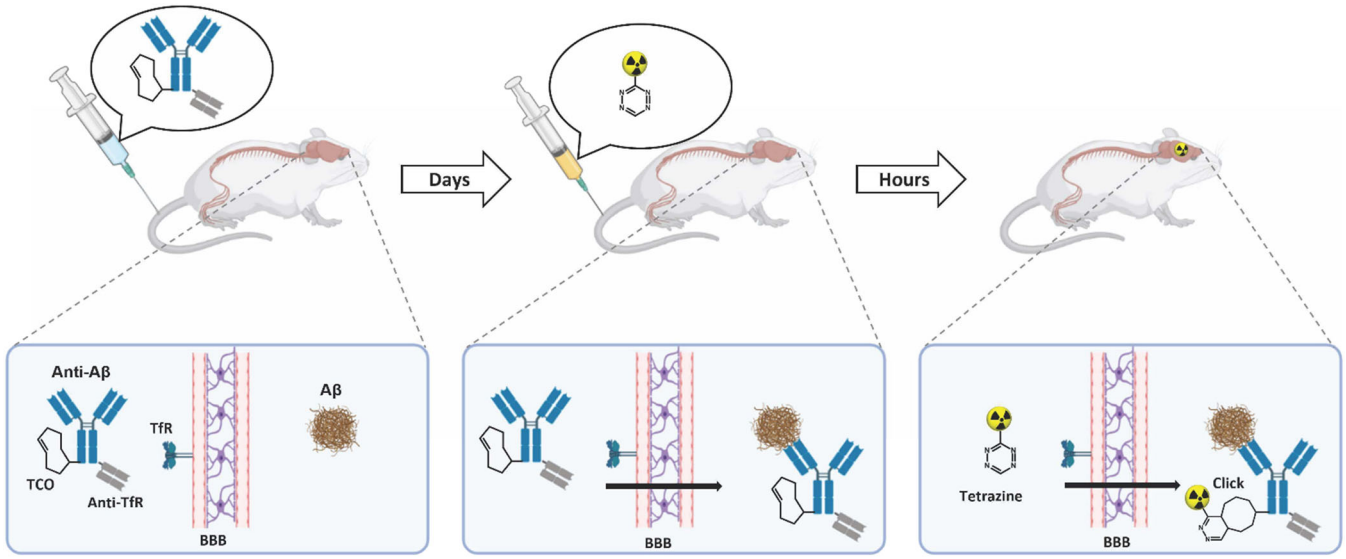

FIGURE 2 Pre-targeting scheme showing injection of targeting antibody, allowing for accumulation in the brain and binding to the target over multiple days, before administration of a radiolabeled clickable molecule. Reproduced from Bredack et al.[97] under the terms of a Creative Commons CC-BY license.

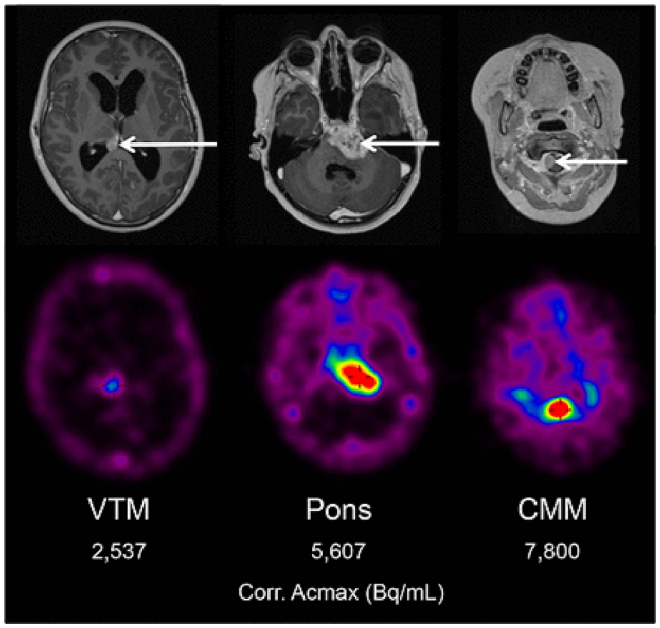

FIGURE 3 Gadolinium-enhanced T1-weighted MR images obtained 8 days before death (top row), and corresponding PET slices obtained 4 days before death (145 h after ^{89}Zr-bevacizumab injection) (bottom row), showing primary pontine tumor and metastases in the right ventricular trigone (VTM) and cervicomedullary junction (CMM). Arrows represent areas of disease. Corr. Acmax = corrected maximum activity concentration. This research was originally published in JNM,[85] © SNMMI, reproduced with permission.

fragments to subsequently be connected via a click reaction. Chemical ligation is advantageous when using commercially available antibodies as it can drastically speed up initial studies including preclinical target validation, but this approach is unlikely to be profitable once scale-up and translation efforts begin because of the high cost of commercial antibodies. Most bispecific antibodies rely on the expression of one protein genetically encoded to contain both targeting functionalities, though this approach comes with its own challenges.[37,39] Expression of bispecific antibodies necessitates knowledge of the primary sequence of the complementarity determining regions, which are often not disclosed in publications. Antibodies can be sequenced, but the very high cost—ca. 5000 USD minimum—makes sequencing only feasible for well-funded projects, inherently limiting early exploration and potential advances. On the other hand, with advances in DNA sequencing technology, sequencing hybridomas is extremely economical, and high-quality antibody literature supports the field by including sequence data. Patents should contain the primary sequences of antibodies, but often large numbers of antibodies are discussed and the ideal candidates can often only be discerned via the expression of the whole library of antibodies and screening. This process can take months with uncertain results. Yet when a sequence is known—from prior work or a publication/patent—taking an antibody from known sequence to antibody-in-hand takes about a month under ideal conditions.

Two of the most significant weaknesses of chemically ligated bispecific systems are huge boons of bispecific antibody expression: (1) commercially available antibodies are exceedingly expensive, essentially prohibitively so for scale-up to clinical use, and (2) chemical ligation

often relies on attaching linkers to lysines or cysteines at random, with uncertain effects on the function of the resulting labeled antibodies. For early-stage (pre)clinical studies, scale-up of antibody expression in academic labs is much more monetarily feasible than acquiring grams of antibodies from commercial vendors. The potential problems arising from random labeling of residues are minimized because the two sequences are expressed together in one protein; no residues are altered in attaching the antibodies to each other. Unfortunately, though, the issue of random modification is not completely avoided with expressed bispecific antibodies. In order to attach a radiometal chelator to an antibody, typically the same modification of random lysine or cysteine residues is used; the number of random modifications is reduced if the process is used only for chelator groups and not for both chelator groups and a second antibody group, but the issue is not completely eliminated unless additional strategies are applied.

To address the problems arising from random modification of antibodies, many methods exist for site-specific labeling. Specific sequences of amino acids can be encoded into the primary sequence of an antibody that can later be recognized by enzymes to perform site-specific modifications, such as with sortase modifying an LPXTG amino acid sequence[107,108] or mushroom tyrosinase activating intentionally exposed tyrosine residues.[109,110] There are also some methods of site-specific modification of antibodies that do not require engineering of the primary amino acid sequence and, thus, can even be applied to commercially purchased antibodies.[111,112] These site-specific modification methods are important as they help avoid or minimize the negative effects of stochastic labeling, such as loss of affinity. In comparing site-specifically labeled to randomly labeled antibodies, site-specific labeling tends to have fewer deleterious effects.[113,114] Stochastic labeling tends to increase non-specific binding. For example, two differently site-specifically ^{89}Zr-labeled pertuzumab constructs exhibited lower off-target, healthy tissue binding than the same antibody when random lysines were labeled.[115,116]

An important factor to consider in PET imaging is the pharmacokinetics of the radiopharmaceutical, even more so when passage across the BBB is involved. Plenty of work has been done to understand antibody passage into the brain, especially with regard to levels reached under therapeutic dosing schemes.[117] With PET, though, the administered doses are much lower and, additionally, the *kinetics* of brain access are of critical importance. In most studies so far, brain PET scans with antibody-based tracers take place multiple days after injection, but there is potential for more widespread utilization if antibodies can access CNS targets in a shorter timeframe, especially as it relates to simplicity for clinical workflows and patient compliance. A variety of factors affect the kinetics of this process,[118] including which brain endothelium transporters are targeted,[119,120] the affinity and avidity toward these transporters, the size and format of the antibody,[121] presence or absence and various modifications to the Fc region of the antibody,[73] including the effector function domain (particularly important for bispecifics that target TfR as it relates to safety[117,122]), and additional methods such as pre-injection with drugs to modify antibody lifetime. These variable properties will have different impacts in diagnostic uses (e.g., PET) versus therapeutic uses, so antibody-based theranostics may involve modification of not just the bound radionuclide but also structural features of the antibody itself.

One factor that has received a lot of attention is that of antibody format. There is an ever-growing list of antibody formats,[123,124] including Fab fragments, F(ab′)$_2$ fragments, nanobodies,[125] diabodies, minibodies, camelid antibodies,[126] shark antibodies,[127] and numerous others (Figure 4). An in-depth discussion of the various formats is beyond the scope of this review (see previous studies[124,125,128,129] for overviews on the topic), but all have their advantages and disadvantages, and some have begun to be explored for CNS PET, whereas others remain ripe for exploration. In one interesting (non-PET) example, camelid single-variable domain VHH antibodies were able to penetrate the BBB in untreated mice and enter astrocytes.[130] In broad terms, the smaller formats tend toward faster kinetics in the body and shorter half-lives,[124,131–133] but they can have lower affinities and/or avidities from IgGs. For example, even an scFab fragment versus an scFv, theoretically different in just the former's presence or the latter's absence of the C_H1 and V_H regions, can have large differences in their K_d values toward the same antigen.[134] Some formats lack an Fc region, which can have a large influence on the pharmacokinetics of antibodies/antibody-fragment pharmaceuticals. The Fc region is important for promoting a longer circulatory half-life, can alter which organs process the antibodies,[124] is critical to release from endothelial cells, and relates to efflux of antibodies from the brain.[135] The complex set of kinetic contributions seen with the Fc region are emblematic of how modifications to antibodies are far from straightforward in their effect on both kinetics and tissue uptake.

Additionally, with bispecific antibodies, any combination of formats is possible, again, with varying advantages and disadvantages. For example, a bivalent format toward the diagnostic/therapeutic target may be optimal for the strongest binding, whereas there is evidence that monovalent binding to BBB receptors leads to greater release into the parenchyma.[136] In another example, a

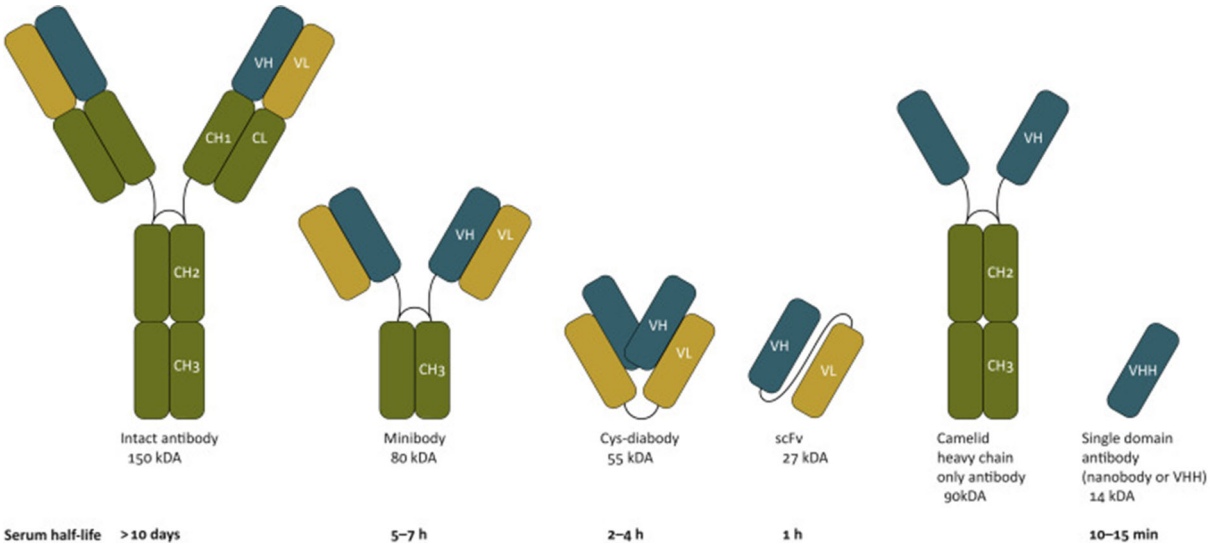

FIGURE 4 Various antibody formats and their serum half-lives. Reproduced from Rashidian and Ploegh[125] under a Creative Commons Attribution—NonCommercial—NoDerivs (CC BY-NC-ND 4.0) license.

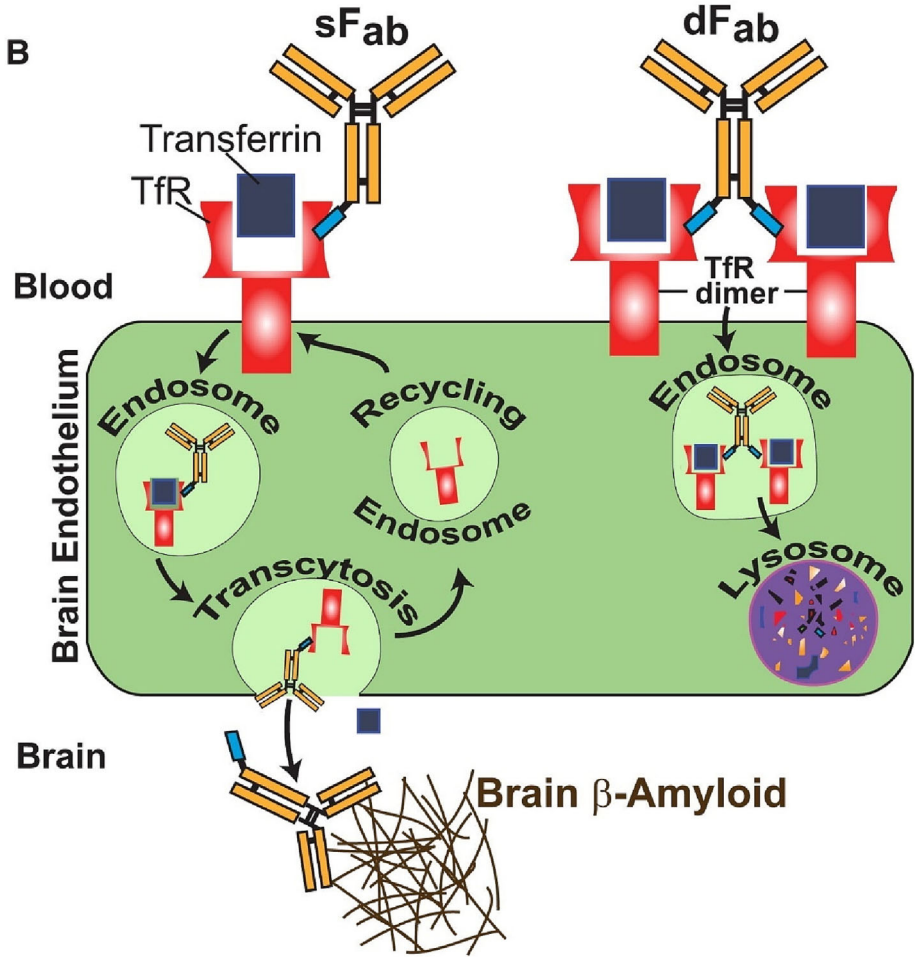

FIGURE 5 Cartoon illustrating the different pathways antibodies that are monovalent (sFab) against transferrin receptor (TfR) may take versus those (dFab) that are divalent. Monovalent binding to TfR enables release into the brain parenchyma because the antibody is able to dissociate from the receptor more easily. Reproduced with permission from Bell and Ehlers,[139] with permission from Elsevier, © 2014.

bivalent full IgG against TfR that was chemically ligated to an $F(ab')_2$ fragment against amyloid-β protofibrils was compared with a recombinantly expressed bivalent full IgG against amyloid-β with only monovalent scFv fragments against TfR (with the scFv fragments expressed at the C terminus of the IgG light chains).[137] The

recombinantly expressed bispecific had a fivefold higher brain concentration three days post-injection in transgenic mice possessing increased amyloid-β expression; the avidity toward TfR (vide infra), antibody formats (with potential affinity changes),[134] and specific versus non-specific attachment of the two antibody moieties[114] are all (competing) factors in the difference in performance. How the two antibody units are attached can also affect performance. For example, a bispecific format could have an IgG with an scFv or Fab fragment toward a BBB target attached at the C-terminus of one or both light chains or one or both heavy chains, and these alterations can result in performance differences as it relates to release into the brain parenchyma from endothelial cells.[136]

A number of studies have focused specifically on how antibody format affects receptor-mediated transcytosis into the brain. The mechanism by which this process occurs involves binding of an antibody to the antigen receptor on the luminal side of the BBB followed by internalization into the endothelium cells. Once inside, some antibodies will dissociate from the receptor and potentially gain access to the parenchyma of the brain by exiting through abluminal side of the cells, but others will stay attached to the receptor.[138] Those that stay attached are ferried to the lysosome for degradation (Figure 5). This delicate balance relies on many features, including at least the affinity (and avidity) the antibody has for the receptor[138] and pH-related changes to the affinity.[140] Another antibody-engineering strategy for optimizing antibody pharmacokinetics, particularly addressing the long half-lives and related high background signal of antibody-based agents, is to attach agents to the antibody that promote its clearance from the blood.[141] Fine tuning of these factors will no doubt prove to be an important feature of antibody-based CNS radiopharmaceuticals. The ability to modify and optimize this large list of antibody structural features is a huge advantage of the modality and yet also adds to an already complex landscape.

With many CNS targets, such as G protein-coupled receptors (e.g., dopaminergic, muscarinic, and opioid receptors), PET agent agonist/antagonist pairs are used to gather complementary data, with the hypothesis that antagonists reflect expression levels and agonists indicate only active receptors.[142] Antibodies are typically designed to behave as antagonists, with strong affinities and binding to a structural motif that is not related to a functional state. This is strongly beneficial for targets with low B_{max} or where effectively irreversible binding is desired, but this can limit the development of agonist antibody radiopharmaceuticals. Antibodies can be designed as agonists, but it is less straightforward than antagonist design; Mayes et al. summarize the problem well: "Unlike antagonists—for which the general rule is to design an antibody with the highest strength of binding (affinity) possible to interact competitively with the ligand-binding domain of the receptor—there are no hard-and-fast rules that can easily predict the potential of an antibody to act as an agonist."[143] In this way, the discovery process for agonist antibodies more closely resembles that of small molecules, potentially minimizing the benefits of an antibody-based approach. However, with growth in the study of agonist antibodies, including applications in cancer treatment,[144] antibody-based CNS PET agents may still hold promise in their use as agonist/antagonist pairs.

Final issues to consider include the challenge in utilizing antibodies in CNS PET for the detection of intracellular targets. Some antibodies have been shown to pass into cells from the extracellular space,[76] whereas others seem to not be internalized.[58] Critically, given current research interests, antigens within human neurons are inaccessible as these cells do not possess the neonatal Fc receptors (FcRn) needed to internalize antibodies[145]; antibodies can still access the intracellular space of neurons via incidental pinocytosis,[146] but, given the low extracellular concentrations in the CNS, this process is unlikely to result in sufficient levels of intracellular antibodies in neurons. As with penetration of the BBB by antibodies, internalization of antibodies into cells is a rich area of inquiry (for a recent review, see Slastnikova et al.[147]), including the development of engineered solutions,[148] and is one that will largely benefit the targeting of CNS targets such as the intracellular proteins TDP-43 and α-synuclein.

A last inherent limitation to antibodies is that they cannot *trace*, they cannot monitor a process in the way that, for example, FDG does. In this way, antibodies behave like small molecule inhibitors that allow for quantification of, for example, enzyme expression, rather than small molecule substrates like FDG that can give measures of enzyme activity. FDG truly monitors the action of hexokinase in cells, with the FDG-6-phosphate trapped metabolite as the main component being detected when a region of uptake is noted on an FDG PET scan. Antibodies can be designed to bind enzymes, but they do not have a mechanism by which they would monitor enzymatic transformations. A similar limitation exists for monitoring transporter function. For example, if one wanted to probe where or in what level serotonin transporters are active, antibodies would not allow direct investigation of this question; at best, antibodies could potentially probe transporters that exist in a topography that is correlated with an active functional state, but they cannot probe the activity of the transporters.

4 | SUMMARY

The development of diagnostic (and therapeutic) radiopharmaceuticals for neurological applications remains an area of intense research and development given both the increasing use of molecular imaging in CNS drug hunting and the emergence of theranostics for the potential treatment of neuro-oncological malignancies. Although much of this work to date has focused on small molecules, this approach relies on ready access to appropriate chemical space that can be radiolabeled for a given target and presents challenges garnering selectivity between targets in the case of overlapping binding mechanisms. Reflecting this, there is much interest in capitalizing on the benefits of PET (and therapy) using antibody-based radiopharmaceuticals for neuroscience and neuro-oncology applications, such as ease of accessing antibodies for new targets, straightforward radiolabeling via chelation of radiometals or pre-targeting approaches, and exquisite selectivity that can be achieved over biologically similar targets. Considerable work has gone into investigating mechanisms by which antibody-based radiopharmaceuticals can be coerced into the CNS, including application in disease states with compromised BBBs, recruitment of cells that can infiltrate the brain, or engineering features such as bispecific antibodies that can utilize transporters on the BBB for transport mediated transcytosis. These efforts have enabled imaging of CNS targets such as misfolded proteins and neuroinflammatory biomarkers, admirably demonstrating proof-of-concept. Although challenges with the approach certainly still remain, there is an abundance of both promise and questions in the application of antibodies to PET imaging and radiotherapy of CNS targets, indicating further development of the approach is warranted and encouraged.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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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