REVIEW ARTICLE

Quantitative Rodent Brain Receptor Imaging

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Abstract

Positron emission tomography (PET) is a non-invasive imaging technology employed to describe metabolic, physiological, and biochemical processes *in vivo*. These include receptor availability, metabolic changes, neurotransmitter release, and alterations of gene expression in the brain. Since the introduction of dedicated small-animal PET systems along with the development of many novel PET imaging probes, the number of PET studies using rats and mice in basic biomedical research tremendously increased over the last decade. This article reviews challenges and advances of quantitative rodent brain imaging to make the readers aware of its physical limitations, as well as to inspire them for its potential applications in preclinical research. In the first section, we briefly discuss the limitations of small-animal PET systems in terms of spatial resolution and sensitivity and point to possible improvements in detector development. In addition, different acquisition and post-processing methods used in rodent PET studies are summarized. We further discuss factors influencing the test-retest variability in small-animal PET studies, *e.g.*, different receptor quantification methodologies which have been mainly translated from human to rodent receptor studies to determine the binding potential and changes of receptor availability and radioligand affinity. We further review different kinetic modeling approaches to obtain quantitative binding data in rodents and PET studies focusing on the quantification of endogenous neurotransmitter release using pharmacological interventions. While several studies have focused on the dopamine system due to the availability of several PET tracers which are sensitive to dopamine release, other neurotransmitter systems have become more and more into focus and are described in this review, as well. We further provide an overview of latest genome engineering technologies, including the CRISPR/Cas9 and DREADD systems that may advance our understanding of brain disorders and function and how imaging has been successfully applied to animal models of human brain disorders. Finally, we review the strengths and opportunities of simultaneous PET/magnetic resonance imaging systems to study drug-receptor interactions and challenges for the translation of PET results from bench to bedside.

Key words: Rats, Mice, Brain, PET imaging, Receptor quantification

Introduction

The demographic change has led to a continuously increasing number of aging people around the world who suffer from neurodegenerative or neuropsychiatric diseases such as

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Parkinson's disease (PD), Alzheimer's disease (AD), major depression, and anxiety. Therefore, research on those disorders with regard to diagnosis, staging, and the development of novel therapies is of great medical and sociopolitical importance.

Due to ethical considerations and legal requirements, many research questions—including some questions relevant to the development of novel therapies, drugs, and biomarkers—cannot be directly addressed in humans. Therefore, disease models using animals, *e.g.*, mice, rats, and non-human primates, are indispensable for biomedical research. In particular, transgenic mouse and rat models of neurological disorders have greatly advanced our understanding of many brain disorders and may provide new avenues to therapy development [1–6].

Non-invasive imaging bridges the gaps between basic, preclinical, and clinical science and, furthermore, links interdisciplinary approaches and methods from pharmacology, physics, chemistry, physiology, and genetics to gain complementary information about the tissue of interest. The technical progress of dedicated small-animal positron emission tomography (PET) systems along with the development of novel radiotracers has led to the use of PET in small laboratory animal models in basic, preclinical, and translational settings. The main advantage of small-animal PET in preclinical and fundamental science is that studies can be performed *in vivo* using longitudinal study designs in the same animals, minimizing the number of animals needed per cohort and maximizing the statistical utility of the data, since the same animals can be measured at several time points; by contrast, immunohistochemical or other hybridization experiments require animals to be sacrificed at each measurement time point. Small-animal PET led to advancements in the fundamental understanding of molecular mechanisms of diseases in basic research, resulting in the development of therapies and new disease models in the preclinical field; however, the successful translation of these advancements to the clinical field has often proven to be difficult. This is partially related to interspecies differences in genetics and physiology between humans and rodents [7]. However, it also involves a qualitative and quantitative issue related to a superficial description of methods and models used in a certain study leading to results which are not reproducible. Furthermore, inaccurate study designs as well as incorrect data analysis and interpretation will largely impact outcome parameters.

In addition, small-animal PET technology has inherent physical limitations that can affect the accuracy of quantification [8, 9]. Moreover, the limited time resolution of these systems makes the detection of fast molecular and functional changes in dynamic processes difficult. In addition, the pharmacokinetics of a PET tracer can be described only by very simplified models and methods, which insufficiently account the complex biology. The inaccuracies and deficits of quantification must be taken into account for data analysis and interpretation. This includes the estimation of binding

values from bolus injection experiments, the mass effect, and the partial-volume effect (PVE). In addition, animal handling, data acquisition, reconstruction, and corrections must be carefully evaluated and standardized before PET experiments can be performed in a quantitative manner (Fig. 1). This review aims to give an overview of the challenges, opportunities, and restrictions in rodent brain receptor PET imaging to provide the reader with a clear understanding of its limitations, as well as its unique benefits and strengths in the field of small-animal brain imaging.

Dedicated High-Resolution Small-Animal PET Systems

Numerous small-animal receptor studies have been performed using commercially available preclinical PET systems [10–16]. However, resolving small structures within rodent brains using PET imaging is feasible but nevertheless challenging due to the relatively low spatial resolution of small-animal PET systems compared to the tiny size of structures in rodent brains. In contrast to human PET scanners, which have a spatial resolution of approximately 4–8 mm (corresponding to a volumetric resolution of ~ 64 – 512 mm³) [17, 18], currently available small-animal PET systems achieve spatial resolutions in a range of 1.3–1.5 mm full width at half maximum (FWHM) (corresponding to a volumetric resolution of ~ 2.2 – 3.4 mm³) in the center of the field of view (FOV) [19, 20]. The spatial resolution is directly linked to the crystal size, with smaller crystals providing improved spatial resolution, which, due to the parallax effect, usually degrades outside the center of the FOV if long crystals are used [19]. However, shorter crystals decrease the detection sensitivity of the PET system [21]. Therefore, the choice of the detector crystal geometry is always a trade-off. However, most importantly in order to achieve quantifiable results, the systems need to provide reliable and reproducible data in order to enable receptor quantification in small animals. A comparison of currently commercially available small-animal PET scanners can be found in the literature [19, 22].

While the human brain has a volume of approximately 1200 cm³, the rat and mouse brains measure ~ 2 and ~ 0.5 cm³, respectively, which makes them approximately 600- and 2400-fold smaller than the human brain (Fig. 2). In contrast, the volumetric resolution of preclinical scanners is increased only by a factor of about 30 compared to that of human scanners. Downscaling from the whole brain to particular brain regions, the resulting volumes of interest (VOIs) measure only a few cubic millimeters. Especially in mice, small brain structures with volumes below the volumetric spatial resolution such as the substantia nigra (~ 2 mm³), the globus pallidus (~ 2 – 3 mm³), the nucleus accumbens (~ 5 – 6 mm³), the prefrontal (~ 1.8 mm³) and somatosensory cortex (~ 6 mm³), the hypothalamus (~ 6 mm³), and the dorsal (~ 7 – 8 mm³) and ventral hippocampus (~ 6 mm³) [23–25], which are of particular interest with regard to receptor quantification, cannot be fully distinguished from surrounding

Fig. 1. Technical, methodological, biological, and radiochemical aspects influencing the quantification accuracy of PET brain studies in rodents.

regions. Thus, for a reliable quantification of small brain structures, imaging technologies used to quantify receptor availability changes in the mouse brain need to offer increased spatial resolution, which, in the case of PET, usually results in a loss of sensitivity. However, low sensitivity is a serious issue, especially in small-animal PET studies. Indeed, only a small fraction of the injected tracer accumulates in the rodent brain, and the portion of specifically bound radiotracer (SB) is derived mostly from the ratio of the tracer uptake in a target brain region to the uptake in a reference region over a time period of 30 to 60 min after injection, when a large proportion of the initial radiotracer dose (usually C-11 tracers with a half-life of ~20 min) has already decayed. Increasing the injected dose, however,

would lead to a violation of the tracer principle ($< 1\%$ receptor occupancy), leading to a wrong quantification [26].

Thus, to offer quantitatively correct imaging data of rodent receptor functions, small-animal PET systems require both high sensitivity and high spatial resolution. To solve the trade-off between sensitivity and resolution and to improve current PET technology, an ongoing field of research focuses on the optimization of several aspects of the hardware. By taking the fundamental restrictions into account [27], the spatial resolution of a PET system can be optimized by decreasing the ring diameter of the system to the size of a rodent's head. Furthermore, resolving the exact position of the γ -photons within the crystals allows an

Fig. 2. Comparison between the brain sizes of humans and rodents.

increased sampling rate within the FOV. This improvement leads to an increase in spatial resolution and helps to keep it constant over the entire FOV [28]. Thus, the depth-of-interaction resolution addresses the parallax error and enables the use of long crystals in a high-resolution PET system, which increases the sensitivity [28].

Additionally, the FOV of the PET system should be easily accessible, *e.g.*, for providing anesthesia and injecting a tracer or pharmacological substance during the measurement without moving the animal bed or necessitating a long catheter. In particular, the combination of stimulation techniques, such as optogenetic light stimulation or forepaw stimulation, with PET requires space and access to the animal during the measurement.

Furthermore, the combination of high-resolution, high-sensitivity small-animal PET systems and high-field magnetic resonance imaging (MRI) technology can provide anatomical and functional data from MRI and molecular information from PET and MRI with high spatial and temporal resolution. This is a unique advantage, since functional information from MRI can be correlated with molecular receptor availability changes detected by PET. Several dedicated high-resolution PET/MRI systems have been developed [29–37].

Detector development is an active ongoing field of research and, undoubtedly, will further improve the resolution and sensitivity of preclinical PET systems in years to come.

Data Acquisition, Corrections, and Reconstruction

Rodent receptor imaging requires dedicated hardware and software, as well as proper physiological monitoring of the animals, to enable a reliable and quantitatively accurate measurement of receptor availability [8, 38, 39]. PET in general has limited spatial resolution and, depending on the setup, can be subject to relatively high noise levels, especially in dynamic acquisitions of small structures using short half-life tracers [40]. Therefore, the reliability and reproducibility of the acquired data are strongly dependent on standardization of the data acquisition, correction, and reconstruction.

To determine receptor availability using kinetic modeling, dynamic *in vivo* PET experiments are often conducted. The majority of the PET tracers used for receptor imaging are radiolabeled with the isotope C-11 [41], which enables a simpler labeling process than other isotopes due to the substitution of a methyl group on the tracer molecule with the radioactive isotope C-11. However, more and more F-18-labeled radiotracers have been developed over the last years to circumvent specific disadvantages of C-11. These include a short half-life of 20.4 *versus* 110 min for F-18 and hence the necessity of an on-site production of high molar activities to avoid mass effects from the injection of large volumes as well as a lower spatial resolution due to the

larger positron range of C-11. In addition, with one F-18 synthesis, a larger number of animals can be scanned, reducing synthesis time and costs. Depending on the tracer kinetics, dynamic acquisitions are usually performed over the course of 60–90 min. The temporal resolution of the time–activity curve (TAC) is an important factor for kinetic modeling and is strongly affected by the chosen dynamic framing of the PET acquisition. Shorter frames are desired at the beginning of the acquisition to fully resolve the perfusion peak and fast tracer kinetics, while longer frames are needed at the end of the acquisition to account for the lower count statistics due to the decay of the isotope. Hence, the scanner needs to be able to deal with a relatively large dynamic range of radioactivity and count rate which in turn requires a high detection sensitivity and count rate performance.

Tracer injection can be performed either by bolus injection or by a combination of bolus injection and continuous tracer infusion. Combined bolus infusion protocols are conducted to ensure a continuous equilibrium, in which the tracer is infused at the same rate as it is cleared from the tissue without specific binding sites [42]. By performing such experiments, one can simply derive specific binding from the ratio of the target and reference regions without kinetic modeling, reducing the dependency on a kinetic model [43]. In addition, bolus infusion protocols enable researchers to perform interventional studies within one animal and one single PET experiment. The specific binding at equilibrium represents the baseline condition and can be compared to the specific binding after an intervention (*i.e.*, a drug application). Thus, using such a protocol baseline and intervention can be performed in the same animal and scan which leads to more reliable results and largely reduces the number of animals needed in a study.

The acquired and histogrammed PET data are reconstructed into 3D imaging stacks using analytical or iterative reconstruction methods. While analytical methods, such as the common filtered backprojection (FBP) algorithm, are fast and straightforward to implement, the iterative reconstruction algorithms, *e.g.*, ordered subset expectation maximization (OSEM) algorithm, can include corrections (scanner geometry, *etc.*), as well as model the statistical noise [40]. As shown in Fig. 3a, different reconstruction algorithms lead to very different outcome measures (Table 1).

Multiple studies have focused on the evaluation of reconstruction parameters for different applications [40, 45–48]. However, up to now, there is, at least to our knowledge, no gold standard reconstruction algorithm available for brain receptor imaging and quantification.

Regardless of the reconstruction method, several physical limitations hamper the quantification accuracy of the acquired data and several basic corrections need to be applied to ensure good reliability and quantification of the data [49, 50]. A normalization of the system to correct for PET detector inefficiencies shall be applied, along with decay, dead-time, and random corrections. In addition, the quantity of attenuated

Fig. 3. Comparison of $[^{11}\text{C}]\text{raclopride}$ mouse brain PET images after using different 2D and 3D reconstruction algorithms (**a**), CT versus Co-57 attenuation correction (**b**), increasing matrix sizes (**c**), increasing injected activity (**d**), and increasing injected doses (**e**). In **a**, **b**, **c**, and **e**, scans were performed in the same mouse, but in **b**, two separate scans were performed for the first two (wo ATT and CT ATT) and last two (wo ATT and Co-57 ATT) columns. Different mice of the same strain and age were used in **d**. Abbreviations: FBP = filtered backprojection, OSEM = ordered subset expectation maximization, MAP = maximum a posteriori, ATT = attenuation, wo ATT = without attenuation correction.

and/or scattered photons should be determined and corrected for by using either an attached CT scanner or an external source to acquire the attenuation data (Fig. 3b and Table 1) [49, 51–54]. This is especially important in small-animal studies, since most of the animal beds are relatively thick due to the integrated heating system. Furthermore, motion correction of the PET data has been studied extensively over the last few decades, and several approaches are available [55–59]. However, to our knowledge, this is currently not implemented on a routine basis for preclinical scanners and remains an important research topic.

In most preclinical PET brain studies, anatomical reference images are not available and PET data are co-registered to a common MRI brain template [24, 60–62]. This is however a challenging and error-prone process, since many PET tracers do not show uptake in major brain regions, such as cortex, cerebellum, and midbrain, and therefore tracer-specific anatomical reference points are often missing.

One important advancement in data post-processing is the availability of tracer-specific standard brain templates,

Table 1. [^{11}C]Raclopride quantitative values derived from images shown in Fig. 3

Reconstruction	FBP2D	OSEM2D	OSEM3D	OSEM3D/MAP
BPND (SRTM, striatum)	1.7	1.7	2.0	2.5
Attenuation	wo ATT	CT ATT	wo ATT	Co-57 ATT
striatum [kBq/cm ³]	252	275	189	228
cerebellum [kBq/cm ³]	83	91	81	106
BPND (SRTM, striatum)	1.2	1.2	0.9	1.1
Matrix size	64 × 64	128 × 128	256 × 256	512 × 512
BPND (SRTM, striatum)	1.4	1.6	1.8	1.6
Injected Activity [MBq]	7.4	14.8	22.2	29.6
BPND (SRTM, striatum)	1.7	1.8	1.8	1.2
Injected Dose [nmol/kg]	4	17	50	226
BPND (SRTM, striatum)	1.8	1.5	0.7	0.3

The binding potential (BP_{ND}) was calculated using the simplified reference tissue model (SRTM) [44] with the cerebellum as the reference tissue

aligned to a standard reference space for several PET radioligands [63, 64]. This enables the use of an automatic normalization and the application of predefined VOIs, minimizing the user-dependent variability. This is particularly important in small-animal PET brain studies, if a voxel-based analysis is performed and small deviations from the standard space will largely impact the results.

Quantification Accuracy: Partial-Volume Effect, Spillover, Mass Effect, and Data Reproducibility

In addition to the abovementioned points, the PVE needs to be taken into account when accurate quantitative data are desired. The PVE occurs due to the finite spatial resolution of the PET system and to the activity spillover from the surrounding tissue by either spill-in or spill-out to the target region. As a consequence of the PVE, the measured activity concentration does not reflect the real activity concentration in a given tissue [65–67], altering the PET signal and hence the quantification accuracy [66]. Figure 3c and Table 1 illustrate this effect, which is also referred to as the tissue fraction effect, by comparing different matrix sizes for an [^{11}C]raclopride mouse brain PET scan. The resulting voxel value is a combination of different tissue activity levels within the voxel. If a small matrix size is used, different tissue activity levels will contribute to the PET signal within one voxel. However, if the voxel size is too small, the activity concentration within one voxel is very small, resulting in high noise levels that may affect the quantification accuracy. The matrix and corresponding voxel size will consequently have an impact on the PVE and a trade-off has to be accomplished between smaller voxel sizes with less spill-in and spill-out of activity of surrounding tissues compared to higher noise statistics within smaller voxels. The magnitude of the PVE in general is dependent on many individual factors, such as the positron range of the selected isotope, the size of the target, the contrast ratio of the target to the background region, the reconstruction and correction algorithms and parameters, the position of the voxel within the FOV, and the count rate of the acquisition [54, 68, 69].

In small-animal PET scanners, in which the length ratio of the axial FOV to the transaxial FOV is greater, the PVE seems to be much more distinct than in clinical PET scanners [54, 68].

Over the past few years, multiple methods have been developed to correct for the PVE, most of them based on either simulations, MR- or CT-acquired anatomical reference images or phantom experiments [70, 71]. However, multiple challenges come with attempting to correct for the PVE, such as animal motion, dynamic tracer accumulation (and, hence, changing contrast ratios in the target region compared to the background), sequential multimodal imaging (*i.e.*, registration errors due to a mismatch of the images based on motion), and low count rate statistics [70]. Thus, to our knowledge, no automatic PV correction is available for preclinical data at present.

Another important factor for receptor quantification using PET is the dose of radiotracer injected (Fig. 3d). Due to the limited sensitivity of the scanner, the injected dose of the radiotracer needs to be sufficient to obtain decent count rates. A high injected dose, however, comes with an increased amount of non-labeled ligand, which is dependent on the molar activity of the radiotracer (the ratio of tracer activity to mass) and can vary widely by synthesis (Fig. 3d and Table 1). High injected doses at low molar activity, especially in neuroreceptor studies, lead to high receptor occupancy and thus to competition of the radiotracer and the non-labeled ligand at the binding site (Fig. 3e). As previously mentioned, this can lead to pharmacological effects, thus violating the tracer principle [26], and can also compromise quantification accuracy due to reduced specific binding of radiolabeled compound. In order to obtain high quantification accuracy in rats and mice, it is highly important to keep the injected dose very low. Therefore, it is crucial to know over what range the specific binding is unaffected by the injected dose. These evaluations can be carried out using saturation binding experiments, in which an increasing mass is added to the tracer solution by keeping the injected activity constant. Using this dose-response relationship, the dose required to obtain < 1 % receptor occupancy (tracer principle) can be determined from non-linear regression analysis. Through saturation binding experiments for [^{11}C]raclopride, an injected

dose of < 1 nmol/kg for rats [72] and 0.5 nmol/kg for mice [12] was estimated by others and in our studies. For [^{11}C]DASB, we observed an injected dose of < 1 nmol/kg for rats, while low receptor occupancy could not be obtained in mice using high molar activities up to 116 GBq/ μmol [11]. In those experiments, receptor occupancy of less than 5–10 % could not be achieved; therefore, the values were obtained only by interpolation of the occupancy plot and need to be confirmed by *in vivo* experiments at higher molar activities. Ultra-high molar activities of 4000–7000 GBq/ μmol have been obtained by Nogushi et al. [73]. However, the molar activity levels in most published receptor studies are still between 50 and 200 GBq/ μmol . Enhancing the molar activity through improved tracer synthesis modules is therefore a major goal for PET receptor studies in small laboratory animals; this area of research will increase the receptor quantification accuracy of PET.

One major advantage of PET is the possibility to perform longitudinal investigations in the same animal. Thus, differences between animals due to inter-individual variations can be excluded. For this, it is assumed that differences between measurements in the same subject come from a pathological change or intervention. However, this assumption can only be made if the test-retest variability between two scans is low. The test-retest variability is heavily dependent on the radiotracer used in a study and therefore an important criterion to select a radiotracer for a particular target [74]. Low signal-to-noise ratios and off-target binding will lead to a higher variability between scans and thus differences of the same magnitude cannot be reliably detected. In addition, errors, which result from the post-processing of data, including data reconstruction, correction, and co-registration to a standardized template as well as the estimation of binding parameters from simplified models have to be taken into account. If the test-retest variability for a given tracer, target region, and analysis method in the brain is high, changes cannot be reliably detected. Hence, higher numbers of animals are needed to gain statistically reliable results. To assess the influence of these limitations on the estimation of quantitative binding values and to determine the optimal quantification approach for a given PET tracer in small laboratory animals, test-retest experiments have been performed for several CNS PET ligands [11–13, 75–83]. In addition, physiological parameters can also vary within one animal at two different scan time points and may influence receptor availability, limiting the ability of PET to detect differences in an interventional study where a baseline and a treatment scan are compared. As a consequence, to increase the test-retest stability in a PET study, it is important to keep factors influencing the animal physiology including anesthesia, body temperature, heart rate, breathing rate, and stress constant and standardize imaging and post-processing protocols for a particular PET tracer.

Moreover, one important parameter in small-animal PET brain studies is the effect of scan duration on parameter stability. While longer scan times increase the signal-to-noise ratio and reduce the variability of parameter estimates, shorter scan times are desirable to reduce the effect of anesthesia on the animal physiology which can negatively impact binding parameter

estimates. For example, a reduction in scan time from 120 to 70 min was proposed for the quantification of adenosine A1 receptors using [^{18}F]CPFPX [84].

Quantification of Receptor Binding

Currently, many PET datasets are based on static PET measurements conducted at a fixed time point after a bolus injection of a PET tracer ignoring the holistic information about the tracer's pharmacokinetics. Therefore, the conclusions from these studies are drawn on the basis of semi-quantitative approaches, *e.g.*, using the standardized uptake value ratio (SUV_R) as an outcome measure [85]. Although these semi-quantitative approaches may be useful and convenient to perform, they lack true image quantification and may be influenced by differences in tissue perfusion, vascularization, or differing levels of non-specific PET tracer uptake between or within experimental groups, the length of the uptake period, plasma glucose levels, plasma protein binding levels, and partial-volume effects [86, 87].

By contrast, dynamic PET scans together with mathematical compartment models describing the *in vivo* pharmacokinetics of the PET tracer allow quantification of the rates of physiological processes, such as perfusion or binding of a ligand to a neuroreceptor [44, 88, 89]. One of the standard compartmental models for the characterization of brain receptor PET tracers is the two-tissue compartment model (2TCM), which is, in fact, a simplification of the three-tissue compartment model (3TCM) (Fig. 4). The 3TCM assumes that the PET tracer freely diffuses from the plasma to the tissue, where it can be free (C_F), bound non-specifically (C_{NS}), or bound to a specific receptor (C_B). However, kinetic modeling using three-tissue compartments is difficult due to the high number of rate constants to be estimated. Moreover, the C_F and C_{NS} fractions are kinetically indistinguishable, and thus, they are combined in a single compartment in the 2TCM [90]. This simplification requires an assumption that the equilibrium between the free and the non-specifically bound compartments is achieved rapidly. The 2TCM is described with the following differential equations:

$$\begin{aligned}\frac{dC_{F+NS}(t)}{dt} &= K_1 C_p(t) - (k_2' + k_3') C_{F+NS}(t) + k_4 C_B(t) \\ \frac{dC_B(t)}{dt} &= k_3' C_{F+NS}(t) - k_4 C_B(t)\end{aligned}$$

where $C_p(t)$ is the plasma concentration of the tracer over time and K_1 , k_2' , k_3' , and k_4 are the transfer coefficients.

The total of $C_{F+NS}(t)$ and $C_B(t)$ corresponds to the tissue TACs and can be derived from the PET image, while $C_p(t)$ must be measured in the blood during the PET measurement. The rate constants of the pharmacokinetic reactions (K_1 , k_2 , k_3 , and k_4) are estimated in the data analysis process, and the obtained values can be compared between the experimental groups [91, 92]. However, not only the rate constants but also the outcome parameter *volume of distribution* (V_T) can

Fig. 4. Three-tissue compartment model for brain receptor studies with reversible radioligand binding. The PET signal is composed of different components: radiotracer in plasma (C_P), free radiotracer in tissue (C_F), radiotracer specifically bound to a receptor (C_B), and non-specifically bound radiotracer in tissue (C_{NS}) (a). Since kinetic modeling using three-tissue compartments is difficult due to the large number of rate constants estimated, the model can be simplified to two-tissue compartments assuming a rapid equilibrium between the free and non-specifically bound compartments (b).

be derived from the ratio of compartmental rate constants and is generally used as primary outcome parameter as it is directly proportional to the receptor availability [93, 94]. For a two-tissue compartment model, V_T is derived as:

$$V_T = \frac{K_1}{k_2} \left(1 + \frac{k_3}{k_4} \right)$$

and is defined as the ratio of the radiotracer concentration in one region of the brain C_T to that in plasma C_P .

$$V_T = \frac{C_T}{C_P} \\ C_T = C_B + C_{NS} + C_F$$

However, despite the undeniable benefits of using fully quantitative methods for PET data analysis, these approaches impose also some challenges. As mentioned above, $C_P(t)$ must be measured using blood samples during PET

acquisition. While blood sampling is relatively easy in patients, the procedure becomes difficult in rats and even more so in mice due to the limited blood volume (~ 20 ml in rats and ~ 1.5 ml in mice). If too much blood is taken, physiological disturbances may occur [95].

As a way of circumventing this problem, a proposal has been made to record the radioactivity level in the blood circulating in an arterial-venous shunt with a blood counter [95] (Fig. 5). With the blood counter, the volume is preserved, the animal's physiology should remain stable, and the PET tracer remains in the system. Additionally, the high sampling rate of blood counters ensures accurate capturing of the peak activity following a bolus injection, which may be missed with manual sampling.

However, automated blood counting has also some disadvantages. Primarily, insertion of the arterial-venous shunt is a relatively complex procedure. Secondly, since the blood counter records the activity in the whole blood, the ratio of the PET tracer concentration in plasma to that in the whole blood ($\frac{p}{wb}$) over time needs to be known in order to extract $C_P(t)$ [95, 97]. Thus, if the technique is used to evaluate pharmacokinetics of a new PET tracer whose $\frac{p}{wb}$ ratio is unknown, collecting manual blood samples is unavoidable. In this respect, the recently introduced “CD-Well” has the advantage that small whole blood samples of 2–3 μ L can be collected into tiny U-shaped capillaries in which they are subsequently centrifuged to separate the plasma. The radioactivity concentration is measured using autoradiography [98]. This approach allows the $\frac{p}{wb}$ ratio to be calculated in mice.

Nevertheless, additional blood samples are still necessary to correct for the fraction of the tracer that has been metabolized in the plasma. This can be done with high-performance liquid chromatography (HPLC) or thin layer chromatography (TLC). Depending on the method and available equipment, relatively large volumes of plasma, and thus more animals, may be needed for this purpose. Preparation of samples for the chromatography analysis includes careful precipitation of plasma proteins. Moreover, in case of TLC, the measurement of the concentration of the metabolized tracer is not perfectly accurate, and hence, an error may be introduced. Consequently, in order to reduce the animal number and the workload, or to limit possible sources of errors, the metabolite correction is not always performed.

Finally, measuring blood and plasma activity, whether in a well-counter or an automated blood counter, requires careful cross-calibration of the external device with the PET scanner, including exact time matching. This procedure, as well as blood sampling and metabolite analysis described above, certainly makes full kinetic modeling more challenging compared to the semi-quantitative approaches, particularly by introducing more possibilities of additional errors to occur. Therefore, in small-animal imaging, a compromise is sometimes required between applying corrections to reduce errors and to omit corrections which may introduce too much bias.

Fig. 5. Schematic illustration of an online blood counter setup and manual blood sampling to obtain an arterial input function from a rat. After the start of the PET acquisition, the radiotracer is injected *via* an infusion pump as a fast bolus, and the activity in the whole blood is counted using an online blood counting system. For this, a catheter is placed in the femoral artery of the animal and subsequently passes through the blood counter, in which the radioactivity from the blood is counted. The peristaltic pump ensures a constant and continuous blood flow through the arterial-venous shunt. Manual blood sampling can be conducted using an arterial catheter to correct the blood curve for the plasma free fraction and the contribution from metabolites (figure from [96]).

Alternatively to using invasive blood sampling, a PET image-derived arterial input function (AIF) can be applied with certain constraints [99]. This is done by extracting the TAC of the blood from a region of interest drawn on the left ventricle of the heart. However, it is crucial to remember that spillover and the PVE may substantially confound the measured values [97] and that appropriate corrections must therefore be applied [100]. Furthermore, as with the blood counter method, the value of $\frac{p}{wb}$ is necessary.

If the PET tracer binds to its receptor reversibly and if a region within the brain devoid of specific binding sites with similar non-specific binding as in the target region (K_1/k_2 is the same) is available, then the input function from such a reference region can be used instead of $C_p(t)$. The specific binding can then be calculated from the volumes of distribution (V_T) of the target and reference region. This is, especially in small-animal imaging studies, a decisive advantage over modeling approaches using the plasma input function as it is non-invasive, less labor-intensive and a less error-prone approach, since plasma metabolite corrections are not necessary. However, before a reference tissue model can be applied for a given PET tracer, blocking experiments are needed to ensure that a reference tissue has no specific binding sites and comparable non-specific binding. If a reference tissue is available, specific binding can be calculated from reference tissue compartment modeling [101]. To reduce the complexity of fitting four parameters

with a full reference tissue model, simplified reference tissue models have been introduced which calculate only three parameters (R_1 , k_2 , and BP_{ND}) [44]. The binding potential (BP_{ND}) equals the ratio of the concentration of specifically bound tracer in tissue to the non-displaceable (ND) concentration.

The BP_{ND} relates to the V_T :

$$BP_{ND} = \frac{V_T - V_{ND}}{V_{ND}} = \frac{V_T}{V_{ND}} - 1 = DVR - 1$$

where V_{ND} is the distribution volume of the non-displaceable compartment and DVR is called the distribution volume ratio [94].

For example, the analysis of dynamic PET data from [^{11}C]raclopride, which targets striatal D2/D3 receptors, can be performed using the simplified reference tissue model (SRTM) [44], in which the cerebellum is used as the reference region. The SRTM allows the level of specific binding to be evaluated by fitting the model function to the dynamic PET data, yielding estimates for fitting constants, such as the non-displaceable binding potential (BP_{ND}) [94]. While this very elegant approach to obtaining quantitative values for the specific binding of a receptor ligand is adequate for [^{11}C]raclopride, it cannot be used in every model organism without accounting for errors. For instance, it is not suitable for analyzing [^{11}C]DASB, a reversibly

binding serotonin transporter (SERT) ligand, because of the lack of an appropriate reference region. The entire cerebellum cannot be used in this case due to the presence of serotonergic projections in the cerebellar white matter [11]. Since the rat brain, and consequently the rat cerebellum, is larger in size, the obstacle of specific binding in the reference region can be surmounted by deriving TACs specifically from the cerebellar cortex, which lacks SERT; this solution, however, is not feasible for the mouse brain [11]. A reference region in the brain is also not available for the synaptic vesicle protein 2A (SV2A) tracer [^{11}C]UCB-J. In such cases, using an invasively generated or image-derived AIF, as described above, is inevitable [102, 103].

Another important factor in small-animal imaging studies is the blood–brain barrier (BBB) penetration of PET tracers. If a PET tracer is a substrate of drug efflux transporters such as P-glycoprotein (P-gp) or multidrug resistance proteins (MRPs) that are expressed at brain capillary endothelial cells and astrocytic end-feet, the brain uptake can be very low leading to low signal-to-noise ratios and thus low reliability. The expression of these transporters has been shown to be higher in rodents than in humans and thus PET tracers, which have shown reasonable brain uptake in humans, only showed negligible brain uptake in rodents [104–107]. P-gp inhibition has shown increased brain uptake for several brain PET tracers in rodents using cyclosporine A (CsA) [105, 106, 108, 109]. One example is [^{18}F]altanserin, a PET tracer for imaging 5-hydroxytryptamine type 2a receptors (5-HT_{2A}R). Brain uptake in rats was only 10 % [110, 111] in comparison to humans [112] and CsA treatment resulted in a two- to three-fold increased cerebral uptake of [^{18}F]altanserin [106]. It has been assumed that the effects of efflux transporters are similarly distributed across different brain regions. In terms of reference tissue modeling, the BP_{ND} should therefore not change due to an increase of the radioligand uptake. However, changes of the BP_{ND} between drug-naïve animals and CsA-treated animals have been reported, likely related to differences in the regional expression of the efflux transporter [104, 106]. Therefore, regional differences of drug-efflux transporter expression have to be carefully taken into account for the tracer quantification in rodents. In addition, if a tracer is a substrate of drug efflux transporters, altered P-gp function under pathological conditions in some diseases may influence the quantification and interpretation of tracer binding data and needs to be carefully evaluated.

Finally, species-dependent tracer metabolism has to be taken into account for PET studies in rodents [113, 114]. If a PET tracer is administered intravenously, several enzymes from blood and tissue will lead to metabolism of the parent PET tracer. Lipophilic radio-metabolites that enter the brain are undistinguishable from the parent PET tracer and will lead to biased parameter estimates. As the enzymatic profile between species is fairly different, with higher metabolism in lower organism, metabolic analysis across different species is important [115]. In rodents, metabolite formation is

clearly detrimental as the determination is invasive and laborious. *In vivo* imaging of [^{18}F]FDOPA to quantify dopamine (DA) turnover for example has been limited due to the complex formation of metabolites in the rat brain. However, Walker and colleagues [114] demonstrated the feasibility of [^{18}F]FDOPA PET imaging using a data-driven graphical analysis after applying inhibitors of the two main enzymes. Tracer defluorination is another major issue and species differences have also been reported for several PET tracers. While the mGluR5 receptor tracer [^{18}F]SP203 is defluorinated in rats and monkeys, it shows no defluorination in humans [116, 117].

Separate Determination of B_{avail} and $\text{app}K_d$ in PET Receptor Studies

Since the BP is defined as the product of receptor availability (B_{avail}) and radiotracer affinity ($1/\text{app}K_d$) *in vivo*, a change in BP can reflect a change of B_{avail} , $\text{app}K_d$, or both [94]. Several methods have been described in the PET literature to determine the two values separately in small laboratory animals. The multiple ligand concentration receptor assay (MLCRA) is a multiple-injection approach derived from *in vitro* saturation binding experiments. The tracer and increasing amounts of the non-labeled compound are administered to the same animal, and at least four *in vivo* PET scans need to be performed. Hence, B_{avail} and $\text{app}K_d$ can be obtained from the hyperbolic saturation curve by plotting the bound over the free tracer concentration (Fig. 6a), which can be calculated from the TACs of the target and reference regions [119, 120]. However, if only four values are obtained from the *in vivo* experiments, linearization approaches are commonly used to avoid erroneous estimates of the curve fit (Fig. 6b). One major drawback of the MLCRA is that B_{avail} and $\text{app}K_d$ can be obtained only from multiple tracer injections, which must be performed on separate days and within a time window in which no receptor availability changes or occupancy from previous tracer injections are expected and animal recovery from anesthesia between scans is guaranteed. However, this can lead to inter-day variation of physiological conditions, such as cerebral blood flow and receptor availability states, affecting the quantification accuracy of B_{avail} and $\text{app}K_d$.

For the purpose of avoiding inter-day variations between scans, a multiple-injection simplified reference tissue model (MI-SRTM) was developed to determine B_{avail} and $\text{app}K_d$ from a single PET scan with multiple tracer injections [121, 122]. Radiotracer injections are performed at intervals of 50 min using decreasing molar amounts of raclopride (1.5, 10, and 30 nmol/kg), and data are acquired for 150 min. Quantitative values (R_1 , k_2 , and BP_{ND}) are then estimated using the MI-SRTM. This approach reduces the stress for the animals, the variability, and the overall costs [122].

To further circumvent the problem of multiple tracer injections, Delforge et al. introduced a single-injection approach for the quantification of benzodiazepine receptors

Fig. 6. Binding studies for the separate determination of B_{avail} and $appK_d$. **a** The bound tracer concentration (B) is plotted over the free tracer concentration (F) and results in a hyperbolic saturation curve with asymptote B_{avail} . $appK_d$ is the amount of free ligand, necessary to saturate 50 % of receptors. **b** Linearization of data points to display data by plotting B/F over B , resulting in a straight line with x-axis intercept B_{avail} , y-axis intercept BP , and $appK_d$ being the negative reciprocal of the slope (Scatchard plot) (adapted from [118]).

with [^{11}C]flumazenil [123]. The method relies on the assumption that a dynamic equilibrium occurs during the time course of a PET experiment, resulting from the natural linear decrease in the bound concentration. This approach can be applied to PET tracers with fast kinetics and has already been used in rats [124] and mice [125, 126]. One advantage of partial-saturation over multiple-injection approaches is that the natural decrease in the bound tracer concentration over time leads to a comparably high number of data points, which reduces error proneness in the estimation of B_{avail} and $appK_d$.

However, one general challenge of all approaches is that the radiotracer must be injected at high doses leading to > 30 % and sometimes up to 80 % receptor occupancy, which violates the tracer principle and may bias the quantification due to compensatory changes in the receptor system under investigation. In addition, when a pharmacological challenge is applied, the receptor may be saturated from the endogenous neurotransmitter, leading to low numbers of available receptor sites and, therefore, to high tracer saturation. Moreover, after a pharmacological challenge, the system under investigation may not be in a steady state over the time frame of the PET experiment, and the quantification may therefore be biased. Thus, separate quantification of B_{avail} and $appK_d$ with the methods described above is still very difficult, and results must be interpreted with care.

Quantification of Neurotransmitter Release in Rodents

Dysregulation of neurotransmitter signaling is involved in many neuropsychiatric and neurodegenerative disorders. Currently, the gold standard method for quantifying neurotransmitter changes in the synaptic cleft is *in vivo* microdialysis, which allows the quantification of neurotransmitter variations in the range of 15–20 % from baseline with nanomolar sensitivity. However, in contrast to PET, microdialysis experiments are very invasive, can only be used to measure neurotransmitter changes in distinct areas of the

brain, and have severely limited temporal resolution (one sample every 10 to 20 min). Therefore, *in vivo* PET imaging of neurotransmitter release is increasingly being used to non-invasively quantify neurotransmitter concentration changes in the rodent brain. PET quantification of neurotransmitter changes, however, can be obtained only by indirect methods, as a direct detection of endogenous neurotransmitters is not possible. Two main models to describe the PET signal changes after a pharmacological challenge have been described in the literature: the occupancy model, in which a PET receptor tracer and the neurotransmitter compete for the same binding site on the receptor [127], and the internalization model, in which increased neurotransmitter concentrations lead to agonist-induced receptor internalization at the postsynaptic membrane [128].

Using the high-affinity D2/D3 receptor antagonist PET ligand [^{18}F]fallypride in occupancy studies with increasing amphetamine doses (0.1 to 2.5 mg/kg), a dose-dependent decrease in [^{18}F]fallypride BP_{ND} ranging from 2.4 % to 17.9 % after the release of 38.3 to 434.3 fmol of DA (~1.5- to 17-fold increase) in the striatum was observed [129]. This decrease in [^{18}F]fallypride BP_{ND} followed a hyperbolic saturation curve. Thus, changes in DA release greater than 20-fold will not lead to higher PET signal changes. Similar experiments have been performed for the D2/D3 receptor antagonist PET ligand [^{11}C]raclopride using a RATPET small-animal scanner (spatial resolution of 2.4 mm FWHM) [130]. In microdialysis experiments, Hume et al. observed a 16 % reduction of [^{11}C]raclopride BP_{ND} , which was associated with an approximately 25-fold increase in extracellular DA [131]. A maximum reduction of 21 % in [^{11}C]raclopride BP_{ND} was observed at a high amphetamine concentration of 4 mg/kg, which induces an increase in extracellular DA by approximately 30-fold [132]. However, this change was not significantly different from the one induced by a 2-mg/kg dose. Using a higher-resolution quad-HIDAC system with a spatial resolution of ~1.5 mm FWHM, a maximum reduction of

approximately 30 % in [^{11}C]raclopride BP_{ND} was observed, pointing to a possible impact of scanner spatial resolution and sensitivity [130]. In contrast to the DA concentration, which usually peaks approximately 10 min after intravenous injection, changes in [^{11}C]raclopride BP_{ND} have been shown to persist over several hours, suggesting that receptor internalization is involved in amphetamine-induced changes in radiotracer binding [130, 133]. To distinguish DA receptor occupancy from receptor internalization, Skinbjerg et al. used arrestin-3 knockout mice, which are incapable of internalizing D2 receptors [134]. Four hours after amphetamine administration, radiotracer binding returned to baseline in knockout but not wild-type mice, confirming receptor internalization after DA displacement by amphetamine.

In order to increase the sensitivity of PET radiotracers to DA, agonist radiotracers such as [^{11}C]NPA [135], [^{11}C]PHNO [136], and [^{11}C]MNPA [137] have been developed. These agonist radiotracers bind to the D3 receptor and the high-affinity state of the D2 receptor and show higher sensitivity to extracellular DA changes than the antagonist radiotracer [^{11}C]raclopride in animals and humans [138–140].

In contrast to the dopaminergic system, imaging of other neurotransmitter systems has been more challenging in the past. Regarding the serotonergic system, several promising candidates have been shown to be ineffective for quantifying serotonin (5-HT) release [128]. However, recent studies using PET radiotracers for the 5-HT_{2A} receptor [141] and the 5-HT_{1B} receptor [142] in the pig brain have shown promising results concerning the quantification of 5-HT release. Pharmacological intervention with 0.5 mg/kg fenfluramine, which induces an average six- to eightfold increase in cerebral interstitial 5-HT release compared to baseline in the medial prefrontal cortex [141, 143], resulted in only a 16 % change in [^{11}C]AZ10419369 [142] but a 46 % change in [^{11}C]Cimbi-36 [141]. This means that [^{11}C]Cimbi-36 is over three times more sensitive to 5-HT than [^{11}C]raclopride is to DA. Studies in rats or mice will need to confirm whether it is feasible to use this PET tracer to measure 5-HT release in rodent models of neurological disorders.

In the last few years, quantification of endogenous noradrenaline (NA) levels has increasingly come into focus. Recent studies have focused on PET radiotracers for α 2-adrenoreceptors such as [^{11}C]yohimbine [144] and [^{11}C]ORM-13070 [145]. [^{11}C]Yohimbine, for example, has been shown to be a surrogate marker of NA release in rats [146] and pigs [144] by showing a significant decrease in the V_T in response to amphetamine challenge. For example, acute amphetamine injection of 2 mg/kg induced a BP_{ND} change of ~ 38 % in rats [146]. However, the quantification of α 2-adrenoreceptors and endogenous NA in rodent models with kinetic modeling still remains difficult due to the distribution of the receptors throughout the whole brain, leaving no appropriate reference region.

Adenosine is an extracellular signaling molecule, which is released from neurons and glia cells during inflammation [147] and modulates the release of other neurotransmitters [148]. Adenosine fulfills its function by stimulating G protein-coupled adenosine receptors and shows highest affinity to A₁ and A_{2A} receptors [148]. Selective PET targeting A₁ and A_{2A} receptors have been developed [149–153], but to our knowledge, sensitivity to endogenous adenosine has not been observed *in vivo*. However, in binding assays, using human brain homogenates and CHO cells transfected with the A₁ receptor [^3H]CPFPX was displaced by high adenosine concentrations to the level of non-specific binding [154]. In a recent study, Guo et al. evaluated a partial agonist radiotracer for the adenosine A₁ receptor in rats, which showed good BBB penetration, a high specificity, and subtype selectivity being a promising candidate to measure variations of the endogenous neuromodulator adenosine *in vivo* [155].

As the number of novel PET receptor ligands increases, more promising candidates will become available to quantify other neurotransmitter systems, such as the GABA and glutamate (GLU) systems, in the brain. This will be an enormous gain for future applications of *in vivo* neurotransmitter-release PET imaging.

Animal Models

Recent years have brought rapid development of transgenic and toxin-induced animal models, which provide many insights in the pathophysiology, progression, and treatment of neurological diseases [156–159]. The latest advances in genome engineering methodologies based on the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system provide a novel molecular tool that, *via* knockout, overexpression, and mutation of genes, resemble disease phenotypes with increased accuracy and link specific genes to molecular changes [160–162]. CRISPR/Cas9 expedites the generation of transgenic animals, and several tools are under investigation to optimize the *in vivo* delivery to the brain. Currently, adeno-associated viral (AAV) vectors represent the most tempting approach due to their tissue spreading, stable transgene expression, versatility, mild toxicity, and translational potential [160, 161, 163]. Genetic modifications have been demonstrated by direct injections in the mouse brain [161, 164–166] and recently in the rat brain [167]. Indeed, a novel Huntington disease (HD) mouse model has been generated by AAV-CRISPR striatal injections efficiently targeting and inactivating the mutant Htt allele [168]. Furthermore, this powerful gene-engineering technology expands the possibilities for disease modeling to large animal species such as pigs and non-human primates [169–171], in which gene editing has been challenging until now.

Chemogenetics might be used if a more naturalistic stimulation is preferred to complete protein expression. Indeed, Designer Receptors Exclusively Activated by Designer Drugs

(DREADDs) have been engineered from native receptors to respond solely to a specific ligand, mimicking the endogenous stimulation of a receptor through neurotransmitters. This approach allows for the selective regulation and identification of neuronal circuitry and signals that specify social behaviors [172], addiction [173], and feeding [174–176]. Major drawbacks of the system are the off-target effects on wild-type DA, HIS, and 5-HT receptors arising from the *in vivo* retro-conversion of the DREADD-ligand Clozapine-*N*-Oxide (CNO) to Clozapine [177, 178]. These can be avoided injecting the CNO intracranial [179] or using the compound 21 as DREADD-ligand [180]. Provided with the appropriate control group, which does not express DREADDs but receives the ligand, DREADDs can be considered a powerful strategy to dissect pathways involved in neurodegenerative diseases [181–184]. Recently, DREADD technology has been coupled to [^{18}F]FDG-PET (DREAMM) to map non-invasively the regional or global effects mediated by the DREADD stimulation in targeted cells [185, 186].

Among all neuroimaging modalities, PET offers the highest degree of sensitivity and allows the quantitative evaluation of receptors, transporters, neurotransmitters, and aggregated proteins in the brains of transgenic animals [187, 188]. Imaging of aggregated proteins has been successfully shown in mouse models of AD [189–192]. In contrast to the situation in AD, however, there is not yet an available PET tracer to assess alpha-synuclein pathology in the brains of humans and animal models of PD. Thus, research has focused on pre- and postsynaptic dopaminergic transporters and receptors to distinguish PD from related disorders or to quantify therapeutic effects [193, 194]. Compensatory mechanisms in PD, which occur to counterbalance the dopaminergic loss, can be observed with PET before the symptoms occur. Indeed, decreased binding of presynaptic markers such as [^{11}C]methylphenidate, [^{18}F]DOPA, [^{11}C]CFT, and [^{11}C]DTBZ and increased [^{11}C]raclopride binding to postsynaptic D2/D3 receptors have been reported in asymptomatic toxin-lesioned [195, 196] and transgenic rats [197].

[^{18}F]FDG and [^{11}C]raclopride PET highlights pathological alterations before the onset of clinical symptoms in HD [198, 199]. Toxin-induced HD rodent models have been extensively characterized by PET, showing reductions in DA D2 and adenosine 2A receptor occupancy [153, 200]. Conversely, no PET *in vivo* imaging studies have been performed to date in preclinical rodent models of schizophrenia, with only one recent imaging publication using fMRI [201]. With several reports showing that the GLU excitatory neurotransmission, *via* NMDA receptor activation, contributes to schizophrenia [202], PET imaging represents a major research area in this field and has very likely a huge potential for clinical translation.

PET imaging plays also a key role in evaluating the efficacy of gene therapy, which currently constitutes one of the most appealing treatment approaches [203, 204]. Hence, PET radionuclides coupled to reporter genes have been successfully developed to monitor gene expression over time [205–208].

Application of Simultaneous PET/MRI to Investigate Neurotransmitter-Receptor Interactions

A major strength of multimodal imaging is the concerted application of a variety of imaging methods to measure similar or distinguished biochemical and morphological states. The combination of PET and MRI (PET/MRI) devices was proposed in the 1990 [209–211]; however, fully elaborated small-animal systems [212, 213] were developed 10 years later. Historically, the development of multimodal imaging strategies originates from the need to match functional and morphological information. Due to the availability of valid morphological rodent brain atlases and the fact that the brain is well embedded in the skull, simultaneous data acquisition may not always need to study static receptor availability. However, if functional processes change dynamically during the acquisition, for example, as a result of interventions altering brain function, simultaneous acquisitions can be inevitable for obtaining complementary information of both imaging modalities [214].

In a preclinical setting, this was first demonstrated by Wehrl et al. [214], where our group reported a simultaneous PET/MRI study of brain function in response to a whisker stimulation in rats. Here, [^{18}F]FDG-PET was applied to trace changes in glucose metabolism on a slow time scale, while functional MRI (fMRI) simultaneously assessed fast vascular and oxygenation changes. Based on this multifunctional dataset, Wehrl et al. reported spatial and quantitative discrepancies between the PET and the fMRI activation profiles, revealing comprehensive and complementary information of both modalities.

The unique benefit of simultaneous PET/MRI with regard to functional neuroimaging is the high temporal correlation of data revealed from both imaging modalities. This has a variety of advantages, since functional neurotransmitter processes can be monitored at the same time using PET and fMRI techniques to investigate multiple stages of neurotransmitter-receptor plasticity [215] (Fig. 7). Thus, PET/MRI is capable of monitoring *in vivo* processes on various levels, *e.g.*, brain activation can be monitored using the blood oxygen level-dependent (BOLD) effect with fMRI while changes in receptor plasticity or blood flow are being monitored by PET [216, 217]. A simultaneous PET/MRI study in non-human primates by Sander et al. [217] investigated the relationship between changes in D2/D3 DA receptor occupancy measured by PET and changes in brain activity obtained by fMRI. Here, similar temporal profiles were observed for specific [^{11}C]raclopride binding estimates and changes in cerebral blood volume inferred by fMRI. Overall, this multifunctional dataset demonstrated the utility of simultaneous PET/fMRI to correlate neurochemical profiles with hemodynamic changes, paving the way for *in vivo* studies of different receptor systems in the healthy and diseased rodent brain.

Fig. 7. Schematic illustration of mapping neuronal activity using simultaneous PET/BOLD fMRI. After a stimulus is applied, the neuronal response can be obtained from changes in receptor occupancy levels using PET imaging of synaptic neurotransmitter release. Images show changes in [^{11}C]raclopride PET signal, a D2/D3 receptor antagonist, in the striatum after pharmacological stimulation with the dopamine-releasing drug amphetamine in one rat. The hemodynamic response of the same animal is obtained at the same time from changes in cerebral blood flow, blood volume, and blood oxygenation using blood oxygen level-dependent (BOLD) fMRI contrasts. *T*-map (baseline versus stimulation) is shown in the right image after a pharmacological stimulation with D-amphetamine.

However, from a methodological point of view, simultaneous imaging might be limited by the fact that PET tracers have certain uptake characteristics that often make direct, truly simultaneous acquisition difficult. Furthermore, the cost and space requirements of simultaneous PET/MRI systems need to be considered. Nevertheless, simultaneous PET/MRI holds great potential to further decode brain function on multiparametric levels, which might directly contribute to the investigation of neurological pathologies, especially in the field of neurodegenerative and psychological disorders.

Quantitative Receptor Imaging in Clinical Translation

The technological advancement of preclinical systems and the development of specialized neurotracers have positively translated into the clinical field. However, in order to successfully translate these findings into the human scenario, it is necessary to maintain constant scrutiny of experimental conditions in humans and animal experiments in order to accurately progress in the translational field. The clinical neuroimaging field has advanced substantially since the implementation of [^{18}F]FDG for evaluation of glucose metabolism in the brain [218–220]. Over 5000 PET/CT systems have been installed worldwide, which will likely lead to implementation of more experimental tracers and hopefully help the development of personalized medicine. The field has been further boosted by an increased number of clinical PET/MRI systems, including until recently a

whole-body human PET system. This has motivated multidisciplinary groups composed of radiochemists, neurobiologists, and neuroimaging scientists in the preclinical and clinical areas to focus on “bench-to-bedside” evaluations of specific receptor subtypes in multiple tissues and diseases. Thus, clinical neuroimaging has certainly matured due to these technological milestones and the synergistic advances of these fields.

Beyond the glucose analog [^{18}F]FDG, there is a large number of dedicated neuroimaging tracers in the preclinical pipeline that have been translated into Phase I clinical trials. There have been remarkable increments in the application of radiotracers in the fields of clinical neuroscience, neurology, psychology, and psychiatry in the last 10 years. However, it is still seldom the case that a PET tracer reaches financial stability and enters clinical diagnostic routine. Here, we mention several examples of different targets that have translated, reaching clinical routine and others that have made it to the experimental human setting. After years of preclinical development, [^{18}F]florbetaben, [^{18}F]florbetapir, and [^{18}F]flutemetamol were approved by the U.S. Food and Drug Administration and the European Medicines Agency to evaluate β -amyloid neuritic plaque density in patients with cognitive impairment [221–223]. Also, recently, [^{18}F]MK-6240, which was designed for quantifying the brain burden of neurofibrillary tangle (NFT) pathology and originally evaluated in brains of non-human primates [224], has also completed Phase I of clinical trials. It has consistently shown increased uptake in the regions associated with NFT deposition in AD [225]. Other radiotracers have been

developed and characterized in animal models but have taken a significant amount of time to reach human experiments.

Tracers synthesized for neuroimaging have a wide range of usages, *e.g.*, specifically targeting neurotransmitter synthesis, receptor, release or transport, BBB permeability, or neuroinflammation to measure enzymatic activity and general ligand availability [226]. Some tracers are better known than others. For instance, due to the early production availability in radiopharmacies of DA D2 receptor tracers such as [^{11}C]raclopride and later [^{18}F]fallypride for D2/3 receptors, there is a relatively robust understanding of the dopaminergic system [227]. This knowledge comes to a very large extent from preclinical evaluations performed in rodents [12, 82, 228, 229].

Some other tracers have allowed a good understanding of the physiological roles of important receptor families, such as the 5-HT receptors. Plenty of work has been performed preclinically in order to characterize this family of receptors, which has slowly translated into clinical experiments. It has been 10 years since [^{11}C]SB207145 was first shown in the brains of pigs *in vivo* [230]. Recently, it has been revealed that reduced binding to the cerebral 5-HT₄R shows a positive correlation with aggressive behavior in men [231]. Likewise, [^{11}C]AZ10419369, originally characterized in non-human primates [232], has also recently been used to find a positive correlation between reduced binding of 5-HT_{1B} and the time since the last migraine onset [233]. GLU tracers have shown reduced availability of the type 1 metabotropic GLU receptor (mGluR1) in patients with hereditary and sporadic cerebellar ataxia, which now is proposed as a specific imaging biomarker of the disease [234]. A tracer targeting the κ -opioid receptor was evaluated in non-human primates for the first time, potentially providing a novel tool to investigate this system in humans [235]. Another example is the 18-kDa translocator protein (TSPO) tracer, which has been used to provide preliminary imaging data on neuroinflammation in non-human primates [236] and in patients [237, 238]. Overall, a wide range of neurotransmitter systems, neuropsychological diseases associated with neurotransmitter imbalance, and brain-specific targets can be studied using PET, providing an increasingly detailed understanding of the normal and pathological human brain. However, in order to ensure the congruent translation of these receptor availabilities between species, we must attempt to close the gap between the experimental conditions between humans and animals.

A major complication for translation in preclinical studies is the use of anesthetics. Anesthetics have effects not only in vasodilation or perfusion [239] but also on receptor binding in the brain [240–242]. Animal brain receptor experiments are often performed under anesthesia due to the difficulty or impossibility of training an animal to remain immobile during the scan. This mismatch between preclinical and clinical experiments can potentially lead to a high range of uncertainty in translational studies. In order to circumvent

this challenge, new technologies have been developed in the past years to evaluate animals in an awake state.

Takuwa et al. [243] developed a system for imaging an awake mouse by restraining the cranium onto an acrylic head-holder, while the animal was able to move its legs freely on a Styrofoam ball. This innovative approach effectively showed that there were significant differences in the binding potential of [^{11}C]raclopride in free-walking awake mice, in comparison to isoflurane-anesthetized mice and whole-body-restrained awake mice. Another method was developed by Miranda et al. in 2017 [244], consisting on the tracking of a rodent head by attaching four-point sources onto it, in combination with a complex reconstruction algorithm. The animal was placed on a cylindrical transparent tube and allowed move freely in the container during the scan. The approach was further validated using mice, where the authors found that mementine led to a 2.6 larger [^{18}F]FDG uptake on awake animals in comparison to anesthetized animals [245]. Kyme et al. [246] further investigated the field of awake-animal imaging by using an automated motion-tracking robot in combination with motion correction approaches and placing a visual marker on the animal's head. The robot performed accurate and responsive movements in order to real-time correct the animal's alignment to the center of the FOV. In these experiments, open-field observations were also annotated after the administration of amphetamine, which was successfully corrected for motion artifacts. The behavioral data was obtained simultaneously to the PET acquisition which provided congruent real-time information. Altogether, the field of awake-animal scanning has the potential to modify many receptor tracer paradigms and should be followed closely. The novel techniques will likely further develop the field of animal brain imaging and help reduce the incongruences between animal and human data. Before PET tracers can be routinely applied in clinical diagnostics, convergence of the results among different human trials is required. To improve this translational process, clinical neuroreceptor imaging studies must attempt to meet the standards of experimental design that the preclinical field proposes. Clinical research is inevitably less standardized than the controlled environment of the preclinical field, leaving plenty of work for translational scientists. This is exemplified by a recent meta-analysis of human schizophrenia studies performed with [^{11}C]flumazenil, a tracer targeting GABA_A receptors, which showed inconsistencies in reduced brain region uptake among the studies [247]. The authors remark that these results contrast with those of preclinical studies, which suggest that benzodiazepines can help prevent the neuroanatomical and neurophysiological dysfunctions related to schizophrenia [247]. Of course, there are inherent differences between species, but the variance among human studies suggests inconsistency of experimental design in the clinic. Likewise, preclinical scientists must consciously improve methods and implement more accurate animal models in order to have a

close pathophysiological resemblance to the human disease and obtain translatable results.

Conclusion and Outlook

The clinical application of tracers that have been translated to the clinical field from preclinical laboratories shows that preclinical research can deliver clinical solutions. Despite some physical limitations inherent to PET imaging, it is now possible to quantify receptor-binding parameters in laboratory animals in a reproducible and reliable manner. An absolute quantification in rats and mice is difficult, but BP changes larger than 10 % can be reproducibly quantified *in vivo* in the picomolar range in large brain regions with high receptor availability, such as the striatum, cortex, and thalamus [12, 13]. One crucial point in rodent imaging studies is the standardization of the experimental protocols and analysis [248]. Different evaluation and analysis methods of the same data can lead to very different results, explaining the large discrepancies among results and often rendering it very difficult to compare results from different laboratories. For this reason, future PET studies should apply strict standardization not only to the study conditions, including protocols, image reconstruction, and correction methods (Table 1), type of anesthesia, and injected tracer dose, but also to the analysis methods to improve the comparability of the performed experiments.

A major gap between preclinical imaging research and clinical translation is the required anesthesia for PET and MR preclinical imaging studies. Changes in anesthetic states can influence imaging outcomes, especially if multimodal functional and molecular information are acquired [249]. The same applies to the role of body temperature stabilization. Indeed, changes in body temperature can influence cerebral blood flow and, hence, the results obtained in PET and MR functional measurements [250]. For this reason, animal PET scanners for freely moving animals are under development to reduce the influence of different anesthesia regimes.

Although the use of PET alone does not allow the absolute quantification of receptor and neurotransmitter changes, its application in combination with other *ex vivo* methods, such as immunohistochemistry, autoradiography, HPLC and microdialysis, has provided an enormous gain in scientific knowledge. In addition, it allows longitudinal *in vivo* investigation in the same subject, thus reducing the number of experimental animals involved in the study, increasing the statistical reliability of the data, and supporting the translation of preclinical results into clinical applications [251, 252].

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflicts of interest.

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