PET as a Translational Tool in Drug Development for Neuroscience Compounds

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In central nervous system drug discovery programs, early development of new chemical entities (NCEs) requires a multidisciplinary strategy and a translational approach to obtain proof of distribution, proof of occupancy, and proof of function in specific brain circuits. Positron emission tomography (PET) provides a way to assess in vivo the brain distribution of NCEs and their binding to the target of interest, provided that radiolabeling of the NCE is possible or that a suitable radioligand is available. PET is therefore a key tool for early phases of drug discovery programs. This review will summarize the main applications of PET in early drug development and discuss the usefulness of PET microdosing studies performed with direct labelling of the NCE and PET occupancy studies. The purpose of this review is also to propose an alignment of the nomenclatures used by drug metabolism and pharmacokinetic scientists and PET imaging scientists to indicate key pharmacokinetic parameters and to provide guidance in the performance and interpretation of PET studies.

Positron emission tomography (PET) is a molecular imaging technique widely used to measure the distribution of radiolabeled compounds, as well as functional parameters (e.g., blood flow and glucose metabolism), and the availability of different biological targets (e.g., receptors and enzymes). PET is a key experimental tool used in neuroscience drug discovery and development for assessment of exposure of new chemical entities (NCEs) in the central nervous system (CNS) and for quantitative assessment of target occupancy. The quantitative properties of PET and the ability to assess biological functions are also applied in other therapeutic areas. For instance, whole-body hybrid PET imaging combined with computed tomography and/or magnetic resonance is used for the diagnosis and staging of solid tumors, and the development of new radioligands for specific targets (e.g., [68Ge]DOTATOC or [68Ge]DOTATATE for neuroendocrine tumors or [68Ge]PSMA for prostate cancer) has enabled the evaluation of tumors that would be otherwise difficult to access.1-3

The importance of PET in CNS drug development was highlighted by Morgan et al.,4 where the term “three pillars of survival” was coined based on a systematic evaluation of phase II failures, uncovering that in almost 50% of the drug development programs investigated it was not possible to conclude whether the drug mechanism had been tested adequately due to unknown target site, drug exposure or target occupancy.4 The three pillars have undoubtedly been implemented in many pharma companies. In this context, it will be called (i) proof of distribution; (ii) proof of occupancy, and (iii) proof of function. The last term can be further subdivided into proof of mechanism, proof of principle, etc. However, for our purpose proof of function indicates exposure of the drug at the site of action as well as target occupancy, which leads to engagement of the target and downstream functional effect.

PET radioligands developed for binding to specific targets associated with CNS disorders are developed as biomarkers for disease diagnosis, patient stratification, and assessment of disease progression (Figure 1). One example is the development of amyloid and tau PET radioligands for in vivo identification of Alzheimer pathology, Braak staging (tau), and assessment of a biological effect of a compound (reduction of amyloid plaques or tau accumulation).5

A comprehensive evaluation of the different applications of PET in drug development is beyond the scope of this review. Therefore, this review will focus on the application of PET in relation to the translatability of NCEs from experimental animals to humans and not on the application of PET for disease diagnosis, progression, etc. (Figure 1). For CNS drugs, brain exposure and target occupancy are key parameters that influence decision making in early drug development programs and are considered two important pillars for survival4 (Table 1). PET studies intended to measure brain exposure are part of the development plan and characterization of NCEs, which requires continuous interaction between PET and drug metabolism and pharmacokinetic (DMPK) experts. Key pharmacokinetic (PK) parameters of NCEs are often described differently in publications that present PET or DMPK studies. In addition, PET occupancy studies are keys not only to define target engagement but also to enable decisions on therapeutic doses applied in early drug development studies. Overall, this review aims to provide an update on the application of PET in early drug development neuroscience programs with a specific focus on the assessment of drug brain exposure and target occupancy, as well as to propose an alignment of the nomenclatures used in PET and DMPK.

CONCEPT OF MICRODOSING STUDIES BY RADIOLABELING OF DRUG MOLECULES

Brain exposure and distribution of an NCE can be evaluated with direct radiolabeling of the compound with a short-lived...
radioactive isotope, such as $^{11}$C (half-life ~ 20 minutes) or $^{18}$F (half-life ~ 2 hours). The possibility to perform such studies is dependent on whether such isotopes can be installed late in the synthesis of the target molecule by radiosynthesis and in practice often as the last step. This process is not always possible for many drug molecules and needs to be evaluated for each individual drug candidate. For instance, only some molecules can be labeled with $^{18}$F because fluorine is not part of all drug molecules. In other cases, the radiochemistry may not be feasible for either $^{11}$C or $^{18}$F.

If successful radiolabeling of the drug candidate can be achieved, this can be used for PK and distribution studies in animals and humans. This type of approach is referred to as microdosing, because it consists of the administration of a negligible amount of the radiolabeled compound, without any pharmacological effect. According to the European Medicines Agency (EMA) guidelines, a dose is considered a microdose when it is < 100 micrograms. In PET studies, the amount of radiolabeled compound in most cases is < 10 micrograms. This method is typically used in early drug development when a PET radioligand for the target of interest is not available for direct assessment of target occupancy. In such cases, the assessment of brain distribution is an important parameter that can be measured in vivo (proof of distribution). Under the assumption that the in vitro and in vivo affinity ($K_i$) are the same, the microdosing approach can also be used to estimate the occupancy of the drug to the target.

The microdosing PET studies are typically validated preclinically, by examining the kinetic and distributional properties of the radiolabeled drug in experimental animals, such as rats, pigs, and non-human primates (NHPs), using both invasive methods and PET to build confidence in extrapolating the findings into the clinic. This translational phase is key to advance the program to phase 0/first-in-human (FIH) studies. The confirmation of sufficient brain exposure in humans is an important stage gate for the decision to advance the program into the next phases of development.

These microdosing studies can also be performed in combination with the administration of a pharmacological dose of the compound. The purpose with this approach is to examine whether the uptake of the radiolabeled compound is influenced by, for example, the activity of transporters located on the blood-brain barrier (BBB) and/or to make sure that only the

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**Figure 1.** Positron emission tomography (PET) can be applied for different purposes, in relation to the characterization of the drug or to the study of different aspects of the disease. This review will focus on the methods/endpoints highlighted in the right-hand side of the circle.

**Table 1.** Three pillars of survival of new chemical entities, according to (Morgan, P. et al., Drug Discovery Today 2012, 17, 419–424)

| Animal | Human |
|--------|-------|
| Exposure at site of action (brain exposure) | Bioanalysis vs. PET labeling of drug candidate | PET labeling of drug candidate |
| Binding to target (occupancy) | PET study using target specific PET ligand | PET study using target specific PET ligand |
| Pharmacodynamic effect$^a$ | Not discussed here | Partially discussed here |

PET, positron emission tomography.

$^a$In the original publication, this pillar was referred to as “Expression of pharmacology.” However, it has been changed to “Pharmacodynamic effect” for alignment with the content of the Review.
nondisplaceable signal of the radiolabeled compound is used for measuring the ratio of brain to plasma. The evaluation of the brain distribution of the muscarinic M_1 positive allosteric modulator [^{11}C]GSK1034702 in the living human brain is a good example of using PET microdosing to demonstrate brain uptake and blood-brain barrier (BBB) passage consistent with passive diffusion or active influx, providing information to progress the molecule into the next stage of clinical development. Another example comes from the observation that [^{11}C]osimertinib, a tyrosine kinase inhibitor of the mutated epidermal growth factor receptor, that has shown clinical efficacy in the treatment of brain metastasis from non-small cell lung cancer, displayed favorable PK profile in healthy volunteers, with rapid brain distribution and higher uptake in grey than in white matter. PET microdosing studies can also be performed with peripheral drug molecules to demonstrate limited brain availability, avoiding potential side effects on the CNS, that can be important for progressing molecules for peripheral indications. Such a study was done in NHPs with the cannabinoid receptor agonist AZD1940 that was confirmed to have limited brain availability.

**CONCEPT OF TARGET OCCUPANCY STUDIES**

Target occupancy is the other key parameter that influences the chance of clinical success. The proof that the compound interacts with the target of interest following drug administration (proof of occupancy) is needed in order to establish a link between target occupancy and pharmacological effect in vivo. Preclinically, these pharmacodynamic (PD) studies are often run in parallel with the occupancy studies that can be performed in experimental animals using both invasive ex vivo binding methods and noninvasive PET. The translational value of this approach with initial occupancy studies in experimental animals (rodents, pigs, or NHPs) followed by confirmatory studies in human subjects is the ideal de-risking strategy to ensure that the drug has been tested at an adequate dose/exposure level in relation to the project hypothesis. This approach is described later in this article for the multimodal drug molecules to demonstrate limited brain availability, PET microdosing studies can also be performed with peripheral drug molecules to demonstrate limited brain availability, avoiding potential side effects on the CNS, that can be important for progressing molecules for peripheral indications. Such a study was done in NHPs with the cannabinoid receptor agonist AZD1940 that was confirmed to have limited brain availability.

**DMPK PARAMETERS, PET OUTCOME MEASURES, AND METHODOLOGICAL CONSIDERATIONS**

The nomenclature used by DMPK scientists to refer to parameters that characterize CNS compounds is different from the nomenclature used by PET scientists. Because the terminology is different depending on the context, it is important to align the DMPK and PET nomenclature (Table 2).

Table 2: Definition of the key PK parameters of CNS candidate drugs according to the DMPK and PET nomenclatures

| Parameter | DMPK | PET | Calculation |
|-----------|------|-----|-------------|
| Total concentration of drug in the brain | C\text{brain} | C\text{T} | |
| Free fraction of drug in the brain | f\text{u,brain} or f\text{u,b} | f\text{ND} | |
| Free concentration of drug in the brain | C\text{u,brain} | C\text{FT} | C\text{brain} · f\text{u,brain} |
| Concentration of drug in the plasma | C\text{plasma} | C\text{P} | |
| Free fraction of drug in plasma | f\text{plasma} | f\text{P} | |
| Free concentration of drug in plasma | C\text{u,plasma} | C\text{FP} | C\text{plasma} · f\text{plasma} |
| Brain to plasma ratio of the drug | K\text{p} | V\text{ND} | C\text{brain}/ C\text{plasma} |
| Ratio of free concentration of drug in brain to free concentration of drug in plasma | K_{p,uu} | C_{P}/C_{FP} | f\text{u,brain}/ f\text{plasma} · K\text{p} |

CNS, central nervous system; DMPK, drug metabolism and pharmacokinetics; PET, positron emission tomography; PK, pharmacokinetic.

f\text{u,brain} can be measured using brain homogenates (V\text{u,brain}) or on brain slices (V\text{u,brain}).
transfer of the tracer from plasma to brain (mL · min⁻¹ · g⁻¹) and \( k_2 \) is the rate of transfer from brain to plasma (min⁻¹).

In most of the cases, though, the tracer does not equilibrate rapidly, and the brain uptake and washout can then often be described by a two-compartment model. In this case, \( V_T = K_1 / k_2 \left( 1 + k_3 / k_4 \right) \). In this model, two additional rate constants describing the transfer of the molecule from a fast equilibrating to a slow equilibrating compartment (\( k_3, \text{min}^{-1} \)) and vice versa (\( k_4, \text{min}^{-1} \)) are required to be able to fit the data. It is important to clarify that \( k_3 \) and \( k_4 \) are conventionally used in PET quantification to describe the transfer of a radioligand from the nondisplaceable to the specific compartment (\( k_3 \)) and vice versa (\( k_4 \)). According to the PET nomenclature, the concentrations in the two compartments are referred to as \( C_{ND} \) and \( C_S \).

Therefore, the nondisplaceable distribution volume of the tracer is \( V_{ND} = C_{ND} / C_p \) and the specific distribution volume is \( V_S = C_S / C_p \). \( V_{ND} \) can be estimated from the measurement of the brain radioactivity of the tracer in a region of the brain devoid of receptors (e.g., cerebellum for the D₂/D₃ ligand \([11C]\text{raclopride}\), which is referred to as the reference region.

Because \( V_{ND} \) represents the ratio at equilibrium between the free and nonspecific concentration in the brain and the concentration of the compound in plasma, \( V_{ND} \) is the distribution volume that most appropriately represent \( K_p \). If specific binding is negligible under the microdosing conditions and no regional differences in brain distribution of the radiolabeled compound are observed from the analysis of the brain PET data, \( V_T \) can be considered as \( V_{ND} \) and equivalent to \( K_p \) (Figure 3). If specific binding can be detected in the brain, a possible approach to estimate \( V_{ND} \) is to block the binding of the radiolabeled compound with a pharmacological dose of the cold compound (Figure 3). In case of drugs, such as selegiline (MAO-B inhibitor) and doxepin (H₁ receptor antagonist), the experiments performed with pharmacological doses of the cold compounds showed that the binding of the radiolabeled drug was blocked by the concomitant administration of the cold drug, indicating the presence of specific binding. Because both selegiline and doxepin have been developed as MAO-B and H₁ receptor radioligands, the results obtained after co-administration of the cold drug are not surprising, but are relevant to indicate that the co-administration of the cold drug can be important to demonstrate the presence of specific binding and for obtaining \( V_{ND} \) that can be used to estimate \( K_p \).

It is not always possible to estimate the distribution volume in the brain using standard compartmental modeling. In such cases, an alternative approach is to use the ratio of the area under the curve of the brain (AUCbrain) to the metabolite corrected plasma (AUCplasma). Although the ratio AUCbrain/AUCplasma can be used as a proxy of \( V_T(V_{ND}) \), it is important to clarify that the ratio consistently underestimates \( V_T(V_{ND}) \).

### Radioactive metabolites

One important aspect to consider for the quantification of data from PET studies is whether there are brain penetrant metabolites formed during the experimental investigations that will complicate the quantification of \( V_T \). In the early phase of drug development, a comprehensive understanding of metabolites across species is seldom available, but in vivo preclinical studies together with in vitro metabolite profiling in liver microsomes and/or hepatocytes across species will give some valuable information about the potential risk. Combining such in vitro and in vivo studies with metabolic soft-spot analysis using high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) can provide early indications of likely major metabolites. In general, radiometabolites that are more lipophilic than the parent compound and that are formed during the time window of the PET study are more likely to enter the brain and affect PET quantification. Less lipophilic radiometabolites may have less chance to cross the BBB, although this cannot be excluded unless metabolite analysis is conducted on plasma and brain homogenates after the administration of the NCE (e.g., in rodents).

In case of radioligands, such as \([11C]\text{PE2I (DAT)}, [11C]\text{PBB3 (tau), and [11C]SMW139 (P2X7R)}, \) radiometabolite peaks that were less lipophilic than the parent compounds were observed from the analysis of the plasma samples. The same radiometabolite peaks were also observed from the analysis \emph{ex vivo} of brain extracts. In PET radioligand development and microdosing studies, the extent to which brain penetrant metabolites affect the estimation of the distribution volume of the parent will depend on the molecular structure of the metabolite that can help predicting the relative abundance of the metabolite in the brain. The identification of the structure of the radiometabolites requires the use of HPLC-MS/MS. The synthesis of the metabolite might be needed to confirm the identity of the metabolite from the analysis. A recent review has summarized the main findings of studies on the radiometabolism of well-known PET ligands. Ex vivo experiments in rodents using carrier-added PET radioligands (i.e., the unlabeled ligand in concentrations above

| DMPK \emph{ex vivo} biodistribution | Pre-clinic | Clinic |
|---|---|---|
| | \( K_p \) | \( V_T \) |

| \emph{PET in vivo} microdose biodistribution | | \( V_T \) |
|---|---|---|
| | | |
microdoses is added to the radioligand formulation) might be helpful to elucidate which radioactive metabolites are present in the brain.20

In case of potential brain penetrant metabolites of an NCE, the generation of radioactive metabolites will depend on the route of metabolism and the position of the radioisotope in the NCE. The two different positions of labeling the 5-HT1A receptor antagonist PET ligand WAY-100,635 illustrates the principle that the labeling of the molecule in the position that generates the more hydrophilic and non-brain penetrant radioactive metabolite leads to the cleanest brain signal of the parent.22

In case potentially brain-penetrating radiolabeled metabolites are generated, an indirect way to assess the presence of potential radioactive metabolites is to examine the time stability of V_T.23

In general, the contribution of brain penetrant radioactive metabolites is more relevant toward the later part of the dynamic PET measurement, in which the contribution of the parent radioligand to the total PET signal is lower. If the estimate of V_T progressively decreases by reducing the duration of image analysis, this pattern might be indicative of potential brain penetrant radioactive metabolite(s). Methods for estimation of distribution volume of the parent in presence of a brain penetrant radioactive metabolite are not used conventionally and have been described for radioligands such as [123I]epidepride24 and [11C]PBB3,25 based on the experimental evidence of the presence of lipophilic metabolites in the brain.

**ASSESSMENT OF BRAIN EXPOSURE BY MICRODOSING**

The main interest from a neuroscience drug development perspective is to assess the extent of brain penetration in quantitative terms according to the free drug hypothesis.26 In DMPK, the relevant distribution parameter is referred to as K_p,uu, which is the ratio between the free drug concentration in the brain to the free drug concentration in the plasma.27

In preclinical species, the unbound concentrations are typically derived from the measurement of total concentrations in vivo multiplied by the free fraction (f_u) obtained in vitro in the brain and plasma. The accuracy of the measurement of f_u in the brain and plasma is critical for the calculation of K_p,uu and this can be challenging for compounds that are highly bound.

Generally, the free fraction of the compound in the brain, f_u(brain) (DMPK) or f_uND (PET) is measured through in vitro experiments in brain homogenates (f_u(brain)) or brain slices (V_u(brain)) from rodents and is assumed to be similar across species.8,28 The free fraction of the compound in plasma is measured through the ultrafiltration method for the radiolabeled compound and with the equilibrium dialysis method for the non-radiolabeled compound. This latter measurement is considered most accurate, because the ultrafiltration method
is sensitive to the physicochemical properties of the compound and the recovery from the filter, which might be low for lipophilic compounds.

The free concentration in the brain (C_{FT}) and the free concentration in the plasma (C_{FP}) are assumed to be equal if a compound only distributes in the brain by passive diffusion.\textsuperscript{13,17} In this case, C_{FT}/C_{FP} = 1, which is the general assumption for PET radioligand quantification (similar to K_{p,u} of 1 in DMPK nomenclature). In case of compounds developed as drugs, C_{FT}/C_{FP} can be < 1 in case the molecule is substrate for BBB efflux transporters, such as P-glycoprotein (P-gP) or breast cancer resistance protein (BCRP), or > 1 in case the passage of the molecule to the brain is mediated by active transport.\textsuperscript{17}

According to the standard PET nomenclature:

\[
C_{FT}/C_{FP} = f_{ND} \cdot C_{ND}/f_{P} \cdot C_{P}
\]  

In the general case, because V_{ND} = C_{ND}/C_{P}

\[
C_{FT}/C_{FP} = f_{ND}/f_{P} \cdot V_{ND}
\]

Therefore, K_{p,u} can be calculated as:

\[
K_{p,u} = C_{FT}/C_{FP} = f_{ND}/f_{P} \cdot V_{ND}
\]

if f_{ND} and f_{P} can be measured reliably and V_{ND} can be estimated by compartmental modeling.\textsuperscript{17}

In case of passive diffusion:

\[
C_{T}/C_{P} = f_{P}/f_{ND}
\]

Therefore,

\[
V_{ND} = f_{P}/f_{ND}
\]

For the equivalence of the parameters used in DMPK and PET, see Table 2.

The estimation of K_{p,u} is key to make decisions on advancing the early development plan. There is no established threshold of K_{p,u} and the decision to progress a potential drug candidate is based on project specific parameters, linked to the overall feasibility of the target, drug, and disease for a given program.

In a study assessing the K_{p,u} measured in rats, only 7 out of 57 marketed CNS drugs had K_{p,u} < 0.3.\textsuperscript{29} In a microdosing PET study in NHPs, the K_{p,u} of 10 reference CNS drugs, calculated using the free fraction measured on brain slices (V_{u,brain}), ranged from 0.42 (caffeine) to 4.8 (clomipramine).\textsuperscript{16}

The measurement of f_{ND} or V_{ND} is a critical element for the calculation of C_{ND}/C_{P}. The C_{ND} has been shown to be the parameter that best predicts the D_{2} receptor occupancy by established antipsychotics and is superior to the concentration of the drug in the cerebral spinal fluid or in the blood.\textsuperscript{30} The ability of C_{ND} to predict target occupancy has also been observed in studies evaluating the relationship between occupancy and PK parameters of inhibitors of dopamine and serotonin transporters.\textsuperscript{31,32} In those studies, the animals were given drug and \textsuperscript{3}H-labelled radioligands in vivo, but the target occupancy by the drugs was measured after the animals were killed by decapitation. The brains were quickly removed, and the drug occupancy was either determined using counts from target specific homogenates or by using brain sections and autoradiography. The C_{ND} was measured from brain homogenates and f_{ND} was measured with the dialysis method. The relationship between C_{ND} and target occupancy was evaluated by normalizing C_{ND} by the in vitro K_{i}. The in vitro K_{i} was measured with classical competition experiments with \textsuperscript{3}H-labelled tracers using brain homogenates.

To our knowledge, a similar approach using in vivo measurement of receptor occupancy with PET instead of ex vivo using, for example, autoradiography has not been reported. From a theoretical standpoint, if C_{ND} and K_{i} are available, receptor occupancy can be estimated as: RO\% = C_{ND} 100 / (K_{i} + C_{ND}).\textsuperscript{8,9} This calculation can be used to make predictions on the potential activity of the drug in early development phase. However, in vivo measurement of receptor occupancy with PET is still the preferred method, if a PET radioligand is available, to advance early candidates to FIH studies (see section on receptor occupancy).

### ASSESSMENT OF TARGET OCCUPANCY

The possibility to perform occupancy studies relies on the availability of a suitable PET radioligand. For some brain targets, such radioligands exist but for most new drug targets this is not the case. Therefore, a key aspect to consider in new drug project is whether an imaging ligand exists for target occupancy studies and whether there is need for further qualification of a radioligand to be used for both preclinical as well as clinical studies. If an imaging ligand does not exist, it is important to start the search as early as possible and optimally in parallel with the discovery of the drug candidate. In practice, this means that the medicinal chemists will initiate both hit-to lead and hit-to imaging ligand programs in parallel. However, as drug molecules and successful PET ligands often display different molecular properties, this is two parallel tracks in the medicinal chemistry program.

A comprehensive discussion of the optimal properties of a PET radioligand is beyond the scope of this review, but the process includes the measurement of in vitro target affinity across species, selectivity, and the establishment of the quantification and test-retest properties of the radioligand in vivo in experimental animals and in human subjects. Overall, the identification of a successful clinical PET ligand for a new target is a major endeavor.

The outcome measure used to calculate target occupancy in case there is a reference region in the brain, is BP_{ND}, which is calculated from the distribution volumes or estimated using reference region models (Table 3). In the general case, when no reference region is present, V_{T} is the outcome measure of choice. In this case, the revised Lassen plot\textsuperscript{13} is the method used to derive an occupancy measure.

The revised Lassen plot uses a linear regression analysis approach, in which the difference between estimates of V_{T} Post drug (V_{T, post}) and V_{T} at baseline (V_{T, baseline}) obtained across several brain regions is plotted against V_{T, baseline}. The method is applied under the assumption that V_{T, baseline} is the same across regions, as well as the fractional occupancy (\%) at the given target. The
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Table 3 PET outcome measures for calculation or estimation of target occupancy

| Outcome measure | Definition | Rate constants | Distribution volumes | Occupancy calculation |
|-----------------|-----------|----------------|----------------------|-----------------------|
| $BP_{ND}$ (specific to nondisplaceable binding potential) | $B_{max}/K_{D} \cdot f_{ND}$ | $k_{3}/k_{4}$ | $V_{T}/V_{ND} \approx 1$ | $BP_{ND \text{ baseline}} = BP_{ND \text{ drug}} / BP_{ND \text{ baseline}}$ |
| $V_{T}$ | $V_{S}+V_{ND}$ | $K_{1}/k_{2}(1+k_{3}/k_{4})$ | - | $V_{T \text{ drug}} - V_{T \text{ baseline}} = OCC \cdot (V_{T} - V_{ND})$ |
| $\lambda k_{3}$ | $\lambda = k_{3}/k_{2}$ | $K_{p2}/k_{2} \cdot k_{3}$ | - | $\lambda k_{3 \text{ baseline}} = \lambda k_{3 \text{ drug}} / \lambda k_{3 \text{ baseline}}$ |

Explanation of symbols:
- $B_{max}$: max number of available receptors (targets); $K_{D}$: dissociation rate constant; PET: positron emission tomography; $V_{ND}$: nondisplaceable distribution volume (usually measured in a reference region); $V_{T}$: distribution volume of the radioligand bound to the target.
- $V_{S}$: volume of the brain regions from which the signal is not able to be measured, which is usually assumed to be the volume of the cerebrospinal fluid.
- $V_{T}$: total volume of distribution of the radioligand.

Examples of successful applications of target occupancy studies in translational PK/PD assessments: Serotonin transporter occupancy by multimodal serotonergic drug vortioxetine

Vortioxetine that is available worldwide as an antidepressant drug was brought into phase II studies based on clinical PET occupancy data. The project was paused due to challenges in setting the dose for the phase II study. The dose was initially based on efficacious plasma exposure in experimental animals, but this could not be directly translated into the clinical situation in this case. This stipulates that important species-dependent parameters need to be accounted for when translating plasma exposure levels between species for a given drug. Firstly, target affinity may differ between species, requiring information on in vitro $K_{i}$ in both humans and in the relevant species used in the preclinical pharmacology studies. Second, it is widely accepted that the unbound concentrations are responsible for engaging the target and are therefore the relevant exposure metric to consider. Third, species differences in drug uptake and/or efflux transporters at the BBB level may lead to species differences in the extent of CNS penetration.

As vortioxetine is an inhibitor of serotonin (5-HT) reuptake at the serotonin transporter (SERT), a SERT occupancy PET study was undertaken using the selective PET ligand [11C]3-amino-4-[[2-((dimethylamino)methyl)phenyl]thio]benzonitrile ([11C]DASB). The PET study was suggested based on an extensive preclinical data package linking the SERT occupancy to serotonin release, as measured using microdialysis. Acutely, vortioxetine dose-dependently occupied the SERT in rats, thus verifying its brain penetration. Following 3 days of continuous dosing in rats, a significant increase in brain extracellular 5-HT was observed, whereas only 41% SERT occupancy was observed. This indicated that relevant functional effects at the neurotransmitter level could be obtained at low SERT occupancy compared to classical selective serotonin reuptake inhibitors and serotonin norepinephrine reuptake inhibitors, which typically require 80% SERT occupancy for a functional and therapeutic effect. In a PET study of vortioxetine in healthy volunteers performed at different oral doses, the relationship between plasma concentrations and SERT occupancy showed that doses of 5–10 mg/day resulted in occupancies around 40–55%. Later, a wide range of efficacy studies in depressed patients confirmed that these doses were therapeutically effective and well-tolerated. However, vortioxetine is also an agonist at 5-HT$_{1A}$ receptors, a partial agonist at 5-HT$_{1B}$ receptors, and an antagonist at 5-HT$_{3}$ and 5-HT$_{2C}$ receptors. SERT and 5-HT$_{3}$ receptors are primarily occupied at 5 mg, whereas at 20 mg, all targets are likely occupied at functionally relevant levels. The multimodal profile of vortioxetine has been demonstrated in vitro and in vivo in rats using ex vivo binding studies but because no PET ligands exist for most of these targets, clinical PET studies have only been performed on 5-HT$_{1A}$ besides from SERT. Thus, the clinical SERT occupancy study was prioritized as PET ligands for SERT were made available at the time but the translation using PET could in principle have been done using one of the other targets as well.

For vortioxetine, retrospective PK/PD evaluation showed that ~ 10-fold higher total plasma concentrations were required in rats compared with humans to achieve the same SERT occupancy. Detailed evaluation of the species-dependent parameters showed that vortioxetine displayed approximately a six-fold weaker SERT $K_{i}$ in rats compared with humans, thus increasing the predicted required equivalent exposure in rats correspondingly. As to plasma protein binding, vortioxetine is highly bound to plasma proteins in both rats and humans (around 99% bound), making it difficult to quantitatively assess whether this parameter would have implications for translating effective drug exposure between these species.

Regarding extent of brain penetration, low involvement of active efflux at the BBB from P-gp was suggested from in vitro permeability assessment and in vivo brain distribution studies in P-gp knock-out mice. Overall, it seems likely that the species difference in
SERT affinity is the main contributor to the observed difference between rats and humans in the SERT occupancy PK/PD relationship for vortioxetine. Hence, whereas the applicability of CNS target occupancy as quantitative translational biomarker between animal and humans have been widely implemented in the industry, a rigorous evaluation of potential interspecies differences is important in the prospective prediction of CNS occupancy in humans.\(^5\)

### \(D_2/D_3\) receptor occupancy by antipsychotic drugs

Several studies in healthy volunteers and patients with schizophrenia have been conducted to measure the occupancy of first- and second-generation antipsychotics to \(D_2/D_3\) receptors using PET and single-photon computed emission tomography (SPECT).\(^4\) The classical antipsychotics (e.g., haloperidol) are known to produce extrapyramidal side effects and these effects have been related to the high (> 80%) receptor occupancy to striatal \(D_2/D_3\) receptors.\(^4\) On the other hand, therapeutic efficacy is achieved with striatal receptor occupancy between 70% and 80%, making receptor occupancy studies extremely important to provide guidance on dose setting.\(^5\)\(^,\)\(^6\)

The pharmacological profile of atypical or second-generation antipsychotics has been found to be different, because these drugs provide clinical efficacy at a lower striatal \(D_2/D_3\) receptor occupancy and at higher \(D_2/D_3\) occupancy in extrastriatal regions, such as the temporal cortex. In a meta-analysis, PET and SPECT studies in patients with schizophrenia (total \(n = 139\)) have been reviewed and articles were selected based on the reported occupancy in striatum and temporal cortex after chronic dosing (steady-state).\(^3\) The classical antipsychotics (e.g., haloperidol) are known to produce extrapyramidal side effects and these effects have been related to the high (> 80%) receptor occupancy to striatal \(D_2/D_3\) receptors.\(^4\) On the other hand, therapeutic efficacy is achieved with striatal receptor occupancy between 70% and 80%, making receptor occupancy studies extremely important to provide guidance on dose setting.\(^5\)\(^,\)\(^6\)

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5-HT\(_{2A}\) receptor occupancy and clinically relevant doses, suggesting that 5-HT\(_{2A}\) antagonism by second-generation antipsychotics is less likely related to clinical efficacy.

Another meta-analysis of 12 PET studies (11 with \([\text{11C}]\text{raclopride}\) and 1 using both \([\text{11C}]\text{raclopride}\) and \([\text{11C}]\text{FLB457}\) for striatal and extrastriatal \(D_2/D_3\) receptors) including 82 subjects, specifically examined the relationship between \(D_2/D_3\) receptor occupancy and severity of extrapyramidal side effects (65 subjects) as well as treatment response (70 subjects) assessed through clinical scales (25% or greater or 50% or greater reduction in the Positive and Negative Syndrome Scale (PANSS) or the Brief Psychiatric Rating Scale (BPRS)).\(^5\) The mean dopamine \(D_2/D_3\) receptor occupancy was significantly higher in subjects who presented extrapyramidal side effects (\(n = 12; 77 \pm 9\%\) vs. \(n = 53; 63 \pm 17\%, P = 0.011\). Higher \(D_2/D_3\) receptor occupancy (66 \pm 14\%) was observed in patients who presented a 25% reduction in the PANSS or BPRS (\(n = 43\)) as compared with the occupancy observed in patients who did not show symptom improvement (\(n = 27, 58 \pm 19\%\)). The difference did not reach statistical significance (\(P = 0.054\)), but the greatest accuracy in predicting a 25% and 50% of reduction in PANSS or BPRS corresponded to occupancy measures of 60% and 78%, respectively, confirming that this is the range of occupancy associated with a clinical effect.

On the contrary, third-generation antipsychotics like aripiprazole, cariprazine, and brexpiprazole exhibit a higher \(D_2/D_3\) receptor occupancy at clinically relevant doses (closer to 90% or 95%), without increasing the risk of extrapyramidal side effects.\(^4\)\(^,\)\(^5\)\(^,\)\(^6\)\(^,\)\(^7\) This has been rationalized based on the partial \(D_2\) receptor agonism of those compounds that are different from the antagonism of both first- and second-generation antipsychotics.\(^5\)\(^,\)\(^7\) Finally, a special consideration should be given to clozapine, a highly efficacious antipsychotic used for the treatment of schizophrenia. The efficacy of clozapine is achieved at much lower \(D_2/D_3\) receptor occupancy than first- and second-generation antipsychotics.\(^4\) This “atypical” property has been linked to the higher \textit{in vitro} affinity of clozapine for \(D_4\) receptors as compared with \(D_2/D_3\) receptors and to the evidence from human PET studies of equivalent occupancy of clozapine for the \(D_1\) and \(D_2/D_3\) receptors.\(^5\)\(^,\)\(^6\)

### ASSESSMENT OF PHARMACODYNAMIC EFFECT

The PD effect of a drug can be studied \textit{in vivo} either by assessing the direct effect on the modulation of neurotransmitter release or by evaluating the downstream effect of the drug on neuronal activity or specific brain circuits. The first approach benefits from the capability of PET to measure changes in endogenous neurotransmitter levels, whereas the second approach uses a multimodal evaluation of PET receptor occupancy and pharmacological-induced changes in specific brain circuits measured with functional magnetic resonance imaging (fMRI).

The downstream effect related to target occupancy or to neurotransmitter release can be close in time with drug administration or occur more distant in time. The latter situation is especially relevant for disease-modifying drugs, which recently have attracted much attention by the approval of aducanumab for Alzheimer’s...
disease. In this case, amyloid-$\beta$ (A$\beta$) PET ligands that bind to A$\beta$ plaques$^{64}$ were developed in parallel with ongoing drug discovery programs targeting A$\beta$ and used to show reduction in A$\beta$ plaques. A recent paper exemplifies, for example, that aducanumab shows a dose- and time-dependent reductions in the amyloid PET standard uptake value ratio as measured by $^{18}$F-labeled flurbiprofen.$^{62}$ Thus, PET as a technology has had major impact on the approval of aducanumab by the US Food and Drug Administration (FDA). Neurobiological data that are aggregates of hyperphosphorylated tau is another important target for ongoing drug development programs with disease modifying potential. For these aggregates, PET ligands have also been successfully developed and will most likely have impact on future approvals of drug for AD and other tauopathies.$^{61,63}$

With regard to measuring endogenous neurotransmitters, a classical occupancy model has been described to explain the effects of changes in endogenous dopamine on the binding of a PET radioligand (e.g., $[^{11}C]$raclopride) to the D$_2$/D$_3$ receptors.$^{64,65}$ According to the model, the radioligand, at tracer doses, occupies a fraction of the receptors on the postsynaptic site. The increase in the levels of endogenous dopamine, induced pharmacologically by the administration of a dopamine releasing drug, such as amphetamine,$^{66}$ will lead to an increased occupancy of dopamine to the D$_2$/D$_3$ receptors and a displacement of the PET radioligand from the D$_2$/D$_3$ receptor, resulting in a decrease of $B_P^{ND}$. The opposite effect (i.e., an increase) on $B_P^{ND}$ is observed in cases of a decrease in dopamine levels, induced for instance by administration of a dopamine depleting drug, such as alpha-methyl-para-tyrosine.$^{67,68}$

Studies in NHPs and in human subjects using agonist ($[^{11}C]$MPNA or $[^{11}C]$PHNO) and antagonist ($[^{11}C]$raclopride) PET radioligands have shown that changes in striatal $B_P^{ND}$ of $[^{11}C]$MPNA or $[^{11}C]$PHNO induced by amphetamine were larger than the changes of $B_P^{ND}$ observed with $[^{11}C]$raclopride,$^{69,70}$ indicating a higher sensitivity of D$_2$ agonist PET radioligands to measure endogenous dopamine levels. PET imaging with $[^{11}C]$MPNA or $[^{11}C]$PHNO before and after the administration of a drug modulating dopamine release will enable the assessment of the pharmacological effect of the drug on synaptic neurotransmitter release. An example of such an approach is the examination of the effect of the orphan Gprotein-coupled receptor GPR139 agonist TAK-041 on the attenuation of the amphetamine-induced release of endogenous dopamine in the human brain using $[^{11}C]$PHNO PET.$^{71}$ A dose of 20 or 40 mg TAK-041 administered 2 hours prior to the oral administration of 0.5 mg/kg d-amphetamine, significantly attenuated the decrease of $B_P^{ND}$ of $[^{11}C]$PHNO in the putamen, ventral striatum, and substantia nigra, with 40 mg being associated with less reduction of $B_P^{ND}$ that the 20 mg dose.$^{71}$ A mechanism postulated to explain these findings has been that TAK-041 modulates D$_2$ autoreceptors, reducing dopamine synthesis and hence synaptic dopamine release. The pharmacological modulation of neurotransmitter release has been applied also to other monoaminergic and non-monoaminergic receptor and enzyme systems, as reviewed by Finnema et al.$^{65}$ It should be noted that the same technology can be used to investigate disease-related changes in endogenous neurotransmitter release. For instance, studies conducted in patients with schizophrenia examining the changes of D$_2$ receptor availability after dopamine depletion or amphetamine-related dopamine release have suggested that patients with schizophrenia have increased synaptic dopamine concentration and increased dopamine release as compared with controls.$^{72-74}$

The second approach has been used to evaluate the relationship between receptor occupancy and functional hemodynamic changes measured with fMRI, by measuring the changes in cerebral blood volume (CBV; neurovascular response) or the changes in blood oxygen level-dependent (BOLD) response. With the advent of the hybrid PET/MRI systems, studies have been conducted to address the relationship between pharmacological modulation of neurotransmitter systems and functional brain changes. In an early study performed in anesthetized NHPs, the relationship between D$_2$/D$_3$ receptor occupancy and changes of CBV was examined.$^{75}$ Receptor occupancy was measured using $[^{11}C]$raclopride as PET radioligand and pharmacological doses of raclopride. Increasing D$_2$/D$_3$ receptor occupancy by pharmacological doses of raclopride (4.5 to 40 µg/kg) was associated with increased CBV changes. The relationship between D$_2$/D$_3$ receptor occupancy and CBV changes was monotonically increasing, with the putamen exhibiting approximately twice the CBV magnitude compared with caudate. The increase of CBV observed with increased receptor occupancy was explained with the fact that the fractional occupancy of dopamine to D$_2$/D$_3$ receptors is the parameter that drives the fMRI signal. The occupancy of D$_2$/D$_3$ receptors by raclopride reduces the fractional occupancy of dopamine to D$_2$/D$_3$ receptors, producing an increase in CBV. This interplay between D$_2$/D$_3$ receptor occupancy and changes in CBV suggest a link between fractional occupancy of dopamine and neuronal activity in the striatum.

On the contrary, in a study in which the D$_2$/D$_3$ receptor agonist ropinirole was used, the occupancy of D$_2$/D$_3$ receptors measured by the degree of displacement of $[^{11}C]$raclopride, was associated with a decrease of CBV.$^{76}$ A similar inverse relationship has been found between displacement of $[^{11}C]$carfentanil ($\mu$ opioid receptor agonist PET radioligand) by naltrexone ($\mu$ opioid receptor antagonist) and a decrease in CBV.$^{77}$

Effects of naltrexone and other opioid antagonists on the dopamine system have been linked to their ability to attenuate reward properties. In a combined PET and fMRI study, receptor occupancy of the $\mu$ opioid receptor antagonist GSK1521498 and its effects on brain function were assessed and compared with naltrexone.$^{77}$ $\mu$-opioid receptor occupancy of GSK1521498 and naltrexone was measured with $[^{11}C]$carfentanil PET. An fMRI paradigm comparing BOLD-response associated with a palatable stimulus vs. purified water was used to measure food-related activation in limbic brain regions (amygdala and nucleus accumbens). The 50% effective dose for GSK1521498 and naltrexone were 1.5 and 5.6 mg. The relationship between plasma concentration of GSK1521498 and receptor occupancy was time-independent. On the other hand, for naltrexone and its metabolite 6-b-naltrexol, at the same plasma concentration, the receptor occupancy was time-dependent, suggesting that probably other metabolites or a longer residence time at the $\mu$ opioid receptor could contribute to the time-dependent occupancy relationship. The oral administration of GSK1521498 was associated with a significant decrease of BOLD-response in the amygdala,
whereas no differences in any of the brain regions were observed for naltrexone. Overall, the higher selectivity for μ opioid receptors, in association with the direct plasma occupancy relationship and the functional effect observed (attenuation of food-related activation in the amygdala), indicated improved pharmacological properties of GSK1521498 compared with naltrexone.

CONCLUSIONS
The strategy needed to advance NCEs from research to early stages of development is multidisciplinary and takes advantage of several in vitro, in vivo, and ex vivo methods. Molecular imaging with PET is a methodology that supports the development strategy by providing in vivo evidence of NCE brain exposure and proof of occupancy of the NCE to the target of interest. Standard methods of quantification used for PET radioligands can be applied to describe the PKs of radiolabeled NCEs and obtain key parameters of brain exposure as $K_p$ and $K_{pu}$. The availability of suitable PET radioligands is a prerequisite to be able to demonstrate proof of occupancy, but if receptor occupancy studies are not possible, microdosing studies can be used to make predictions of brain exposure to support early decisions. Multimodal imaging approaches combining PET with FMRI can be used to link target occupancy of NCEs with PD effects on specific brain regions or circuits.

CONFLICT OF INTEREST
A.V., C.B., and B.B.-A. are employed at H. Lundbeck A/S.

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