Bolus infusion scheme for the adjustment of steady state $[^{11}\text{C}]$Flumazenil levels in the grey matter and in the blood plasma for neuroreceptor imaging

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ABSTRACT

The use of hybrid PET/MR imaging facilitates the simultaneous investigation of challenge-related changes in ligand binding to neuroreceptors using PET, while concurrently measuring neuroactivation or blood flow with MRI. Having attained a steady state of the PET radiotracer using a bolus-infusion protocol, it is possible to observe alterations in ligand neuroreceptor binding through changes in distribution volumes. Here, we present an iterative procedure for establishing an administration scheme to obtain steady state $[^{11}\text{C}]$flumazenil concentrations in grey matter in the human brain. In order to achieve a steady state in the shortest possible time, the bolus infusion ratio from a previous examination was adapted to fit the subsequent examination. 17 male volunteers were included in the study. Boli and infusions with different weightings were given to the subjects and were characterised by $k_{bol}$ values from 74 min down to 42 min. Metabolite analysis was used to ascertain the value of unmetabolised flumazenil in the plasma, and PET imaging was used to assess its binding in the grey matter. The flumazenil time-activity curves (TACs) in the brain were decomposed into activity contributions from pure grey and white matter and analysed for 12 vol of interest (VOIs). The curves highlighted a large variability in metabolic rates between the subjects, with $k_{bol} = 54.3$ min being a reliable value to provide flumazenil equilibrium conditions in the majority of the VOIs and cases. The distribution volume of flumazenil in all 12 VOIs was determined.

1. Introduction

The use of hybrid PET/MR imaging facilitates the simultaneous investigation of challenge-related changes in ligand binding to neuroreceptors and of neuroactivation or blood flow. The first measurement is achieved by using PET and the latter with the concurrent use of MRI. An advantageous method for the analysis of the neuroreceptor binding status is to establish a steady state of the radiotracers in the compartments under investigation. For example, the ratio of the tissue tracer concentration and its metabolite-corrected plasma concentration can be used to calculate the total distribution volume of a ligand (Carson et al., 1993). This means that the receptor binding potential of a particular radioligand can be determined without being impaired by the effects of a potentially varying radioligand influx. This is crucial for PET/MR experiments where the subjects undergo neuronal stimulation, for example in modern fMRI-measurements. Furthermore, in such experiments, it must be ensured that the measured changes in radionuclide binding are only due to the stimulation and are not the result of any other factors. Both requirements, the steady state of the radiotracers and the avoidance of interfering influences can be achieved with a PET/MR investigation using a bolus infusion protocol, where, after reaching a steady state, the first study part looks at the control situation and the second verum part occurs during neuronal stimulation. This one-stop-shop approach enables direct comparison of the binding of a neuroreceptor ligand with the

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additional advantage of being able to record MR-based data, such as fMRI data, simultaneously. Compared to the alternative approach of two separate studies with bolus injections for control and verum, a single bolus infusion study conveniently ensures that the measurements with and without neuronal stimulation take place under almost identical physiological, mental and emotional states. Moreover, a single bolus infusion set-up is much more convenient for the subject, particularly if the subject is a patient, as well as being easier and cheaper from an organisational point of view as only one scanning session and one radiopharmaceutical synthesis is necessary. Furthermore, arterial blood sampling is not required with the bolus infusion protocol since, at the equilibrium condition, arterial and venous blood do not differ in respect to radioligand concentration.

Flumazenil binds to the benzodiazepine allosteric binding site between the α and γ subunits on γ-aminobutyric acid (GABA_A) receptors (Hadingham et al., 1993). It acts as a partial GABA_A antagonist with a positive GABA shift (Frankle et al., 2012). Therefore, ¹¹C-labelled flumazenil has been used for probing GABAergic neuronal activity in a wide range of studies (Bouvard et al., 2005; Lingford-Hughes et al., 2005; Pearl et al., 2009).

In human experiments, it is necessary to minimise the radiation dose to the subject while maximising the evaluable measurement time. Consequently, equilibrium must be gained as quickly as possible. This is possible to achieve using complicated approaches, such as time variable infusion rates, which may even be driven in a feedback-controlled mode (Ohba et al., 2013). Such schemes for the administration of ¹¹C-flumazenil have been provided by computer-controlled pumps and were initially applied to experiments on animals in neuroscience (Erissson et al., 2008a) and anaesthesiology (Erissson et al., 2008b). Recently, the concept of a programmed infusion of ¹¹C-flumazenil has been simplified into an application procedure with a constant ratio between the initially applied bolus and the infusion, hereafter named the “bolus infusion scheme” and has been transferred to human applications (Feng et al., 2016). Despite being able to achieve equilibrium quickly, in all these approaches, the time-dependent responses to the flumazenil bolus injections were measured in a precursor experiment, which also exposed the volunteer to radiation. Moreover, determining a time-dependent impulse response in a bolus injection study requires the sampling of arterial blood (Carlson et al., 1993). The required bolus and infusion activities could be determined by exploiting the convolution of the impulse response with the metabolite-corrected plasma input function. The development of the bolus infusion protocol presented here bears two major advantages. First, it offers an easier and reliable alternative by administering a radiotracer bolus, followed by an infusion with a constant rate, which provides the maintenance of the radiotracer. Second, without the need for any precursor experiments, complete subject data sets, which include modalities requiring the steady state, can be acquired immediately. When the initially estimated and subsequently adjusted ratio between bolus and infusion establishes a steady state radio ligand concentration in the plasma, the data of the respective adjustment is included in the study analysis. Otherwise, the data requiring the steady state will be discarded and the weighting between bolus and infusion will be further adapted towards steady state plasma levels in the following examinations on other subjects.

¹¹C-Flumazenil binds to GABA_A receptors, which are widely spread in the grey matter of human brains. Therefore, data analysis must apply volumes of interest (VOIs), defined based on MR images with anatomical atlases, all over the grey matter so that time activity curves (TACs) can be obtained from the dynamic PET images. Due to the imperfect coregistration of the atlas based VOIs to the cortex and due to the limited image resolution of even high-resolution PET devices (la Fougère et al., 2011) such as the BrainPET (Herzog et al., 2011) used in this study, anatomical VOIs might capture signals from adjacent areas of grey and white matter, which corresponds to the mixing of signals from GABA-receptor rich and receptor depleted tissue types with possibly different TAC slopes. In order to assess whether this blurring effect has a relevant impact on the slope of the TACs, the activity curves were decomposed into the individual contributions from grey and white matter and all subsequent analyses were performed separately for both tissue classes.

Specific brain regions may show distinct TACs due to different activation patterns, different radiotracer influx velocities or dispersion effects, depending on the supporting arterial tree. Standard analysis approaches of the TACs used large VOIs, which were composed of multiple small VOIs. The extent to which the inherent averaging procedure is responsible for flat TACs or whether the underlying, multiple pure grey matter VOIs are already characterised by flat TACs remains unclear. Consequently, because specific TACs of brain regions may be more relevant for specific activation patterns than only the average cortical curves of the various lobes, data from 59 regions of interest were analysed for similar time courses in adjacent areas and were merged into 12 regions of interest.

The 17 investigations of the present report originate from an ongoing study on the correlation of brain function with GABA_A receptor occupancy in depressive patients. The aims of these investigations were: (i) the iterative determination of the optimum kbol value for reaching the steady-state of ¹¹Clumazenil kinetics in GM areas with similar uptake kinetics, which requires (ii) the decomposition of signal intensities into contributions from GM and WM, (iii) the merging of detailed anatomical VOIs of GM with equal ¹¹C-lumazenil uptake curves into larger GM VOIs, and (iv) the sampling of venous blood to proof constant flumazenil plasma levels. Finally, the calculation of the distribution volume of ¹¹C-flumazenil is presented as an example.

2. Material and methods

General procedure. The measurements were repeated under interindividual variation of the bolus infusion weighting until a sufficiently constant flumazenil concentration was detected in the plasma and confirmed in the grey matter of the brain. The flumazenil concentration was measured by means of the delay of its radioactive label ¹¹C. The data sets were acquired in two hybrid fMRI PET studies, in which the volunteers underwent either an auditory stimulation or an emotional judgement task. The auditory stimulation reflected response inhibition in signal processing, known from the loudness dependence of auditory evoked potentials (LDAEP) experiments (Wyss et al., 2018), and occurred between 30 and 70 min after bolus injection. The emotional judgment task used visual stimulation between 40 and 81 min following bolus injection (Heinzel et al., 2005; Grimm et al., 2006). The presented pictures comprised stimuli from the International Affective Picture System (Lang et al., 2008).

Subjects. All measurements were approved by the local ethics committee/federal authorities and were in accordance with the Declaration of Helsinki. Written informed consent was obtained from all volunteers before the measurement. A series of 20 healthy, Caucasian volunteers with no history of neurological or psychiatric disease (assessed by the Mini-International Neuropsychiatric Interview (M.I.N.I) (Sheehan et al., 1998)) underwent simultaneous PET/MR measurements and venous blood samples were taken. On average, 404 ± 16 MBq of ¹¹C-flumazenil was administered per volunteer. Three subjects were excluded because of insufficient atlas registration in the analysis and irregular plasma time-activity curves, indicating a delayed ingress of the infusion. 17 males aged 27.5 ± 5.4 years and 77.7 ± 10.3 kg in weight were included in the analysis.

Radiochemistry. The synthesis of ¹¹C-flumazenil (ethyl 8-fluoro-5-[¹¹C]methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazipine-3-carboxylate, ¹¹C]RO 15-1788) was performed by a modified literature method (Suzuki et al., 1985). ¹¹C]Carbon dioxide was produced by the ¹⁴N(p,α)¹¹C nuclear reaction in a gas target filled with ¹⁴N₂ containing 0.5% O₂ using a GE PETtrace cyclotron. ¹¹C]CO₂ was converted to ¹¹C]CH₃ using a literature method (Holshbach and Schüller, 1993). The ¹¹C]CH₃ was transferred into cold solution (−20 °C) of...
0.3 ± 0.2 mg desmethylumazenil (ABX, Radeberg, Germany) and 0.1–0.2 mg sodium hydride in 500 µl in N,N-dimethylformamide (Sigma-Aldrich, Schnelldorf, Germany) and heated to 50 °C for 1 min. The final HPLC-purification was carried out with a Kromasil 100-10 C18 (250 × 8 mm) column (CS-Chromatographie Service, Langerwehe, Germany) using MeOH/H₃PO₄ (c = 0.06 mol/l) 35/65 (v/v) as the eluent. For formulation, [¹¹C]umazenil was fixed on a SEP-PAK C18 cartridge (Waters, Eschborn, Germany) and eluted with ethanol in isotonic sodium chloride solution (B. Braun, Melsungen, Germany) and filtered through a 0.22 µm Millex® filter. The average molar activity at the injection time was 190 ± 60 GBq/µmol.

**Administration of the bolus and the infusion.** Two syrings for the bolus and for the infusion radioactivity were prepared with an excess of [¹¹C] activity exactly 9 min prior to administration so that the remaining activity after these 9 min met the targeted dose and bolus/infusion ratio. To ascertain the actual applied ratio, the values were corrected for the activity remaining in the syringes. On average, subjects were given 404 MBq [¹¹C]umazenil, but not more than 425 MBq (approved by the Federal Office for Radiation Protection). The percentage of the tracer administered by bolus injection was given by its kbol value, which defines the ratio of the bolus to 1 h’s worth of infusion. Beginning with an initial kbol value of 74.4 min, the weighting was progressively corrected from study to study, with continual feedback from the metabolite analysis and time-activity curves of the brain. This procedure was repeated until the slope of the plasma and brain tissue curves scattered closely around a constant line, indicating almost flat profiles. The bias which is induced by applying the population-wide kbol value to a group of subjects with individual metabolisation rates was analysed by assessing the variability of TAC slopes at a given kbol value.

**MR and PET data acquisition and reconstruction.** The data were measured using a Siemens Hybrid 3T MR-BrainPET. Immediately following the administration of the [¹¹C]umazenil bolus and after starting the infusion pump at a rate of 1 ml/min, voxel-wise data were acquired for 100 min. Simultaneously, T₁-weighted MPRAGE data with 1 mm isotropic resolution and full brain coverage were acquired for 4 min. After 40 min of infusion time, when the beginning of the steady state interval was expected, several functional scans were performed with an echo planar imaging (EPI) sequence for 50 min. The scan duration under the steady state condition was determined by the time demand of the functional measurements. The PET images were iteratively reconstructed using PRESTO (120 iterations) (Scheins et al., 2011, 2015) and a framing of 20 × 300 s, including corrections to obtain quantitative images, i.e. for normalisation, dead-time, attenuation (Rota Kops et al., 2015), scattered and random events. Post-processing comprised motion correction and registration to the T₁-weighted MPRAGE data using the Fmod software package (Version 3.5).

**Analysis of the brain tissue activity profiles.** TACs were exported from cortical and subcortical brain regions, based on the definition given by the Harvard-Oxford cortical and subcortical structural atlas, were numerically separated into the contribution from grey and white matter, and analysed for the value range within the time frame of 40–100 min. In more detail, the following steps were performed: the Harvard-Oxford cortical and subcortical structures atlases (Desikan et al., 2006) were warped to the T₁ data using the linear and non-linear registration provided by FSL (Jenkinson and Smith, 2001; Jenkinson et al., 2002). As GABA receptors are predominantly present on the cell membranes of neurons, specific umazenil enhancement mainly occurs in the grey matter, whereas the signal from benzodiazepine receptor-depleted white matter (Abadie et al., 1992) is mostly caused by unspecific background uptake that may have a different time course to that in grey matter. Although, due to the nature of the cortical structural atlas, its anatomical volumes mainly capture grey matter voxels; the signal from white matter and cerebrospinal fluid also contribute to the signal of the anatomical VOIs to a certain extent. The analogue situation holds for the areas defined in the subcortical structural atlas. The signal from grey matter was numerically separated from the white matter signal by applying the multivariate curve resolution approach, which is known for disen- tangling MR spectra that are mixed due to the partial volume effect (Huo et al., 2004; De Edelenyi et al., 2005; Goryawala et al., 2018). The total signal intensity, Iₙ, of the voxel, n, with the partial volumes/tissue probabilities for grey matter (GM), white matter (WM), cerebrospinal fluid (CSF), SGM and SWM, was given by the value ranges obtained from SPM8 and smoothed with the point spread function of the BrainPET detector (Gaussian kernel, 4 mm full width at half maximum in average (Hertzog et al., 2011)(Ja Fougeré et al., 2011), was decomposed into the signal contributions SGM and SWM from grey and white matter, respectively. The grey matter term essentially represents the specific enhancement given by umazenil-receptor binding and the background signal from the blood volume, whereas the white matter term almost completely relates to unspecific uptake, signal from the blood and scatter effects. Because the activity of CSF has not been included in the model, voxels with a CSF probability greater than 5% were excluded. For each anatomical area defined in the atlas, each with a different voxel number N, the overdetermined linear system of equations

\[
I = G W [S_{GM} S_{WM}]]
\]

where \(G^{n×n}, W^{n×1}\) are vectors, and \(S_{GM}, S_{WM}\) are scalars, was solved using an in-house developed MATLAB code, yielding the values for umazenil uptake in theoretically pure grey and pure white matter.

Initially, 48 cortical and 11 subcortical brain region specific time-activity curves (including three non-disjunct curves) were analysed in terms of their shapes. For this purpose, the time activity curves of the subset of 56 disjoint VOIs and all subjects were centred, normalised and decorrelated by using the principal component analysis (PCA). The number of PCA-components, n, was chosen based on the PCA score (log-likelihood average) in a cross-validation scenario (stratified K-Fold, \(n\text{-splits} = 5\)). These components were further decomposed by using the independent component analysis (ICA) approach fastICA (Hyvärinen and Oja, 2000). Finally, each column of the ICA weight matrix was back-transformed into data space. The individual n contributions of the 56 VOIs to the n independent components were interpreted as weights of n basic, abstract tissue types. Tissue classes with a similar weighting of basic tissue types were identified by means of the k-means clustering method, of which, the optimum number, k, of clusters was determined by using the silhouette method (Rousseeuw, 1987). In order to facilitate comparison with results from the literature, the final merging included VOIs that were used by other groups (Koepppe et al., 1991; Frankle et al., 2012; Feng et al., 2016). The brainstem was included in the group of anatomical areas because it can be used as a reference region for a tissue VOI with a depleted number of GABA receptors. The cerebral WM was included for comparison only. The TACs of all VOIs were normalised to their average values from 40 to 100 min after bolus injection and the range of the activity values within this interval was calculated. Increasing activity profiles were represented by positive, decreasing values by the negative sign of the derivative. Confounded white matter (kboil) was corrected for the umazenil uptake in theoretically pure grey and pure white matter.

**Metabolite analysis.** Following bolus injection, venous blood samples were taken at 2, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min. The blood cells were spun down and the radioactivity of the supernatant plasma was measured. Thin-layer chromatography (TLC) was used to determine the proportion of unmetabolised umazenil in the plasma. After protein precipitation, shaking and centrifugation, the supernatant metabolite fractions were separated from each other on normal phase 60 Å silica plates. The migration distances of the metabolites remained significantly behind the umazenil parent compound. The plates were developed and read with a beta/gamma imager (PerkinElmer/Packard Instanmager). The parent compound fraction on the TLC plate and the plasma total
radioactivity concentration were fitted by 3-exponential curves. The concentration of the parent compound was calculated based on the product of the total plasma radioactivity concentration and the parent fraction in the extract. All of the flumazenil parent compound TACs were normalised to their average values from 40 to 100 min, and the corresponding normalised radioactivity ranges were evaluated.

**Distribution volume.** The Pearson’s correlation coefficients between the plasma curve range and the TAC range were calculated for all VOIs. For all anatomical areas where both the tissue TAC and the metabolite-corrected plasma curve were sufficiently flat, indicating a steady state, the distribution volume of flumazenil $\text{V}_T = \frac{C_p}{C_T}$ was calculated at the respective times, where $C_T$ denotes the flumazenil concentration in the brain tissue and $C_p$ represents the unmetabolised flumazenil parent compound in the plasma. The profiles were accepted as being flat if the normalised range values from 40 to 100 min varied less than 10%. In VOIs where this could not be accomplished, the distribution volume was estimated based on the higher range of 20%.

3. Results

The flumazenil parent substance activity in the plasma was determined for each application of the bolus infusion schema with $k_{bol}$ values from 74.4 min down to 42 min, corresponding to normalised activity ranges of ±0.23 (Fig. 1). The nearly vertically aligned range values at around $k_{bol} = 46.7$ min indicate the large interindividual variability of the plasma curves. Flat plasma curves between 40 and 100 min after the injection of the tracer are most likely to be obtained with $k_{bol} = 51.5$ min.

Numerical separation between signal contributions from grey and white matter was successful for all 48 and 11 VOIs of grey and white matter, respectively, for all volunteers (see supplementary material). The volunteers who underwent the emotional judgment task under visual material, respectively, for all volunteers (see supplementary material). The volunteers who underwent the emotional judgment task under visual material, respectively, for all volunteers (see supplementary material). The volunteers who underwent the emotional judgment task under visual material, respectively, for all volunteers (see supplementary material).

On the basis of the signal contributions from grey and white matter, the TACs of the detailed anatomical areas were analysed for similarity by using the previously described processing pipeline comprising the PCA, ICA and k-means clustering method (Table 1). Depending on the subject, this mostly resulted in 2–3 different tissue classes, corresponding to two large VOIs, which did not match the common VOIs in the literature. Data from volunteers 2 and 4 allowed no coarser merging than 21 and 22 numerical tissue classes, respectively. Such overall diverging clustering results did not allow for valid consensus clustering for all cases. In order to facilitate comparison with results from other groups, 6 larger cortical and subcortical volumes of interest were chosen (Table 2 and supplemental material), so that the TACs of grey and white matter in these merged VOIs could be determined. Although the resulting TACs showed negligible enhancement in WM, uptake in GM was strong enough to calculate the AUC values. The brainstem region of the Harvard-Oxford atlas was included in the set of VOIs as it comprises the pons, which is used as a reference region in flumazenil studies. In this VOI, WM and GM were found to be homogeneously distributed. Owing to the fact that the reference tissue approach aims at a tissue type devoid of GABA receptors, the WM component of the brainstem VOI was selected for the subsequent analyses. These included analysis of the range of the $[^{11}\text{C}]$flumazenil time-activity curves (Fig. 2) and analysis of the brain-to-plasma TAC correlation (Fig. 4). In order to prove the non-negligible $[^{11}\text{C}]$flumazenil uptake in the brainstem GM compartment, its corresponding AUC is listed in addition to the WM AUC in Table 2.

All analysed brain areas are characterised by a wide spread of TAC ranges (Fig. 2), caused by the broad variety of the underlying curve patterns in spite of similar $k_{bol}$ values. The zero crossings of the best-fit lines, indicating flat profiles at the transition from increasing to decreasing TAC profiles, shows an overall optimum $k_{bol}$ value of 54.3 ± 2.1 min (average ± standard deviation, Tab. 2) for GM. This value does not significantly change if one narrows the calculation of the average value ($k_{bol} = 55.1 ± 1.9$) exclusively to the VOIs of the cerebral cortex, where equilibrium is crucial for GABA receptor studies.

The bias, which was introduced by using a population-wide $k_{bol}$ value for subjects with individual metabolism rates, was analysed for six volunteers and were measured with the approximately constant $k_{bol}$ value of 46.67 ± 0.27 min (Fig. 2). The associated standard deviations of the normalised activity ranges spanned a range from 0.04 to 0.13 (Table 3). Using the example of two volunteers, the high variability of the activity ranges from 40 to 100 min at similar $k_{bol}$ values is shown in Fig. 3. Although the value $k_{bol} = 46.9$ min is far off the optimum value $k_{bol} = 54.3$, the corresponding plasma and cortex time-activity curves are flat. However, owing to the spread of metabolism rates, the value $k_{bol} = 53.2$ min, which is much closer to the group optimum, did not lead to constant curves.

### Table 1

Results of the numerical clustering after PCA, ICA and k-means clustering.

| Case no | Number of PCA/ICA components | Explained variance [%] | k at silhouette max |
|---------|-------------------------------|------------------------|---------------------|
| 1       | 4                             | 94.97                  | 2                   |
| 2       | 4                             | 90.06                  | 2                   |
| 3       | 5                             | 96.67                  | 2                   |
| 4       | 3                             | 85.79                  | 22                  |
| 5       | 4                             | 87.46                  | 3                   |
| 6       | 2                             | 84.09                  | 2                   |
| 7       | 2                             | 90.04                  | 2                   |
| 8       | 2                             | 88.88                  | 2                   |
| 9       | 2                             | 84.34                  | 3                   |
| 10      | 3                             | 93.08                  | 3                   |
| 11      | 4                             | 91.77                  | 6                   |
| 12      | 4                             | 90.98                  | 2                   |
| 13      | 2                             | 89.65                  | 2                   |
| 14      | 3                             | 91.90                  | 3                   |
| 15      | 2                             | 84.55                  | 2                   |
| 16      | 4                             | 96.42                  | 4                   |
| 17      | 6                             | 97.58                  | 2                   |
| Avg ± SD| 3.29 ± 1.21                  | 90.48 ± 4.35           | 4.88 ± 6.34         |

![Graph](image-url)
Table 2
Segmentation of the volumes of interest of 17 data sets into grey/white matter and corresponding activities. The larger the fraction of peripheral voxels of a particular volume of interest is, the less GM and WM content add up to one. This is because smoothing with a gaussian kernel spreads in zero intensity values from the outside of the brain. The AUCs are either calculated from the pure GM or WM signal of the VOIs.

| Volumes of interest | Grey matter content | White matter content | AUC (40-100 min) |
|---------------------|---------------------|-----------------------|------------------|
|                      | Avg±SD              | Avg±SD                | Avg±SD [kBq/cm³] |
| Frontal Lobe GM     | 0.55 ± 0.03         | 0.30 ± 0.04           | 29.24 ± 3.53     |
| Insular Cortex GM    | 0.76 ± 0.02         | 0.16 ± 0.02           | 23.91 ± 3.05     |
| Temporal Lobe GM    | 0.67 ± 0.02         | 0.24 ± 0.02           | 26.23 ± 3.92     |
| Parietal Lobe GM    | 0.52 ± 0.03         | 0.33 ± 0.04           | 30.06 ± 3.63     |
| Occipital Lobe GM   | 0.56 ± 0.03         | 0.32 ± 0.03           | 30.77 ± 4.50     |
| Limbic Lobe GM      | 0.69 ± 0.02         | 0.21 ± 0.02           | 24.76 ± 3.46     |
| Thalamus GM         | 0.43 ± 0.04         | 0.52 ± 0.04           | 12.99 ± 1.91     |
| Hippocampus GM      | 0.81 ± 0.01         | 0.14 ± 0.01           | 17.58 ± 3.07     |
| Amygdala GM         | 0.86 ± 0.02         | 0.09 ± 0.02           | 15.09 ± 2.02     |
| Basal Ganglia GM    | 0.52 ± 0.03         | 0.45 ± 0.03           | 11.53 ± 1.28     |
| Brainstem GM        | 0.29 ± 0.10         | 0.59 ± 0.10           | 7.73 ± 2.33      |
| Brainstem WM        | 0.29 ± 0.10         | 0.59 ± 0.10           | 2.70 ± 0.93      |
| Cerebral WM         | 0.19 ± 0.02         | 0.79 ± 0.02           | 3.40 ± 0.59      |

The degree of flatness of the normalised plasma curves shows a high correlation with the flatness of the accordingly normalised TAGs of the various brain regions (Fig. 4). The correlation coefficients \( r \) spanned a range from 0.18 to 0.82. It was possible to find cases and anatomical areas where both the plasma profile and the TAC were sufficiently flat. The distribution volumes \( V_T \) were calculated for all VOIs of which the normalised TAGs and the corresponding, normalised plasma TAGs stayed within a range of 0.1. This condition could not be fulfilled for the GM of the thalamus and amygdala, nor for the WM of the brainstem. For these VOIs, subjects with normalised ranges of 0.2 within the relevant interval of the brain TAGs were included in the calculation of the distribution volume, as shown in Table 4. The distribution volumes are compared to the literature values in Fig. 5.

4. Discussion
The concept of the study aimed to ameliorate two major restrictions that frequently occur in neuroscientific PET studies i.e. minimising the amount of radiation that subjects are exposed to and a paucity of volunteers. The first restriction, pertaining to the applicable dosage per volunteer, was, in this case, limited to 425 MBq (enforced by federal authorities and the local ethics committee) and meant that volunteers could not be measured a second time with any validation method, such as compartmental modelling with arterial-derived input function. In terms of the second factor, due to the limited number of volunteers admitted to the study, complete study datasets consisting of fMRI and steady-state flumazenil PET data were obtained in as many volunteers as possible. Therefore, no subgroup of the subjects could be used in a separate bolus-injection study with arterial sampling, as published by Feng et al. (2016). The presented procedure addresses the aforementioned conditions and shows how the bolus infusion schema for flumazenil can be established in an iterative way in a population-based approach such that constant drug concentrations are reached in the plasma and in the brain tissue from

![Fig. 2](image-url) Range of the [11C]flumazenil time-activity curves of various anatomical brain areas depending on the bolus infusion weighting \( kbol \). The TACs were normalised to their average value from 40 to 100 min and the corresponding range of values was evaluated. The positive and negative sign of this value indicates an increasing or decreasing slope, respectively. The zero crossing (\( * \)) of the best-fit line indicates the optimum bolus value \( kbol_{opt} \) which gives rise to the flat plasma profile in the respective anatomical area. The graphs showing the two VOIs from which the GM signal was numerically removed, the brainstem and the cerebral white matter, have different scales.
between 40 and 100 min after injection of the bolus. This approach does not require additional bolus response measurements. Furthermore, if the initially estimated and then iteratively adapted bolus infusion ratio has led to steady-state conditions, it offers the option to acquire full data sets which are valid for the analysis of the underlying functional experiment. Additionally, it shows how an existing bolus infusion schema can be improved during a series of measurements.

The segmentation of the tissue showed a white matter component of up to 33% in the cortical VOIs and grey matter contributions of up to 19% to cerebral WM, whereby the latter was considered to be pure white matter. Hence, the standard atlas- or VOI-based analyses employed in the

Table 3

| Volumes of interest | Variability of the normalised range Avg±SD [1] | Variability relative to the Avg SD/Avg [1] |
|--------------------|-----------------------------------------------|------------------------------------------|
| Frontal Lobe GM    | 0.042 ± 0.082                                 | 1.94                                    |
| Insular Cortex GM  | 0.125 ± 0.041                                 | 0.33                                    |
| Temporal Lobe GM   | 0.055 ± 0.078                                 | 1.40                                    |
| Parietal Lobe GM   | 0.046 ± 0.073                                 | 1.58                                    |
| Occipital Lobe GM  | 0.080 ± 0.018                                 | 0.23                                    |
| Limbic Lobe GM     | 0.105 ± 0.028                                 | 0.27                                    |
| Thalamus GM        | 0.090 ± 0.183                                 | 2.02                                    |
| Hippocampus GM     | 0.068 ± 0.225                                 | 3.31                                    |
| Amygdala GM        | −0.096 ± 0.216                                | −2.26                                   |
| Basal Ganglia GM   | 0.053 ± 0.181                                 | 3.43                                    |

between 40 and 100 min after injection of the bolus. This approach does not require additional bolus response measurements. Furthermore, if the initially estimated and then iteratively adapted bolus infusion ratio has led to steady-state conditions, it offers the option to acquire full data sets which are valid for the analysis of the underlying functional experiment. Additionally, it shows how an existing bolus infusion schema can be improved during a series of measurements. The segmentation of the tissue showed a white matter component of up to 33% in the cortical VOIs and grey matter contributions of up to 19% to cerebral WM, whereby the latter was considered to be pure white matter. Hence, the standard atlas- or VOI-based analyses employed in the

Fig. 3. $^{11}$C-Activity as a function of time while applying the bolus infusion scheme on two subjects, A and B, measured with $k_{bol} = 53.2$ min and $k_{bol} = 46.9$ min, respectively. The TACs show the activity in the grey matter of the occipital lobe, the plasma curves represent only the not metabolised $^{11}$C-flumazenil. The curves exemplify the widespread of the individual metabolisation rates. Although the $k_{bol}$ value of volunteer A is close to the group optimum $k_{bol} = 54.3$ min, the curves show a decreasing slope. Volunteer B, where the underlying $k_{bol}$ value is far off the optimum, is characterised by almost flat curves.

Fig. 4. Correlation between the range of the $^{11}$C-flumazenil parent substance activities in the plasma with the range of the time-activity curves in different brain regions. All of the curves were calculated based on signal contributions from either pure grey or pure white matter. They normalised to their average value from 40 to 100 min and the corresponding range of values was evaluated. The positive and negative sign of these values indicates an increasing or decreasing slope, respectively. The Pearson correlation coefficients (see lower right corner of each plot), ranging for $r = 0.18$ to $r = 0.82$, indicate very low to high levels of correlation. Note the change of scale in the ordinate of the brainstem and cerebral white matter graphs, which are based on the signal contribution from pure WM.
Table 4

\( k_{bol} \), correlation between the flatness of the plasma and brain profiles, and the distribution volume of the volumes of interest of 17 data sets. The VOIs show the results either from the pure GM or WM content of the respective anatomical areas. The distribution volumes \( V_f \) were calculated for the minimum existing \( N \) cases with the listed TAC range.

| Volumes of interest | \( k_{bol} \) [min] | Correlation plasma/brain, \( p(p) \) | Distribution volume \( V_f \) Avg±SD(range, number) |
|---------------------|-------------------|-----------------|-----------------|
| Frontal Lobe GM     | 53.1              | 0.73 (0.001)    | 8.65 ± 0.73 (0.1, N = 8) |
| Insular Cortex GM    | 58.5              | 0.73 (0.001)    | 7.11 ± 0.90 (0.1, N = 2) |
| Temporal Lobe GM     | 55.3              | 0.75 (0.001)    | 6.87 ± 0.69 (0.1, N = 9) |
| Parietal Lobe GM     | 54.7              | 0.72 (0.001)    | 8.15 ± 1.01 (0.1, N = 9) |
| Occipital Lobe GM    | 55.0              | 0.82 (0.000)    | 8.34 ± 0.85 (0.1, N = 10) |
| Limbic Lobe GM       | 53.9              | 0.82 (0.000)    | 6.87 ± 0.58 (0.1, N = 8) |
| Thalamus GM          | 52.3              | 0.51 (0.037)    | 3.31 ± 0.00 (0.1, N = 1) |
| Hippocampus GM       | 55.4              | 0.55 (0.023)    | 4.65 ± 0.24 (0.2, N = 4) |
| Amygdala GM          | 50.5              | 0.52 (0.032)    | 4.02 ± 0.29 (0.2, N = 7) |
| Basal Ganglia GM     | 54.4              | 0.56 (0.020)    | 3.09 ± 0.36 (0.2, N = 8) |
| Cerebral White Matter| 75.3*             | 0.51 (0.035)    | 0.95 ± 0.02 (0.2, N = 5) |
| WM                  | 61.1*             | 0.18 (0.498)    | 1.11 ± 0.00 (0.3, N = 1) |

* Values are listed for comparison only as WM does not express a significant number of GABA receptors, unlike GM where a flat TAC is preferable.

Fig. 5. Correlation of the distribution volumes, \( V_f \), with values from the literature, which were found after arterial blood sampling and modelling. The comparison comprises results obtained from (a) in increasing order: frontal cortex, temporal cortex, parietal cortex, occipital cortex, hippocampus, thalamus, amygdala, caudate/putamen vs. basal ganglia) the three injections method and modelling used by Millet et al. (Millet et al., 2000), from the slow bolus method (a, parietal cortex, occipital cortex, amygdala, hippocampus) applied on nine (Frankle et al., 2012) and (g) 22 subjects (Frankle et al., 2015), and measured by using the bolus-infusion method (i, occipital cortex, hippocampus, thalamus, cerebral WM) on five subjects (Feng et al., 2016).

literature tend to mix signals from GABA receptor rich and receptor-depleted tissue types. In order to avoid distortions of the analysis of the slope of the TACs arising from this blurring, the individual contributions from GM and WM to the total activity were numerically determined and all subsequent analyses were performed separately for GM and WM.

Another source of mixing potentially different TACs exists in the use of large VOIs, which comprise smaller anatomical areas with possibly varying TAC profiles. As Feng et al. (2016) did not examine this effect, it was studied here by analysing the large number of VOIs defined in the Harvard-Oxford atlas. The TACs of the detailed 48 cortical and 11 subcortical structures exhibited similar slopes that they could not be uniformly fused into larger distinct VOIs for all subjects using the PCA-, ICA-, and k-means clustering pipeline. Instead, the VOIs were manually merged into 12 larger VOIs according to the literature.

In all predominantly grey matter VOIs, the area under the curve AUC-\( GM \) exceeded the AUC-\( WM \) of pure cerebral white matter by up to a factor of nine following the separation of white matter signal contributions (Table 2). The highest value was found in the occipital lobe and corresponded to the highest GABA-receptor density (Kujala et al., 2015). Since white matter does not express GABA receptors in significantly large quantities, only a low and constant presumable background signal was found, most likely owing to unspecific binding and signal from the perfusing blood. The accuracy of the AUCs depends on the accuracy of the GM and WM maps that were computed from MPRAGE data, and secondly, it depends on the accuracy of the activity data obtained from the PET measurements. WM, which was used for GM/WM segmentation, recognises GM and WM with a sensitivity of 0.67 and 0.81, respectively (Kazemi and Noorizadeh, 2014). Moreover, the fact that the BrainPET detector has an optimum resolution of about 3 mm (Herzog et al., 2011) results in the PET signal being blurred within this range, which is in the order of the thickness of the cerebral cortex. In order to minimise the underestimation of the activity in grey matter and the overestimation of the signal from white matter at the transition from grey matter the GM/WM maps were convoluted with a gaussian kernel representing the point spread function of the PET detector, before the data were introduced into the system of equations (Eqn. (1)). In addition to this effect, a possibly imperfect scatter correction may leave a background signal which adds a constant intensity value up to both, grey and white matter. Although the PET data were reconstructed using the PRESTO method, which outperforms the accuracy of the manufacturer provided OSEM3D algorithm, the reconstructed intensity values are still slightly different from the ground truth (unpublished results of our group). Overall, this means that a certain percentage of misclassified voxels, blurred and elevated intensity values were introduced into the system of equations (Eqn. (1)). These limitations of the measurements lower the accuracy of the separation between signals from GABA-receptor rich and receptor-depleted tissue. However, the major difference in signal intensity between grey and white matter could be retrieved.

The scatter of the TAC range values for very similar \( k_{bol} \) values confirms the known broad range of metabolisation rates among the subjects (Ishiwata et al., 1998; Feng et al., 2016). However, due to radiation protection considerations, it was not possible to perform the preparative impulse response measurements individually for the subjects. In order to obtain the \( k_{bol} \) value which has the highest probability of inducing steady state flumazenil levels in the grey matter of all analysed VOIs of all volunteers from 40 to 100 min after injection, the zero crossings of the best-fit lines in the TAC range versus \( k_{bol} \) plots were determined and revealed \( k_{bol,opt} = 54.3 \pm 2.1 \) min. Given the \(^{11}C\) half-life of 20 min, data acquisition later than 100 min after injection is no longer reasonable. Owing to the fact that the determination of the individual \( k_{bol} \) values based on the measurement of a ratio of two activities of the same order of magnitude (the bolus and the infusion activities), the error of the \( k_{bol} \) values is negligible compared to the impact of the metabolisation rates.

The correlation between the range of the flumazenil plasma profiles normalised to the average value from 40 to 100 min and the range of the appropriately normalised TACs in the brain was large, but still not perfect (0.18 ≤ \( r \) ≤ 0.82 for all 12 VOIs). Nevertheless, the correlation was large enough to find cases (1 ≤ \( N \) ≤ 10, depending on the VOI) with steady state conditions from 40 to 100 min in both profiles so that the corresponding distribution volumes could be calculated (Table 4). The results, ranging from 0.95 to 8.34, deviate between −23% and 32% from the
distribution volumes found by modelling after slow bolus injection and arterial blood sampling (Frankle et al., 2012, 2015). The three injections method used by Millet et al. (2000) resulted in distribution volumes from which the presented values differ to a much larger extent - by up to 91%.

Generally, the VOI-specific distribution volume values show a correlation with results from the literature (Fig. 5), which is indicative of the correctness of the presented approach. Differences in the methodology, such as different delineations of the VOIs or the correction for WM and GM-content, may have contributed to the obvious deviations in the correlation. As a result, the proposed iterative method of applying the bolus infusion protocol provided enough cases with sufficiently flat TACs so that they could already be included in the analysis of the functional experiment (to be published in a subsequent manuscript).

An alternative approach to calculating the distribution volume of a radio ligand employs the reference tissue model and requires the identification of an anatomical area devoid of the respective receptors. In the case of flumazenil/GABA receptors, the penis is frequently used for that purpose, although its use is questionable as significant specific uptake has been reported (Abi-Dargham et al., 1994; Frankle et al., 2012), which was confirmed by our findings (see AUC of the Brainstem GM VOI in Table 2). In contrast to the reference tissue approach, the employed steady state approach with metabolite corrected plasma as reference avoids the problem to find a reference tissue area for the calculation of the distribution volume of the radiotracer - without the necessity of arterial blood withdrawal.

Due to the given constraints regarding the administered activity and number of subjects, no alternative methods for validating the result could be used. The discussion of the different steps of the analysis pipeline has focused on possible errors on the way to the final result. The latter is consistent with the independently found result from the literature: by following the standard method with arterial blood sampling and modelling, the result $kbol = 55$ min, which is almost identical to $kbol = 54.3$ min, was found (Feng et al., 2016). The fact that the bolus response was measured on a limited number of subjects ($n = 5$) in that study, may have led to some uncertainty in the $kbol$ value. The concordance of the results in both studies supports the validity of our results.

The suggested $kbol$ value was found in a population-based approach. Therefore, it is most likely to provide flat TACs in subjects when their flumazenil metabolisation rate is unknown. Nevertheless, a bias may be induced if the individual metabolisation rate deviates from the average value. In order to assess this bias, the variation of the TAC profiles was analysed at six almost constant values around $kbol = 46$ min, as not enough data were available at around $kbol = 54.3$ min. Due to the fact that only the deviation from the average slope of the TACs is relevant in this analysis, the assessment at $kbol = 46$ min is equivalent to $kbol = 54.3$ min. Six subjects showed standard deviations of the normalised TAC range from 0.04 (frontal lobe) to 0.13 (insular cortex).

A limitation of the concept of the presented approach lies in the fact that one needs to estimate the time at which the steady state will have been reached. It is only from this time onwards that fMRI data can be acquired, which are synchronous with the PET data, and, therefore, there is a tendency to start the fMRI measurement rather late. This results in a somewhat lower signal-to-noise ratio in the simultaneously acquired PET data because of the radioactive decay of the $^{11}$C-flumazenil. As all volunteers underwent auditory or visual stimulation in the fMRI paradigm, possible side effects must be addressed. For this analysis, the framing of the PET data was set such that the frame length of 5 min significantly exceeded the total time of 41 s, required for presenting 13.6 different visual stimuli, so that possible changes of the receptor binding during the sequence of visual stimulations and resting conditions were averaged out.

A significant difference between the slopes of the TACs in GM and WM was found under the given resolution of the BrainPET detector, GM/WM segmentation method and atlas co-registration. This implies that the numerical separation of the signal contributions from both tissue types would be advisable for future studies.

5. Conclusions

The metabolism of flumazenil shows a wide distribution of rates among the subjects in the examined group. This impedes determination of a universal $kbol$ value for all subjects. However, under the given constraints, $kbol = 54.3$ min is a reliable value that covers the majority of the cases in a sufficient way. The numerical decomposition of the $^{11}$C TACs into the contributions emanating from grey and white matter revealed different slopes. Therefore this step is recommended for future analyses.

Data and code availability

The ethical approval of the study does not allow the data and code (which contains information about the volunteers) to be made publicly available.

CRediT authorship contribution statement

Jörg Mauler: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Visualization, Project administration. Alexander Heinzel: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - review & editing, Supervision, Project administration. Andreas Matusch: Methodology, Formal analysis, Investigation, Writing - original draft. Hans Herzog: Conceptualization, Methodology, Validation, Writing - review & editing. Irene Neuner: Methodology, Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition. Jürgen Scheins: Software, Validation. Christine Wyss: Investigation, Resources, Writing - review & editing, Funding acquisition. Jürgen DAMMERS: Methodology, Software, Formal analysis, Writing - review & editing. Markus Lang: Investigation. Johannes Ermert: Resources, Writing - original draft, Funding acquisition. Bernd Neumaier: Investigation, Resources, Writing - original draft, Project administration, Funding acquisition. Karl-Josef Langen: Writing - review & editing, Supervision, Project administration. N. Jon Shah: Methodology, Validation, Resources, Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

There are no known conflicts of interest.

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Fifty-nine brain region specific time-activity curves were analysed for TAC shapes and were merged into 12 larger regions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroimage.2020.117160.

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