Impact of Neurodegenerative Diseases on Drug Binding to Brain Tissues: From Animal Models to Human Samples

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Published online: 19 April 2018

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Abstract

Drug efficacy in the central nervous system (CNS) requires an additional step after crossing the blood–brain barrier. Therapeutic agents must reach their targets in the brain to modulate them; thus, the free drug concentration hypothesis is a key parameter for *in vivo* pharmacology. Here, we report the impact of neurodegeneration (Alzheimer's disease (AD) and Parkinson's disease (PD) compared with healthy controls) on the binding of 10 known drugs to postmortem brain tissues from animal models and humans. Unbound drug fractions, for some drugs, are significantly different between healthy and injured brain tissues (AD or PD). In addition, drugs binding to brain tissues from AD and PD animal models do not always recapitulate their binding to the corresponding human injured brain tissues. These results reveal potentially relevant implications for CNS drug discovery.

Keywords Unbound drug fraction · Central nervous system · Brain tissue binding · Neurodegeneration · Parkinson · Alzheimer

Introduction

The discovery of therapeutic agents that are able to reach and effectively modulate targets in the brain makes central nervous system (CNS) research one of the most challenging endeavors in drug discovery [1]. CNS-targeting drugs must cross the blood–

brain barrier (BBB) to reach their therapeutic receptors inside the brain. More than 98% of small molecules intended for therapeutic use in the CNS never reach the market because of their inherent inability to cross the BBB [2]. In addition, once therapeutic agents cross the BBB, they must reach their corresponding targets and interact with them to exert the pharmacological actions.

The free drug hypothesis is a fundamental principle for *in vivo* pharmacology [3–7]. This hypothesis states that the free (unbound) drug concentration at the site of action leads to pharmacological activity and that the steady-state free drug concentration is the same across any biomembrane (e.g., the BBB) when drug transporters are not involved in the distribution process [6, 8]. Consequently, the unbound drug concentration in the brain must be measured to guide drug design, lead optimization, and candidate selection in drug discovery.

Direct [9–14] and indirect measurements of the unbound drug concentrations in the brain or by CSF sampling [3, 5, 15, 16], respectively, are analytically challenging and not suitable for high-throughput formats. The most common strategy used in the pharmaceutical industry consists of measuring the unbound drug fraction (f_u) in the brain and the total concentration in the brain [17]. The f_u in brain ($f_{u, \text{brain}}$) is routinely measured using equilibrium dialysis of brain homogenates [18–20].

Traditionally, the unbound drug fraction has been measured in brain tissues from different species. However, after studying the

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13311-018-0624-5>) contains supplementary material, which is available to authorized users.

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unbound fraction of 47 diverse drugs in brain tissues from 7 species and strains, Di and coworkers [17] concluded that $f_{u, \text{brain}}$ is species-independent. The lack of species differences may be due to the higher lipid content in the brain, which is similar across the species tested, leading to high nonspecific binding, or the lack of sufficient concentrations of brain proteins that selectively bind the compounds of interest.

Considering the important changes observed in the brains of patients with neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, we wondered whether $f_{u, \text{brain}}$ is affected by these alterations. Alzheimer's disease (AD) is a neurodegenerative disorder that affects wide areas of the cerebral cortex and hippocampus. AD is associated with the accumulation of insoluble forms of amyloid- β (A β) in plaques in extracellular spaces, as well as in the walls of blood vessels, and the aggregation of the microtubule protein tau in neurofibrillary tangles in neurons [21]. The pathogenesis of Parkinson's disease (PD) is characterized by the loss of dopaminergic nigral neurons and the presence of intraneuronal inclusions known as Lewy bodies [22].

Because the composition of the brain tissues differs, at least in certain localized areas, between neurodegenerative diseases as well as compared with healthy controls, we examined whether $f_{u, \text{brain}}$ also varied in the present study. Thus, f_u was experimentally determined for a small set of diverse drugs in the brain tissues of healthy controls and subjects affected by AD and PD in different species to assess whether the neurodegenerative damage impacts the abilities of these compounds to bind brain tissues. Moreover, this information will also allow us to evaluate whether animal models of neurodegenerative diseases (Parkinson's disease and Alzheimer's disease) are useful for estimating the binding of drugs to brain tissues in human patients.

The obtained f_u values were thoroughly evaluated and statistically analyzed; these results might have important implications in the CNS drug discovery process: from the identification of an adequate pharmacological tool compound for *in vivo* proof of concept to the selection of a clinical candidate and its adequate dosage.

Methods

Test Compounds Test compounds (Fig. 1) were purchased from Sigma-Aldrich (Taufkirchen, Germany) purity > 95%.

Brain Tissues Brains of Alzheimer's model mice, parkinsonian rats, and AD and PD humans, together with their corresponding controls, have been used in this study.

The mouse line used, APP/PS1 transgenic mice, expressed human amyloid precursor protein (APP) 695 isoform with the Swedish double mutation (K670N/M671L), in conjunction with the exon-9-deleted variant of human presenilin 1 (PS1-

dE9) [23]. This double transgenic mice have visible plaque deposition at 6 months of age with an even greater amyloid burden at 18 months and show impaired memory and learning performance from the age of 6 months [24, 25]. Due to ethical reasons, whole mice brains ($n = 4$, 6 months of age) were used to prepare brain homogenates.

The 6-hydroxydopamine (6-OHDA) rat model of Parkinson's disease was obtained as follows: animals were deeply anesthetized via an intraperitoneal injection of a mixture of 4 parts of Ketaset (1% solution of ketamine, 75 mg/kg) and 3 parts of Rompun (2% solution of xylazine, 10 mg/kg). The rats were then placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA), and a total of 8 μg of the neurotoxin 6-OHDA (2 $\mu\text{g}/\mu\text{L}$) was delivered at a rate of 0.5 $\mu\text{L}/\text{min}$ into the medial forebrain bundle (MFB). Twenty-eight days postlesioning, the rotational behavior of the lesioned animals was tested on a computerized rotometer (Panlab, Barcelona, Spain) following intraperitoneal injection of 1 mg/kg apomorphine (Sigma, St. Louis, MO, USA). Circling behavior exhibited after apomorphine treatment is directly related to the extent of the nigrostriatal damage induced with 6-OHDA [26, 27]. In this regard, only animals showing more than 400 net turns over a period of 90 min were considered to be valid for the study ($n = 4$). Lesioned area on left hemisphere was used to prepare brain homogenates, and unlesioned right hemisphere of the same rat was used as control for each compound.

Postmortem human brain tissues were obtained from the Neurological Tissue Bank of Navarra Health Service. To prepare Parkinson's disease brain homogenates, caudate nucleus and putamen were sampled, since these areas are associated with PD. In the case of Alzheimer's disease, hippocampus and entorhinal cortex were obtained, the parts of the brain involved in memory (Fig. 2). For each disease, samples from the same areas of a healthy subject were used as controls. Clinical data of the subjects are summarized in Table 1 (below).

All procedures were approved by the corresponding committees of the University of Navarra, the Ethical Committee for Animal Testing and Research, and the Institutional Review Board of the University Hospital for using human biological samples (refs. 152/12 and 104/2014).

Preparation of Brain Homogenates Brain homogenates were prepared from frozen brain tissue in our laboratory. First of all, tissue was chopped and rinsed twice with 0.9% NaCl solution to remove blood traces. Then, 4 mL of Dulbecco's phosphate-buffered saline (DPBS) was added per gram of tissue (fivefold dilution) and mixed with a high-speed tissue grinder. The homogenates were further processed with a glass Dounce homogenizer and finally frozen at -80°C before use.

Rapid Equilibrium Dialysis Rapid Equilibrium Dialysis (RED) device (Thermo-Fisher Scientific, Waltham, MA) was

Fig. 1 Chemical structures of selected drugs

Fig. 2 Schematic illustration of the areas involved in Parkinson's disease (A) and Alzheimer's disease (B) examined in this study: the caudate nucleus (Cau) and putamen (Put), and hippocampus (Hp) and entorhinal cortex (EC) are illustrated in A and B, respectively. In both cases, because of their anatomical locations, the thalamus (T) and amygdala (A) are depicted. (C) Chemical structures of neurotransmitters acting in the Cau/Put (GABA, glutamate and dopamine) and Hp/EC (glutamate)

Table 1 Clinical data of the subjects

Case	Region	Age (years)	Sex	PMI (h)	pH	Illness description
BCN-219	Caudate and putamen	90	Male	12.3	7.4	Braak PD stage IV
BCN-376	Hippocampus and entorhinal cortex	93	Male	2.45	7.7	Braak AD stage VI
BCN-362	Caudate, putamen, hippocampus and entorhinal cortex	72	Male	9	7.4	Control

PMI, postmortem interval; AD, Alzheimer's disease; PD, Parkinson's disease

prepared prior to experiments following vendor instructions. The base plate was placed in 20% ethanol for 10 min, then rinsed twice with ultrapure water and allowed to dry. The inserts (with a membrane MWCO of 8 kDa) were soaked in ultrapure water for 10 min before discarding the water and soaking for a further 10 min. Water was removed by inverting and shaking gently and the membranes used without allowing them to dry.

An aliquot of compound dissolved in dimethyl sulfoxide (DMSO) stock solution (10 mM) was used to make 100 μ M experimental stock solution. The experimental stock solution was added (1/100 v/v) to the brain homogenate and mixed thoroughly. The final compound concentration for the equilibrium dialysis experiment was 1 μ M with 1% DMSO. Then, a 220- μ L aliquot of brain homogenate spiked with 1 μ M of compound was added to 1 side of the chamber (donor), and 350 μ L of DPBS was added to the other side of the dialysis insert (receiver). Compounds were assessed in triplicate (using 3 RED inserts for each experiment) to determine $f_{u, \text{brain}}$ in mice and rats and in 5 independent occasions in human samples, as we considered these samples more relevant. The RED plate was covered with a sealing tape obtained from Thermo-Fisher Scientific and placed on a shaking plate at 350 rpm and incubated for 4 h at 37 °C.

After incubation, 15 μ L of the brain homogenate from the donor wells and 45 μ L of dialyzed DPBS from the receiver wells were taken in 1.5-mL polypropylene tubes. All samples were matched with an equal volume of opposite blank matrix, and then 180 μ L of cold acetonitrile was added. The sampling cleanup procedure created analytically identical sample matrices (matrix matching), avoiding any potential errors from varying background or ionization efficiency during LC-MS/MS analysis. The tubes were vortex mixed, then mixed for 5 min at 1000 rpm on an Eppendorf Thermomixer, and centrifuged at 13200 rpm and 4 °C for 5 min. Finally, 100 μ L of the supernatant was transferred to a 96-well plate and evaporated at 37 °C under a stream of nitrogen. Residues were dissolved in 100 μ L of initial mobile phase conditions prior to LC-MS/MS analysis except for rifampicin which was dissolved in water:methanol (50:50).

LC-MS/MS Analysis A Xevo-TQ MS triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and an Acquity UPLC (Waters, Manchester,

UK) was used. Chromatographic separation was performed by gradient elution at 0.45 mL/min using a XSelect CSH C18 column (50 $\times$ 2.1 mm, 2.5 μ m particle size; Waters). The mobile phase consisted of A: water with 0.1% formic acid and B: methanol with 0.1% formic acid. Gradient elution started with 10% B, increased to 98% over 3 min, and maintained for 0.5 min, and then a linear gradient to initial conditions was applied for 0.1 min and maintained for 2.4 min to re-equilibrate the column, giving a cycle time of 5 min. The autosampler temperature was set at 10 °C and column temperature at 40 °C. The injection volume was 5 μ L. The compounds were detected using multiple reaction monitoring (MRM). Mass spectrometric settings are summarized in the Supplementary Material (Table S1). For detection and quantification, the electrospray ionization operated in the positive mode, and the collision gas used was ultrapure argon at a flow rate of 0.14 mL/min. Source and desolvation temperatures were 150 and 500 °C, respectively. Capillary voltage was set at 0.5 kV, and ultrahigh purity nitrogen was used for cone gas (50 L/h) and desolvation gas (1000 L/h). Data collection and processing were performed with MassLynx MS software.

Calculation of Fraction Unbound Fraction unbound (f_u) was calculated using Eqs. 1 and 2 as described by Kalvass and Maurer [18] which correct for homogenate dilution (D). Masses in receiver and donor were determined from the area counts in these samples obtained from LC-MS/MS analysis corrected to account for sampling volumes. A dilution factor of 5 ($D = 5$) was applied in the calculation that reflects the dilution of the brain tissue homogenates.

$$\text{Diluted } f_{u,d} = \frac{\text{receiver mass}}{\text{donor mass}} \quad (1)$$

$$\text{Undiluted } f_u = \frac{I/D}{((1/f_{u,d}) - 1) + 1/D} \quad (2)$$

Statistics Data were summarized as mean and standard deviation in tables, and means with 95% confidence interval for the mean in graphs, and analyzed with 2 $\times$ 2 (disease status and species) factorial ANOVA. When interaction was significant, simple effects contrasts were used to study the differences

between disease statuses within each species. Normality was assessed on the ANOVA models residuals using Shapiro–Wilk test. The statistical software used was Stata 12.1.

Results

A small set of 10 drugs covering a wide range of the unbound fraction in brain tissue, human $f_{u, \text{brain}}$ data available in the literature [17], and a broad scope of physicochemical properties was selected for this study (Fig. 1). Chemical diversity was focused on those descriptors that play a role in plasma protein binding, as guidance for brain protein binding. Thus, we considered 7 descriptors to predict binding (via Bayesian classifier) to human serum albumin (HSA, the most ubiquitous plasma protein) with good accuracy, 70% on average [28]: molecular weight (MW), the predicted octanol/water partition coefficient (AlogP), number of charged acceptor groups (CAG, e.g., carboxylic acid groups, a group known to contribute to HSA binding), hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), molar refractivity (MR, a measure of the polarizability of the molecule), and polar surface area (PSA). The covered range of fraction unbound to brain tissue spans > 2 log units, from 0.003 to 0.583; on the other hand, the relevant chemical descriptors also cover a broad range (e.g., MW, from 151 to 823; AlogP, from -0.72 to 4.24; TPSA, from 15.3 to 220.1) (Table 2). According to relevant descriptors for a related biological response (HSA binding), the use of a representative set of chemically diverse compounds covering a range of $f_{u, \text{brain}}$ data should allow us to explore a wide spectrum of potential responses.

The $f_{u, \text{brain}}$ data for the 10 selected drugs were experimentally determined in brain tissues affected by neurodegenerative diseases, such as PD and AD. For the human samples, we

focused on the areas that are predominantly affected by the disease to prepare brain homogenates: for PD, the caudate nucleus and putamen (brain regions associated with movement control) and for AD, the hippocampus and entorhinal cortex (brain areas involved in memory) (Fig. 2).

In the caudate nucleus and putamen, the vast majority of output neurons use GABA as a neurotransmitter (Fig. 2C), an endogenous neutral zwitterionic ligand, with an inhibitory effect on its targets [31]. The dopaminergic input from the substantia nigra and the glutamatergic input from the cortex and the thalamus to the caudate/putamen modulate GABA transmission [32]. The caudate/putamen presents the highest levels of dopamine receptors, dopamine (Fig. 2C, positively charged at physiological pH), and its metabolites in the brain [33, 34]. On the other hand, the majority of synapses in the cortical and hippocampal regions utilize glutamate (Fig. 2C), endogenous substrate bearing a net negative charge at physiological pH, as a neurotransmitter that activates glutamate and *N*-methyl-D-aspartate (NMDA) receptors [35–37]. The hippocampus contains the highest density of glutamate receptors, particularly *N*-methyl-D-aspartate (NMDA) receptors, which mediate some forms of learning and memory. Thus, due to the different natures of the neurons and neurotransmitters, as well as the heterogeneous distribution of neurotransmitter receptors among the different brain regions, we expect that the $f_{u, \text{brain}}$ values also differ between the 2 brain areas analyzed in healthy donors.

To determine the impact of the brain tissue area on ligand binding, $f_{u, \text{brain}}$ values from healthy controls were calculated in the post-mortem human brain regions affected by PD (caudate/putamen) and AD (entorhinal cortex/hippocampus) and compared. Results obtained for the 10 selected drugs using rapid equilibrium dialysis (RED) with diluted ($5\times$) brain homogenates are shown in Table 3.

Table 2 Physicochemical properties and published $f_{u, \text{brain}}$ data for the test compounds

Compound	MW	AlogP	CAG	HBD	HBA	MR	TPSA	Ionic Label ^a	Therapeutic indication	$f_{u, \text{human brain}}^a$
Amantadine	151	1.50	0	1	1	4.5	26.0	Base	Antiparkinson ^b	0.171 ± 0.060
Carbamazepine	236	2.68	0	1	1	7.1	46.3	Neutral	Antiepileptic ^b	0.171 ± 0.021
Chlorpropamide	277	2.06	1	2	3	6.8	75.3	Acid	Hypoglycemic agent ^b	0.583 ± 0.274
Clinafloxacin	366	-0.72	1	2	6	9.0	86.9	Zwitterion	Antibiotic ^c	0.157 ± 0.100
Desipramine	266	3.85	0	1	2	8.4	15.3	Base	Antidepressant ^b	0.011 ± 0.000
Entacapone	305	1.66	2	2	6	7.8	130.4	Acid	Antiparkinson ^b	0.025 ± 0.003
Indomethacin	358	4.24	1	1	4	9.6	68.5	Acid	Anti-inflammatory agent ^b	0.055 ± 0.005
Metoclopramide	300	1.78	0	2	4	8.2	67.6	Base	Antiemetic ^b	0.295 ± 0.036
Rifampicin	823	3.26	1	6	15	21.7	220.1	Zwitterion	Antibiotic ^b	0.119 ± 0.012
Tolcapone	273	3.13	2	2	5	7.1	103.4	Acid	Antiparkinson ^b	0.003 ± 0.000

^a Previously reported data [17]

^b Data obtained from www.drugbank.ca [29]

^c This molecule has not yet been approved by the FDA, but it is annotated in the clinical drug library of 1524 drugs [30]

Table 3 Comparison of the unbound fraction (f_u) in homogenates of healthy postmortem human brain tissues from different areas: affected in Alzheimer's disease ($f_{u, \text{control-ADarea}}$; hippocampus and entorhinal cortex) and affected in Parkinson's disease ($f_{u, \text{control-PDarea}}$; caudate nucleus and putamen); p values for the statistical analysis are also shown

Compound	$f_{u, \text{control-ADarea}} (n = 5)^a$	$f_{u, \text{control-PDarea}} (n = 5)^{a,b}$	p value
Amantadine	0.389 ± 0.032	0.277 ± 0.051	0.014*
Carbamazepine	0.237 ± 0.017	0.203 ± 0.024	0.031*
Chlorpropamide	0.752 ± 0.247	0.733 ± 0.091	0.891
Clinafloxacin	0.610 ± 0.197	0.606 ± 0.096	0.972
Desipramine	0.013 ± 0.002	0.013 ± 0.001	0.847
Entacapone	0.045 ± 0.008	0.029 ± 0.003	0.007**
Indomethacin	0.092 ± 0.027	0.076 ± 0.014	0.278
Metoclopramide	0.487 ± 0.080	0.584 ± 0.133	0.199
Rifampicin	0.160 ± 0.025	0.191 ± 0.030	0.110
Tolcapone	0.011 ± 0.005	0.005 ± 0.004	0.094†

^a Each independent RED measurement (n) was determined in duplicate

^b Except for compounds 1, 3, 4, 5 and 10, for which $n = 4$ due to limited sample availability

* $p < 0.05$ and ** $p < 0.01$: statistically significant differences between different control brain areas

† $0.05 < p < 0.1$: tendency toward significant differences between different control brain areas

Amantadine, carbamazepine, and entacapone showed statistically significant differences between the $f_{u, \text{brain}}$ values determined in the hippocampus/entorhinal cortex and $f_{u, \text{brain}}$ values determined in the caudate nucleus and putamen. In addition, tolcapone exhibited a tendency toward a significant difference (with $0.05 < p < 0.1$). Interestingly, 3 of the drugs (amantadine, entacapone, and tolcapone) that show a high bound fraction in the caudate/putamen are antiparkinson agents (Table 2). Different brain areas have diverse compositions; thus, as we might expect, compounds binding to brain tissues ($f_{u, \text{brain}}$) vary from 1 CNS area to another.

Consequently, as the composition of the same brain tissue region differs (at least in some CNS areas) between healthy controls and patients suffering neurodegeneration, the next step was to determine experimentally the impact of neurodegenerative diseases (PD and AD) on drug binding to brain tissues. Thus, brain tissues from human patients with AD and PD and from rodent models of AD and PD (described above, “Methods”) were utilized to measure the $f_{u, \text{brain}}$ values for the 10 selected drugs. All these $f_{u, \text{brain}}$ data and the corresponding comparisons (healthy controls compared with AD or PD) are presented in Tables 4 and 5.

A 2×2 (disease status and species) factorial ANOVA was conducted to evaluate whether significant differences existed in the $f_{u, \text{brain}}$ between healthy and injured brain tissues (AD or PD). In cases where the interaction was significant, simple effects contrasts were used to study the differences between disease statuses within each species. The obtained p values are displayed in Tables 4 and 5.

A detailed analysis of Table 4 shows the significant impact of postmortem human AD tissue on the binding of carbamazepine, as well as a clear trend (with $0.05 < p < 0.1$) for entacapone and rifampicin. In fact, for these 3

drugs, the unbound fraction is higher in the postmortem healthy human tissue from the AD-related regions. Then, considering that the total drug concentration in the brain is equal in a healthy control and AD patient, according to these results, the latest will have lower free drug concentration in entorhinal cortex/hippocampus than a healthy person; thus, it is relevant implications for therapeutic response and dosage. On the other hand, although there is a clear significant impact of brain tissues from the mouse AD model on the binding of entacapone and rifampicin, their behaviors were opposite; the unbound fraction for these 2 drugs was remarkably reduced in brain tissues from control mice. A similar result was also obtained for tolcapone; this drug tends to display a higher unbound fraction in postmortem brain tissues from the mouse AD model.

Regarding the impact of postmortem human PD brain tissue on drug binding, Table 5 clearly highlights entacapone; a significantly lower unbound fraction (31%) was observed in the brain tissue from a patient with PD than in the postmortem brain tissue, PD-related regions, from a healthy donor. Indomethacin also shows a tendency toward a significantly higher unbound fraction in PD-related brain regions from a postmortem healthy control. These results are similar to those observed above for AD patients, over-estimating the free drug concentration in the injured brain region of PD patients.

Finally, as indicated for the mouse AD model (Table 4), no correlation was observed between the results obtained with postmortem samples from human patients with PD and animal PD models (Table 5). Only carbamazepine showed a statistically significant difference (a higher unbound fraction in control rats); in fact, brain tissue from the rat model of PD had no impact on the

Table 4 Unbound fraction (f_u) and standard deviations for binding to homogenates of healthy ($f_{u, \text{control}}$) and Alzheimer's disease brain tissues ($f_{u, \text{Alzheimer}}$) from postmortem human and mouse subjects. The corresponding p values obtained from the statistical analysis are also displayed

Compound	Human			Mouse		
	$f_{u, \text{control}} (n = 5)^a$	$f_{u, \text{Alzheimer}} (n = 5)^a$	p value	$f_{u, \text{control}} (n = 3)^a$	$f_{u, \text{Alzheimer}} (n = 3)^a$	p value
Amantadine	0.389 ± 0.032	0.362 ± 0.105	0.565	0.302 ± 0.021	0.339 ± 0.083	0.542
Carbamazepine	0.237 ± 0.017	0.207 ± 0.029	0.039*	0.258 ± 0.017	0.268 ± 0.010	0.554
Chlorpropamide	0.752 ± 0.247	0.728 ± 0.239	0.868	0.749 ± 0.107	0.961 ± 0.185	0.255
Clinafloxacin	0.610 ± 0.197	0.579 ± 0.367	0.849	0.951 ± 0.112	0.903 ± 0.091	0.815
Desipramine	0.013 ± 0.002	0.011 ± 0.002	0.229	0.013 ± 0.001	0.014 ± 0.002	0.387
Entacapone	0.045 ± 0.008	0.034 ± 0.009	0.052 [†]	0.029 ± 0.003	0.060 ± 0.010	0.001***
Indomethacin	0.092 ± 0.027	0.084 ± 0.010	0.488	0.093 ± 0.005	0.081 ± 0.002	0.385
Metoclopramide	0.487 ± 0.080	0.553 ± 0.210	0.479	0.649 ± 0.083	0.600 ± 0.117	0.681
Rifampicin	0.160 ± 0.025	0.137 ± 0.019	0.082 [†]	0.184 ± 0.005	0.313 ± 0.010	0.001***
Tolcapone	0.011 ± 0.005	0.010 ± 0.004	0.624	0.006 ± 0.000	0.011 ± 0.002	0.092 [†]

^a Each independent RED measurement (n) was determined in duplicate

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$. Statistically significant differences between controls and patients or AD mice

[†] $0.05 < p < 0.1$: tendency toward significant differences between controls and patients or AD mice

binding of entacapone and indomethacin compared with the controls.

Discussion

As the composition of the brain tissue differs, at least in certain localized areas, between human patients with

neurodegenerative diseases and healthy controls, $f_{u, \text{brain}}$ values also vary for some molecules. Based on our analyses, neurodegenerative damage significantly impacts on the binding of some compounds to the postmortem brain tissue corresponding to regions affected in patients with AD and PD. Thus, at least for some drugs, our results clearly indicate that $f_{u, \text{brain}}$ is affected by these alterations. Therefore, taking also into account the total drug

Table 5 Unbound fraction (f_u) and standard deviations for binding to homogenates of healthy ($f_{u, \text{control}}$) and Parkinson's disease brain tissues ($f_{u, \text{Parkinson}}$) from postmortem human and rat subjects. Rat controls come from the contralateral hemisphere of the brain of 6-OHDA rat model

(unlesioned right hemisphere, as described in “[Methods](#)”). The corresponding p values obtained from the statistical analysis are also displayed

Compound	Human			Rat		
	$f_{u, \text{control}} (n = 5)^{a,b}$	$f_{u, \text{Parkinson}} (n = 5)^a$	p value	$f_{u, \text{control}} (n = 3)^a$	$f_{u, \text{Parkinson}} (n = 3)^a$	p value
Amantadine	0.277 ± 0.051	0.294 ± 0.043	0.601	0.306 ± 0.059	0.338 ± 0.041	0.437
Carbamazepine	0.203 ± 0.024	0.196 ± 0.009	0.490	0.269 ± 0.007	0.235 ± 0.009	0.020*
Chlorpropamide	0.733 ± 0.091	0.888 ± 0.389	0.363	0.882 ± 0.073	0.998 ± 0.081	0.572
Clinafloxacin	0.606 ± 0.096	0.494 ± 0.180	0.294	0.833 ± 0.145	0.673 ± 0.163	0.223
Desipramine	0.013 ± 0.001	0.013 ± 0.002	0.949	0.011 ± 0.001	0.012 ± 0.000	0.808
Entacapone	0.029 ± 0.003	0.020 ± 0.004	0.002***	0.045 ± 0.002	0.044 ± 0.005	0.744
Indomethacin	0.076 ± 0.014	0.063 ± 0.005	0.068 [†]	0.072 ± 0.010	0.079 ± 0.009	0.407
Metoclopramide	0.584 ± 0.133	0.516 ± 0.060	0.252	0.584 ± 0.029	0.538 ± 0.062	0.531
Rifampicin	0.191 ± 0.030	0.146 ± 0.026	0.114	0.238 ± 0.056	0.286 ± 0.066	0.186
Tolcapone	0.005 ± 0.004	0.006 ± 0.005	0.775	0.016 ± 0.002	0.018 ± 0.003	0.532

^a Each independent RED measurement (n) was determined in duplicate

^b Except for 1, 3, 4, 5 and 10, for which $n = 4$ due to limited sample availability

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$. Statistically significant differences between controls and patients or PD rats

[†] $0.05 < p < 0.1$: tendency toward significant differences between controls and patients

concentration in the brain, these results suggest an impact on drug efficacy in human patients with AD and PD: we might be under- or over-estimating the free concentration of utilized drugs in the region of action. These findings have important implications in the CNS drug discovery process: from the selection of a clinical candidate to the definition of an appropriate dosage to achieve the pharmacological response.

On the other hand, taking into account that unbound drug fractions measured using brain homogenates from AD and PD animal models do not recapitulate results obtained with human samples, these data suggest focusing on postmortem human samples to obtain a reliable conclusion for each tested compound. Although a small amount of human sample is required and this analysis might be only required for the last prioritization step (clinical candidate designation), a predictive in silico model is currently under construction.

As a next step, we will expand the number of CNS drugs examined and will increase the number of human samples, in terms of the number of patients and different disease statuses. These additional studies are needed to confirm these findings, natural variability is usually observed in human samples, and fine-tune their implications.

If these results are confirmed, the roadmap for the CNS drug discovery process might require an update in terms of clinical candidate selection and compound dosage. Thus, in addition to classical requirements (e.g., related to crossing BBB and total drug concentration), (i) using postmortem human brain samples (healthy controls and patients with neurodegenerative disorders) to perform reliable measurements of $f_{u,brain}$ on the areas of interest and (ii) prioritizing molecules which unbound drug fraction in the brain are high and are minimally affected by neurodegeneration. If injured brain tissue has any significant impact on selected for selected candidate, $f_{u,brain}$, then, its corresponding dosage should be adjusted accordingly.

Acknowledgments We thank the Neurological Tissue Bank of Navarra Health Service for providing us the postmortem human brain samples. We also thank Carmen Molina for technical support.

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Funding This study was supported by Foundation of Applied Medical Research (Spain), University of Navarra, by a Torres Quevedo Grant (Ana Ugarte) from Ministerio de Economía, Industria y Competitividad (MINECO) (PTQ-12-05641) and by the ISCIII-FEDER (PI14/02070).

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Abbreviations 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; BBB, blood-brain barrier; CSF, cerebrospinal fluid; PD, Parkinson's disease; RED, rapid equilibrium dialysis; TPSA, topological polar surface area

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