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An ex Vivo Model for Evaluating Blood–Brain Barrier Permeability, Efflux, and Drug Metabolism

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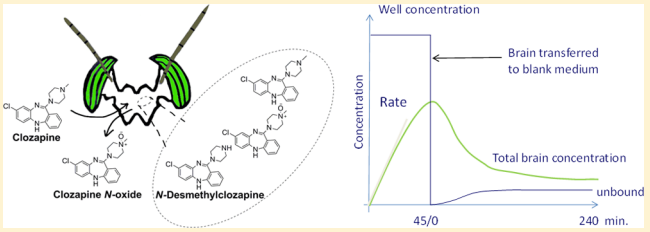
Supporting Information

ABSTRACT: The metabolism of drugs in the brain is difficult to study in most species because of enzymatic instability in vitro and interference from peripheral metabolism in vivo. A locust ex vivo model that combines brain barrier penetration, efflux, metabolism, and analysis of the unbound fraction in intact brains was evaluated using known drugs. Clozapine was analyzed, and its major metabolites, clozapine *N*-oxide (CNO) and *N*-desmethylclozapine (NDMC), were identified and quantified. The back-transformation of CNO into clozapine observed in humans was also observed in locusts. In addition, risperidone, citalopram, fluoxetine, and haloperidol were studied, and one preselected metabolite for each drug was analyzed, identified, and quantified. Metabolite identification studies of clozapine and midazolam showed that the locust brain was highly metabolically active, and 18 and 14 metabolites, respectively, were identified. The unbound drug fraction of clozapine, NDMC, carbamazepine, and risperidone was analyzed. In addition, coadministration of drugs with verapamil or fluvoxamine was performed to evaluate drug–drug interactions in all setups. All findings correlated well with the data in the literature for mammals except for the stated fact that CNO is a highly blood–brain barrier permeant compound. Overall, the experiments indicated that invertebrates might be useful for screening of blood–brain barrier permeation, efflux, metabolism, and analysis of the unbound fraction of drugs in the brain in early drug discovery.



KEYWORDS: *Locust, brain, drug metabolism, blood–brain barrier, efflux, drug–drug interactions*

Optimizing drugs for central nervous system (CNS) diseases include designing compounds with high efficiency for brain penetration, quantified as drugs with high permeability, high unbound concentration in the brain, high uptake transport activity, low efflux transport activity, and low brain metabolism. However, in the early phase of drug discovery, the focus has been on whether drugs reach the brain or not, and not on the fate of drugs beyond "the wall." We report on an ex vivo model for evaluation of brain barrier penetration efficiency with a focus on drug metabolism and the unbound fraction in the brain. Screening of brain permeation[1] and efflux transport activity[2] using the same model have previously been reported.

Drug metabolism by cytochrome P450 enzymes (CYPs) takes place primarily in the liver, but CYPs are also found in many other organs, including the brain.[3−5] Because the amount of CYPs present in the brain is just a fraction of that found in the liver, drug metabolism in the brain has largely been ignored during the drug discovery process. However, CYPs in the brain are localized within specific cells[6] (Table 1) where specific CYPs can reach concentrations comparable with the ones found in the liver.[7] Thus, cell-specific drug metabolism can generate significant effects if it takes place within the vicinity of the site of action. In addition, unique CYPs are present in the brain that are not present in the liver, which leads to different metabolite profiles in the brain and liver. An example is the metabolism of the anxiolytic drug alprazolam that shows different metabolite profiles in the brain and liver.[8] Another important aspect of metabolism in the brain is the generation of toxic metabolites in the brain; the pesticide chlorpyrifos can cause neurologic effects in humans probably through the metabolite chlorpyrifos-oxon. However, chlorpyrifos-oxon formed peripherally is reactive and quickly inactivated and unlikely to reach the brain, suggesting that a direct transformation in the brain causes the neurological effects.[9] Moreover, it has been brought forward that drugs such as carbamazepine might cause affective and cognitive malignancies in patients by interfering with neurosteroid homeostasis via region-specific CYP induction in the brain.[6]

Studies on the function of brain CYPs are challenging for several reasons. In the whole organism, CYPs in the periphery produce many of the same metabolites as those in the brain, and many of these metabolites can cross the blood–brain barrier (BBB) from the periphery, which makes the relative contributions of hepatic and brain metabolism in vivo difficult to study. Additionally, ethical considerations limit the use of mammals in the initial phases of drug discovery when numerous compounds are evaluated.

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Table 1. CNS-Acting CYPs and Endogenous Substrates and Metabolites[a]

enzyme[b]	brain region (cell type)[c]	endogenous substrate and metabolites[d]
CYP2B6	cerebellum, hippocampus (N, A)	estradiol, arachidonic acid, estrone, serotonin, testosterone
CYP2D6	cortex, cerebellum, midbrain, striatum, hippocampus, thalamus (N)	progesterone, tyramine
CYP2E1	frontal cortex, hippocampus, cerebellum (N)	estradiol, arachidonic acid, estrone, prostaglandin, arachidonic acid, linoleic acid, oleic acid/HETE, hydroxylinoleic acid, hydroxyoleic acid
CYP1A1	abundant (N, A)	estradiol, arachidonic acid, progesterone, all-*trans*-retinal/l, retinol, all-trans-retinoic acid
CYP1B1	temporal lobe (N)	melatonin, estradiol, hydroxyestradiol
CYP2B	blood–brain barrier, frontal cortex, striatum, olfactory tubercle (N, A)	arachidonic acid, testosterone, serotonin, all-*trans*-retinoic acid /hydroxytestosterone, nitric oxide, hydroxyretinoic acid, oxoretinoic acid
CYP2C	blood–brain barrier, abundant (N, A)	testosterone, progesterone, arachidonic acid, serotonin, harmaline, harmine, linoleic acid, melatonin, all-*trans*-retinoic acid/hydroxytestosterone, hydroxyprogesterone, nitric oxide, harmalol, harmol, hydroxyoctadecadienoic acid, *N*-acetylserotonin, hydroxyretinoic acid
CYP2D	olfactory bulb, striatum, pons, medulla oblongata, substantia nigra, cerebellum	octopamine, synephrine, tyramine, progesterone, harmaline, harmine /noradrenaline, adrenaline, dopamine, deoxycorticosterone, hydroxyprogesterone, harmalol, harmol

[a]Data combined from refs 6, 11, 14, and 15. [b]Human CYPs. [c]Brain regions in mammals. Cell type A = astrocytes, N = neurons. [d]Substrates/metabolites identified in humans and insects.

In our search for a model that can be efficiently used in early drug discovery to evaluate the pharmacokinetic properties of potential drugs and toxins in the central nervous system, we sought out alternative animal models based on insects and zebrafish. We chose the desert locust, *Schistocerca gregaria*, which has the potential to combine blood–brain barrier penetration, transporter activities, drug metabolism, and fraction of unbound drug concentrations in the brain in one ex vivo model. This model is a further development of a recently validated locust model for assessment of blood–brain barrier permeability.[1] As in mammals, the insect brain has conserved the essential mechanisms for protecting the CNS, that is, the requirements for tightness and control (transporters) of elements entering the brain.[10] Furthermore, the difference in liver and brain metabolism has led to the hypothesis that CYPs in neurons may have specific functions other than metabolism of xenobiotics. CYP function in neurons is about cell-specific maintenance of endogenous neurochemicals, and CYPs in astrocytes help the blood–brain barrier protects against xenobiotics. Although the insect CYPs identified thus far share limited sequence identity with human CYPs, mammals and invertebrates share a large number of neurochemicals (Table 1).[11] Thus, it is still likely that in mammals and insects these neurochemicals share endogenous pathways of maintenance. Furthermore, the interplay between CYP3A4 and P-glycoprotein (P-gp),[12] a pathway for brain clearance of metabolites due to the overlapping pharmacophores, seems to be conserved across species, as both proteins have close homologues in *Schistocerca gregaria*.[2,13]

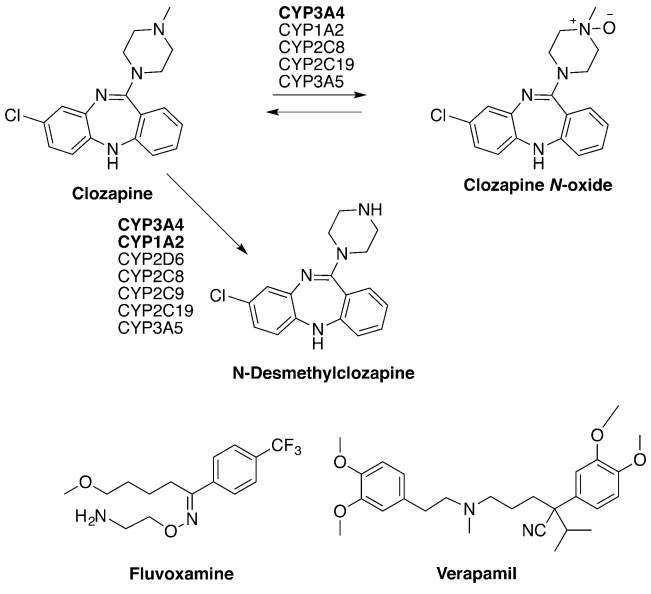
Here, we report experiments in which we compare data using the locust model and data extracted from the literature for the reference compounds.

RESULTS AND DISCUSSION

A Brief Overview of the Method. In the locust ex vivo model, the brain is dissected from the insect and placed in a well containing the test compound of interest. After exposure, the concentration in the brain is measured with high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) after homogenizing the brain followed by centrifugation. The absence of a vascular system in insects makes the ex vivo model independent of blood flow through the brain. Thus, the locust ex vivo model uses controlled in vitro–like exposure conditions that provide direct comparison of chemical compounds. The locust brain is designed for compensating for the absence of a vascular system, with a small size but relatively large surface of ($1.5 \times 1.5 \times 0.7$ mm^3) with tracheoles (blind ended tubes). This makes the insect brain unique compared to mammals. The insect brain is enclosed within an intact brain barrier after dissection, resembling a compressed golf ball.

The Clozapine and Metabolites. In 2003–2004, researchers from Merck & Co[17] and ACADIA Pharmaceuticals[18] independently suggested that *N*-desmethylclozapine (NDMC), the major metabolite of clozapine (Scheme 1), was behind the unique efficacy of clozapine to improve

Scheme 1. Metabolic Pathways of Clozapine, and Compounds to Study Drug–Drug Interactions[a]



[a]Structures of clozapine, clozapine *N*-oxide (CNO), and *N*-desmethylclozapine (NDMC). The CYPs involved in each pathway are indicated, and the major ones are bolded.[16] Fluvoxamine and verapamil herein used to study drug–drug interactions.

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Table 2. Analysis of Clozapine, NDMC, and CNO[a]

test compd	concn	incubation	clozapine (μM)	NDMC (μM)	CNO (μM)
clozapine (brain)	1 μM	15 min	0.1 ± 0.02	2.6 ± 0.14	nd
well			0.34 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
clozapine (brain)	1 μM	45 min	0.29 ± 0.05	4.0 ± 0.80	0.04 ± 0.00
well			0.33 ± 0.01	0.05 ± 0.01	0.04 ± 0.00
clozapine (brain)	3 μM	15 min	1.3 ± 0.30	3.8 ± 0.46	0.05 ± 0.01
well			1.32 ± 0.02	0.02 ± 0.01	0.11 ± 0.01
clozapine (brain)	3 μM	45 min	3.9 ± 0.9	8.7 ± 1.7	0.13 ± 0.02
well			1.2 ± 0.23	0.12 ± 0.05	0.11 ± 0.01
NDMC (brain)	1 μM	15 min	nd	1.2 ± 0.22	nd
well			nd	0.6 ± 0.07	nd
NDMC (brain)	1 μM	45 min	nd	4.3 ± 0.83	nd
well			nd	0.6 ± 0.05	nd
NDMC (brain)	3 μM	15 min.	nd	6.2 ± 1.11	nd
well			nd	2.3 ± 0.06	nd
NDMC (brain)	3 μM	45 min	nd	15.4 ± 3.4	nd
well			nd	1.8 ± 0.20	nd
CNO (brain)	1 μM	15 min	nd	0.04 ± 0.00	nd
well			nd	nd	1.0 ± 0.18
CNO (brain)	1 μM	45 min	nd	0.13 ± 0.01	0.06 ± 0.01
well			nd	nd	1.1 ± 0.03
CNO (brain)	3 μM	15 min	nd	0.05 ± 0.01	0.15 ± 0.04
well			nd	nd	3.2 ± 0.3
CNO (brain)	3 μM	45 min	0.27 ± 0.76	0.36 ± 0.26	0.2 ± 0.1
well			0.01 ± 0.00	nd	2.8 ± 0.6

[a]Values represent the mean ± SD of three independent experiments (n = 3), two locust brains pooled in each experiment, from one male and one female. nd = not detected (concentrations in the brain < 0.02 μM, concentrations in wells < 0.002 μM).

cognition and negative symptoms in the treatment of patients with schizophrenia. The metabolite NDMC was detected in concentrations 20−150% of that observed for clozapine in humans. NDMC was evaluated in a phase IIb trial for the treatment of schizophrenia. Unfortunately, NDMC demonstrated no improved efficacy compared to placebo at two doses, and evaluation at higher doses was discouraged because of peripheral dose-dependent side effects. Thus, the NDMC hypothesis remains to be tested. Replacing the *N*-methyl with hydrogen, that is, adding a hydrogen bond donor, theoretically would decrease blood−brain barrier penetration and could be the reason for NDMC's lack of efficacy in the trial. The other major clozapine metabolite in humans, clozapine *N*-oxide (CNO), is used as an inert ligand in chemical genetics using the Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology and is interesting itself.[19,20]

In the present study, clozapine, NDMC, and CNO were incubated with an isolated locust brain, and the brain content was analyzed for clozapine, NDMC, and CNO (Table 2). In addition, the test solutions and the actual concentrations of the compounds in the wells after the experiments were measured. The test solutions were analyzed to confirm the initial concentrations and to exclude contamination by the other two compounds. Atenolol, which does not readily penetrate the blood−brain barrier, was included as a quality control reference in the experiments to ensure that the experimental procedure did not damage the brain barrier (results not shown). The test solutions used in this study were made as 1 and 3 μM solutions, but the actual concentrations measured in the well after the experiments were lower than the initial concentration. This is commonly seen for several compounds tested in the ex vivo model. In-house studies have shown that the reduced well concentration most likely is due to the compounds sticking to the wall in the well. This is a common problem, which in general is more pronounced for compounds that are more lipophilic. The selection of 1 and 3 μM solutions was based on previous studies; concentration lower than 1 μM showed increased variability in the exposure concentrations because of adsorption to the well, and concentrations about 3 μM are starting to show saturation of the biological systems.[1,2]

As observed from the ex vivo experiment results shown in Table 2, clozapine permeated the locust brain barrier, and concentrations of 0.1 μM or higher were obtained at all conditions used in this study. When the incubation time was increased from 15 to 45 min, the concentration of clozapine in the brain increased approximately three times, and increasing the incubation concentration 3-fold, from 1 to 3 μM, rendered at least a 10-fold increase in the clozapine concentration in the brain. Thus, the concentration in the brain increased more than linearly after the exposure concentration was increased, indicating a saturation of the enzymatic system. Together with clozapine, the two major clozapine metabolites, NDMC and CNO, were identified in all of the experiments in which locust brains were incubated with clozapine. The two metabolites were also found in the well solutions after the experiments. To rule out that NDMC and CNO came from contamination, the test solutions were analyzed, and only clozapine was found in the test solutions used in the experiments. As the volume of the test solution in the well was about 150 times larger than the insect brain volume (250 μL versus 1.6 μL), large quantities of metabolites may have crossed the brain barrier from the brain and out in the well solution. In particular, at 1 μM and 15 min of incubation, the amount of CNO in the brain was below the detection limit (<0.02 μM) while the CNO concentration in the well was 0.04 μM. Thus, although low CNO concentrations were detected in

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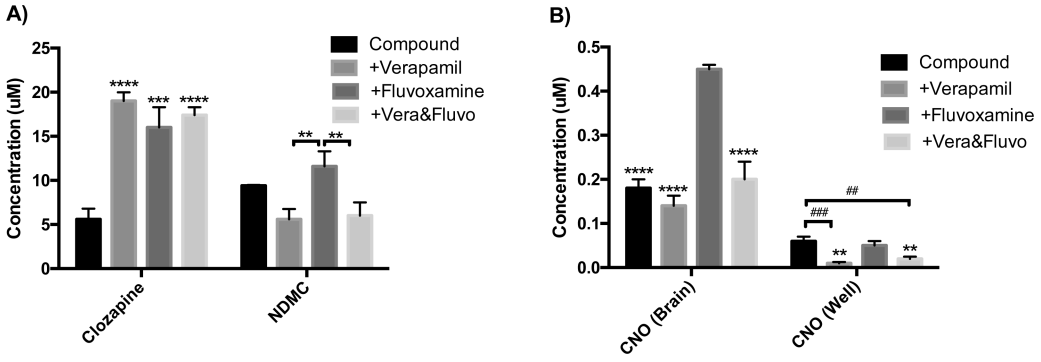

Figure 1. Interaction experiments of clozapine with verapamil, fluvoxamine, or both. Incubation time (45 min) with clozapine (3 μM) alone or together with verapamil (25 μM), fluvoxamine (25 μM), or both. Values represent the mean ± SD of three independent experiments (n = 3), two locust brains pooled in each experiment, from one male and one female. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. (A) Statistically significant differences from clozapine and differences marked with brackets are indicated by * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). (B) Statistically significant differences from coadministration of fluvoxamine are indicated by (*) and significant differences marked by brackets are indicated by (#), ** or ## (p < 0.01), *** or ### (p < 0.001), and **** (p < 0.0001). Vera = verapamil, Fluvo = fluvoxamine.

the brain, a substantial amount of CNO was formed in the brain and transferred across the barrier to the well solution. This could have occurred because of active transport, passive diffusion because of a large unbound fraction in the brain, or both.

NDMC, the main metabolite, was present in higher concentrations than clozapine itself. After 15 min of incubation at 1 μM, the NDMC concentration in the brain reached levels more than 20 times higher than the clozapine concentration in the brain. After 45 min at 1 μM, the NDMC level was around 10 times higher than clozapine. Increasing the exposure concentration from 1 to 3 μM reduced the difference between the clozapine and NDMC concentrations in the brain. After 15 min of incubation at 3 μM, the NDMC level was approximately three times higher than clozapine, and after 45 min, the NDMC concentration was approximately twice that of clozapine. This indicates that the metabolism of clozapine to NDMC is concentration dependent and influences the difference between the clozapine and NDMC concentrations in the brain.

Similar to CNO, NDMC was found in the brains and in the well solutions. The NDMC concentrations measured in the wells after 15 or 45 min at 1 or 3 μM were around 0.1 μM or less. Although the NDMC concentrations found in the brain were 100-fold higher than the CNO concentrations in the brain, the well concentrations of the two compounds were similar magnitudes.

Incubation of the brains with NDMC showed that the compound crosses the brain barrier. Neither CNO nor clozapine was detected in the brains or wells after NDMC exposure. Interestingly, at 1 μM the concentration of NDMC in the brain was lower than the NDMC concentration obtained when the brains were incubated with clozapine. This occurred although the well concentration of NDMC was higher than that of clozapine. This observation indicates that clozapine is more likely to cross the brain barrier than NDMC and that in addition the conversion of 1 μM of clozapine to NDMC is fast. NDMC was not converted to CNO or clozapine suggesting that the conversion of clozapine to NDMC is an irreversible process, which is in line with results for humans and rodents.

Treatment of the brains with CNO showed that CNO is less likely to permeate the brain barrier and only low concentrations of CNO were detected in the brain. Despite the low CNO concentration in the brain, detectable levels of NDMC in the brain were reached in the experiments. Clozapine was detected only at the highest concentration (3 μM) and after 45 min of incubation.

The data in Table 2 suggest that there is equilibrium between the interconversion of clozapine and CNO while the formation of NDMC from clozapine is irreversible; thus, NDMC acts as a metabolic sink. This could explain why the well concentration of CNO was high when the brains were incubated in 3 μM clozapine. At this concentration, the conversion of clozapine to NDMC has started to reach a maximum, and CNO may be transported out of the brain before reverting to clozapine. Thus, the data suggest that NDMC is a product of the metabolism of clozapine and indirectly of CNO. The reversible metabolism of clozapine and CNO in patients with schizophrenia and guinea pigs has been described previously.[21,22] However, in mice no significant back-transformation to clozapine was found in plasma after intraperitoneal injection of CNO.[23] However, in our study at a low dose, clozapine was not detected in the locust brain, but a fast conversion to NDMC was found as sign of the interconversion. In the reference often cited to show the high blood–brain barrier penetration of CNO, the biodistribution of carbon-11-labeled clozapine and CNO was determined.[24] However, the procedure used in the study neither discriminated between the brain uptake of CNO and the amount CNO formed in the brain from clozapine and vice versa nor included peripheral metabolism as the researchers measured the total radioactivity of all C11-labeled compounds that permeated the blood–brain barrier and not the administered compounds per se. This makes it difficult to interpret the interesting time-dependent slight increase in the CNO concentration in the brain, which could be a slow uptake of CNO or any other C11-labeled compound formed peripherally (e.g., clozapine). Another study, in which clozapine was intraperitoneally injected in rats, showed only NDMC and clozapine in the brain, no CNO, although the serum contained all three compounds.[25]

Clozapine Interaction Experiments. Next, we performed interaction experiments with coadministration of clozapine (3 μM) with verapamil (25 μM), fluvoxamine (25 μM), or both (Scheme 1). After 45 min of incubation the concentrations of clozapine, NDMC, and CNO in the brains and the wells were determined (Figure 1). Verapamil is an L-type calcium channel blocker, an inhibitor of P-gp,[26] and a moderate inhibitor of

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CYP3A, especially CYP3A4. The more selective inhibitors, cyclosporine A and ketoconazole have previously been used to evaluate P-gp and CYP3A4/5 interactions, respectively, in locust.[2,13] Fluvoxamine functions as a selective serotonin reuptake inhibitor with antidepressant properties. In vitro, fluvoxamine has been reported to inhibit a number of CYPs with high to medium affinity, CYP1A2, 2C19, 2C9, and 2D6.[27] Fluvoxamine has also been reported to have intermittent P-gp inhibition.[28] Administration of fluvoxamine to patients who receive clozapine therapy may increase the steady-state serum concentrations of clozapine by a factor of 5−10.[27] In fact, clozapine has been suggested to be coadministrated with fluvoxamine, to enhance efficacy and minimize side effects by increasing the clozapine:NDMC ratio.[29] As described previously, a homologue to P-gp has been identified in the locust brain,[2] and a functional homologue of the mammalian CYP3A4 has also been identified in locusts.[13] The effects of the coadministration of clozapine with fluvoxamine or verapamil are shown in Figure 1.

Coadministration of clozapine with verapamil, fluvoxamine, or both increased the clozapine concentration in the brain three to four times compared to the concentration obtained when clozapine was administered alone. The formation of NDMC did not follow the same trend. Despite clozapine concentration increased, NDMC remained at approximately the same concentration when verapamil was coadministered alone or in combination with fluvoxamine, suggesting that the metabolism of clozapine to NDMC was reduced by verapamil. The same was observed in the formation of CNO: no increase in CNO concentrations in the brain despite the higher clozapine concentration. However, the CNO well concentrations were reduced with verapamil coadministration also suggesting that verapamil reduces the production of CNO. Fluvoxamine alone did not have the same effect as verapamil, the concentration of NDMC and CNO(brain), shadow the increase in clozapine concentration, which is logically related to the fluvoxamine and verapamil difference in potency at CYP3A4 in mammals, K_i 24 μM vs 3−6 μM, respectively. The increase of both clozapine and NDMC levels corroborates with plasma levels found in schizophrenic patients during coadministration of clozapine with fluvoxamine. However, clozapine plasma levels are also increasing more than NDMC, giving rise to a higher clozapine/NDMC ratio, which also seen in the locust model.[29] Furthermore, fluvoxamine was much less effective as an inhibitor of CNO compared to NDMC corroborates with previous studies.[27] In human liver microsomes, fluvoxamine has been reported to reduce the formation of CNO by 10−25% and the formation of NDMC 40−60% by from clozapine.[30] In the present study, although the well concentration remained at the same level as when no inhibitor was added, the relative CNO concentration in the well was reduced by fluvoxamine, which might be because of the moderate inhibition of the P-gp. The results in Figure 1 imply fast metabolism of clozapine by one or more pathways, and they may be inhibited by verapamil. The main metabolite was NDMC, which reached levels higher than those reached by clozapine itself. Fluvoxamine inhibited metabolism of clozapine and had little or no impact on the pathway producing CNO. The diffusion of CNO out of the brain seemed low. However, as the solution volume in the well was large compared to the brain, the actual amounts of metabolites that efflux in moles are substantial.

Structure−Brain Exposure Relationship. To evaluate whether the model is useful for establishing the structure relationships of blood−brain barrier permeation (Figure 2), two compounds, N-PCAP1 and O-PCAP1, from a drug discovery project aimed at developing procognitive antipsychotics, were tested with clozapine and NDMC in the locust model.[31]

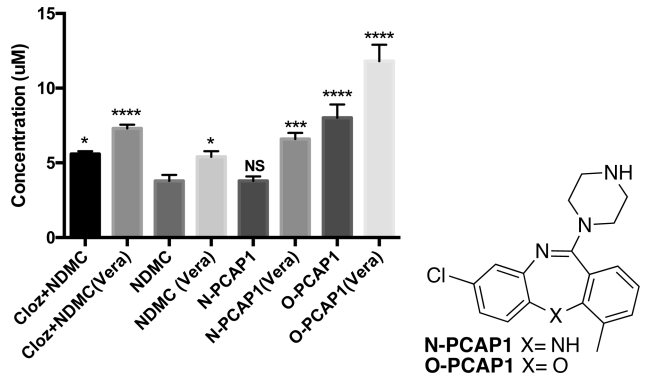

Figure 2. Structure−Brain exposure relationship of clozapine and NDMC analogues. Incubation with 3 μM test compound for 15 min. Verapamil 25 μM. Bars represent the mean ± SD of three independent experiments (n = 3), two locust brains pooled in each experiment, from one male and one female. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistically significant differences from NDMC are indicated by *(p < 0.05), *** (p < 0.001), and **** (p < 0.0001). NS = not significant, Vera = verapamil, Cloz = clozapine.

As previously observed, NDMC was present in the brain in similar amounts independent of whether clozapine or NDMC was the test compound. However, shown in Figure 2 is the total concentration of clozapine and NDMC, to evaluate the relative brain uptake of clozapine. Clozapine penetrated the blood−brain barrier more readily, and the clozapine and NDMC concentrations together were considerably higher than the NDMC concentration than after exposure with NDMC. The lower brain barrier penetration with NDMC compared with clozapine might be because of the additional hydrogen donor present in NDMC, because it is slightly less lipophilic, or both. Adding verapamil to the incubation solutions of clozapine or NDMC influenced the metabolism and increased the concentrations of the two test compounds. Verapamil inhibited the transformation of clozapine to NDMC and the further metabolism of NDMC. As verapamil is a P-gp inhibitor, that part of the increase might be because clozapine and NDMC are P-gp substrates. Adding a methyl to the 4-position in NDMC (N-PCAP1) had no effect on the drug concentration in the brain. However, during the coincubation with verapamil, higher brain concentrations of N-PCAP1 was observed compared with NDMC, which indicates that the methyl gives a higher brain barrier permeability, but at the cost of higher metabolism. However, this is expected as the N-PCAP1 has a benzylic position, a soft spot for oxidation not present in NDMC. Reducing the number of hydrogen bonds by replacing one of the nitrogens with oxygen (O-PCAP1) increased the brain uptake considerably better than clozapine and close to 2-fold compared with N-PCAP1 and NDMC.

Identification of Known Metabolites of Risperidone, Fluoxetine, Haloperidol, and Citalopram. To test whether drugs other than clozapine are metabolized in the locust brain, we investigated the brain penetration, efflux, and metabolism of the CNS-acting drugs risperidone, fluoxetine, haloperidol, and

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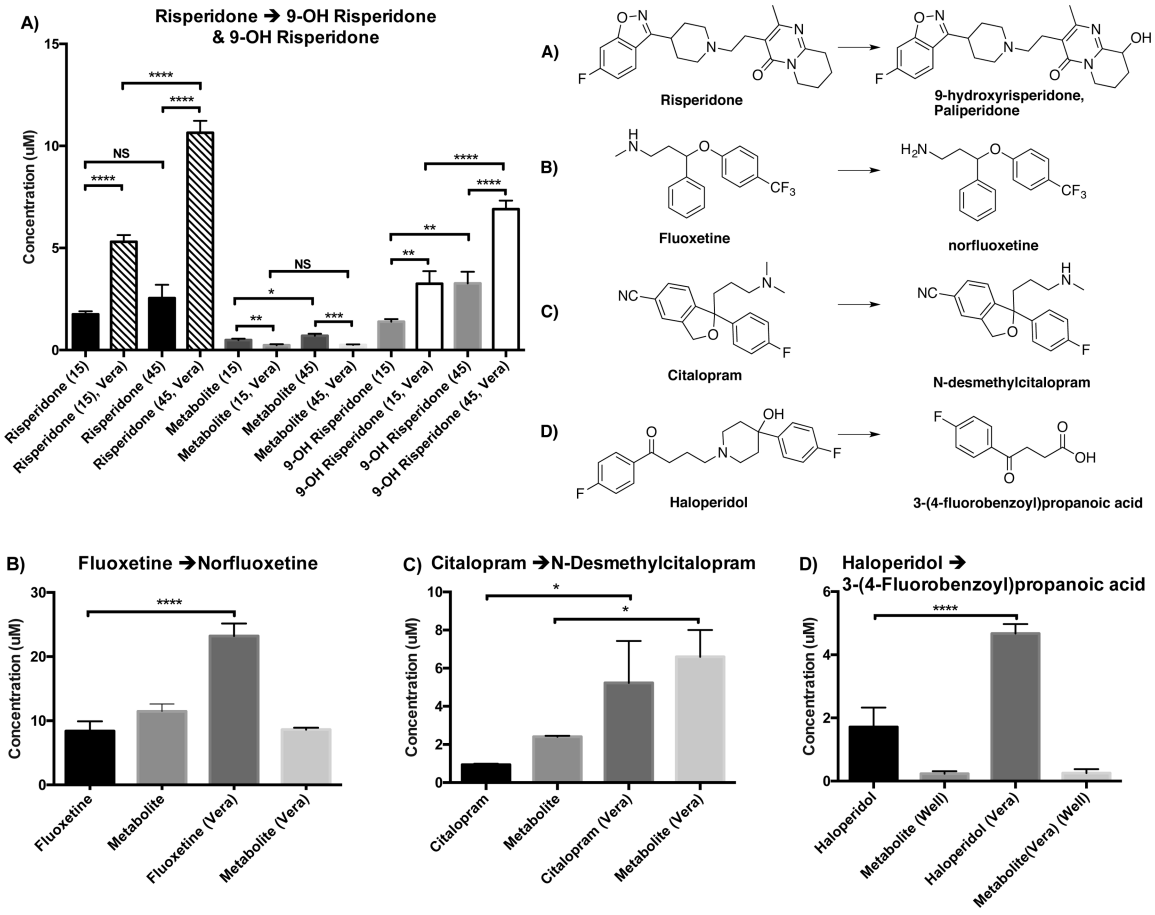

Figure 3. Brain uptake and metabolism of risperidone and 9-OH risperidone (A), fluoxetine (B), citalopram (C), and haloperidol (D). Values represent the concentration in the brain, and the bars represent the mean ± SD of three independent experiments (n = 3), two locust brains pooled in each experiment, from one male and one female. In (D), the concentration of the metabolite was measured in the well; no detectable levels were found in the brains. Incubation with 3 μM of risperidone or 9-OH risperidone, incubation time in parentheses (minutes) (A). Five minute incubation with 3 μM fluoxetine (B), citalopram (C), or haloperidol (D). Metabolites: 9-OH risperidone (A), norfluoxetine (B), *N*-desmethylcitalopram (C), and 3-(4-fluorobenzoyl)propionic acid (D). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons within each compared group. Statistically significant differences are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). NS = not significant; Vera = verapamil.

citalopram. Moreover, we preselected one known major metabolite based on the commercial availability of the metabolites of each CNS-acting compound and investigated whether it was possible to identify and quantify the metabolites; see Figure 3.

In the periphery, risperidone is metabolized by CYP2D6 and CYP3A4 to 9-OH risperidone, and both compounds are substrates for P-gp.[16] As shown in Figure 3A, risperidone and 9-OH risperidone were detected in the locust brain when risperidone is the test compound. Moreover, the risperidone concentrations in the brain increased whereas the 9-OH risperidone concentrations marginally decreased when risperidone was coincubated with verapamil. This indicates that verapamil reduces efflux of risperidone by interacting with the P-gp and at the same time inhibits conversion of risperidone to 9-OH risperidone leading to higher risperidone concentrations in the brain. Support for this is provided by comparing the data obtained after 15 and 45 min of exposure. When the exposure time was extended from 15 to 45 min, there was no significant difference in the levels of risperidone in the brain while the 9-OH risperidone concentration in the brain increased slightly. However, when risperidone and verapamil are added together in the well, the risperidone concentration in the brain increased substantially while the 9-OH risperidone concentration decreased slightly. Thus, the metabolism to 9-OH risperidone is a minor pathway for the lower amount of risperidone without verapamil. Treatment of the brain with 9-OH risperidone showed that the compound permeates the brain barrier, and significant amounts were measured in the brain. The brain uptake of 9-OH risperidone increased when the exposure time was increased from 15 to 45 min. Moreover, the 9-OH risperidone concentration increased when the compound was coadministered with verapamil, which supports that 9-OH risperidone is an efflux substrate.

Exposing the brains to fluoxetine alone showed that fluoxetine permeates the blood–brain barrier (Figure 3B) and that the metabolite norfluoxetine is formed in the locust brain.[32] Coadministration of fluoxetine with verapamil increased the concentration of fluoxetine in the brain while the concentration of norfluoxetine remained the same. However, with an increase in the fluoxetine concentrations the metabolite concentration should increase as well if verapamil has no effect on the formation of norfluoxetine. Thus, verapamil partly inhibits the formation of norfluoxetine. In addition, norfluoxetine showed no indication of being a P-gp substrate, as it was not detected in the wells.

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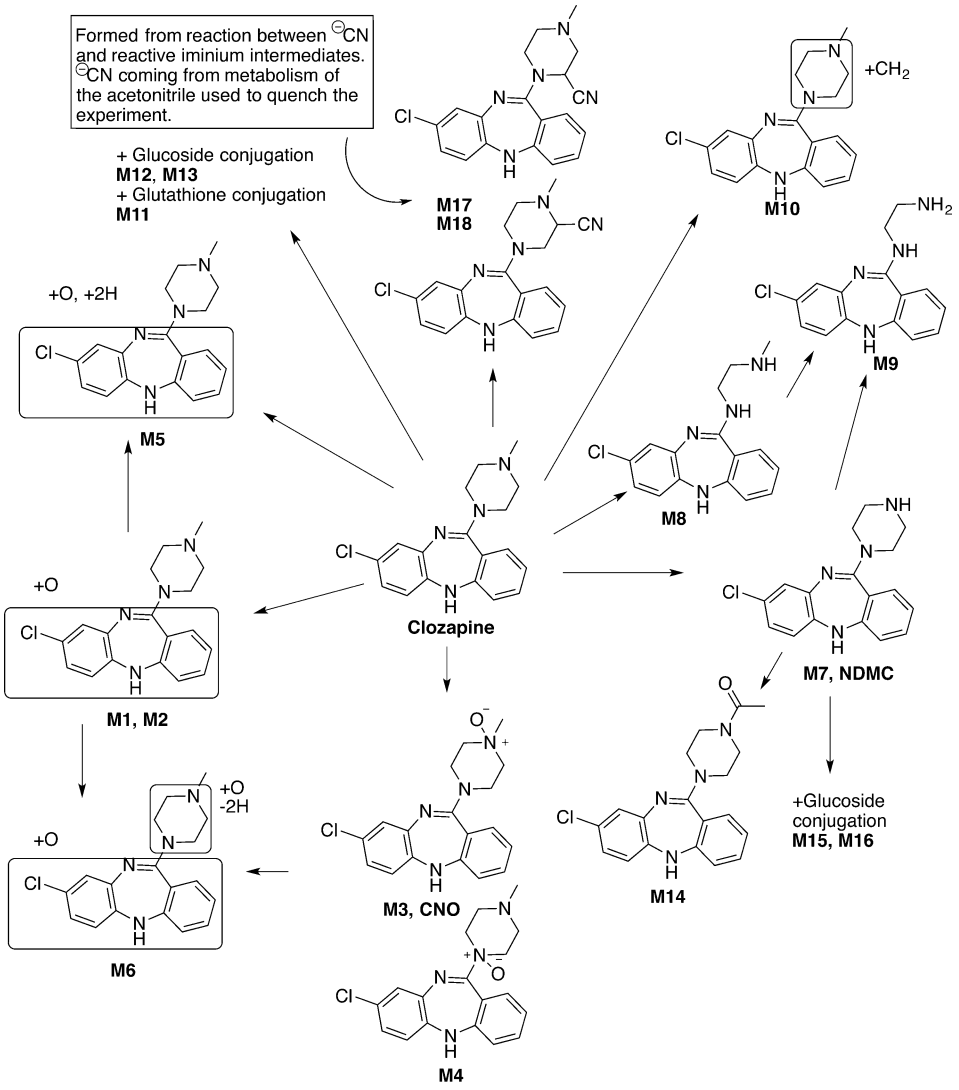
Scheme 2. Clozapine Metabolites Found in the Locust Brain



Citalopram is metabolized by CYP2C19 and CYP3A4 to the major metabolites *N*-desmethylcitalopram, and *N*,*N*-didesmethylcitalopram.[33] Exposing the insect brain to citalopram for 45 min showed that the compound enters the brain and the metabolite *N*-desmethylcitalopram is formed in concentrations approximately two to three times higher than the parent compound. Co-administration of citalopram with verapamil increased the concentration of citalopram in the brain more than 5 times whereas the *N*-desmethylcitalopram concentration increased only approximately three times, rendering an equal concentration of both. This suggests that citalopram is a CYP3A4 substrate, P-gp substrate, or both, which reflects the case in mammals. Minor concentrations of the formed metabolite *N*-desmethylcitalopram were found in the wells (40 nM), which did not increase with an increasing concentration in the brain indicating a slight inhibition of efflux in the presence of verapamil. Thus, the high increase in *N*-desmethylcitalopram in the presence of verapamil could be because of the inhibition of efflux and inhibition of further metabolism forming *N*,*N*-didesmethylcitalopram.

The analysis of haloperidol showed that it passed the blood−brain barrier and that significant concentrations were detected in the brain. No significant concentrations in the brain of the metabolite 3-(4-fluorobenzoyl)propionic acid were detected. However, the measured levels in the wells of the metabolite were around 0.25 μM. This indicates that 3-(4-fluorobenzoyl)-propionic acid is formed in the brain and subsequently efficiently transported out. Coincubation of haloperidol with verapamil increased the concentration of haloperidol in the brain almost three times while the 3-(4-fluorobenzoyl)-propionic acid metabolite was detected in the wells but below the detection level in the brain. The concentrations in the well remained the same despite an increase in haloperidol levels that indicates that verapamil inhibits the formation of 3-(4-fluorobenzoyl)propionic acid, the efflux, or both. These findings support the observations in mammals in which CYP1A2 and CYP3A4 metabolized haloperidol forming 3-(4-fluorobenzoyl)propionic acid. After intramuscular administration, plasma contained 3-(4-fluorobenzoyl)propionic acid in concentrations comparable to those of haloperidol. However, only haloperidol was found in the rat brain.[34] In contrast, using brain homogenate in in vitro studies showed that haloperidol is converted into 3-(4-fluorobenzoyl)propionic acid.[35] In the latter study, the metabolite was trapped in the homogenate. Thus, taken together, these findings suggest that haloperidol in mammals is converted to 3-(4-fluorobenzoyl)propionic acid in

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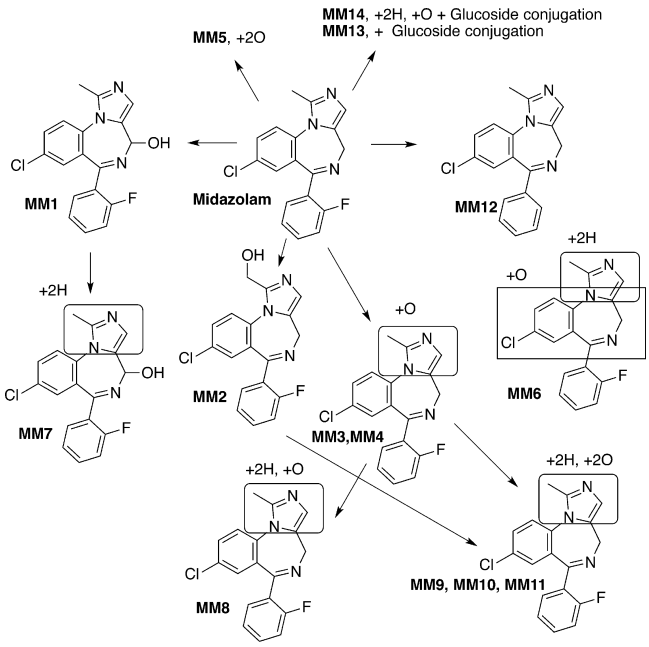
the brain and that this metabolite is efficiently transported out of the brain and excreted peripherally.

Metabolite Identity Experiments of Clozapine and Midazolam. During the early drug discovery phase, the metabolites of a potential drug candidate are generally not known. Therefore, in the present study, traditional metabolite identity experiments were performed. Clozapine and midazolam were selected as the test compounds for comparison with the available literature, as most of their metabolites are known, at least the ones formed in the periphery. The two compounds were incubated separately at three concentrations (1, 3, and 10 μM) for 45 min, and then the intact brains were sent to a contract research laboratory (Admescope, Oulu, Finland) for bioanalysis. The LC/MS data obtained for clozapine, midazolam, and their metabolites are summarized in the Supporting Information. All detected metabolites were tentatively identified according to accurate mass data, retention times, and high resolution fragment ion data.

For clozapine, a total of 18 metabolites (M1−M18) were observed (Scheme 2, for a full discussion, see the Supporting Information). Briefly, NDMC (**M7**) was the most abundant clozapine metabolite (53% at 1 μM) compared to the parent clozapine (43%), and direct glucoside conjugation (M13) was the second most abundant metabolite (2%). CNO (**M3**) was the third largest metabolite formed in 0.7% relative clozapine. Metabolites **M2**, **M3**, **M5**, **M7**, **M8**, **M10**, **M12**, and **M13** were observed in the brain and incubation well samples, while the metabolites **M1**, **M4**, **M6**, **M9**, and **M14**−**M18** were not observed in any of the incubation well samples. The relative abundance of the metabolites for clozapine decreased as a function of the test concentration, suggesting saturation of the metabolic enzymes. Except for **M5**, **M10**, and **M14**, the identified metabolites were found in earlier studies.[36−38] **M10** is an addition of CH2, corresponding to adding a methyl to the piperazine and was detected in only minor amounts 0.01% at the two highest concentrations only. **M5** is two subsequent oxidations, first hydroxylation and followed by oxidation. This not an unlikely metabolite as the primary oxidation products (**M1** and **M2**) might be trapped in the brain compartment and susceptible to further transformation. This could be noted as these metabolites were not readily detected in the wells. **M14** is a conjugation, the *N*-acylation of NDMC. Interestingly, the incubations were quenched by acetonitrile in large excess. Acetonitrile forms cyanide as a metabolite that could react with reactive metabolic intermediates. **M17** and **M18**, two nitrile metabolites, were formed that corroborates the metabolites identified in a metabolic trapping experiment using potassium cyanide. Although one metabolite from direct S-glutathione conjugation was identified (**M11**), several glutathione metabolites have been reported to be formed via a reactive nitrenium intermediate. After further inspection of the chromatograms, three additional LC/MS peaks with exact mass fitted to S-glutathione conjugates were observed, but their abundance was very low, and no supporting MS/MS data were obtained. In addition, a similar very minor LC/MS peak with intensity close to the detection limit was observed for m/z fit to the exact mass of S-glutathione replacing the chlorine atom (m/z 598.2442), as well as replacement of chlorine by S-thiomethyl (m/z 339.1638), but no confirming MS/MS data were obtained.

For midazolam, 14 metabolites were observed (Scheme 3; for full discussion, see the Supporting Information). The known hydroxy metabolites 4-hydroxymidazolam (**MM1**) and 1-hydroxymidazolam (**MM2**) were observed, the combination

Scheme 3. Midazolam Metabolites Found in the Locust Brain



of the two oxidation sites in **MM5**, although the structure of the latter was not definitely determined.[39] A number of additional oxidation/hydroxylation products alone or in combination with a reduction in methylimidazole were found. Two metabolites from glucoside conjugations were found, one directly to midazolam (**MM13**) in 2% and the second in minor amounts of 0.05% relative midazolam after hydroxylation and hydrogenation (**MM14**). Almost all of the observed metabolites were present at all test concentrations in the brain and incubation well samples. Similarly to clozapine, the relative abundance of metabolites compared to midazolam decreased as a function of the incubation concentration, suggesting the enzymatic reactions were saturated. However, less than 10% of the midazolam was metabolized compared with >55% for clozapine. Thus, a longer incubation time could lead to more pronounced metabolism. Interesting, the metabolism of midazolam was investigated by Olsen et al.[40] in vivo in locusts in order to evaluate the presence of an enzyme with functionality similar to human CYP3A4/5. In addition to phase I metabolites, the main metabolites were a number of glucose and glucose-phosphate conjugated metabolites. This differentiates the metabolic profiles in the brain and the periphery in the locust. In contrast to the periphery, only one phase II metabolite was found in the brain in a relative amount comparable with the individual hydroxylated metabolites. However, six metabolites found in the brain had undergone a reduction in methylimidazole. These metabolites have not been previously identified. Whether these metabolites are brain specific or produced because of species differences remains to be evaluated.

Time-Dependent Depletion Studies. Drugs are normally administered in a bolus dose. The initial drug concentration in the plasma and the brain are reduced over time depending on the drug's half-life. To mimic this and to find out how long the locust brain is viable without adding external energy in the form of glucose, for example, the brains were incubated with clozapine, and after 45 min, they were transferred to a blank

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Table 3. Time-Dependent Depletion Studies of Clozapine, CNO, and NDMC[a]

clozapine	concn	time[b]	clozapine (μM)	CNO (μM)	NDMC (μM)
brain	1 μM	45 min	1.78 ± 0.42	<0.02	6.06 ± 0.46
well			0.84 ± 0.05	0.05 ± 0.01	0.05 ± 0.02
brain	1 μM	+30 min	0.42 ± 0.05	<0.02	7.2 ± 1.74
well			< 0.02	<0.02	<0.02
brain	1 μM	+60 min	< 0.02	<0.02	8.12 ± 0.40
well			< 0.02	<0.02	<0.02
brain	1 μM	+90 min	< 0.02	<0.02	7.33 ± 1.22
well			< 0.02	<0.02	0.05 ± 0.00
brain	3 μM	45 min	9.26 ± 1.63	<0.02	12.9 ± 5.21
well			1.67 ± 0.18	0.07 ± 0.01	0.06 ± 0.01
brain	3 μM	+30 min	2.81 ± 0.98	<0.02	18.8 ± 0.40
well			0.80 ± 0.02	<0.02	0.07 ± 0.03
brain	3 μM	+60 min	2.00 ± 0.26	<0.02	17.60 ± 2.20
well			0.11 ± 0.03	<0.02	0.11 ± 0.03
brain	3 μM	+90 min	1.60 ± 0.30	<0.02	15.06 ± 3.20
well			0.10 ± 0.02	<0.02	0.13 ± 0.02

[a]Values represent the mean ± SD of three independent experiments (n = 3), two locust brains pooled in each experiment, from one male and one female. [b]Time after 45 min of incubation with clozapine.

buffer solution, and analyzed after 30, 60, and 90 min (Table 3).

After 45 min of incubation with the 1 μM clozapine solution, the same trend as in previous studies was observed. NDMC was formed in high amounts, and CNO was detected in the wells but not in the brains. At 60 min in a blank buffer, clozapine was not detectable in either the brains or the wells, while the amount of NDMC increased slightly. Incubation with 3 μM clozapine followed the same trend as for 1 μM. Metabolism is an energy-consuming process, and the metabolism decreased probably because most of the energy for maintaining metabolic activity was consumed after 60 min. An alternative explanation could be that the high concentration of NDMC has an inhibitory effect on metabolic enzymes. The reduction of clozapine over time in the brain correlated well with what was observed in a previous study in mice that used 11-C labeled clozapine. However, the present study showed that the metabolism was quite active at least during 45 min incubation and 30 min in blanks buffer without the addition of external of energy.

Unbound Fraction of the Drug in the Intact Brain (ibrain). With energy-dependent processes including metabolism and active transport declining over time, an opportunity was provided to evaluate whether the intact brain, as a passive membrane, could be used to estimate the unbound fraction of the drug in the brain. Unbound fraction values are important to measure as they can be used to derive the free drug concentrations from the total concentrations in the brain. Furthermore, it is generally accepted that the unbound drug concentration exerts a pharmacological effect or is susceptible to biotransformations, and not the total tissue concentrations.[41] Brain tissue binding is highly correlated with lipophilicity; consequently, the unbound fraction in the brain is reported to be species and brain region independent. Thus, single species brain binding can be used to predict brain binding for all species, including humans.[42]

A basic assumption in pharmacokinetics is that unbound drug concentrations are equal on both sides of a physiological membrane at steady state (Figure 4). This may not be the case for brain tissue since there are active efflux and influx processes at the BBB (free drug plasma and free drug interstitial fluid

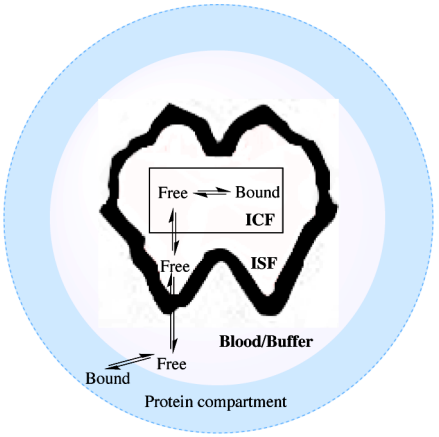

Figure 4. Schematic picture of the locust brain showing bound and free concentrations of drugs and equilibrations between different compartments. Interstitial fluid (ISF), intracellular fluid (ICF).

(ISF)) and intercellular (free drug ISF and intracellular fluid (ICF)). The method for directly measuring the concentration of unbound drug in the brain ISF ($C_{u,brainISF}$) is microdialysis.[43] Unfortunately, use of this method in early drug discovery programs is limited by the complexity and the cost and time requirements. Alternative methods to microdialysis have used the unbound fraction in the brain ($f_{u,brain}$) and the volume of distribution intrabrain ($V_{u,brain}$), using brain homogenate equilibrium dialysis[42] and brain slice uptake methodology,[44,45] respectively. Both $V_{u,brain}$ and $f_{u,brain}$ describe the same property to estimate and relate $C_{u,brainISF}$ to whole concentrations in the brain (C_{brain}). The brain slice method has been used as a model with an increased physiological basis compared to the brain homogenate technique, preserving much of the complex cellular integrity. However, the largest difference between the two methods seems to be because of lysosomal trapping of basic compounds, taking into account the pK_a of the drug and the pH values of the cellular environments to correct for pH partitioning the two methods provides basically equivalent results.[46]

Like the brain slice method, the intact locust brain model has the cellular integrity conserved, but with the addition of an

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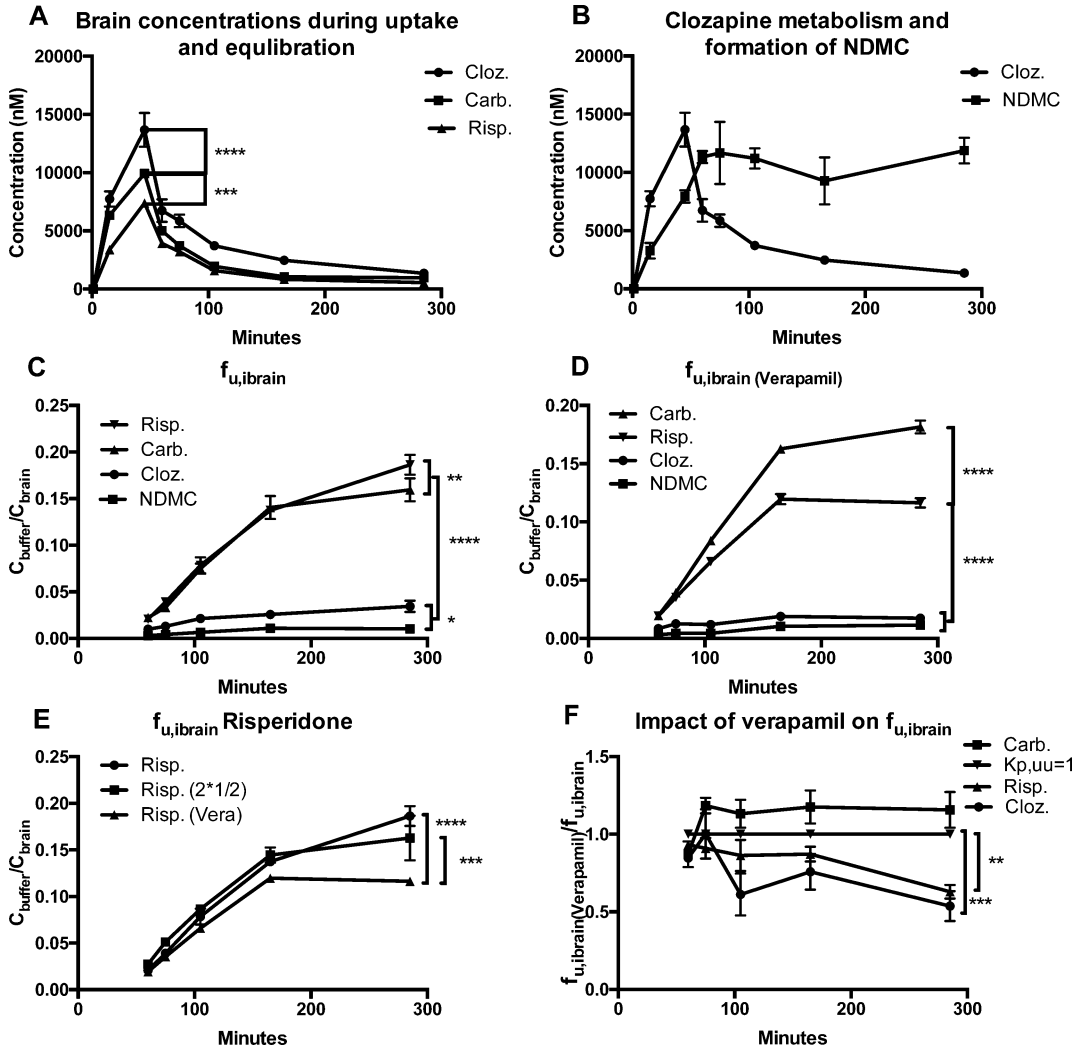

Figure 5. Results for the $f_{u,ibrain}$ studies. (A) 45 min of exposure followed by 240 min of equilibration in a blank buffer. (B) Time-dependent formation of the metabolite NDMC in the locust brain during the exposure and equilibration phases. (C) Fraction of the unbound drug in the intact brain during the equilibration phase. (D) The impact on $f_{u,ibrain}$ of adding verapamil to the buffer during the equilibration phase. (E) Differences in $f_{u,ibrain}$ for the different conditions used. (F) $f_{u,ibrain(verapamil)}/f_{u,ibrain}$ show the impact of verapamil on $f_{u,ibrain}$. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistically significant differences are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Cloz = clozapine; Carb = carbamazepine; Risp = risperidone; NDMC = *N*-desmethylclozapine; Vera = verapamil; (2*1/2) = two halves of the brain.

intact brain barrier. The key assumption in the setup is that the unbound brain-to-plasma concentration ratio (Kp,uu) equals unity at steady state. Assuming that compounds cross the brain barrier only by passive diffusion and that the free drug concentration ISF is equal to the drug concentration in the well:

$$\text{Kp,uu} = 1 \Rightarrow C_{u,brainISF} = C_{u,plasma/well} \quad (1)$$

This assumption is obviously not always true but can efficiently be manipulated toward unity by using selective molecules to inhibit specific biological functions (e.g., CYPs and P-gp), without affecting the brain barrier integrity. In the present study, the brain barrier integrity over time was evaluated experimentally using 3 μM atenolol, a compound that does not permeate the blood–brain barrier. Two different experimental conditions were applied, constant exposure for 360 min and exposure for 60 min intervals, and the 0–60, 60–120, 120–180, 180–240, 240–300, and 300–360 min intervals were compared. Although no significant increase in the uptake of atenolol was seen up to 360 min, a trend was evident showing a larger variation of the standard deviation at the last time point 300–360 min (see the Supporting Information). Thus, the blood–brain barrier integrity was reasonably preserved for up to 300 min in the buffer, which makes it possible to use similar equilibration times as used in the brain homogenate and brain slice assays.

In the initial experiment of the unbound fraction using the intact brain model, clozapine, risperidone, and carbamazepine were selected as test compounds. Clozapine is extensively metabolic to NDMC, risperidone is a known P-gp substrate, and carbamazepine is a high brain uptake compound without strong association with active transport. The brains were incubated at 3 μM for 45 min. The first point at 15 min during the loading phase was included to calculate the uptake rate. Thereafter, the brains were transferred to a blank buffer and incubated for 15, 30, 60, 120, and 240 min. Two additional conditions were then used. After the first 45 min, 25 μM of verapamil was included in the blank buffer to inhibit P-gp and

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metabolic enzymes, or the brains were divided into two halves to disrupt the intact brain barrier, the latter condition to resemble the brain slice assay.[47] The exposure profile of the three compounds showed a significant difference in uptake after 45 min, whereas after 15 min carbamazepine and clozapine had similar uptakes (Figure 5A). The maximum concentration of NDMC formed in the brain was reached after 60 min and thereafter was constant. Thus, the formed NDMC reach equilibrium with additional metabolism and buffer concentration (Figure 5B). The $f_{u,ibrain}$ (ibrain was used to denote the intact brain) was calculated with eq 2 after 240 min of equilibration:

$$f_{u,ibrain} = C_{brain}/C_{buffer} \quad (2)$$

The following $f_{u,ibrain}$ values were calculated: NDMC (1%), clozapine (3%), followed by carbamazepine (16%) and risperidone (19%) (Figure 5C). $f_{u,brain}$ in mammals was previously reported for NDMC (0.5%,[48] 1.2%[49]), clozapine (1.0%,[50] 1.4%[49]), risperidone (10%,[49] 7−11%[50]), and carbamazepine (12−19%[42,50]). Notably, risperidone and clozapine had the largest deviations compared to the literature data; however, both have been reported to be efflux substrates, and clozapine is extensively metabolized in the locust brain, which may impact the equilibration. Adding verapamil to inhibit P-gp and metabolic activity had a significant impact on $f_{u,ibrain}$ for risperidone ($f_{u,ibrain(verapamil)}$ 11.6%) and clozapine ($f_{u,ibrain(verapamil)}$ 1.7%), but no effect on $f_{u,ibrain}$ for carbamazepine and NDMC. Thus, using verapamil gave $f_{u,ibrain}$ results in the same range and hierarchical order as previously reported for these four compounds (Figure 5D). Dividing the brain into two halves had no significant outcome compared to using an intact brain, as shown with risperidone (Figure 5E). We divided the locust brain symmetrically in the middle to easily reproduce the cut, which might have caused less damage to the brain barrier. The ratio $f_{u,ibrain(verapamil)}/f_{u,ibrain}$ gives an indication of the effect of $f_{u,ibrain}$ when verapamil is used. A compound not affected by the inclusion of verapamil in the blank buffer during equilibration would give $f_{u,ibrain(verapamil)}/f_{u,ibrain} = 1$ (Figure 5F). A compound with $f_{u,ibrain(verapamil)}/f_{u,ibrain} < 1$ experiences efflux, metabolism, or both during equilibration whereas $f_{u,ibrain(verapamil)}/f_{u,ibrain} > 1$ has an active uptake. Risperidone and clozapine had ratios lower than 1 statistically significantly deviating from the Kp,uu = 1; carbamazepine had a ratio > 1 (1.15). Although it has been reported that compounds can decrease the uptake of carbamazepine indicating an active uptake, the current result is not statistically significantly different from Kp,uu = 1.

The combination of the brain barrier intrinsic permeability and brain tissue binding in early drug discovery is useful for rank-ordering compounds and is more appropriate to correlate with unbound drug concentrations than either property alone. Structural modifications for optimizing the unbound fraction alone make little sense, as the free drug concentration correlates with therapeutic efficacy, and increasing the unbound fraction does not necessarily increase the unbound concentration. Measuring the unbound fraction becomes important as the lipophilicity of the compounds increases, and permeability seems to be an important factor for compounds characterized by low brain tissue binding.

The metabolism of drugs in the brain has been mostly ignored during early drug discovery. This is probably because brain metabolism is difficult to study in most species without interference from peripheral metabolism. With the drugs evaluated, the locust model seemed to show a correlation with the results obtained in mammals. This might be because of the conservative use of the CYPs in the brain to produce neurochemicals. In addition, the P-gp and CYP3A4 interplay reported to be used to eliminate xenobiotics seems to be conserved between locusts and mammals. Results indicated clozapine is a great prodrug for NDMC. An equal or higher concentration of NDMC was reached when treating with clozapine compared with NDMC itself depending on the exposure concentration. In addition, in the locust model, CNO was not as highly blood−brain barrier permeant as reported. This could, of course, be because of a discrepancy between the insect-based model and mammals. However, in the literature, to our knowledge, there is no compelling evidence (PK or PK−PD studies on CNO in the brain when given peripherally) that CNO actually is highly blood−brain barrier permeable in mammals. In addition to the clozapine studies, risperidone, citalopram, fluoxetine, and haloperidol were tested, and one preselected known peripheral metabolite for each drug was identified and quantified. Interestingly, in the case of haloperidol the metabolite 3-(4-fluorobenzoyl)propionic acid was found only in the well solutions and not in the brain samples. This corroborates two previous studies in mammals if they are combined. Each study alone did not recapitulate the full event suggesting that 3-(4-fluorobenzoyl)propionic acid is probably formed in the brain but is efficiently transported from the brain to the blood.

The locust brain is highly metabolically active after dissection (ex vivo) for about 1 h. The metabolite identification experiments with clozapine and midazolam identified 18 and 14 metabolites, respectively. Interesting, by serendipity it was found that by using acetonitrile in the quenching of metabolic activity, the trapping of reactive clozapine metabolites was possible that otherwise would require the addition of potassium cyanide.

Transporter activities and metabolism are difficult components to study. Not only are there species differences in the animal models used, but there are also significant interpatient variations. Furthermore, differences in disease states and circadian time points are important factors to account for when treating individual patients. Although predicting the exposure to drugs in the brain in patients seems to be an overwhelming task, it is important to early on during the discovery phase to get indications of potential issues with compounds and compound classes. Issues can then be confirmed or refuted using more sophisticated methods than the initial screening models. In line with this, we suggest to evaluate permeability and fu,brain, parameters that show little species differences, separately from parameters indicating drug−drug interactions (e.g., interactions with transporters and metabolic enzymes) that have potential significant species differences. This separation amenable also the individual parameters for optimization and evaluation, especially drug−drug interactions can be deduced using specific inhibitors and if it pertains to humans.

METHODS

Experimental Preparation. Desert locusts, *Schistocerca gregaria* (L), used in the present study were obtained from a commercial animal wholesaler (Petra Aqua, Prague, Czech Republic). On arrival, the locusts were housed under crowded conditions in insect cages supplied by Small-Life Supplies (Peterborough, Great Britain) and adapted to a 10:14 h dark/light cycle with a temperature of 25−34 °C

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depending on locust proximity to the light bulb. The animals were fed Chinese cabbage and wheat bran ad libitum. All experiments were carried out 2−3 weeks after adult emergence. The insect brains used in the ex vivo experiments were dissected by cutting off the frontal part of the head, above the esophagus. The brain was removed from the cuticle, dissected free from fat and tissue using fine forceps, and placed in a microwell plate (96U Microwell; Nunc) that contained 250 μL of the compound of interest dissolved in an insect buffer.

Chemicals. All drugs used in the insect ex vivo studies and HEPES were purchased from Sigma-Aldrich (Stockholm, Sweden). All other chemicals were analytical reagent grade. The test compounds were purchased from Sigma-Aldrich (Stockholm, Sweden).

Test Solution Preparation. Stock solutions were prepared by dissolving the test compounds in dimethyl sulfoxide (DMSO). The final test solution concentrations were obtained by diluting stock solutions with insect buffer [NaCl 147 mM, KCl 10 mM, $CaCl_2$ 4 mM, NaOH 3 mM, and HEPES 10 mM pH 7.2].

Experiments were performed by incubating the locust brains in a solution of insect buffer and test compound(s). The ex vivo studies were performed in triplicate tests with two locust brains pooled in each test tube (i.e., a total of six brains in each experiment) as described in previous publications. Each brain was transferred to a well containing 250 μL of a solution containing the compound of interest diluted in insect buffer. The brains were removed from the compound solutions after fixed exposure periods and washed twice in ice-cold insect buffer. Brains from two animals were placed in 150 μL 75% solution of acetonitrile (in Milli-Q water), and the brain tissue was homogenized with ultrasonication (UIS250 V, Hielcher−Ultrasound Technology, Germany) for 8 pulses at an amplitude of 70%. After centrifugation at 10 000*g* for 5 min at 4 °C, 50 μL of the supernatants were further diluted with 150 μL Milli-Q water (approximately 20% acetonitrile final concentration) in glass vials and analyzed with high-performance liquid chromatography mass spectrometry (LC-MS). Additionally, when the brains were removed from the wells the compound concentrations in the wells were measured. Volumes of 25 μL of each of the two solutions corresponding to the brain duplicates were mixed with 950 μL of 20% acetonitrile in a test tube.

Analysis of drug concentrations in brains and wells was performed by Red Glead Discovery AB (Lund, Sweden). The supernatant from the brain homogenates were analyzed with LC-MS/MS using HPLC coupled to a triple quadrupole mass spectrometer. The chromatographic column was either a Phenomenex Synergi Polar, 4 μm, 2 × 50 mm or a Symmetry Shield RP8, 5 μm, 2.1 × 50 mm. Mobile phase A was 95% water in acetonitrile with 0.1% formic acid, and mobile phase B was 5% water in acetonitrile with 0.1% formic acid. The analysis was performed using gradient elution from 0% to 100% mobile phase B in 4 min. Detection was performed in MRM mode either in positive or negative mode.

Statistical Analysis was performed using GraphPad Prism version 6.0h.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.6b00024.

Additional experimental details as described in the text (PDF)

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Author Contributions

R.O. designed the study. K.H. performed the ex vivo locust model. K.H, P.A.N., F.E., and R.O. analyzed the data and wrote the paper.

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Notes

The authors declare the following competing financial interest(s): R.O. has submitted a patent application for the locust model.

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