In Vitro and In Vivo Evidence for Active Brain Uptake of the GHB Analog HOCPCA by the Monocarboxylate Transporter Subtype 1

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ABSTRACT

γ-Hydroxybutyric acid (GHB) is a recreational drug, a clinically prescribed drug in narcolepsy and alcohol dependence, and an endogenous substance that binds to both high- and low-affinity sites in the brain. For studying the molecular mechanisms and the biologic role of the GHB high-affinity binding sites, ligands with high and specific affinity are essential. The conformationally restricted GHB analog HOCPCA (3-hydroxycyclopent-1-enecarboxylic acid) is one such compound. The objective of this study was to investigate the transport of HOCPCA across the blood-brain barrier in vitro and in vivo and to investigate the hypothesis that HOCPCA, like GHB, is a substrate for the monocarboxylate transporters (MCTs). For in vitro uptake studies, MCT1, -2, and -4 were recombinantly expressed in Xenopus laevis oocytes, and the previously reported radioligand [3H]HOCPCA was used as substrate. HOCPCA inhibited the uptake of the endogenous MCT substrate L-[14C]lactate, and [3H]HOCPCA was shown to act as substrate for MCT1 and 2 (Km values in the low- to mid-millimolar range). Introducing single-point amino acid mutations into positions essential for MCT function supported that HOCPCA binds to the endogenous substrate pocket of MCTs. MCT1-mediated brain entry of HOCPCA (10 mg/kg s.c.) was further confirmed in vivo in mice by coadministration of increasing doses of the MCT inhibitor AR-C141990 (R)-5-(3-hydroxypyrrolidine-1-carbonyl)-1-isobutyl-3-methyl-6-(quino-lin-4-ylmethyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione) which inhibited brain penetration of HOCPCA in a dose-dependent manner (ID50 = 4.6 mg/kg). Overall, our study provides evidence that MCT1 is an important brain entry site for HOCPCA and qualifies for future in vivo studies with HOCPCA.

Introduction

The neuroactive substance γ-hydroxybutyric acid (GHB) is a recreational drug (Fantasy, liquid x), but it is also a clinically prescribed drug in narcolepsy [sodium oxybate (Xyrem; Jazz Pharmaceuticals, Palo Alto, CA)] (Robinson and Keating, 2007) and alcohol dependence (Addolorato et al., 2009; Keating et al., 2014), and an endogenous compound with an unknown biologic role (Wong et al., 2004). After exogenous intake, GHB readily enters the brain and produces several effects, ranging from mild euphoria, muscle relaxation, hypothermia, and sedation, and, at higher doses, coma and death (Bernaseoni et al., 1999; Wong et al., 2004). As a drug of abuse, GHB was recently ranked ninth on a list of drugs that abuse, GHB was recently ranked ninth on a list of drugs that can reach its targets and elicit its effects (Vogensen et al., 2013). The most well described and prominent target is the GABAB receptor, which produces many of the known pharmacologic effects of GHB (Kaupmann et al., 2003; Carter et al., 2009; Vienne et al., 2010; Black et al., 2014); however, since GHB is only a low-affinity, weak partial agonist at this target (Mathivet et al., 1997; Lingenhoehl et al., 1999), millimolar concentrations of compound are required to elicit these effects; therefore, effects of lower GHB concentrations mediated by other more sensitive targets cannot be ruled out. These can be known or unknown targets that bind GHB with much higher affinity (Bay et al., 2014), such as certain subtypes of ionotropic GABA\textsubscript{A} receptors (Absalom et al., 2012), although their physiologic importance in relation to GHB effects have been debated (Connelly et al., 2013).

To study the role of the high-affinity GHB sites in further detail, compounds have been developed that are devoid of affinity for GABA\textsubscript{A} receptors (Wellendorph et al., 2005;...
One of these compounds is the conformationally restricted GHB analog, HOCPCA (3-hydroxypropenyl-1-ene-carboxylic acid) (Wellendorph et al., 2005) (Fig. 1A), which is highly selective for the GHB high-affinity binding sites, binds with 27 times better affinity than GHB itself to these, and readily penetrates the brain (Vogensen et al., 2013). These properties make HOCPCA an attractive tool compound for studying molecular mechanisms pertaining to the specific GHB high-affinity binding sites that may hold promise as therapeutic targets.

As more than 99% of GHB (pKᵦ = 4.7) is ionized at physiologic pH, the distribution of GHB to various tissues, including the brain, relies on specific transporters (Wang and Morris, 2007). Of the known transporters, GHB is a substrate for a family of transporters known as monocarboxylate transporters (MCTs). The family consists of 14 members, of which subtypes 1–4 mediate proton-coupled transport of endogenous monocarboxylates, such as lactate and pyruvate (Halestrap, 2013). GHB has previously been shown to interact with MCT1 and -2 (Carpenter and Halestrap, 1994; Wang and Morris, 2007; Wang et al., 2007), whereas uptake by MCT4 is not reported consistently (Manning Fox et al., 2000; Wang and Morris, 2007). MCTs are expressed in a wide range of tissues, including the liver, kidney, intestines, and brain. Specifically, MCT1 is suggested to be involved in the transport of GHB across the blood-brain barrier (BBB) (Bhattacharya and Boje, 2004), as its brain entry is inhibited by known MCT substrates and inhibitors (Bhattacharya and Boje, 2004), most recently shown with the highly potent MCT1-prefering inhibitor AR-C155858 (Vijay et al., 2015). Because of the structural resemblance between GHB and HOCPCA, we hypothesized that HOCPCA would use the same transport system as GHB to enter the brain. Accordingly, the objective of this study was to investigate HOCPCA as an MCT substrate in vitro and in vivo. For in vitro studies, MCT1, -2 and -4 were recombinantly expressed in Xenopus laevis oocytes, a well established and superior expression system for studying MCTs (Halestrap and Wilson, 2012). The in-house developed radioligand [3H]HOCPCA was used as substrate (Vogensen et al., 2013). The molecular mechanism of HOCPCA transport into the brain was further confirmed by assessing the effects of the MCT inhibitor AR-C141990 ([R]-5-(3-hydroxypropyrlidine-1-carbonyl)-1-isobutyl-3-methyl-6-(quinolin-4-ylmethyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione) (Pålman et al., 2013) (Fig. 1A) on the brain distribution of HOCPCA in vivo.

Materials and Methods

Chemical Compounds. GHB and L-lactate were purchased from Sigma-Aldrich (St. Louis, MO). Racemic HOCPCA (sodium salt) was prepared as previously described (Vogensen et al., 2013). AR-C141990 was synthesized by a modification of the published procedure (Guile et al., 2007). [14C]Lactate was purchased from PerkinElmer (Waltham, MA), and [3H]HOCPCA was synthesized and radiolabeled as previously described (Vogensen et al., 2013).

Generation of MCT-Encoding DNA Constructs. Constructs encoding rat MCT1, -2, and -4 or rat embigin in the pGHJ vector were kindly provided by Professor A. Halestrap, University of Bristol, UK. All initial uptake studies were conducted with these constructs, but for mutational studies, the cDNAs were subcloned by polymerase chain reaction into the pUNIV vector (Addgene, Cambridge, MA), reported to ease cRNA transcription and give higher expression levels in Xenopus oocytes (Venkatachalan et al., 2007). All forward primers contained the alfalfa mosaic virus sequence segment (in italics), the MCT coding sequence (in bold), as well as the Nhel restriction site. All reverse primers contained the MuI restriction site. MCT1 forward: 5’-CGCGG-CTAGC-CTTTCAATCTCTCCACCCATGCAACTTCGCGG-CTAGC-’; MCT2 forward: 5’-CGCGG-CTAGC-CTTTCAATCTCTCCACCCATGCAACTTCGCGG-CTAGC-’; MCT2 reverse: 5’-CGCGG-CTAGC-CTTTCAATCTCTCCACCCATGCAACTTCGCGG-CTAGC-’; MCT2 reverse: 5’-CGCGG-CTAGC-CTTTCAATCTCTCCACCCATGCAACTTCGCGG-CTAGC-’.

The absence of mutations was verified by DNA sequencing (GATC Biotech AB, Konstanz, Germany). Point mutations chosen based on molecular determinants for lactate transport (MCT1 mutants: K38Q, R306K; MCT2 mutants: K44Q, R301K) were generated by mutagenesis employing an internal sequence segment (in italic) and the MCT coding sequence (in bold) as primers (Manning Fox et al., 2000; Wang and Morris, 2007).

MCT1-Mediated Brain Uptake of HOCPCA 167
concentrations determined using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific).

**Xenopus Oocyte Injection and Handling.** Defolliculated *Xenopus laevis* oocytes stage V–VI, ready for injection, were purchased from EcoCyte Bioscience (Castrop-Rauxel, North Rhine-Westphalia, Germany). Oocytes were injected with 20 ng of the relevant cRNA. Oocytes to express MCT2 were injected with a mixture of 10 ng cRNA encoding MCT2 and 10 ng cRNA encoding the ancillary protein embryos (Ovens et al., 2010b). Control oocytes were injected with the equivalent volume of water (36.8 nl). Injected oocytes were stored individually in 96-well plates at 18°C in Barth’s solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.91 mM CaCl₂, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.1 mg/ml gentamycin, pH 7.5). Transport activity was assayed 3 to 4 days post-injection.

**In Vitro Radioactive Uptake Assays in Xenopus Oocytes.** For L-[¹⁴C]lactate competition studies, the assay conditions described by Ovens et al. (2010a) were followed with slight changes. After a 15- to 60-minute preincubation in uptake buffer [20 mM MES (4-morpholineethanesulfonic acid), pH 6.0, with 75 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 0.82 mM MgCl₂], the oocytes were incubated for 2.5 minutes at room temperature (r.t.) in uptake buffer containing 50 μM L-[¹⁴C]lactate with or without the addition of unlabeled inhibitor. Incubation was carried out in 2-ml Eppendorf tubes (six oocytes per condition; 100 μl total volume). Uptake was terminated by adding 1 ml of ice-cold uptake buffer to each Eppendorf tube, and oocytes were quickly transferred to Corning-Net-wells inserts (Sigma-Aldrich) for washing. The oocytes were quickly washed three times with fresh ice-cold uptake buffer. Intact individual oocytes were then carefully placed in a 24-well PicoPlate (PerkinElmer), and excess buffer was carefully removed. Oocytes were then lysed by the addition of 50 μl of 2% SDS and vigorously shaken for 5–10 minutes before the addition of 450 μl of MicroScint 20 scintillation liquid (PerkinElmer), shaking for 1 hour, and quantification of radioactivity (CPM values) on a TopCount NXT reader (PerkinElmer). Competition uptake using [³H]HOCPCA (100 nM) was carried out in a similar fashion. Initially, the incubation time was investigated by testing total and nonspecific uptake (in the presence of 10 mM L-lactate) from 0–15 minutes and set to 2 minutes for competition and saturation assays. Based on reports that related analogs act from an intracellular site (Ovens et al., 2010a), AR-C141990 studies included a 45-minute preincubation step with compound. After incubation and washing as described here, individual oocytes were transferred to a 96-well Optiplate (PerkinElmer), lysed by the addition of 20 μl of 2% SDS, and vigorous shaking for 5 minutes, followed by addition of 180 μl MicroScint 20 scintillation liquid, shaking for 1 hour, and determination of CPM values. Saturation transport experiments at MCT1 and -2 were conducted with [³H]HOCPCA up to a maximum concentration of 1000 nM. To measure transport at higher concentrations, [³H]HOCPCA was diluted with various concentrations of unlabeled HOCPCA. DPM values were counted to allow conversion to molar values. Nonspecific transport was determined in water-injected oocytes and subtracted from the total uptake. To study uptake in point-mutated MCT1 and -2, [³H]HOCPCA was increased to 300 nM. Otherwise, the experiment was carried out as described for the [³H]HOCPCA competition assay. In addition, a kinetic analysis of MCT1 was also performed at pH 7.4, using a 3-minute incubation time (linear uptake), [³H]HOCPCA up to a maximum concentration of 4000 nM, and a pH 7.4 uptake buffer (20 mM HEPES, pH 7.4, with 75 mM NaCl, 2 mM KCl, 1 mM CaCl₂, and 0.82 mM MgCl₂).

**In Vivo Exposure Studies.** Plasma and brain exposures of HOCPCA and AR-C141990 were evaluated in male NMRI mice (18–22 g; Charles River Laboratory, Kent, UK). Both compounds were administered subcutaneously in solution in volume of 10 ml/kg. HOCPCA and AR-C141990 were dissolved in 0.9% sodium chloride and 10% (2-hydroxypropyl)-β-cyclodextrin, respectively. Pharmacokinetic assessment of HOCPCA and AR-C141990 was performed by dosing each compound 10 mg/kg in individual groups of animals (n = 3/time point). Plasma and brain concentrations were measured at 15, 30, 60, and 120 minutes. Evaluation of the influence of AR-C141990 exposure on the brain-to-plasma distribution ratio (B/P ratio) of HOCPCA was assessed 30 minutes after the administration of both compounds. HOCPCA was dosed at 10 mg/kg together with vehicle or AR-C141990 at six different doses (0.3–90 mg/kg, n = 3–6 in each dose group). During the in vivo interaction studies, AR-C141990 was used in a dose range of 0.3–90 mg/kg. From pilot studies, this dose range was found to be adequate to describe the complete dose-response relationship. Under isoflurane anesthesia, cardiac blood was obtained in EDTA-coated tubes and centrifuged for 10 minutes at 4°C, after which plasma was harvested. After decapitation, the brain was removed and gently rinsed on filter paper and frozen together with plasma specimens at −80°C until analysis. Before bioanalysis, brain homogenate was prepared by homogenizing the whole brain with purified water:2-propanol:dimethylsulfoxide (50:30:20 v/v/v) followed by centrifugation and collection of the supernatant.

Ethical permission for the procedures used in this in vivo study was granted by the Danish Animal Experiments Inspectorate, and all animal procedures were performed in compliance with Directive 2010/63/EU of the European Parliament and of the Council, and with Danish Law and Order regulating animal experiments (LBK no. 253, 08/03/2013 and BEK no. 88, 30/01/2013).

**Brain and Plasma Bioanalysis.** Quantitative determination of HOCPCA and AR-C141990 concentrations in mouse plasma and brain tissue homogenates was performed using ultra-performance liquid chromatography (UPLC) (Waters, Milford, MA) coupled to tandem mass spectrometry (Sciex 4000, AB Sciex, Foster City, CA). Data acquisition and analysis were performed using Analyst software, version 6.1 (AB Sciex).

The HOCPCA samples were prepared by mixing 50 μl plasma or brain homogenate sample with 200 μl methanol containing internal standard and 0.2% formic acid. After centrifugation (6200 rpm at 4°C for 20 minutes), 200 μl supernatant was transferred to a new plate and placed in the autosampler; 10 μl was injected onto the UPLC. Separation of the analyte was achieved on a HILIC Waters Acquity BEH Amide column (2.1 × 50 mm, 1.7 μm). The mobile phase consisted of methanol/water containing 10 mM ammonium formate and 0.1% formic acid pumped through the column at flow rate of 0.6 ml/min as a gradient from 25% to 90% methanol over 5 minutes. Tandem mass spectrometry quantitation of HOCPCA was determined using electrospray ionization source operated in negative ion mode. Multiple reaction monitoring mode was used to characterize fragmentation of singly charged analytes with transitions m/z 127.2/82.9. The limits of quantitation were 10 ng/ml in plasma and 50 ng/g in brain.

AR-C141990 samples were precipitated by adding 25 μl plasma or brain sample with 175 μl acetonitrile containing internal standard and 0.1% ammonium hydroxide. After centrifugation, 100 μl supernatant was diluted with 100 μl of water, and 10 μl was injected onto the chromatographic column (Acquity UPLC Phenyl BEH 2.1 × 30 mm, 1.7 μm) applying a gradient mobile phase with acetonitrile/water containing 0.1% formic acid. Electrospray ionization source was used operated in positive ion mode with multiple reaction monitoring fragmentation transition m/z of 493.3/406.0. The limits of quantitation for AR-C141990 was 1 ng/ml in plasma and 5 ng/g in brain. The interday precision of the assay, based on the coefficient of variation of quality controls, ranged from 5% to 8%. The assay accuracy was within 15% of the nominal concentrations for all quality control samples.

**Membrane Permeability and Plasma Protein Binding.** The bidirectional permeability of AR-C141990 was measured in the Madin-Darby canine kidney (MDCK) cell line expressing human multidrug resistance protein (MDR1; P-glycoprotein) (referred to as MDR1-MDCK cells) in triplicate as described previously (Risgaard et al., 2013). The efflux ratio was calculated as the ratio between the permeability in the basal-to-apical direction divided by the permeability in the apical-to-basal direction. The free fraction of AR-C141990 in mouse plasma was determined in vitro at 37°C in
triplicate using equilibrium dialysis as described by (Redrobe et al., 2014). Attempts were made to measure plasma protein binding of HOCPCA by in vitro equilibrium dialysis. However, the binding was found to be very low (<1%) and could therefore not be quantitatively determined.

Data Analysis. All in vitro and in vivo data were analyzed using Prism 6.0 (GraphPad Software, San Diego, CA). Concentration-inhibition curves were fitted by nonlinear regression using the following equation:

\[
\text{response} = \text{Min} + \frac{(\text{Max} - \text{Min})}{1 + 10^{\left(\log IC_{50} - \log[I]\right)\text{H}}},
\]

where Max and Min are the upper and lower plateaus, respectively, \([I]\) is the concentration of the inhibitor, \(IC_{50}\) is the concentration of inhibitor needed to reduce the response by 50%, and \(n_H\) is the hill slope that describes the steepness of the curve. The time courses for \(^{3}H\)HOCPCA uptake were fitted to a one-phase association equation describing pseudo-first-order association kinetics:

\[
Y = Y_0 + \text{plateau} \times X / (K_m + X),
\]

where \(Y_0\) is the value of \(Y\) when \(X\) (time) is zero. In this study, \(Y_0\) was constrained to zero. The plateau describes the value of \(Y\) at an infinite time, and \(K\) is the rate constant for the reaction. Data from the saturation experiments were analyzed to obtain the kinetic parameters \(K_m\) and \(V_{max}\) using the Michaelis-Menten equation:

\[
Y = V_{max} \times X / (K_m + X),
\]

where \(Y\) is the uptake velocity, \(X\) is the substrate concentration, \(V_{max}\) is the maximum uptake velocity in the same unit as \(Y\), and \(K_m\) is the Michaelis-Menten constant in the same unit as \(X\). The curve fitting was compared with a fit based on a modified Michaelis-Menten equation containing a diffusional component, \(k \times X\), using an extra sum-of-squares \(F\) test, and a significance level of 0.05. The simpler model was preferred in all cases. In all experiments, extreme outliers were identified and removed using Grubb’s test and significance level \(a = 0.01\). Statistical analysis (unpaired Student’s \(t\) test or one-way
analysis of variance followed by Dunnett’s test) was performed when appropriate and indicated in the figure captions, with the following statistical significance levels: *P < 0.05, **P < 0.01, and ***P < 0.001.

The in vivo B/P ratios of HOCPCA were assessed relative to the doses and plasma exposures of the inhibitor by similar nonlinear regression analysis as for the in vitro data analysis. Individual pairs of HOCPCA, B/P ratios and AR-C141990 exposures from each animal were used as input for the regression analysis.

### Results

#### Functional Characterization of [1-14C]Lactate Uptake in Xenopus Oocytes Expressing MCT Subtypes.

Before the functional characterization of HOCPCA uptake at the MCTs, the ability of Xenopus oocytes to express MCT1, -2, or -4 and transport lactate in vitro was validated using the established MCT substrate [1-14C]lactate (Ovens et al., 2010a). As expected, oocytes injected with cRNA encoding each of the three subtypes were able to transport lactate during 2.5 minutes of incubation time at r.t. The uptake was specific, as 10 mM cold l-lactate inhibited the response by 75% ± 2.6%, 88% ± 0.39%, and 70% ± 3.2%, at MCT1, -2, and -4, respectively (n = 5–7). By comparison, water-injected oocytes displayed minimum lactate uptake (Fig. 1B).

The ability of GHB and the analog HOCPCA to compete with [1-14C]lactate uptake was examined at all three MCT subtypes using 10 mM concentrations. In accordance with other studies (Wang et al., 2007; Wang and Morris, 2007), we found that GHB at 10 mM significantly inhibited the uptake at MCT1 and -2. Similarly, the GHB analog HOCPCA inhibited the uptake at MCT1 and -2, with seemingly higher potency at MCT2 and no apparent inhibition of MCT4 (Fig. 1B).

**Development of a [3H]HOCPCA Uptake Assay at MCT1 and -2.** To investigate whether HOCPCA is a substrate for MCT subtypes, an assay was developed using titrated HOCPCA (Vogensen et al., 2013). Initially, the time dependence of uptake of 100 nM [3H]HOCPCA was studied with uptake in the presence of 10 mM unlabeled l-lactate, as well as to the uptake in water-injected oocytes. The total and nonspecific [3H]HOCPCA uptake curves were fitted to monoequivalent and linear functions, respectively, showing that [3H]HOCPCA is a substrate for both MCT1 and MCT2, displaying negligible uptake in water-injected oocytes (Fig. 2, A and B). As the uptake was linear up to 2.5 minutes, a 2-minute incubation time was chosen for subsequent competition and saturation experiments. In oocytes expressing MCT4, maximum counts were less than 100 CPM and not significantly different from the uptake in water-injected oocytes (Fig. 2C); thus, no further studies on MCT4 were conducted.

**In Vitro Pharmacologic Characterization of GHB, HOCPCA, and AR-C141990 at MCT1 and -2.** To determine IC_{50} values for GHB and HOCPCA at MCT1 and 2, competition experiments were conducted using 100 nM [3H]HOCPCA. As presented in Fig. 2, D and E and Table 1, both compounds inhibited [3H]HOCPCA uptake in a concentration-dependent manner and yielded potencies in the low millimolar range at MCT1 and in the high micromolar range at MCT2. In accordance with previous studies (Halestrap, 2013), the reference substrate L-lactate followed the same trend, being three times more potent at MCT2 compared with MCT1 (Table 1). The obtained IC_{50} values for GHB are in the expected area compared with K_{m} values reported for GHB using cell lines predominantly expressing MCT1 (Wang et al., 2007). For all compounds tested, the Hill slope coefficients were between −0.9 and −1.2. As expected, the compound AR-C141990 also inhibited the uptake of [3H]HOCPCA in oocytes expressing MCT1 or 2 in a concentration-dependent manner (Fig. 2, D and E), yielding potencies in the high nanomolar range at MCT1 and low micromolar range at MCT2 and steeper slopes (IC_{50} values of −2.3 and −2.9 at MCT1 and -2, respectively). A similar trend was seen when the compound was tested in [1-14C]lactate uptake, which also confirmed a less than 20% inhibition of 10 μM AR-C141990 at MCT4 (data not shown), in accordance with findings by Ovens et al. (2010a). The results are in agreement with previous binding experiments performed with AR-C141990 on recombinant MCT1/2 expressed in yeast membranes, where the compound affinity was found to be 10-fold higher at MCT1 compared with MCT2 (Påhlman et al., 2013).

To further characterize transporter pharmacology, saturation kinetics of the subtypes was compared. By means of isotope dilution with unlabeled HOCPCA, K_{m} and V_{max} values were calculated by fitting data to Michaelis-Menten kinetics (Fig. 2, F and G; Table 2). As illustrated, curves approached saturation at concentrations above approximately 10 mM, K_{m} values for HOCPCA were found to be in the low millimolar range with a slight preference for MCT2 over MCT1, reflecting the respective IC_{50} values. For MCT1, K_{m} and V_{max} values were also determined at pH 7.4 and found to be decreased 4.3 and 1.8 times, respectively, compared with pH 6.0 (Fig. 2, F and G; Table 2).

**Molecular Determinants Involved in MCT-Mediated HOCPCA Uptake.** To study the molecular determinants for transport, single-point mutations known to be essential for l-lactate transport, and not to compromise surface expression, were introduced into MCT1 (K38Q and R306K) and MCT2.

### Table 1

Pharmacological characterization of GHB, HOCPCA, and AR-C141990 at recombinant MCT1 and -2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>IC_{50} (μM) ± S.E.M.</th>
<th>Hill Slope ± S.E.M.</th>
<th>IC_{50} (μM) ± S.E.M.</th>
<th>Hill Slope ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-lactate</td>
<td>3.8 mM (2.4 ± 0.05)</td>
<td>−1.1 ± 0.26</td>
<td>1.2 mM (3.0 ± 0.19)</td>
<td>−0.9 ± 0.13</td>
</tr>
<tr>
<td>GHB</td>
<td>1.8 mM (2.8 ± 0.14)</td>
<td>−1.2 ± 0.17</td>
<td>0.86 mM (3.1 ± 0.07)</td>
<td>−1.0 ± 0.19</td>
</tr>
<tr>
<td>HOCPCA</td>
<td>2.8 mM (2.6 ± 0.10)</td>
<td>−1.0 ± 0.07</td>
<td>0.70 mM (3.2 ± 0.11)</td>
<td>−1.2 ± 0.15</td>
</tr>
<tr>
<td>AR-C141990</td>
<td>0.21 μM (6.7 ± 0.07)</td>
<td>−2.3 ± 0.04</td>
<td>2.32 μM (6.0 ± 0.43)</td>
<td>−2.9 ± 1.0</td>
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</table>
and HOCPCA, indicating that L-[14C]lactate and [3H]HOCPCA efflux ratio of 45. From nonlinear regression, the ED50 value was 4.6 mg/kg for the inhibitor AR-C141990 decreased HOCPCA B/P ratios.

For both compounds, as shown in Fig. 5A, increasing doses of systemic absorption of AR-C141990, in vivo interaction studies were performed with a 30-minute pretreatment time point (15 minutes) in both compartments. Based on the respective area under the curves, the B/P ratio was 0.36. HOCPCA exhibited a first-order elimination profile with a parallel decline in both plasma and brain concentrations with a half-life around 20 minutes.

AR-C141990 was also rapidly absorbed in plasma after subcutaneous dosing (Fig. 4B) with a similar elimination half-life for HOCPCA; however, brain exposure of AR-C141990 was quite low compared with plasma. Hence, from the areas under the curve, the B/P ratio was 0.03. The low brain penetration observed in vivo was in agreement with the data from the in vitro bidirectional permeability assessment in MDR1-MDCK cells, where AR-C141990 displayed low apical to basal permeability of 0.42 ± 0.08 10^-6 cm/s with a high efflux ratio of 45 ± 6.0.

Based on the rapid brain absorption of HOCPCA and systemic absorption of AR-C141990, in vivo interaction studies were performed with a 30-minute pretreatment time point for both compounds. As shown in Fig. 5A, increasing doses of the inhibitor AR-C141990 decreased HOCPCA B/P ratios. From nonlinear regression, the ED50 value was 4.6 mg/kg (95% CI, 2.7–7.7). Application of the corresponding plasma concentrations of AR-C141990 measured in each individual animal resulted in a concentration-dependent decrease in the B/P of HOCPCA with an EC50 of 860 ng/ml (95% CI, 660–1110) (Fig. 5B). As the plasma-free fraction of AR-C141990 was 17% ± 1.8%, the unbound EC50 value was calculated to approximately 300 nM (molecular mass 493 g/mol), very close to the value obtained value in vitro at rat MCT1 (Table 1).

### Discussion

In this study, we have identified MCT1 as a key mediator of the active brain uptake of the GHB analog HOCPCA. Using radiolabeled [3H]HOCPCA, we show that the compound is a substrate for rat MCT1 and MCT2, but not MCT4, when recombinantly expressed in Xenopus oocytes, and it is transported by a molecular mechanism similar to the endogenous substrate L-lactate at MCT1/2 (Manoharan et al., 2006; Wilson et al., 2009). In contrast to previous studies performed using immortalized cell lines, the present studies in Xenopus oocytes more accurately describe MCT subtype molecular pharmacology as oocytes do not endogenously express MCTs (Halestrap and Wilson, 2012). Thus, the obtained IC50 values for GHB, HOCPCA, L-lactate, and AR-C141990 may serve as useful reference values at the individual subtypes. The measured in vitro [3H]HOCPCA uptake at MCT1/2 is both time- and concentration-dependent and effectively inhibited by the synthetic inhibitor AR-C141990, which belongs to a recently reported series of very potent MCT inhibitors (Pålman et al., 2013). As the MCT1-mediated transport is proton coupled, it was less efficient at physiologic pH, as expected, but still saturable and time-dependent. This finding supports the in vivo data that HOCPCA can be transported across the BBB by MCT1. Our studies provide very close alignment between in vitro studies at rat MCT1 and in vivo studies at mouse MCT1. Since protein sequences of rat and mouse are 94% identical and conserved at central positions involved in substrate recognition, species differences appear to be negligible.

Several aspects of the current findings warrant additional research in both the GHB and MCT fields. First, new knowledge about the in vivo profile of HOCPCA will be highly useful in relation to future in vivo studies aimed at understanding the role of the elusive high-affinity GHB binding sites. Additionally, the compound AR-C141990 is identified as

<table>
<thead>
<tr>
<th>MCT1 pH 6.0</th>
<th>MCT1 pH 7.4</th>
<th>MCT2 pH 6.0</th>
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<tr>
<td>$K_m$ (mM) ($pK_m$ ± S.E.M.)</td>
<td>3.8 (2.4 ± 0.10)</td>
<td>16.3 (1.8 ± 0.17)</td>
</tr>
<tr>
<td>$V_{max}$ ± S.E.M. (nmol/min*oocyte)</td>
<td>0.44 ± 0.09</td>
<td>0.25 ± 0.08</td>
</tr>
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</table>

### Table 2

Kinetic characterization of HOCPCA transport in oocytes expressing MCT1 and MCT2. Kinetic analysis was performed using a series of HOCPCA concentrations as described in Materials and Methods. Results are given as mean ± S.E.M. of, respectively two, four, and three independent experiments.

Fig. 3. Effects of single point–mutated MCT1 and 2 on [3H]HOCPCA uptake measured in Xenopus oocytes carried out as described in Materials and Methods. Wild-type (WT) values for total uptake and total uptake plus 10 mM L-lactate are given for reference. Total uptake is reduced to less than 10% of WT values in MCT1/2 carrying the known surface-expressed mutations K38Q/K44Q and R306K/R301K. Data are from a single representative experiment expressed as mean CPM values ± S.D. (n = 2).
a useful in vivo MCT1 inhibitor with a favorable pharmacokinetic profile and hence of promise for future work where MCT-inhibition is specifically desired.

Attempts to experimentally target high-affinity GHB binding sites, abundantly present in brain regions such as cortex and hippocampus (Gould et al., 2003; Wellendorph et al., 2010), have so far been performed using GHB, which, however, is also a weak agonist at GABA_B receptors (Mathivet et al., 1997; Kaupmann et al., 2003). This makes it difficult to exclude involvement of these receptors even at low doses of GHB (Bay et al., 2014). Others have used the compound NCS-382 as a specific antagonist (Maitre et al., 1990), although its intrinsic activity has been questioned (Castelli et al., 2004; Crunelli et al., 2006). To exclude GABA_B receptor-mediated effects, by either pharmacologic or genetic means, GABA_B antagonists or GABA_B1 knockout mice have been used (Kaupmann et al., 2003; Carter et al., 2009; Vienne et al., 2010). Whereas the latter is a very elegant approach, there is always the risk of compensatory mechanisms in these mice that might interfere with or mask the results.

Although GHB and HOCPCA are structurally very similar, using HOCPCA has some clear advantages for studying the high-affinity GHB sites specifically. First, HOCPCA binds with approximately 40 times higher affinity to the specific GHB sites than GHB itself (Wellendorph et al., 2005). More importantly, this compound is devoid of affinity for GABA_B receptors (Wellendorph et al., 2005) as well as 45 other neurotargets (Vogensen et al., 2013). As reported here and earlier, the HOCPCA sodium salt is water-soluble, easily administered either orally or subcutaneously, and readily enters the brain with peak concentrations observed before 30 minutes (Vogensen et al., 2013). In addition, it appears to be well tolerated in mice (no sign of sedation or abnormal behavior in the current studies). Robust synthetic routes to both HOCPCA and [^3H]HOCPCA have been developed (Vogensen et al., 2013), which further encourages not only in vivo pharmacologic studies with HOCPCA, but also in vivo live imaging binding studies with, for example, fast-lived positron-emitting isotopes.

It has previously been reported that GHB is a substrate for MCT1, and the transporter is suggested to take part in the oral absorption and renal reabsorption of GHB, as well as GHB transport across the BBB (Bhattacharya and Boje, 2004; Wang et al., 2006; Lam et al., 2010). Given that GHB is a drug of abuse, for which no antidote is currently available, targeting MCT1-mediated transport holds great possibilities as a potential treatment strategy. Accordingly, administration of the MCT substrate l-lactate increases renal and total clearance of GHB in rats (Morris et al., 2005; Wang et al., 2008). In higher doses, administration of l-lactate also decreases cortex extracellular fluid concentrations by inhibiting GHB brain uptake. A consequent decrease in the sedative effect is observed, indicating that inhibiting transport across the BBB can serve as an additional target for treating GHB overdoses (Roiko et al., 2013). The use of l-lactate in combination with the osmotic diuretic mannitol further verified MCT1 as a drug target by increasing GHB renal clearance in healthy human volunteers (Morris et al., 2011). More recently, a group of much more high-affinity compounds were identified as inhibitors of MCT1 (and -2) (Ovens et al., 2010a; Påhlman et al., 2013). Of these, AR-C155858 was recently shown to produce a significant improvement of GHB-induced respiratory depression through inhibition of both renal reabsorption and brain uptake of GHB in rats. As a result of the high potency of the compound, it represents a more efficient treatment strategy, causing greater increases in clearance compared with previously tested concentrations of l-lactate or other MCT inhibitors (Vijay et al., 2015). The
studies presented here is the first to functionally characterize the analog AR-C141990. By means of the confirmed MCT1 inhibition and inhibition of brain uptake, the compound also holds promise as a potential drug candidate for treatment of GHB toxicity.

In addition to being a drug of abuse, GHB is also an approved drug for treatment of narcolepsy (Robinson and Keating, 2007) and, in some countries, alcohol dependence (Addolorato et al., 2009; Keating, 2014). Being 99% charged at physiologic pH, little passive diffusion of GHB is expected, and the drug relies on MCT-mediated transport across the BBB to reach its brain targets and exert its effect. Likewise, for GHB analogs such as HOCPCA, brain penetration is essential for pharmacologic action at the elusive high-affinity GHB binding sites. Thus, the MCT uptake assay can be a useful and simple in vitro method to predict BBB penetration of potential therapeutic agents.

In this study, MCT-mediated brain uptake of HOCPCA was further verified in vivo by assessment of B/P distribution ratios after coadministration with the inhibitor AR-C141990 at different doses. Based on the physicochemical and pharmacokinetic properties in mice, AR-C141990 was found to be a suitable compound for these in vivo interaction studies. Thus, AR-C141990 exhibited high solubility in (2-hydroxypropyl)-β-cyclodextrin at neutral pH, allowing for appropriate dose-escalation studies after subcutaneous administration using this vehicle. Via this route, systemic absorption of AR-C141990 could be obtained rapidly with dose-proportional increases in plasma concentrations. These pharmacokinetic properties are in line with reported studies with AR-C141990 in rats showing dose-proportional increases in plasma levels (Pålman et al., 2013). In the same study, AR-C155858, a previously identified MCT inhibitor, showed inferior dose-exposure relationship, supporting the choice of AR-C141990 for the current pharmacokinetic interaction studies. Interestingly, AR-C141990 was found to be non–brain penetrant in vivo, likely resulting from a strong P-glycoprotein–mediated efflux as indicated from the bidirectional permeability studies in MDRI-MDCK cells. Despite the lack of brain exposure of AR-C141990, the inhibitory action of MCT at the BBB level was present in vivo for HOCPCA, underscoring the fact that the point of interaction is the MCT1 subtype. Assuming no species differences, this notion is in accordance with previous rat immunohistochemical findings of a symmetrical distribution of MCT1 at the luminal and abluminal membranes of brain capillary endothelial cells (Leino et al., 1999).

In conclusion, we have shown that MCT1 is actively involved in the brain uptake of the GHB analog HOCPCA, which adds to our knowledge about the in vivo pharmacokinetics of this compound as an investigational tool compound. Such molecular mechanistic information will be crucial for further studies with HOCPCA in vivo. It also holds promise for the identification of new structural classes of MCT1 inhibitors and tool compounds among other reported GHB analogs, such as the biaromatic GHB analogs reported earlier (Høg et al., 2008; Sabbatini et al., 2010).

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