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A Mechanism-Based Model to Describe GABAA Receptor Trafficking and Benzodiazepine Pharmacoresistance during Status Epilepticus

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A Mechanism-Based Model to Describe GABA<sub>A</sub> Receptor Trafficking and Benzodiazepine Pharmacoresistance during Status Epilepticus

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Elaine Alice Merrill ENTITLED, A Mechanism-Based Model to describe GABA\textsubscript{A} Receptor Trafficking and Benzodiazepine Pharmacoresistance during Status Epilepticus, BE ACCEPTED IN PARTIAL FULFFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Merrill, Elaine Alice. Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2012. A Mechanism-Based Model to Describe GABA$_A$ Receptor Trafficking and Benzodiazepine Pharmacoresistance during Status Epilepticus.

Status epilepticus (SE) is a perplexing pathology involving a sudden and long disruption of the brain’s normal electrical activity. The study of relevant cellular processes has been useful in identifying therapeutic targets. As a result, many novel drugs are being studied which target receptor systems involved in neuronal membrane excitability. Yet, the standard treatment for SE remains benzodiazepines (BZs), a class of GABA$_A$ agonist drugs. Unfortunately, the targeted receptors undergo a desensitization mechanism via enhanced endocytosis (receptor trafficking), leading to rapidly reduced BZ efficacy (pharmacoresistance) within minutes of seizure onset. A comprehensive understanding of the complex interplay between the anticonvulsant's pharmacokinetics and its effect during SE is still lacking. Quantitative information regarding how the trafficking mechanisms of the targeted receptor contribute to the drug's overall pharmacodynamic profile is especially important for the development and assessment of SE countermeasures. This is because the testing of seizure therapeutics can only be performed in animals. Therefore computational modeling of pharmacodynamics provides a useful approach for extrapolations to humans.

This dissertation links a physiologically-based pharmacokinetic (PBPK) model for the therapeutic agent, with a cellular level pharmacodynamic (PD) model of the targeted receptors. The latter explicitly takes into accounts the targeted receptor's surface expression and disrupted trafficking during seizures and the binding of the therapeutic drug. This approach is demonstrated for the interaction of diazepam and its major active metabolite, with the GABA$_A$ receptor, the major therapeutic target. The GABA$_A$ receptor is known to be rapidly modulated during seizure activity. Hypothetically, by accounting for diazepam's pharmacokinetics and
occupancy of BZ-sensitive GABA$_A$ receptors, as well as the cellular trafficking of those receptors during SE, one should be able to mathematically describe the rapid pharmacoresistance. The model developed suggests that approximately 55% occupancy of the original receptor number is required to reverse ongoing seizures. This is up from a reported 37% occupancy required to prevent seizures in the rat, when diazepam is administered just before seizure onset. The physiological basis of the model allows for extrapolation to humans and dose optimization. In addition, the modeling approach used may serve to explain why some drugs may be more or less effective than BZs in treating SE and to offer suggestions for alternative therapeutics.
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<th>Description</th>
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<tbody>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACSLX</td>
<td>Advanced Continuous Simulating Language Xtreme</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism and excretion</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AP2</td>
<td>adaptor protein 2</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BW</td>
<td>body weight (kg)</td>
</tr>
<tr>
<td>BZ</td>
<td>Benzodiazepine</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CANA</td>
<td>convulsion antidote to nerve agent</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
</tr>
<tr>
<td>$C_T$</td>
<td>concentration in tissue (T)</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DMD</td>
<td>Desmethyldiazepam</td>
</tr>
<tr>
<td>DZ</td>
<td>Diazepam</td>
</tr>
<tr>
<td>FF</td>
<td>free fraction</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td><em>im</em></td>
<td>Intramuscularly</td>
</tr>
<tr>
<td><em>ip</em></td>
<td>Intraperitoneally</td>
</tr>
</tbody>
</table>
iv Intravenously

$K^+_o$ extracellular potassium

$k_a$ rate of unbinding from scaffolding proteins (/h)

$k_b$ rate of binding to scaffolding protein (/h)

$k_c$ rate of lateral movement from synaptic to extrasynaptic region (/h)

KCl potassium chloride

$k_d$ rate of lateral movement from extrasynaptic to synaptic region (/h)

$k_{deg}$ rate of degradation (/h)

$k_{ex}$ rate of exocytosis (/h)

$k_{in}$ rate of endocytosis (/h)

$K_{m1}$ Michaelis constant (diazepam to DMD) (mg/L)

$K_{m2}$ Michaelis constant (diazepam to TZ and other metabolites) (mg/L)

$K_{m3}$ Michaelis constant (DMD to OX and other metabolites) (mg/L)

MAPK mitogen-activated protein kinase

$Mg^{2+}$ Magnesium

M-p4 Myristoylated membrane-permeable p4 peptide

NA nerve agent

NMDA N-methyl-D-aspartase

ODE ordinary differential equation

OP Organophosphate

$op$ Orally

OX Oxazepam

$PA_{ BodDMD}$ cell membrane permeability coefficient for desmethyldiazepam in body (mg/L/h)

$PA_{ Br-DZ}$ cell membrane permeability coefficient for diazepam in brain (mg/L/h)

$PA_{ Br-DZ}$ cell membrane permeability coefficient for diazepam in brain (mg/L/h)

$PA_{ F-DZ}$ cell membrane permeability coefficient for diazepam in fat (mg/L/h)
$PBod_{DMD}$  body to plasma partition coefficient for DMD (unitless)

PBPK  physiologically-based pharmacokinetic

$PBr$  brain to plasma partition coefficient for diazepam (unitless)

$PBr_{DMD}$  brain to plasma partition coefficient for DMD (unitless)

PD  Pharmacodynamic

$PF$  fat to plasma partition coefficient for diazepam (unitless)

$PKid$  kidney to plasma partition coefficient for diazepam (unitless)

$PL$  liver to plasma partition coefficient for diazepam (unitless)

$PL_{DMD}$  liver to plasma partition coefficient for DMD (unitless)

$PMu$  muscle to plasma partition coefficient for diazepam (unitless)

$PR$  richly diffused to plasma partition coefficient for diazepam (unitless)

$PS$  slowly diffused to plasma partition coefficient for diazepam (unitless)

$Q_{Bod}$  blood flow from peripheral body = $1.0 - QL - QBr$ (L/hr-kg)

$Q_{Br}$  blood flow from brain (L/hr-kg)

$Q_{EC}$  cardiac output (blood flow) (L/hr-kg)

$Q_{F}$  blood flow from fat (L/hr-kg)

$Q_{Kid}$  blood flow from kidneys (L/hr-kg)

$Q_{L}$  blood flow from liver (L/hr-kg)

$Q_{Mu}$  blood flow from muscle (L/hr-kg)

$Q_{R}$  blood flow from richly perfused tissues (L/hr-kg)

$Q_{S}$  blood flow from slowly perfused tissues (L/hr-kg)

$R_{b}$  density of bound post synaptic receptors (to scaffolding proteins)

Rd  Rhodopsin

$R_{ESM}$  density of extrasynaptic membrane receptors (receptors/dendritic spine)

$R_{f}$  density of free post synaptic receptors (receptors/dendritic spine)

$R_{in}$  density of internal receptors (receptors/dendritic spine)

$Rs$  total number of surface receptors (receptors/dendritic spine)

SE  status epilepticus
TZ  Temazepam

$V_{Art}$  arterial blood volume (L)

$V_{Bred}$  peripheral body volume (used in DMD model) = 1.0-$v_l$-$v_{lb}$ (L)

$V_{Br}$  brain volume (L)

$V_{BrB}$  blood volume within brain (L)

$V_{F}$  fat volume (L)

$V_{FB}$  blood volume within fat (L)

$V_{Kid}$  kidney volume (L)

$V_{L}$  liver volume (L)

$V_{LB}$  blood volume within liver (L)

$V_{max1}$  maximum velocity (diazepam to DMD) (mg/h/kg)

$V_{max2}$  maximum velocity (diazepam to TZ and other metabolites) (mg/h/kg)

$V_{max3}$  maximum velocity (DMD to OX and other metabolites) (mg/h/kg)

$V_{Mu}$  muscle volume (L)

$V_{MuB}$  blood volume within muscle (L)

$V_{R}$  richly perfused tissues volume (L)

$V_{S}$  slowly perfused tissues volume (L)

$V_{syn}$  rate of synthesis (R/h)

$V_{Ven}$  venous blood volume (L)
ACKNOWLEDGEMENTS

I wish to extend my gratitude to several people. First, I thank Dr. Jeff Gearhart for his encouragement and for sharing his intuition on PBPK/PD modeling. I also greatly appreciate my other advisors, Drs. Gerald Alter, Adrian Corbett, James Lucot and Jim McDougal for educating me, being generous with their time, and for providing constructive comments. In addition several of my work colleagues deserve acknowledgement: Teri Sterner and Chris Ruark for digitizing published data sets; Dr. Peter Robinson for his insightful reviews, and Eric Hack and Tammy Covington for their assistance with ACSLX. I also would like to thank Dr. Jeff Fisher, now at the FDA, for introducing me to PBPK/PD modeling and for his encouragement. Lastly, I acknowledge the Defense Threat Reduction Agency (DTRA) for funding much of this work through contract 2G806-08-AHB-C.
DEDICATIONS

“Loving parents have caring children.”

Chinese Proverb

To Andrew, Helen, Arlene, Jean, Ruth and Edward, thanks for caring for mom and dad in their final years.
Chapter 1. Introduction/Background

Status Epilepticus and Pharmacoresistance to Benzodiazepines

Continuous seizures lasting five minutes or more or distinct seizures, which occur without complete recovery of consciousness in between, are characterized as status epilepticus (SE). There are various causes of SE, some of which include: brain tumors or trauma, systemic infections, metabolic disorders (high blood sugar, low sodium or low calcium), abrupt withdrawal of alcohol or anticonvulsant drugs, organophosphate (OP) poisoning, strokes, or genetic mutations in brain receptors. If the seizures are not controlled within a short period, brain damage or death may occur (Sloviter, 1999; Treiman, 2007). Profound neuropathological changes are usually detected within 20 min of the onset of seizure. This is due to hyperactivity of N-methyl-D-aspartase (NMDA) receptors, causing increased influx of calcium ($Ca^{2+}$), which results in cell death (Lallement et al., 1993; McDonough and Shih., 1995; Leadbeater et al., 1985). In addition, after a single episode of SE, survivors have a high risk of injury to the hippocampus or developing epilepsy or neuropsychological dysfunction (Provenzale et al., 2008). This is because SE can trigger epileptogenesis. Epileptogenesis is a process where after an initial SE episode, the brain undergoes structural and functional alterations, including neuronal loss and reorganization of neuronal circuits, in certain regions that play a key role in epilepsy, later giving rise to spontaneous seizures. Previous studies have shown that 2 weeks after prolonged SE (> 3 hours) there is extensive loss of GABAergic interneurons in the amygdala (Tuunanen et al., 1996). Such neuropathological changes are similar between findings from animal models of SE and patients with temporal lobe epilepsy, especially in regards to neuronal loss in limbic structures (Tuunanen et al., 1999).
One of the most common treatments for SE is the benzodiazepine (BZ) drug, diazepam. Benzodiazepines are central nervous system (CNS) depressants. Diazepam and other BZs act on postsynaptic GABA$_A$ receptors to enhance their function, which is to mediate fast synaptic inhibition (Macdonald and Barker, 1978). The drug is commonly used for treating anxiety, insomnia, seizures including SE, muscle spasms (such as in cases of tetanus), alcohol withdrawal and Ménière's disease. It is sometimes used prior to certain medical procedures (such as endoscopies) to reduce tension and anxiety. It possesses anxiolytic, anticonvulsant, hypnotic, sedative, skeletal muscle relaxant, and amnesic properties (Mandrioli et al., 2008).

Diazepam is highly lipophilic and readily crosses the blood brain barrier (BBB), yielding a rapid onset of action. It also readily crosses the placenta, and it can be transferred to milk. Following absorption, diazepam quickly distributes throughout the body. Repeated doses can quickly build up to high concentrations (mainly in adipose tissue) (Riss et al., 2008). Diazepam can be administered orally (op), intravenously (iv) (requiring dilution, as it is painful and damaging to veins), intramuscularly (im) or as a suppository. When administered orally, it is rapidly absorbed and has a fast onset of action (5-10 minutes). The onset of action is 1–5 minutes for intravenous (iv), 15–30 minutes for im and approximately 10 minutes for rectal administration (Riss et al., 2008). Diazepam has several pharmacologically active metabolites. The main active metabolite of diazepam is desmethyldiazepam (also known as nordazepam or nordiazepam). Diazepam's other active metabolites include temazepam and oxazepam. These compounds are considered minor metabolites as they are conjugated with glucuronide, and quickly excreted primarily in the urine. Because the metabolites are centrally active, the serum values of diazepam alone are not always useful in predicting the effects of the drug, especially from repeated doses. Diazepam has a biphasic half-life of about 1–3 and 2–7 days for the active metabolite desmethyldiazepam (Riss et al., 2008).

In cases of OP poisoning, it has been shown that a combination of atropine and diazepam are more effective in reducing mortality than atropine or diazepam alone. Diazepam should be
given to patients poisoned with OPs whenever convulsions or pronounced muscle fasciculations are present. The recommended dose in cases of OP poisoning is 5-10 mg iv in the absence of convulsions and 10-20 mg iv in cases with convulsions, which may be repeated as required (Antonijevic and Stojilkovic, 2007). To protect against potential nerve agent (NA) exposures, military personnel are provided with “convulsion antidote to nerve agent” (CANA) injectors, which are im injection canisters containing 10 mg of diazepam, designated for administration into the thigh muscle, upon the appearance of fasciculation or convulsions. While im is a convenient way to administer the drug in field conditions, absorption of the drug by this route is often slow, erratic and incomplete (Abbara et al., 2009).

Unfortunately, the efficacy of diazepam to abolish seizures diminishes rapidly with time after the onset of SE, often necessitating the need for more aggressive anticonvulsants to be used. Studies have shown that the diazepam doses found to be effective either before or a few minutes after seizure onset may be less than effective for terminating ongoing seizure/convulsive activity. For example, Shih et al., (1997) found that approximately a six-fold greater dose of diazepam was required to terminate seizure activity (when administered 5 min after seizure onset) than when given 30 min prior to soman challenge, as a pretreatment. Similarly, Walton and Treiman (1996) achieved 100% efficacy with diazepam when pilocarpine-induced seizures were treated quickly after onset, with less success the longer seizure progressed. The rapid loss of anticonvulsant effect with increasing duration of SE is associated with internalization of BZ-sensitive GABA_A receptors and a slight increase in localization of BZ-insensitive GABA_A receptors to the synaptic membrane (Naylor et al., 2005; Goodkin et al., 2005; Feng et al., 2008). Hence, the synapses mediated by the GABA_A receptor are altered dramatically, resulting in a loss of BZ efficacy. The receptor desensitization differs from the gradual down regulation of target receptors known as tolerance, seen with chronic use of a drug. Here, the desensitization mechanism of the targeted GABA_A receptor, appears to be membrane activity dependant and ligand independent.
GABA<sub>A</sub> Receptor Composition

Neuronal inhibition is of paramount importance in maintaining a balance with dynamic excitatory signals in the central nervous system. Gamma-aminobutyric acid A (GABA) represents the most widely distributed inhibitory neurotransmitter in the central nervous system (CNS). When GABA is released, it binds to two main types of molecular receptors: fast-acting ionotropic GABA<sub>A</sub>/GABA<sub>C</sub> receptors and slower acting metabotropic GABA<sub>B</sub> receptors (Borman, 2000), with GABA<sub>A</sub> being the predominant type. It has been estimated that 20-50% of all CNS synapses contain GABA<sub>A</sub> receptors (Nutt, 2006). The neurons upon which these receptors reside are mostly interneurons and therefore uniquely able to alter the excitability of local circuits within a given brain region (Mohler <i>et al.</i>, 2004).

GABA<sub>A</sub> receptors are ligand-gated ion channels. Once activated by GABA, the receptor's central channel opens, generally allowing Cl<sup>-</sup> influx, hyperpolarizing the cell membrane, resulting in neuronal inhibition. However, the effect of activating these receptors depends on the electrochemical gradient for Cl<sup>-</sup> on the postsynaptic membrane (Macdonald and Olsen, 1994). In the nervous system of adult animals, the extracellular Cl<sup>-</sup> concentration is typically maintained higher than the intracellular Cl<sup>-</sup> concentration by the K<sup>+</sup>/Cl<sup>-</sup> cotransporter (Rivera <i>et al.</i>, 1999). Therefore, activation of the GABA<sub>A</sub> receptors, typically, results in Cl<sup>-</sup> entry into the neuron, hyperpolarizing the cell membrane and diminishing the change of a successful action potential from occurring. In immature hippocampal neurons, the cotransporter is absent and therefore intracellular Cl<sup>-</sup> is higher and activation of the GABA<sub>A</sub> receptor, depolarizes the neurons, increasing its chance of triggering an action potential (Rivera <i>et al.</i>, 1999). GABA, like most synaptically released neurotransmitters, often saturate their corresponding post synaptic receptors (Clements, 1996). Therefore, the most effective means of modulating the plasticity of postsynaptic response, be it hyperpolarizing or depolarizing, is to change the density and/or composition of surface receptors on the neuron.
Each GABA$_A$ receptor is composed of five subunits (Figure 1-1). This heteropentameric Cl$^-$ channel is selected from 18 known subunits existing in mammals. The 18 subunits can be divided into seven classes, of which some have multiple members: α(1-6), β(1-3), γ(1-3), δ, ε(1-3), π, and θ. Alternative splicing of some receptor mRNAs (Jacob et al., 2008) increases structural diversity further. Hence, multiple combinations of the 18 subunits are possible. Remarkable receptor heterogeneity exists, with subtype combinations varying in different brain regions and cell types, and during different times in ontogeny (Luscher and Keller, 2004). However, the vast majority of GABA$_A$ receptor subtypes are composed of the following five subunits: two α (α1 or α2) and two β (β2 or β3) in combination with either a γ (mainly γ2) or δ (Figure 1-1) (Jacob et al., 2008). It is believed that γ and δ subunits cannot co-exist in the same receptor (Rudolph and Mohler, 2006).

![Figure 1-1](image)

**Figure 1-1.** The GABA$_A$ receptor structure is composed of five subunits from seven subunit subfamilies (α, β, γ, δ, ε, θ and π), forming a heteropentameric Cl$^-$ channel. Most GABA$_A$ receptors expressed in the brain consist of two α subunits, two β subunits and one γ subunit; the γ subunit can be replaced by δ, ε, θ or π. Two GABA molecules bind at the interfaces between the α and β subunits and trigger the opening of the channel, allowing the rapid influx of Cl$^-$ into the cell. BZ binding occurs at the interface between the α (1, 2, 3 or 5) and γ subunits and potentiates GABA-induced Cl$^-$ flux. Reprinted by permission from Macmillan Publisher Ltd:[Nature](Jacob et al., 2008).

Subunit composition is very important as it determines the receptor subtypes’ distinct pharmacological properties and cellular localization. The subunit composition also appears to
determine the channel’s kinetics of phasic and tonic activation and can influence its desensitization (Bianchi et al., 2001). Phasic and tonic activation refer to both spatially and temporally unique modes of inhibition in which GABAergic transmissions shape neural activity (Mody, 2001). Phasic, or synaptic, inhibition is believed to result from high GABA concentration transients acting on synaptic GABA_A receptors composed of two α subunits (usually α1-α3 or α5), two β subunits, and a γ subunit (Farrant and Nusser, 2005). Phasic receptors respond rapidly to stimulus change, producing action potentials proportional to the rate of change of the stimulus. Therefore the phasic response of the neuron diminishes very quickly and then stops until another dramatic rate change. They do not respond to sustained stimulus. In this way they do not provide information on the duration of the stimulus; instead they convey information on rapid changes in stimulus intensity and rate. In contrast, tonic inhibition arises from low ambient “overflow” GABA acting on extrasynaptic GABA_A receptors containing two α4 or α6 subunits with two β subunits and a δ subunit (Mody, 2001; Farrant and Nusser, 2005). Tonic receptors adapt slowly to stimuli, continuing to fire as long as the stimulus is maintained. They convey information about the duration or presence and intensity of a stimulus. Some tonic receptors are permanently active and indicate a background level (Santhakumar et al., 2006).

The β subunits appear to be critical for functional cell surface expression and therefore are likely to be components of all receptor subtypes (Connolly et al., 1999). With exception of the β subunits, the distinct differences in subunit compositions, mentioned above, indicate that synaptic and extrasynaptic GABA_A receptors have different sensitivities to BZs. A major property of the γ2 subunit is to bestow diazepam sensitivity to subtypes containing the α subunits, mentioned above. The BZ binding site is proposed to lie at the interface between the α and γ2 subunits, with residues from each subunit contributing to the binding site (Smith and Olsen, 1995). Therefore receptors composed of “synaptic” α1, α2, α3, and α5 subunits together with β and γ subunits are BZ-sensitive and are predominantly located within the postsynaptic membrane, with the exception of α5, which is localized extrasynaptically (Fritschy et al., 1998) (Figure 1-2).
Most extrasynaptic $\alpha_5$, $\gamma_2$-containing $\text{GABA}_A$ receptors, contribute to tonic inhibition modulated by diazepam (Glykys et al., 2008) and regulate the excitability of pyramidal cells (Bonin et al., 2007). Consequently, postsynaptic and extrasynaptic receptors formed with $\alpha 1$-$3,5$ and $\gamma 2$ subunit are diazepam-sensitive and contribute to the pharmacological profile of classical BZ-site agonists. The $\gamma 2$ subunit, which is present in the vast majority of $\text{GABA}_A$ receptor subtypes, is also required for postsynaptic clustering of $\text{GABA}_A$ receptors, by interacting with gephyrin, a cytoskeletal protein selectively concentrated in GABAergic and glycineric synapses in the CNS. Gephyrin plays a crucial role in anchoring the $\text{GABA}_A$ receptors in the synaptic cleft (Luscher and Keller, 2004). By contrast, those $\text{GABA}_A$ receptors composed of “extrasynaptic” subtypes ($\alpha 4$ and $\alpha 6$ together with $\beta$ and $\delta$ subunits) are a specialized population that mediate tonic inhibition and are BZ-insensitive (Figure 1-2).

![Figure 1-2](image)

**Figure 1-2.** Localization of surface $\text{GABA}_A$ receptors. $\text{GABA}_A$ receptors composed of $\alpha$ (1–3) subunits together with $\beta$ and $\gamma$ subunits are thought to be primarily synaptically localized, whereas $\alpha 5\beta\gamma$ receptors are located largely at extrasynaptic sites. Both these types of $\text{GABA}_A$ receptors are BZ-sensitive. By contrast, receptors composed of $\alpha (4$ or $6)\beta\delta$ are BZ-insensitive and localized at extrasynaptic sites. Reprinted by permission from Macmillan Publisher Ltd: [Nature](Jacob et al., 2008).
Alterations of GABA$_A$ Receptor expression during SE

The density and composition of GABA$_A$ receptor subunits within the neuronal membranes change rapidly during the course of SE. The changes are reflected by the self-sustaining nature of seizures in SE, and the fact that BZs are effective during the early time course of SE but lose potency by 30 min (Kapur and Macdonald, 1997; Treiman et al., 2007). During SE there is increased neuronal hyperexcitability, miniature inhibitory post-synaptic currents (IPSCs) are reduced and the number of GABA$_A$ receptors per dentate granule cell is also rapidly decreased (Goodkin et al., 2005). Clathrin-dependent GABA$_A$ receptor internalization has been identified as one of the mechanisms involved in this process (Jacob et al., 2008). The events triggered promote a decrease in PKC and PKA activity, resulting in reduced phosphorylation of β3 subunits. The decreased phosphorylation of β3 promotes the interaction between the receptor and the clathrin-adaptor protein 2(AP2) (Figure 1-3), which is involved in recruitment of GABA$_A$ into clathrin-coated pits and its removal from the plasma membrane (Goodkin et al., 2008). The involvement of PKC-mediated phosphorylation in regulating GABA$_A$ receptor trafficking and in effect, synaptic efficacy, has been demonstrated in several studies (Connolly et al., 1999; Brandon et al., 1999). These dynamic processes profoundly affect the strength of GABAergic signaling, neuronal inhibition, and presumably synaptic plasticity.

In vitro studies in hippocampal neurons using low magnesium to promote spontaneous epileptiform discharges demonstrate large reductions in GABA-gated chloride currents that correlate with reductions in GABA$_A$ receptors (Blair et al., 2004). Additionally, GABA$_A$ receptor endocytosis has been identified as an early event in the ischemic response that leads to excitotoxicity and cell death (Mielke and Wang, 2005). In vivo studies using chemoconvulsants have shown that the rapid reduction in the number of BZ-sensitive GABA$_A$ receptors in granule cells correlates with a reduction in the level of β2/β3 and γ2 immunoreactivity present in the vicinity of a presynaptic marker (Naylor et al., 2005). While there is a reduction in the BZ-
sensitive receptors (of α1-3, 5 with β2/β3 and γ2 subunits), extrasynaptic GABA<sub>A</sub> receptor subunits (α4 and δ), have been reported to increase in rat models of temporal lobe epilepsy (TLE) (Fritschy et al., 1998). It is important to note that receptors composed of α4 and δ subunits are BZ-insensitive. In addition, heritable diseases that affect receptor trafficking have been recently recognized and compelling evidence exists that mechanisms underlying SE and acquired epilepsy involve GABA<sub>A</sub> receptor internalization (Bradley et al., 2008). For example, similar alterations in GABA<sub>A</sub> receptor subunit expression patterns noted in hippocampal tissues from humans with TLE (Treiman DM. 2007).

![Figure 1-3. Schematic of GABA<sub>A</sub> receptor trafficking. GABA<sub>A</sub> receptors are synthesized and assembled in the Endoplasmic Reticulum (ER) and matured in the Golgi and reach the surface outside of synapses through the secretory path. Synaptic receptors reach their destination through lateral movement in the plasma membrane, where they mediate phasic inhibition. Phosphorylation affects receptor function and removal from the surface through clathrin mediated endocytosis, by enhancing binding with scaffolding proteins, which stabilize receptors at the post synaptic membrane. From the ER, receptors are either recycled or degraded in lysosomes or through proteasomes after ubiquitylation. (Figure taken directly from Tretter and Moss, 2008).](image-url)
Use of physiologically-based pharmacokinetic/ pharmacodynamic modeling to analyze pharmacoresistance

As discussed previously, the rapid pharmacoresistance to BZs, seen with increasing duration of SE appears to be highly dependent upon the rapid kinetics of BZ-sensitive GABA_A receptors surface expression (Naylor et al., 2005; Goodkin et al., 2005; Feng et al., 2008). Therefore, with specific regard to finding novel therapeutics for preventing or ameliorating NA-induced SE, the role that GABA_A receptor dynamics together with diazepam pharmacokinetics play in the drug’s rapid pharmacoresistance with time after seizure onset deserves further quantitative and mechanistic investigation. Physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling is one way to integrate various data sets to quantitatively assess such interactions.

Physiologically based pharmacokinetic modeling is a mathematical technique for predicting the absorption, distribution, metabolism and excretion (ADME) of a chemical/drug in humans and other animal species. The models are mechanistic by mathematically incorporating the anatomical and physiological structure of the body, as well as physical and biochemical descriptions of the drug involved in the complex ADME processes. The model structures are multi-compartmental, with compartments corresponding to specific tissues, interconnected by blood or lymph flows. Differential equations can then be written to describe the changing distribution of the chemical, being studied, in each compartment, using parameters representing actual blood flows, pulmonary ventilation rates, and tissue volumes. These models are typically validated against in vivo data in animal species. However, the inclusion of measured physiological parameters makes PBPK models very suitable for extrapolations across species (e.g., rat to humans) and dose routes (e.g., iv and im). The capacity for such extrapolations makes PBPK models especially suited for estimating and optimizing the efficacy of anticonvulsants, especially antidotes against OP-induced SE. Most studies that measure efficacy of NA antidotes examine biomarkers of exposure and the antidotes’ impact on them, such as lethality, cell death,
oxidative stress, etc. However, measuring the dose of the therapeutic agent actually reaching a target tissue (i.e., brain) cannot be directly scaled from animal studies and is very important to ensure efficacy. The extrapolation of results from in vitro and animal studies to humans is a basic problem in pharmacological studies. For most drugs, preclinical trials are followed by clinical human trials that corroborate and fine tune the data. But, in the case of assessing therapeutics for exposures to OPs, the problem is especially critical since human toxicity data are not readily available. It is certainly possible to determine the dose of various BZs or other therapeutics that result in no adverse effects in human. But, determining their protective efficacies against seizures induced by OPs have to be estimated based on animal experiments only.  

Pharmacodynamic (PD) models predict the time course of the drug’s effect on the body, through the application of mathematical modeling of the mechanism of action involved at the cellular level. These models are typically validated with in vitro data. In this case, the PD model describes the changing surface expression of GABA$\alpha$ receptors during on-going seizures, which can then be linked with PBPK predictions of the target dose of the anticonvulsant drug via the receptor occupancy theory. The model primarily focuses on the dynamics of GABA$\alpha$ receptor trafficking and does not include mechanistic details of downstream elements, such as Ras or mitogen-activated protein kinase (MAPK). Detailed analysis of receptor trafficking can be well understood in the context of a whole cell model using ordinary differential equations (ODEs). ODE models arise from the description of processes where quantities of interest are changing with respect to time. Linking PBPK and PD models of a drug allow for the prediction of the subsequent temporal pattern of in vivo pharmacological response (Figure 1-4).
Figure 1-4. Linking diverse data sets through PBPK/PD modeling

**Thesis Plan**

The primarily focus of this thesis is to demonstrate an approach for integrating a wide variety of experimental data into a quantitative framework that can be used to extrapolate the efficacy of a particular therapeutic agent against SE. In particular, four specific objectives are performed:

1. Modeling $\text{GABA}_A$ receptor trafficking under both basal and SE conditions (PD model),
2. Modeling the pharmacokinetics of diazepam (PBPK model),
3. Linking the models (PBPK/PD),
4. Extrapolating to humans and optimizing therapeutic window.

Coding of the model was written in ACSLX™ (Advanced Continuous Simulating Language Xtreme, Version 2.4), a software environment for modeling, simulation and analysis of nonlinear systems and processes. The following chapters describe the development and validation of the model components. The final model can be used to make predictions regarding the response (seizure termination) in humans to specific doses of diazepam during SE and identifying an optimal therapeutic dose. In addition, the model may potentially serve as an approach for evaluating similar therapeutics and for generating mechanistic hypotheses for which studies can be designed to test.
Chapter 2. Modeling GABA<sub>A</sub> Receptor Trafficking

Introduction

It is well known that the functional strength, of GABAergic synapses, changes in proportion with the number of postsynaptic GABA<sub>A</sub> receptors (Otis et al., 1994; Nusser et al., 1997). Observations that GABA<sub>A</sub> receptor expression rapidly diminishes following enhanced activity and ischemia underscore the importance of quantitatively understanding the mechanisms regulating the receptor’s endocytosis. The trafficking of GABA<sub>A</sub> receptors has not been modeled previously. This chapter attempts to incorporate what is known regarding the regulation of GABA<sub>A</sub> receptor surface expression (the α1β2/3γ2 subtypes) into a quantitative whole cell PD model which can be used to simulate the rapid trafficking of the receptor during homeostatic and SE conditions. Later in Chapter 4, the cellular PD model is linked with a PBPK model for diazepam to understand the impact on diazepam's overall pharmacodynamic profile and to predict the pharmacoresistance seen during SE. The receptor model may serve to better understand the potential contribution that novel therapeutic targets within the endocytic pathway could play in alleviating SE.

Processes involved in GABA<sub>A</sub> Receptor trafficking

The density of synaptic GABA<sub>A</sub> receptors is in part determined by the exchange of synaptic and extrasynaptic surface receptors while the total number of surface receptors is determined by the rates of internalization, synthesis, recycling and insertion into the plasma membrane (Figure 1-3). Internalization of GABA<sub>A</sub> receptors is in part regulated by clathrin-mediated endocytosis (Kittler et al., 2000; Herring et al., 2003). Clathrin is a specialized protein that forms coated pits at the membrane surface, which recruit and concentrate receptors for
internalization. GABA_A receptors are recruited to clathrin-coated pits via the interaction of the adaptor protein (AP2) with the β and/or γ subunits of the GABA_A receptor complex (when it is dephosphorylated) and with clathrin. Finally, as more receptors are incorporated into the pit, it progressively invaginates until dynamin pinches off the vesicle from the plasma membrane (Kittler et al., 2005). Endocytosis occurs predominantly at peri- and extrasynaptic sites, where clathrin is located.

Following endocytosis the receptor undergoes either recycling to the plasma membrane or degradation. Trafficking of new receptors to the cell surface involves posttranslational modification and maturation through the secretory pathway. The localization of nascent receptors in either synaptic or extrasynaptic regions of the plasma membrane is dictated by receptor subunit composition and other factors, guiding insertion. Once at the cell surface, the receptor may move laterally between the post- and extra-synaptic regions and is subject to both constitutive and regulated endocytosis.

Model Development and Parameterization

To model the movement of major BZ-sensitive GABA_A receptor subtypes (αβ1-3γ2), a simple whole cell model was developed, similar to those developed by Lauffenburger and Linderman (1993) and Earnshaw and Bressloff (2006) for other receptors. The model consists of a series of ODEs describing receptor movement between compartments for the postsynaptic membrane and the internal cytoplasmic region of a single inhibitory synapse on a dendrite shaft (Figure 2-1). The postsynaptic surface membrane consists of two compartments representing the post synaptic density (PSD) region and the extrasynaptic membrane (ESM) of the remaining dendrite spine head and neck. Like most synaptic receptors, within the ESM region, GABA_A receptors diffuse freely within the membrane. However, within the PSD, the movement of GABA_A receptor is more restrained by binding of the β and γ subunits to scaffolding proteins, namely gephyrin (Goodkin et al., 2008; Jacob et al., 2005). GABA_A receptors are internalized
mainly from the ESM, via endocytosis (Bogdanov et al., 2006). Once internalized, the receptors undergo endosomal sorting and are either recycled to the cell surface or delivered to lysosomes for degradation. Rate constants are assigned to each of these major constitutive cycles between membrane and intracellular compartments.

Figure 2-1. Simplified model of GABA<sub>A</sub> receptor trafficking. R<sub>b</sub> and R<sub>f</sub> represent surface GABA<sub>A</sub> receptors within the PSD that are either bound to scaffolding proteins or free, respectively. R<sub>ESM</sub> represents extrasynaptic GABA<sub>A</sub> receptors and R<sub>in</sub> represent internalized receptors. First order rates are used to describe binding (k<sub>b</sub>) and unbinding (k<sub>a</sub>) of receptors to scaffolding proteins within the PSD, lateral movement between the synaptic and extrasynaptic membrane (k<sub>c</sub> and k<sub>d</sub>), internalization (k<sub>in</sub>), recycling and membrane insertion (k<sub>ex</sub>) and degradation (k<sub>deg</sub>). Synthesis of nascent receptors is described with a zero order rate, V<sub>syn</sub>.

The symbols and values of parameters selected to describe GABA<sub>A</sub> receptor trafficking are listed in Table 1.1. To begin parameterization, experimentally measured values for receptor densities were assigned to each compartment of the model. For simplicity, these values are expressed as a number of receptors per post synaptic dendrite spine, thereby treating the dendrite spine much like a whole cell. Using a combined approach of patch-clamp recordings and quantitative immunogold localization, Nusser et al., (1997) estimated post-synaptic GABA<sub>A</sub> receptor numbers in cerebellar stellate cells. They reported that synaptic density is fairly constant at about 1250 functional GABA<sub>A</sub> receptors per um<sup>2</sup>, corresponding to an estimated average of 26 receptors within the PSD area, which is in the range of 0.01 - 0.1 um<sup>2</sup>. A common feature of
synaptic receptors is that they enter and exit synapses through lateral diffusion (Lévi and Triller, 2006). This behavior implies that synaptic receptor numbers may be regulated by movements of extrasynaptic receptors and that pools of extrasynaptic and synaptic receptors are in a dynamic equilibrium. Changes to this equilibrium will alter the amount of receptors present at the synapse. Lévi and Triller also reported that the movement of receptors in or out of synapses also transits through a tiny perisynaptic region (400 nm) where they remained for a longer period than expected from their lateral diffusion coefficient in the extrasynaptic plasma membrane. Hence, diffusion within synapses is confined and likely reflects interactions with scaffolding proteins, setting specific $k_{on}$ and $k_{off}$ values for the receptors binding to gephyrin, in the case of GABA$\alpha$ receptors. Movement of receptors within the synaptic region has been measured for some receptors. For example, approximately 45% of the synaptic glycine receptors are estimated to be highly mobile while the remaining 55% are restricted (Lévi and Triller, 2006). Such values were not available for GABA$\alpha$ receptors. However, based upon measured lateral movements between extrasynaptic and synaptic regions, which are discussed later, a higher percentage of synaptic GABA$\alpha$ receptors appear to be restricted. Bogdanov et al. (2006) reported that approximately 19% of synaptic GABA$\alpha$ receptors are associated with AP2, which recruits them into clathrin coated pits. Therefore, it is possible that the remaining 81% or less of the synaptic GABA$\alpha$ receptors is restricted by interaction with gephyrin or other structural proteins. For the purposes of this model it is assumed that the fraction of synaptic GABA$\alpha$ receptors that are less mobile is similar to that of glycine receptors. Therefore, assuming 26 receptors occur at the synapse during equilibrium, 11.7 would be free ($R_f$) and 14.3 would be bound ($R_b$). Comparing the postsynaptic with extrasynaptic GABA$\alpha$ receptor labeling, it has been reported that 24.6 ± 6.4% of surface GABA$\alpha$ receptor clusters are opposed to synapsin (Bogdanov et al.; 2006). Synapsins are a family of proteins involved in regulating the release of vesicles of neurotransmitters from the presynaptic button. Hence they are located directly across the synaptic cleft from the postsynaptic membrane. From the reported percentage of receptors opposed to synapsin (24.6%),
it can be estimated that approximately 75% of surface \( \text{GABA}_A \) receptors (~79.7 receptors) are extrasynaptic \( (R_{ESM}) \), giving an estimated 105.7 receptors on the surface \( (R_s) \). In terms of receptors per surface area, greater density occurs at the postsynaptic region. However, given that the extrasynaptic region accounts for approximately 98% of the spine’s membrane surface area, whereas the postsynaptic region accounts for approximately 2%, a greater total number of \( \text{GABA}_A \) receptors exist within the extrasynaptic region (Nusser et al., 1995; Lévi and Triller, 2006).

The internal receptor density \( (R_{in}) \) was derived from primary brain cell cultures from 7-day chick embryos using \(^{3}H\)flunitrazepam as an irreversible photoaffinity label for BZ-sensitive \( \text{GABA}_A \) receptors (Czajkowski and Farb, 1989). They found 80% of the labeled \( \text{GABA}_A \) receptors were sensitive to trypsin cleavage and were therefore on the surface, whereas 20% were insensitive, being either intracellular and/or membrane sequestered. Therefore, assuming approximately 105.7 receptors are at the surface, the number of internal receptors is estimated to be approximately 26.4.

The ODEs, solving for each compartment within the model shown in Figure 2-1 can be written as:

\[
\frac{dR_f}{dt} = k_a R_b - k_b R_f + k_d R_{ESM} - k_c R_f \\
\frac{dR_b}{dt} = k_b R_f - k_a R_b \\
\frac{dR_{ESM}}{dt} = k_h (R_f - R_{ESM}) - k_{in} R_{ESM} + k_{ex} R_{in} \\
\frac{dR_{in}}{dt} = k_{in} R_{ESM} - k_{ex} R_{in} - k_{deg} R_{in} + V_{syn}
\]

Equation (2.1)

Equation (2.2)

Equation (2.3)

Equation (2.4)

The concept of steady state (SS) is a mathematical idealization of pseudo homeostasis, which plays an important role in kinetic modeling. A system is in steady state if the concentrations of the compartments do not change, which means the corresponding ODEs are zero. A general
steady state analysis of constitutive receptor trafficking can be performed, using only the model structure and estimated receptor densities, without knowing the rate constants for a particular reaction. When all the rates (accounting for binding, lateral movement, internalization, recycling/exocytosis, synthesis and degradation of receptors) arrive at steady state, we can derive the following system of algebraic equations.

\[
\frac{dR_f^{SS}}{dt} = k_d R_{ESM}^{SS} - k_c R_f^{SS} + k_a R_b^{SS} - k_b R_f^{SS} = 0 \quad \text{Equation (2.5)}
\]

\[
k_b R_f^{SS} + k_c R_f^{SS} = k_a R_b^{SS} + k_d R_{ESM}^{SS}
\]

\[
R_f^{SS} (k_b + k_c) = k_a R_b^{SS} + k_d R_{ESM}^{SS}
\]

\[
R_f^{SS} = \frac{k_a R_b^{SS} + k_d R_{ESM}^{SS}}{k_b + k_c} \quad \text{Equation (2.6)}
\]

\[
\frac{dR_b^{SS}}{dt} = k_b R_f^{SS} - k_a R_b^{SS} = 0 \quad \text{Equation (2.7)}
\]

\[
R_b^{SS} = \frac{k_b}{k_a} R_f^{SS} \quad \text{Equation (2.8)}
\]

Taking equation (2.8) into equation (2.6) yields:

\[
R_f^{SS} = \frac{k_b R_f^{SS} + k_d R_{ESM}^{SS}}{k_b + k_c}
\]

\[
R_f^{SS} \left(1 - \frac{k_b}{k_b + k_c}\right) = \frac{k_d R_{ESM}^{SS}}{k_b + k_c}
\]

\[
R_f^{SS} \left(\frac{k_b + k_d - k_b}{k_b + k_c}\right) = \frac{k_d R_{ESM}^{SS}}{k_b + k_c}
\]

\[
R_f^{SS} = \frac{k_d R_{ESM}^{SS}}{k_c}
\]

\[
\frac{R_f^{SS}}{R_{ESM}^{SS}} = \frac{k_d}{k_c} \quad \text{Equation (2.9)}
\]

Taking equation (2.9) into (2.3):

\[
\frac{dR_{ESM}^{SS}}{dt} = k_c \left(\frac{k_d}{k_c} R_{ESM}^{SS}\right) - k_d R_{ESM}^{SS} + k_{ex} R_{in}^{SS} - k_{in} R_{ESM}^{SS} = 0 \quad \text{Equation (2.10)}
\]

\[
k_{ex} R_{in}^{SS} = k_{in} R_{ESM}^{SS}
\]
Finally, equation (2.11) into (2.4) yields:

\[
\frac{d R_{in}^{SS}}{dt} = k_{in} \left( \frac{k_{ex} R_{in}^{SS}}{k_{in}} \right) - k_{ex} R_{in}^{SS} - k_{deg} R_{in}^{SS} + V_{syn} = 0
\]

\[k_{deg} R_{in}^{SS} = V_{syn}\]

\[R_{in}^{SS} = \frac{V_{syn}}{k_{deg}}\]

From the SS analysis, we can estimate the proportional differences in rate constants to be \(k_d/k_a \approx 1.22, k_d/k_c \approx 0.14, k_{ex}/k_{in} \approx 3.02,\) and \(V_{syn}/k_{deg} \approx 26.4\) receptors/dendritic spine, based upon receptor density values listed in Table 2-1. These ratios were initially applied and resulting simulations were compared to experimental data on GABA_A receptor trafficking. The following sections describe more explicitly how the final rate constants were derived from quantitative in vitro studies, taking care to simulate the data in accordance with the particular experimental design used. For example, in studies where receptors are prelabeled at 4°C and their internalization is turned on at 37°C and measured at designated time points, the internal pool of receptors \((R_{in})\) is initialized with a value of zero. In other words, no internal receptors are labeled at time zero. In comparison, when surface receptors are labeled at each time point, it can be assumed that the change in surface expression reflects not only the loss of labeled surface receptors to endocytosis, but also the reappearance of new or recycled receptors into the membrane via exocytosis, which then become labeled. Such studies reflect all of the endocytic processes occurring simultaneously, with exception of those studies where a chemical or protein is used to enhance or “block” a particular pathway.
Table 2-1. GABA\textsubscript{A} Receptor Trafficking Model Parameters

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Basal</th>
<th>SE</th>
<th>Data References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_f$ (R/dendritic spine)</td>
<td>11.7</td>
<td>*</td>
<td>Nusser et al. 1997; Lévi and Triller, 2006</td>
</tr>
<tr>
<td>$R_b$ (R/dendritic spine)</td>
<td>14.3</td>
<td>*</td>
<td>Nusser et al. 1997; Lévi and Triller, 2006</td>
</tr>
<tr>
<td>$R_{ESM}$ (R/dendritic spine)</td>
<td>79.7</td>
<td>*</td>
<td>Nusser et al. 1995, 1997; Jacob et al., 2005</td>
</tr>
<tr>
<td>$R_m$ (R/dendritic spine)</td>
<td>26.4</td>
<td>*</td>
<td>Czajkowski and Farb, 1989</td>
</tr>
<tr>
<td>$k_a$ (h\textsuperscript{-1})</td>
<td>1.0</td>
<td>2.0</td>
<td>Bogdanov et al., 2006</td>
</tr>
<tr>
<td>$k_b$ (h\textsuperscript{-1})</td>
<td>1.22</td>
<td>1.22</td>
<td>Bogdanov et al., 2006</td>
</tr>
<tr>
<td>$k_c$ (h\textsuperscript{-1})</td>
<td>1.0</td>
<td>2.0</td>
<td>Bogdanov et al., 2006</td>
</tr>
<tr>
<td>$k_d$ (h\textsuperscript{-1})</td>
<td>0.14, 0.18</td>
<td>0.14</td>
<td>Bogdanov et al., 2006</td>
</tr>
<tr>
<td>$k_{in}$ (h\textsuperscript{-1})</td>
<td>0.9, 1.0</td>
<td>6.0</td>
<td>Kanematsu et al., 2007; Connolly et al., 1999; Goodkin et al., 2008; Blair et al., 2004</td>
</tr>
<tr>
<td>$K_{ex}$ (h\textsuperscript{-1})</td>
<td>2.7, 7.0</td>
<td>3.02</td>
<td>Cinar et al., 2001, Joshi and Kapur, 2009</td>
</tr>
<tr>
<td>$k_{deg}$ (h\textsuperscript{-1})</td>
<td>0.28\textsuperscript{a}</td>
<td>0.28\textsuperscript{a}</td>
<td>Kittler et al., 2005; Borden and Farb, 1988</td>
</tr>
<tr>
<td>$V_{syn}$ (R/dendritic spine/h)</td>
<td>7.39</td>
<td>7.39</td>
<td>SS analysis</td>
</tr>
</tbody>
</table>

Notes:
All rate constants initially derived via SS analysis, then optimization to referenced data. Where more than one value is given, the red value represents final value used in model.
Parameters are color coded, as described below in order of confidence, with 1 being the highest:
1. Green – derived from neuronal cultures (final values used in model)
2. Blue - derived from recombinant cell lines (not used in final model)
3. Purple – values used derived from studies on glycine receptors, due to lack of data on GABA\textsubscript{A} receptors
4. Red – value based on SS analysis alone, no supporting time course data available.

\textsuperscript{a}Value derived from one time point.

*Receptor densities change with time from onset of seizure

Basal Conditions

Constitutive Endocytosis

GABA\textsubscript{A} receptors on the surface of cortical neurons at steady state undergo significant constitutive endocytosis. Trafficking of the β2/3 and γ subunit-containing receptors is swift, occurring in a time scale of minutes (Kittler et al., 2000; Goodkin et al., 2005; Bogdanov et al., 2006). Previous studies have revealed that 17-25\% or more of the total cell-surface receptor population, depending on maturity of neurons, are internalized within 15 min at steady state (Kittler et al, 2005). For the purposes of this model, the rate of internalization ($k_{in}$) was optimized.
from experimental data by Katamatsu et al. (2007) and Connolly et al. (1999) (Figure 2-2). Katamatsu and colleagues performed a constitutive receptor internalization assay using human embryonic kidney 293 (HEK293) cells transiently co-expressed with GABA$_A$ receptor $\alpha^\text{myc}/\beta^\text{myc}/\gamma^\text{S\text{myc}}$ subunits. In their experiment, cells were pulse-labeled with radioactive $[^{35}\text{S}/^{35}\text{S}]\text{cysteine/methionine}$ for 4 h, followed by labeling surface receptors with anti-myc antibody on ice prior to chase experiment for up to 4 h at 37°C. The myc-tagged receptors were then precipitated with protein G-Sepharose beads, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography for quantification of remaining myc-tagged surface receptors. To simulate this data, as shown in Figure 2-2a, it was assumed that only the surface level of GABA$_A$ receptors were fully equilibrated with the anti-myc antibodies in initial conditions. Therefore, the simulation was initialized using the basal level of surface GABA$_A$ receptors and zero internal receptors. The decreasing radioactivity, relating to myc-tagged receptors, with chase time, suggest that surface receptors prelabeled with the antibody were constitutively internalized and delivered to the lysosome where they were degraded (Kanematsu et al. 2007).

Connolly et al. (1999) also analyzed the membrane stability of recombinant $\alpha^1\beta^2\gamma^2$ receptors in HEK cells. Cells were prelabeled as described in Figure 2-2 and surface expression was measured up to 2 h. From these data, $k_\text{on}$ value was optimized to fit the amount of remaining surface GABA$_A$ receptors ($R_s$) as shown in Figure 2-2, yielding a value of 0.9 h$^{-1}$. The data from the two studies are very similar, but suggest slightly different rates of internalization. While both utilized the same receptor subunit compositions, within the same cell type, the slight divergence may reflect differences in methods.
Figure 2-2. Constitutive endocytosis of GABA<sub>A</sub> receptors. Model simulation (lines) vs. measure surface levels of GABA<sub>A</sub> receptors as a percent of baseline (solid squares. Data taken from A) pulse-chase analysis (at times indicated) for surface GABA<sub>A</sub> receptors in HEK293 cells, containing the α1β2γ1 subunit receptor composition (X ± SEM, n=3) (Kenamatsu et al., 2007), B) immunolabelled GABA<sub>A</sub> receptors in A293 cell expressing α1β2γ2. All subunits were 9E10-tagged prior to incubation at 37 °C for up to 120 min. Surface receptor levels were quantified using iodinated anti-myc (9E10) antibody and expressed as a percentage of level detected at time zero (X ± SD, n=3) (data from Connolly et al. 1999).

Studies measuring intracellular accumulation of GABA<sub>A</sub> receptors in HEK cells and in cultured neurons were then used to fit the exocytosis rate, \( k_{ex} \), as time course of cytosolic accumulation of labeled receptors reflect both the internalization rate and exocytosis, as well as degradation. Cinar et al. (2001) used to HEK 293 cells, transfected with the α1β2γ2<sup>3x9E10</sup> subunit, to quantify internalized receptors (data shown in Figure 2-3). No endocytic processes were blocked in this study. Therefore, with \( k_{in} \) set to the value established from the above studies and the initial amount of internal receptors set to zero, \( k_{ex} \) was fit to the second phase of the curve with a value of 7.0 h<sup>-1</sup>, resulting in the simulation shown in Figure 2-3.
Intracellular accumulation of GABA$_A$ receptors was also measured by Joshi and Kapur (2009). However, rather than using HEK cells, these researchers compared intracellular accumulation of the $\delta$ and $\gamma2$ subunits in cultured hippocampal neurons using an antibody feeding technique. They found that internalization of the $\gamma2$ subunit was faster than that of the $\delta$ subunit. Intracellular accumulation of the $\delta$ subunit peaked between 3–6 h, whereas, maximum internalization of the $\gamma2$ subunit took 30 minutes. The interest here is in using their data to compare rate constants of neuronal $\gamma2$-containing GABA$_A$ receptors with recombinant forms. It is notable that the percent internalized at one hour in this study, 35±9% ($n=4$), is over three times higher than that measured by Cinar et al., 2001. The $k_{ex}:k_{in}$ ratio estimated by SS analysis was approximately 3.02. In figure 2-4, optimized values of $k_{in} = 1.0$ and $k_{ex}=2.7$ (an approximate ratio in agreement with SS analysis values), were found to simulate the data reasonably well. These observations suggest that GABA$_A$ receptors in neurons undergo a similar rate of constitutive

**Figure 2-3.** Model simulation (line) vs. measured (squares) percent of surface GABA$_A$ receptors internalized in HEK cells transfected with $\alpha1\beta2\gamma2^{9E10}$ or $\alpha1\beta2S^{9E10}\gamma2$ subunits. Receptors were labeled with $^{125}I$-labeled c-Myc(9E10) antibody and placed in fresh medium at 37°C for the times shown. Cells were then stripped at pH 1.5 and extracts and stripping medium were counted. The $^{125}I$ associated with stripped cells is expressed as a percentage of the total activity per well, corrected for binding to cells held continuously at 4°C. ($X \pm SE, n=3$) (data from Cinar et al., 2001).
endocytosis as that of recombinant receptors expressed in HEK293 cells; however the rate of exocytosis in the neuron is slower.

**Figure 2-4.** Model simulation (line) vs. measured data (squares). Intracellular accumulation of the γ2 subunit in organotypic hippocampal slice cultures, expressed as a ratio of intracellular to surface γ2 (X ± SD, n=4). Intracellular accumulation of the γ2 subunit was studied using a biotinylation assay (data from Joshi and Kapur, 2009).

**Degradation of GABA\(_A\) Receptors**

Upon internalization, receptors are subjected to either rapid recycling or targeted for lysosomal degradation. This endosomal sorting of internalized receptors does not occur instantaneously, but over longer periods of time. It is reported that within 6 h 29% of originally internalized receptors are degraded in lysosomes (Kittler et al., 2005). That value is in agreement with data from Borden et al. (1984), which suggest that approximately 24% of receptors are degraded within 6 h. To establish the degradation rate, \(k_{\text{deg}}\), an experimental scenario is assumed where surface receptors are in full equilibrium with a radiolabel and allowed to internalize and be degraded. Because the amount degraded in the model is represented by the integration of \(k_{\text{deg}} \times R_{\text{in}}\), the initial number of internal receptors \((R_{\text{in},0})\) is set to zero before initiating the integration. Then the fraction of receptors degraded equals the amount degraded over the original number of surface receptors. The value of \(k_{\text{deg}}\) was then fitted with an approximate value of 0.28 h\(^{-1}\) to yield between 24 and 29% of labeled receptors being degraded within 6 h (Figure 2-5).
Lateral diffusion of surface GABA<sub>A</sub> Receptors between synaptic and extrasynaptic regions.

In addition to constitutive endocytosis and recycling, GABA<sub>A</sub> receptors also undergo significant rates of lateral movements, which can directly modulate the efficacy of synaptic inhibition (Chen et al., 2007). Lateral diffusion within the plane of the plasma membrane has been demonstrated for almost all excitatory and inhibitory receptors, including GABA<sub>A</sub>. Fluorescence recovery after photobleaching (FRAP) techniques on green fluorescent protein (GFP)-tagged GABA<sub>A</sub> receptors, has been used to demonstrate that synaptic receptors exhibit lower lateral mobility compared to the extrasynaptic receptors (Luscher and Keller, 2001). The mechanisms behind receptor accumulation at the synaptic site are not well defined. But this effect is at least in part due to clusters of the scaffolding protein, gephyrin, which acts to restrict movement of inhibitory receptors within the postsynaptic membrane (Jacob et al., 2005; Luscher and Keller, 2001).

To further understand the movement of receptors to and from synapses, Bogdanov et al. (2006) developed tools to visualize GABA<sub>A</sub> receptor endo- and exocytosis in addition to their accumulation at synaptic sites by creating receptor subunits modified with N-terminal pHluorin reporters, capable of binding α-bungarotoxin (Bgt) with high affinity. Using Bgt derivatives they revealed that the principal sites of both GABA<sub>A</sub> receptor endo- and exocytosis are extrasynaptic
in hippocampal neurons. In addition, they demonstrated that newly inserted extrasynaptic receptors are capable of directly accessing synaptic sites. Therefore, synaptic GABA_\text{A} receptors are derived directly from their extrasynaptic counterparts, and constitute a dynamic mechanism for neurons to rapidly modulate receptor number at inhibitory synapses by controlling the availability and stability of extrasynaptic receptors.

To quantify this apparent loss of extrasynaptic receptors, Bogdanov et al. measured Rd-Bgt staining in 1–2 mm\(^2\) areas of interest representing synaptic receptors and extrasynaptic receptors that were at least 2 μm from the nearest synapses and within the same neuron. The ratio of extrasynaptic-to-synaptic Rd-Bgt staining (Bgt-Extrasynaptic/Bgt-Synaptic) was then determined and ratios at zero time were given an arbitrary value of 1.0. This ratio decreased rapidly with duration of incubation at 37°C, reaching a steady state at 1 h (Figure 2-6). This demonstrates that extrasynaptic GABA_\text{A} receptors containing pHBBS\text{β3} have much shorter residence times on the cell surface as compared to synaptic receptors. To determine whether the time-dependent loss of extrasynaptic receptors represents their preferential endocytosis, the authors preincubated hippocampal neurons expressing pHBBS\text{β3} subunits for 1 h with 50 mM myristoylated membrane-permeable p4 peptide (M-p4), a potent inhibitor of GABA_\text{A} receptor endocytosis (Kittler et al, 2000, 2005) before labeling with Bgt. M-p4 appeared relatively effective at blocking the loss of extrasynaptic receptors, that is seen in the untreated neurons (controls) (Figure 2-6), and therefore the time-dependent decrease in Bgt-Extrasynaptic/Bgt-Synaptic ratio seen in controls (Figure 2-6).

Here, their data was used to validate rates of lateral movement between extrasynaptic and synaptic regions (\(k_c\) and \(k_d\)) within our model. Using values developed from the SS analysis, as well as validated parameters, the model did not predict the shift of receptors from extrasynaptic to synaptic regions (shown by the dashed line in Figure 2.6). Care was taken to replicate experimental conditions by initializing internal receptor density at zero and turning off the internalization rate (\(k_{in} = 0\)) to simulate the data from the endocytosis blocking experiment (e.g.,
M-p4 block pretreatment). Interestingly, the SS values predicted a steady state between the measured Bgt-Extrasynaptic/Bgt-Synaptic ratio with endocytosis blocked. The data suggest that either endocytosis is not completely blocked by M-4 or there is a shift toward the synaptic regions. By increasing $k_d$ from 0.14 to 0.18, the simulations improved.

![Figure 2-6](image-url)  

**Figure 2-6.** Model simulations (lines) vs. data (squares, X ± SD) of movement of synaptic receptors to extrasynaptic region. Data shown expressed as a fraction of baseline extrasynaptic/synaptic ratio for control conditions (red line and lower data) or after 1 h pretreatment with M-p4, which blocks endocytosis (black lines and upper data). The dash lines indicate SS parameters, the solid lines indicate an increase of $k_d$. (Data from Bogdanov et al., 2005).

**Enhanced endocytosis during SE**

The expression of BZ-sensitive GABA$_A$ receptors following pilocarpine-induced SE in 4-7 week rats (40 mg/kg im) was assessed by Naylor et al. (2005). After 1 h from the onset of stage 5 seizures (Racine, 1972), they cultured brain slices and estimated that the dentate granule cells had a 50% decrease of the number of physiologically active GABA$_A$ receptors per granule cell synapse (based upon mIPSC analysis) and a 69 and 76% reduction, respectively, of the number of $\beta_2/\beta_3$ and $\gamma_2$ subunit-like immunoreactivity in the vicinity of the presynaptic marker, synaptophysin. It was suggested that the lower estimate obtained from mIPSC analysis compared with immunocytochemistry may be explained by normalization of synaptic physiology with incubation after slice preparation (which arrests seizures). A corresponding increase in $\beta_2/\beta_3$ and
γ2 subunit-like immunoreactivity in the cytoplasm suggests that the subunits relocate to the cell interior during SE, possibly through endocytosis or decreased recycling to the surface. This loss of physiologically active receptors was mimicked by a 20 min exposure of slices to GABA, suggesting that an increase in GABA release with seizure activity may be a contributing factor. The reduction of synaptic GABA<sub>A</sub> receptors may characterize the key transition in which the potent intrinsic mechanisms that usually stop seizures fail and SE becomes self-sustaining. Of note, the time course for the loss of BZ responsiveness, which fails between 10 and 45 min of SE (Kapur and Macdonald, 1997), parallels the emergence of self-sustaining SE in the lithium-pilocarpine model. Furthermore, a loss of inhibition exceeding 30%, as assessed by paired-pulse inhibition, is predicted to result in spontaneous seizures (Kapur and Lothman, 1989).

**Modeling GABA<sub>A</sub> Receptor Trafficking during SE**

To parameterize the model to simulate changes in GABA<sub>A</sub> receptor surface expression during SE, data from *in vitro* seizure models performed on dissociated hippocampal neurons and hippocampal slice cultures, was used. Epileptiform activity can be induced in neuronal cultures from virtually all epilepsy-sensitive brain regions by elevating extracellular potassium concentration ($K^+o$). The induction coincides with membrane depolarization and blockade of evoked synaptic transmission. It is the result of reduced surface charge screening and reduced Ca$^{2+}$-dependent K$^+$ currents. Activation of NMDA receptors and other glutamate receptors are enhanced. This effect is readily mimicked *in vitro* by raising $K^+o$ in ACSF from 3.0-3.5 mM (standard in most laboratories) to 7.5 mM or higher (Jensen and Yaari, 1988).

Seizure-like events can also be induced by omitting Mg$^{2+}$ from artificial cerebrospinal fluid (ACSF). Induction of this activity depends on increased neuronal excitability resulting from reduced surface charge screening (which increases neuronal excitability), facilitated transmitter release, and activation of NMDA receptors due to removal of the Mg$^{2+}$ block of NMDA receptor-operated ion channels.
These approaches were used by Goodkin et al. (2008) and Blair et al. (2004) to study GABA$_A$ receptor internalization during SE-like conditions. Interestingly, the measured internalization rates from the two studies were consistent, despite the use of different in vitro SE models. In addition, the reduction in surface GABA$_A$ receptor internalization also agrees that measured in rat hippocampus at 1 h after onset of pilocarpine-induce seizures (Goodkin et al., 2008). Given their consistency and agreement with in vivo data, the data from these two studies were used to establish parameters for simulating receptor dynamics during SE. Blair and colleagues used hippocampal cultures treated with low Mg$^{2+}$ to simulate epileptic neurons. The neurons were pre-labeled with β2/3 antibodies at 4°C and then fixed and immunohistochemically analyzed after incubation at 37°C. Goodkin et al. (2008) measured the surface expression of the γ2 and δ subunits in hippocampal slice cultures incubated in an 10 mM KCl external medium for 5 min, 15 min, 30 min or 60 min before fixation and antibody tagging of the γ2 subunit using a primary antibody directed against the N terminus of that subunit. The following paragraphs discuss how the data from these studies were used to parameterize the model for SE conditions.

As described earlier, enhance endocytosis and reduced phosphorylation of the β subunit, resulting in increased lateral mobility, are key components in the rapid internalization of the GABA$_A$ receptor seen during SE. Therefore the parameters that most obviously would be altered upon SE are the rates of endocytosis, $k_{in}$, and the on-rate of binding to gephyrin, $k_b$.

The data from Goodkin et al. (2008) is simulated in Figure 2-7. Here the GABA$_A$ receptors were labeled after fixation at the given time points. Hence, the change in expression of surface GABA$_A$ receptors ($R_{s(t)}$) represents the culmination of all processes occurring simultaneously. The basal $k_{in}$ and other SS values simulated the control data. To simulate the increased level of internalization seen under high external KCl, increasing $k_{in}$ to 6 h$^{-1}$ improved the fit, but lowering $k_b$ had little effect on the simulation. Therefore an increase in lateral movement from the synaptic region to the extrasynaptic regions was examined, resulting in an optimized $k_c$ value of 2 h$^{-1}$ (Figure 2-7).
Figure 2-7. Model simulation (lines) vs. measured percent surface expression (X ±SEM) of GABA$_A$ receptor γ2 subunits (squares) in dissociated hippocampal cultures after incubation in standard or 10[KCl]$_o$ medium for the indicated times. After incubation cultured neurons were fixed and antibody tagged for the γ2 subunit. Each time point pooled from 15 neuronal processes (3 replicates) (Goodkin et al., 2008).

In the study by Blair et al. the receptors were pre-labeled before surface level of receptors was measured. Therefore in simulating their data, the internal receptor density was initialized with a value of zero. The control data was simulated with basal SS values, from Table 2-1. Interestingly, with all other parameters set at SS basal values, it was found that the same increase in the $k_{in}$ and $k_c$ values, which were optimized to Goodkin et al.’s data, also simulated endocytosis under low Mg$^{2+}$ conditions (Figure 2-8). However, while only one timepoint was provided in Blair’s study, the fact that the same model parameters describe receptor internalization from both studies, provides confidence in the model’s ability to predict change surface expression during SE.
Figure 2-8. Model simulation (line) vs. measured data (squares) (X ± SD) of increased endocytosis in low Mg$^{2+}$ treated hippocampal cultures. Viable control and epileptic neurons prelabeled with β2/3 antibodies at 4°C and then fixed at 0 and 1 h after incubation at 37°C for immunohistochemical analysis (n=4, p 0.001). (Data from Blair et al., 2004.)

Sensitivity Analysis of Parameters

To identify important parameters associated with the surface expression of GABA$_A$ receptors and the model’s behavior, a parameter sensitivity analysis was performed. Using the finalizing basal parameters, the model was repeatedly run for 1h to determine the total number of surface receptors ($R_s$). The total number of surface receptors was selected, as it is an endpoint used in Chapter 4 to determine receptor occupancy. During each run, a 1% increase from the baseline value in each parameter was set to determine the resulting change in predicted $R_s$ and sensitivity coefficients for each parameter were then calculated using the equation below:

$$Sensitivity\ Coefficient = \frac{(A - B)}{(C - D)} \frac{B}{D}$$

Where A and B are the endpoint parameter (in this case, $R_s$) with 1% increased in a parameter value or with the original parameter value, respectively. C equals the parameter value increased 1% from original value, and D equals the original parameter value.

The most sensitive parameters were found to be rates for exocytosis and endocytosis, $k_{ex}$ and $k_{in}$. It is not surprising that parameters accounting for binding to scaffolding proteins did not influence total surface receptor levels highly. The individual numbers of free and bound
receptors within the synaptic region ($R_f$ and $R_b$) would be more sensitive to these parameters. It should be noted that a limitation in this analysis is the fact that basal parameters are established by SS analysis, based upon receptor densities found in the literature. Definitive receptor densities were not available, especially for the extrasynaptic and synaptic regions. If a higher degree of receptor densities were assumed to be bound (movement restricted), then $K_b$ and $K_a$ would have a greater influence.

![Figure 2-9. Sensitivity coefficients for Each Parameter](image)

**Conclusions**

A simple mathematical model of receptor trafficking between the postsynaptic and the other regions of a dendritic spine was developed that provides a general theoretical framework for investigating the role of trafficking in the expression of BZ-sensitive GABA$_A$ receptors. The model was constructed similar to a single cell, with mainly first order rates to describe the receptors’ kinetics. Steady state analysis was conducted, using estimated receptor densities from the literature, to derive the proportional relationships between rate constants. The rate constants were then specifically fitted against time course data from studies on GABA$_A$ receptor movement. Differences found between the rate ratios developed from SS analysis and those derived via optimization to *in vitro* data were slight. Sources of these differences are likely to arise from differences in labeling techniques, differences between neuronal and recombinant cell
types and/or the effects of different labels upon receptors’ configuration, movement, and resistance to proteolysis within the lysosome and sensitivity to changes in pH.

For example, the endocytosis and exocytosis rates, $k_{in}$ and $k_{ex}$, established from SS analysis describe relatively well the receptor internalization data seen in neuronal culture by Joshi and Kapur (2009), but the rate of exocytosis observed in HEK cells is nearly double the neuronal value ($k_{ex} = 7.0$ vs. $3.0$). The difference may well be due to the fact that dense synaptic clustering is lacking in non-neuronal cultures (Caruncho et al., 1993). In addition, surface receptor densities are much lower in recombinant cell lines. Bradley et al. (2008) measured wild-type GABA$_A$ receptors expressed in HEK293 cells, using a recombinant N-terminal FLAG epitope-tagged $\alpha_1\beta_2$ construct. The receptor’s cell surface expression averaged $44 \pm 8\%$ of the total cell expression, nearly half of that measured in neuronal cultures by Czajkowski and Farb, 1989 (80% of total receptor expression). Therefore, although constitutive endocytosis is similar between the two cell types, the recombinant cell line may display a greater rate of exocytosis in an attempt to maintain stable surface levels.

It is also important to keep in mind that GABA$_A$ receptor trafficking involves multiple proteins such as clathrin, adaptor protein 2 (AP2) complex, dynamin, GABA$_A$ receptor associated protein (GABARAP), Huntington-associated protein 1 (HAP1), protein linking integrin-associated protein to cytoskeleton-1 (PLIC1), and gephyrin (Kittler et al., 2000; Kittler et al., 2005; Kanematsu et al., 2006) (Figure 1-3). Therefore, each rate constant represents a composite of multiple processes, optimized to produce results consistent with the known experimental data used in this analysis. Yet, the consistency in measured rates observed across a number of different in vitro SE models and in animal models instills confidence in the model’s final parameters.

Together, the trafficking model and the studies used to parameterized the receptor trafficking model demonstrate that the surface stability of GABA$_A$ receptors can be rapidly and specifically regulated, enabling neurons to modulate cell surface receptor number upon the
appropriate cues, such as activity. The kinetics of the model depends upon various composite trafficking parameters that could be targets of second messengers pathways activated during seizures. The model was used to explore the consequence of targeting different parameters to see how these parameter changes can reproduce a wide range of experimental data. To simulate SE conditions, increases in the rates of endocytosis, $k_{in}$, and lateral movement from synaptic to extrasynaptic membrane regions, $k_c$, appeared sufficient to describe the data. Obviously, that does not rule out the possibility that additional parameters are altered significantly enough to impact surface expression. However, for simplicity, the addition of unmeasured parameters was limited to as few as necessary to describe the measured data. In the next chapter, the development and validation of a diazepam PBPK model is discussed. The diazepam model is then linked (in Chapter 4) with the receptor trafficking model described here to predict pharmacoresistance.
Chapter 3. Diazepam PBPK Model Development

The purposes of developing a PBPK model are 1) to demonstrate a means of extrapolating animal studies to humans and 2) to predict the changing concentration of the anticonvulsant and its major active metabolite in the “biophase”, so that it can be linked with the dynamics of surface GABA\(_A\) receptor expression during SE for predicting the time-dependant dose response. Biophase refers to the target tissue where the drug exerts its pharmacological effect. In this case, biophase refers to the brain. Diazepam was chosen as a suitable candidate for illustrating this modeling approach as it is typically the first anticonvulsant used to treat SE and it remains the conventional antidote to NA-induced seizures. In addition, considerable kinetic and dose response data on this compound exist across species (including humans), doses and dose routes, allowing for validation of model parameters and predictions. This model simulates the anticonvulsant concentration in the brains of rats and humans from various dose routes, including intramuscular (im) injections. This is important because kinetics resulting from im administration are known to be highly variable in comparison to other dose routes, and im is the dose route of the diazepam auto injector (CANA), used by the military for NA-induced seizures. Lastly, because PBPK models can be used to predict in vivo doses that result in brain concentrations that are comparable to levels used in published in vitro studies on diazepam's effect on GABA\(_A\) receptors, the model assures equivalent dosimetry when applying in vitro results to predict in vivo effects.

Physiologically-based PK models have been developed previously that describe the absorption and distribution of diazepam in the tissues of both rats and humans (Igari et al., 1983; Gueorguieva et al., 2004). However, these models did not account for diazepam's major active metabolite, desmethyldiazepam (DMD), which extends its half-life to up to 100 h, adding greater nonlinearity to the drug's effects. In addition, these models had not been linked with a cellular PD model accounting for the desensitization of BZ-sensitive GABA\(_A\) receptors occurring during
Therefore, the current model takes previous work further to explore not only the impact of the drug’s pharmacokinetics, but also the impact of the changing expression of the target receptor, upon the drug’s pharmacodynamic profile.

**PBPK Model Structure**

The current PBPK model describes the distribution of diazepam and its major metabolite, DMD (Figure 3-1). The model structure for diazepam includes distinct compartments for the liver, brain, fat and kidney. The remaining tissues are lumped under richly and slowly perfused tissues. From the liver compartment, the model links to a separate compartmental model for diazepam’s major metabolic product (DMD). In selecting the compartments an attempt was made to choose compartments most relevant to diazepam’s distribution and effect, while keeping the model as simple as possible. For example, the liver and brain are important sites of metabolism and pharmacological activity, respectively. Given diazepam’s high lipid solubility, the drug quickly enters the brain and is redistributed to peripheral compartments and readily taken up into fat. Therefore, fat has a major impact on the drug’s distribution.
Metabolism of diazepam occurs mainly in the liver by the cytochrome P450 (CYP) enzyme system. Most of diazepam undergoes oxidative metabolism by desmethylation (via enzymes CYP 2C9, 2C19, 2B6, 3A4, and 3A5) and hydroxylation (CYP 3A4 and 2C19) (Figure 3-2). The metabolites formed further undergo conjugation through glucuronidation in liver microsomes. Glucuronides are secreted in bile and eliminated in urine. Hence, the drug undergoes multiple phases of transformation, making the drug more polar and easier to excrete.

Diazepam has three pharmacologically active metabolites, which bind to the same “benzodiazepine” site on the GABA<sub>A</sub> receptor with similar affinities (Arendt et al. 1987). Its major active metabolite in humans is desmethyldiazepam (DMD) (also known as nordazepam or nordiazepam). While diazepam has biphasic half-life, with a quick distribution half-life of 2-15 min, and a long terminal half-life of about 1-2 d; DMD’s long half-life is approximately 2-7 d (Riss et al., 2008). Minor active metabolites include temazepam (TZ) and oxazepam (OX).
These metabolites are readily conjugated with glucuronide, and are rapidly excreted by humans, primarily in the urine. Less than 1% of the parent compound is excreted unchanged or as DMD (Riss et al., 2008). Therefore, in congruence with the purpose of creating a model for human extrapolations, only the major metabolite is modeled. However, the model still takes into account the depletion of parent compound, resulting from the formation of the other metabolites, mainly TZ. For simplification, compartments for tracking DMD include only the brain, liver and plasma, while the rest of the body is lumped (Figure 3-1). Because DMD is not readily excreted, the depletion of DMD is modeled as a metabolic loss to OX formation.

**Figure 3-2. Major and minor metabolites of diazepam**

The PBPK model is a mix of algebraic and ODEs. The latter are mass balance equations, which describe the rate of change of a chemical within a model compartment. Taken together, the mass balance equations for all compartments describe how the chemical distributes within the body. The amount of a chemical within a single tissue compartment at any time should equal the amount of that chemical entering the compartment in the arterial blood stream, minus the amount
leaving the compartment in venous blood stream, plus the amount taken up within the
compartment, minus any amount excreted (or metabolized).

Below is a mass balance equation for a compartment without metabolism or excretion
and whose concentration is limited by arterial blood concentration and blood flow.

\[ V_T \frac{dC_T}{dt} = Q_T \left( CA - \frac{C_T}{P_T} \right) \]  \hspace{1cm} \text{Equation (3.1)}

Where \( V_T \) represents the tissue volume, \( Q_T \) is tissue blood flow and the subscript, \( T \), represents the
particular tissue. \( CA \) is arterial concentration and \( C_T \) and \( P_T \) are the concentration in tissue and
the tissue/blood partition coefficient, respectively. These compartments are called “flow limited”
and include the liver, kidney, muscle, and richly and slowly perfused compartments. However, as
described later, the amount in the liver compartment is also impacted by metabolism and
elimination.

The rate of change in the amount of chemical in compartments in which the concentration is
limited by diffusion, such as brain and fat, is described as being proportional to the cell
membrane permeability coefficient (\( PA_T \)) for tissue (\( T \)), as shown in the equations 3.2 and 3.3.

Tissue uptake is said to be diffusion-limited when \( PA_T < Q_T \).

\[ V_{TB} \frac{dC_{VT}}{dt} = Q_T (CA - CV_T) + PA_T \left( \frac{C_T}{P_T} - CV_T \right) \]  \hspace{1cm} \text{Equation (3.2)}

Where: \( V_{TB} \) is the volume of blood in the tissue, \( CV_T \) is the concentration in the tissue’s blood
(capillary bed) and \( PA_T \) (cell membrane permeability coefficient, L/h). The tissue concentration
(\( C_T \)) can then be derived from the tissue blood concentration (\( CV_T \)) as follows:

\[ V_T \frac{dC_T}{dt} = PA_T \left( CV_T - \frac{C_T}{P_T} \right) \]  \hspace{1cm} \text{Equation (3.3)}

To model DMD formation and clearance, the diazepam model branches off from the liver
compartment, as \( V_{max1} \), into a separate compartmental model, in which this major metabolite is
tracked in liver, brain and plasma (Figures 3-1 and 3-2) and the other metabolites are lumped as
$V_{\text{max}2}$ below. Hence, the rate of change in DZ concentration within the liver is described using flow limitation and saturable metabolism (forming DMD and TZ):

$$V_L \frac{dC_L}{dt} = Q_L \left( CA - \frac{C_L}{P_L} \right) - \frac{V_{\text{max}1} \times C_L}{K_{m1} + C_L} - \frac{V_{\text{max}2} \times C_L}{K_{m2} + C_L}$$  \hspace{1cm} \text{Equation (3.4)}

Where: $CV_L$ is the concentration of diazepam in the liver capillary bed ($CV_L = C_l/P_L$), $V_{\text{max}1}$ and $K_{m1}$ are the Michaelis Menten (M-M) maximum velocity and affinity constants for the conversion of diazepam to DMD and $V_{\text{max}2}$ and $K_{m2}$ are the M-M constants for the conversion of diazepam to TZ.

Within the DMD model, the rate of change of DMD in the liver is described similarly:

$$V_{LDMD} \frac{dC_{LDMD}}{dt} = Q_L \left( C_{DMD} - CV_{LDMD} \right) + \frac{V_{\text{max}1} \times C_L}{K_{m1} + C_L} - \frac{V_{\text{max}3} \times C_{LDMD}}{K_{m3} + C_{LDMD}}$$  \hspace{1cm} \text{Equation (3.5)}

Where: $C_{LDMD}$ is the concentration of DMD in the liver, $C_{LDMD}$ is the concentration of DMD in liver capillary bed, and $V_{\text{max}3}$ and $K_{m3}$ are the M-M constant for the conversion of DMD to OX, which is glucuronidated and eliminated in feces. Therefore, although the minor metabolites, TZ and OX, are not modeled and tracked specifically, their clearance from the system is accounted for.

Routes of exposures modeled include: iv, ip and im injections. Intravenous infusion was modeled as a zero-order rate into venous blood, based upon the length of the injection time. Intramuscular and im injections were modeled as first-order absorption from a small muscle or peritoneal compartment into the entire muscle or liver, respectively.

**Allometric Scaling**

To account for variation associated with size, all rate constants are scaled by $BW^{0.74}$ as shown below:

$$Q_c = Q_{cC} \times BW^{0.75}$$  \hspace{1cm} \text{Equation (3.6)}

$$PAF = PAFC \times BW^{0.75}$$  \hspace{1cm} \text{Equation (3.7)}

$$V_{\text{max}} = V_{\text{max}C} \times BW^{0.75}$$  \hspace{1cm} \text{Equation (3.8)}
**Model Parameterization**

The rate of entry of any drug into a tissue depends on blood flow to the tissue, the tissue volume, and partition characteristics between blood and tissue. Equilibrium in distribution (when entry and exit rates are the same) between blood and tissue is reached more rapidly in richly perfused areas (e.g., kidney and brain), unless diffusion across cell membranes is the rate-limiting step. After equilibrium, drug concentrations in tissues and in extracellular fluids are reflected by the plasma concentration. Metabolism and excretion also occur simultaneously with distribution, making the process dynamic and complex.

Parameters used to describe these processes within the PBPK model are physiologically and chemical specific. Physiological parameters, describing blood flows and tissue volumes, for rat and man were obtained from experimentally measured data compiled by Brown *et al.*, 1997 (see Table 3-1). Chemical-specific parameters include tissue:plasma partition coefficients ($P_T$s), extent of plasma binding and metabolic and clearance rates. Tissue:plasma partition coefficients are critical parameters in PBPK modeling as they represent steady state chemical distribution and generally are fairly consistent across species. The $P_T$s used here (Table 3-2) for diazepam and DMD represent whole tissue to plasma (unbound) concentration ratios measured *in vivo* at 480 min post *iv* injection of 1.2 mg/kg DZ in rats (Igari *et al.*, 1983), when a pseudo equilibrium is achieved. The unbound fraction in plasma was determined by equilibrium dialysis. The partitioning of diazepam between plasma and erythrocytes in rat was also measured, resulting in a blood to plasma ratio of $1.03 \pm 0.007$ (Igari *et al.*, 1983).
Table 3-1 Physiological parameter values

<table>
<thead>
<tr>
<th>Parameter (Constants)</th>
<th>Name</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td>Body weight (kg)</td>
<td>0.3</td>
<td>70.0</td>
</tr>
<tr>
<td>QC</td>
<td>Cardiac output (blood flow) (L/hr-kg)</td>
<td>15</td>
<td>16.5</td>
</tr>
</tbody>
</table>

**Tissue Blood Flow Constants [fraction of QC]**

| QC       | Liver      | 0.18 | 0.23 |
| QC       | Fat        | 0.09 | 0.05 |
| QC       | Brain      | 0.03 | 0.11 |
| QC       | Kidney     | 0.14 | 0.14 |
| QC       | Muscle     | 0.28 | 0.19 |
| QC       | Richly perfused | 0.12 | 0.11 |
| QC       | Slowly perfused | 0.16 | 0.17 |
| QC       | Peripheral body = 1.0-QL-QBr | 0.79  | 0.66 |

**Tissue Volume Constant [fractions of BW]**

| VC       | Liver      | 0.034 | 0.026 |
| VC       | Fat        | 0.07  | 0.21  |
| VC       | Brain      | 0.0057 | 0.02  |
| VC       | Kidney     | 0.007 | 0.007 |
| VC       | Muscle     | 0.40  | 0.40  |
| VC       | Venous blood | 0.054 | 0.056 |
| VC       | Arterial blood | 0.027 | 0.024 |
| VC       | Richly perfused | 0.21  | 0.16  |
| VC       | Slowly perfused | 0.27  | 0.18  |
| VC       | Peripheral body V_BOD = 1.0-V_L-V_Br | 0.96  | 0.95  |

**Tissue blood volume constants [fractions of tissue volumes]**

| VC       | Blood fraction of liver | 0.21  | 0.11  |
| VC       | Blood fraction of fat   | 0.02  | 0.02  |
| VC       | Blood fraction of muscle | 0.04  | 0.01  |
| VC       | Blood fraction of brain | 0.03  | 0.04  |

Source: All volumes and flows taken from Brown et al., 1997.

- Blood flow constants are multiplied by QC to yield tissue blood flows (L/hr-kg) used in model.
- Tissue volume constants are multiplied by BW to yield tissue volumes (L) used in model.
- Tissue blood volume constants are multiplied by the volume of the corresponding tissue to yield the blood volume of that tissue (L).
### Table 3-2. Chemical Specific Tissue:Plasma (Unbound) Partition Coefficients

<table>
<thead>
<tr>
<th>Tissue:Plasma Unbound 1 for DZ (unitless)</th>
<th>(PT)s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (PBr)</td>
<td>7.03</td>
</tr>
<tr>
<td>Kidney (PKid)</td>
<td>15.9</td>
</tr>
<tr>
<td>Liver (PL)</td>
<td>33.7</td>
</tr>
<tr>
<td>Muscle (PMu)</td>
<td>9.9</td>
</tr>
<tr>
<td>Fat (PF)</td>
<td>88.9</td>
</tr>
<tr>
<td>Slowly diffused (i.e., muscle) (PS)</td>
<td>9.44</td>
</tr>
<tr>
<td>Richly diffused (i.e., kidney) (PR)</td>
<td>15.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue:Plasma Unbound for DMD (unitless)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (PBr\textsubscript{DMD})\textsuperscript{1,2}</td>
</tr>
<tr>
<td>Liver (PL\textsubscript{DMD})\textsuperscript{2}</td>
</tr>
<tr>
<td>Remaining Body (PBod\textsubscript{DMD})\textsuperscript{2}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Sources: (Igari et al., 1982; 1983)
\textsuperscript{2}Optimized to experimental data

---

**Plasma Protein Binding and its impact on tissue:plasma partition coefficients.**

The amount of diazepam distributed into tissues depends on the extent of binding in the blood and tissues. In the bloodstream, diazepam is transported partly in solution as free (unbound) drug and partly reversibly bound to blood components (e.g., plasma proteins, red blood cells). Only unbound diazepam is available for passive diffusion into interstitial fluids, tissue cells as well as to extracellular regions of target sites where the pharmacologic effects of the drug occur. Therefore, the free fraction (\(FF\)) of the drug concentration in systemic circulation typically determines drug concentration at the active site and thus efficacy and this value differs across species. Because total plasma concentrations are being simulated as partitioning into the tissues and measured whole plasma concentrations are being used for validation, the \(P_{Ts}\), which represent tissue:plasma (unbound), must be adjusted to account for the bound fraction which cannot partition into the tissue. To account for this the \(P_{Ts}\) are multiplied by the \(FF\) to yield the species-specific tissue:plasma (total) ratios, as shown:

\[
\frac{\text{Tissue}}{\text{Plasma (total)}} = \frac{\text{Tissue}}{\text{Plasma (unbound)}} \times FF \quad \text{Equation (3.10)}
\]
Diazepam and DMD are both extensively bound to plasma proteins. Moschitto and Greenblatt (1983) quantified the binding extent of normal human plasma to ten different BZs and found that plasma binding was concentration-independent and therefore should not complicate the interpretation of pharmacokinetic studies. The average $FF$ for DZ and DMD were 0.016 and 0.035, respectively. These values did not vary significantly across a range of concentrations from 10.0 to 10,000 ng/ml. With the exception of some cases of massive overdose, plasma levels encountered during therapeutic use do not exceed this upper limit (Greenblatt et al., 1978). In humans, following single iv doses from 5 to 10 mg, the average $FF$ of DZ was found to be 0.015 ± 0.002 % (Greenblatt and Divoll, 1983). Klotz et al., 1976 reported an $FF$ of approximately 0.032 ± 0.008 % in humans. The high level of plasma binding for these compounds is supported by studies in other species. Wala and Sloan (1995) measured the $FF$ in dog plasma. In dog plasma containing 0.5 and 20.0 μg/ml of diazepam or DMD, the $FF$ was 0.038 ± 0.003 and 0.077 ± 0.018 for diazepam, and 0.034 ± 0.004 and 0.048 ± 0.006 for DMD, respectively. Plasma binding occurs to a lesser extent in the rat. Binding studies in rat plasma, indicate $FF$ of diazepam and DMD to be 0.137 ± 0.011 and 0.095 ± 0.01 (Klotz et al., 1976). Here, to simulate the unbound concentration of diazepam and DMD in plasma, $FF$s of 0.14 and 0.095 for the rat and 0.03 and 0.04 for humans, are used respectively, resulting in the adjusted species-specific $Pt$s shown in Table 3-3.
**Table 3-3. Species-Specific Tissue:Plasma (whole) Partitioning and Permeability Coefficients**

<table>
<thead>
<tr>
<th>Tissue:Plasma Unbound for DZ(^1) (unitless)</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain ((PBr))</td>
<td>0.98</td>
<td>0.21</td>
</tr>
<tr>
<td>Kidney ((PKid))</td>
<td>2.2</td>
<td>0.48</td>
</tr>
<tr>
<td>Liver ((PL))</td>
<td>4.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Muscle ((PMu))</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Fat ((PF))</td>
<td>12.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Slowly diffused (i.e., muscle) ((PS))</td>
<td>1.3</td>
<td>0.28</td>
</tr>
<tr>
<td>Richly diffused (i.e., kidney) ((PR))</td>
<td>2.2</td>
<td>0.48</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue:Plasma Unbound for DMD(^1,2) (unitless)</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain ((PBr_DMD))</td>
<td>0.76</td>
<td>0.3</td>
</tr>
<tr>
<td>Liver ((PL_DMD))</td>
<td>2.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Remaining Body ((PBod_DMD))</td>
<td>5.7</td>
<td>2.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell Membrane Permeability Coefficients ((PA)) (mg/L/h)(^2)</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain ((PA_{Br-DZ}))</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Fat ((PA_{F-DZ}))</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Brain ((PA_{Br-DMD}))</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Body ((PA_{ BodDMD}))</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\(^1\)Notes: Tissue:Plasma (whole) values derived from values in Table 3-2 multiplied by the FF in rat and human plasma. The FF for diazepam and DMD were 0.14 and 0.095 for the rat and 0.03 and 0.04 for humans, respectively.

\(^2\)Values optimized to experimental data.

**Metabolic Parameters and Tissue Permeabilities of Diazepam**

Michaelis Menten (M-M) constants \((K_m)\) from various studies in multiple species were evaluated and compared while simulating the metabolism and clearance of diazepam during its conversion to DMD and further metabolic clearance of DMD. Approximate \(K_m\) values collected from \textit{in vitro} rat liver slice studies (Saito et al., 2004a, b) and human liver microsomes (Andersson et al., 1994) were used. However the permeability coefficients \((PAs)\) and maximum velocities \((V_{max})\)’s were visually optimized to the kinetic data from Igari (1983) for the rat, while all other parameters (e.g., partition coefficients and physiological parameters) were set to those in Tables 3-1 and 3-3. With compartments described by diffusion limitation, such as fat, the \(PAs\)
were optimized to the early uptake portion of the corresponding tissue data, while metabolic \( V_{max} \)s were optimized to the later clearance portion of the curve of liver and plasma data. Similarly, the \( V_{max} \) values used for human were optimized to individual human kinetic studies, which are described in the results (see Table 3-5). These values were then averaged and the final metabolic constants are shown in Table 3-4.

**Table 3-4 Metabolic Parameters**

<table>
<thead>
<tr>
<th>( V_{max} ) (mg/h/kg)</th>
<th>Rat</th>
<th>Source</th>
<th>Human</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{max1} ) N-desmethylation</td>
<td>15.0</td>
<td>Optimized(^1)</td>
<td>3.6</td>
<td>Optimized(^2)</td>
</tr>
<tr>
<td>( V_{max2} ) 3-hydroxylation</td>
<td>10.0</td>
<td>Optimized(^1)</td>
<td>0.40</td>
<td>Optimized(^2)</td>
</tr>
<tr>
<td>( V_{max3} ) hydroxylation</td>
<td>3.0</td>
<td>Optimized(^1)</td>
<td>0.36</td>
<td>Optimized(^2)</td>
</tr>
</tbody>
</table>

\(^{1}\)optimized using data from Igari *et al*., 1983 or from human studies

\(^{2}\) see Table 3-5.

\(^{3}\) \( K_{m1} \) and \( K_{m2} \) values for both hydroxylation steps are assumed similar.

**Experimental data**

As described above, a final step in parameterization involved simulating experimental data from an extensive kinetic study by Igari *et al*., (1982). The resulting chemical specific parameters, listed in Tables 3-5, were then tested via simulation of other published kinetic data.

The experimental data from the other studies then served as validation of the model, with exception of the human studies, which also served to optimize \( V_{max} \) values for the metabolism of diazepam. The data presented in this chapter were digitized, from the following published PK studies in rats and humans.

**Rat Data:**

- *Igari et al. (1982):* Male Wistar rats, weighing 250-270g were administered \(^{14}\)C-diazepam diluted with unlabeled diazepam in polyethylene glycol (1.2 mg/kg) by iv injection.
At various times after iv injection, tissues were collected and analyzed for diazepam, DMD, OX and TZ, N = 3-5 rats/time-point. The diazepam and DMD data from this study were used in the development and optimization of model parameters.

- **Gueorguieva et al. (2004):** Tissue and arterial blood were collected following an iv infusion over 5 min, of 1 mg diazepam in each of 24 male Sprague-Dawley rats (weighing approximately 250 g each, 4 mg/kg). Four of these animals were then sacrificed at each of the following time points: 7, 10, 20, 35, 95 and 245 min when various tissues were dissected out for analysis. Plasma samples were taken from the carotid artery at those same times as well as at 2 and 5 min. Samples were quantified for diazepam by a specific and appropriately validated Liquid chromatography–tandem Mass Spectrometry) (LC–MS) method.

- **Friedman et al. (1986):** Male Charles River CD-1 rats were each given a single im injection of 5 mg/kg diazepam. Four rats were sacrificed at 5, 15, 30 min, 1h, 1.5, 2,3,4,5 and 6h. Brain and plasma samples were collected and analyzed for diazepam and DMD by electron-capture gas chromatography.

**Human data:**

- **Mould et al. (1995).** A healthy male volunteer received 0.1 and 0.2 mg/kg diazepam on separate occasions. Plasma was collected over three hours and analyzed for diazepam.

- **Greenblatt and Divoll (1983).** Healthy volunteers, ages 21-78 received a single iv dose of diazepam. Data illustrated was for one male subject given 7.5 mg diazepam (0.1 mg/kg). Following administration, multiple venous blood samples were drawn over the next 5 - 10 d.

- **Ochs et al. (1985):** Healthy volunteers received 5 mg diazepam via rapid iv injection. Blood was collected over 168 h. The unbound plasma fraction was determined using equilibrium dialysis to be 1.42%.


• **Klotz (1976):** Ten healthy male volunteers, ages 23-35 y, were administered diazepam (0.1mg/kg) *iv* over 2 min. Data was illustrated for one subject.

• **Jack and Colburn (1983):** Four healthy male volunteers, ages 25-43, each received an *iv* injection of 10 mg diazepam. Blood was collected at 1, 2.5, 5, 10, 20, 30, and 45 min and at 1, 1.5, 2, 3, 4, 6, 12, 24, 30 and 48 h following administration. Blood samples were analyzed for diazepam and DMD by electron-capture GLC.

• **Lehmann and Wannarka (2008):** Ten mg diazepam was administered by autoinjector in 24 healthy male subjects (mean age = 25.7 ± 2.4 y and BW = 74.5 ± 2.4 kg). All injections were delivered into the midanterolateral thigh, two inches lateral from the midpoint of the midline between the anterior superior iliac spine and the end of the knee bent. Blood samples were collected predose and at several post dose time points to 240 hours and analyzed using gas chromatography with an electron capture detector for plasma diazepam and DMD. The ranges of concentrations for the assay were 5 to 400 ng/mL for diazepam, and 5 to 120 ng/mL for DMD.

**Results**

Model simulations of diazepam kinetics in the rat, using the parameters listed in Tables 2-4, are illustrated in Figures 3-3 – 3-5. As previously mentioned the Igari data shown in Figure 3-3 was used to optimize *PA’s* and *V_max’s*. The simulations shown in Figures 3-4 and 3-5 demonstrate validation of the final rat parameters. Using the final set of parameters, the model is in accord with measured experimental data from a variety of kinetic studies in rats. The reasonable agreement between simulations of brain concentrations and measured experimental data also provides confidence in the use of the model for predicting receptor occupancy in the next chapter. Intraperitoneal injections were simulated in Figure 3-5. The injection and diffusion into systemic circulation were described using a first order rate (*K_ip*) of 3h^−1 from an “intraperitoneal compartment” into the liver compartment. Here the model simulation exceeds
early plasma time-points, but simulates brain concentrations well. However, plasma
concentrations, resulting from *ip* injections, are typically more variable than *iv* injections. This is
because with *ip*, the primary route of absorption is into mesenteric vessels, which drain into the
portal vein and pass through the liver. Therefore, the drug may undergo hepatic metabolism
before entering systemic circulation. This appears to be demonstrated by the measured data in
Figure 3-5, which suggest that diazepam and DMD were metabolized to a greater extent before
entering the blood stream. In some incidences of *ip* injections, the needle may be delivered
directly into a blood vessel within that region, but that does not appear to be demonstrated here.

*Figure 3-3. Predicted (solid lines) vs. observed mean diazepam (black squares) and DMD (red triangles) concentrations in plasma, liver and brain, and DZ alone in kidney, muscle and fat in male Wistar rats following an iv injection of 14C-diazepam diluted with unlabeled diazepam (1.2 mg/kg) N = 3-5 rats/time point ). Data from Igari et al. (1982), SDs were not provided.*
Figure 3-4. Predicted (solid line) vs. observed (squares) diazepam concentrations in plasma and other tissues in Sprague-Dawley rats following iv infusion of 1 mg diazepam (4 mg/kg) over 5 min, N = 4 rats/timepoint. Data from Gueorguieva et al. (2004).

Figure 3-5. Predicted (solid line) vs. observed diazepam (black squares) and DMD (red triangles) concentrations in plasma and brains of Male Charles River CD-1 rats given an ip injection of 5 mg/kg diazepam (n=3-4/time-point. Data taken from Friedman et al. (1986).
Simulations of human studies are shown in Figures 3-6 and 3-7. Diazepam kinetics differ between rats and humans. Equal doses (normalized to BW) result in higher concentrations in humans. Given the higher average body fat content of humans vs. rats (21% vs. 9%), one may initially assume that humans would have a greater capacity to store lipophilic compounds, resulting in lower plasma levels especially initially. However, because fat is poorly perfused, equilibration time is long. The source for the higher plasma levels noted in humans can be attributed to lower capacities of metabolic clearance in the human, as well as differences in plasma binding kinetics.

Individual human plasma concentrations could not be described using a single set of $V_{max}$ values. Therefore, $V_{max}$ values were optimized to each study shown in Figure 3-6 and 3-7 to derive average $V_{max}$ values (see Table 3-4). The appearance of DMD in plasma was used to derive $V_{max1}$, representing the desmethylation of diazepam, while optimizing the remaining clearance of diazepam was used to establish $V_{max2}$, the oxidation of diazepam. Desmethyldiazepam is, to a major extent, metabolized by 3-hydroxylation to oxazepam. Therefore, the total clearance of DMD is possibly a reliable estimate of the rate of 3-hydroxylation ($V_{max3}$) of this compound. Bertilsson et al. (1989) measured an average of 62% of DZ biotransformed to DMD in humans, and this was consistent with our simulations. Their fraction is consistent with other studies, such as a PK analysis of DMD plasma levels by Jack and Colburn (1983), which predicted 50% of DZ is metabolized to DMD.
Figure 3-6. Predicted (lines) vs. observed diazepam (black squares) and DMD (red triangles) concentrations in plasma of a healthy male administered iv 0.1 and 0.2 mg/kg DZ on separate occasions. Data taken from Mould et al. (1995).

Figure 3-7. Predicted (solid lines) vs. observed diazepam (black squares) and DMD (red triangles) concentrations in plasma of individual healthy males, administered the following doses of diazepam iv: A) 0.07 mg/kg (Ochs et al., 1985), B) 0.1 mg/kg (Greenblatt and Divoll, 1983), C) 0.1 mg/kg (Klotz et al., 1976), and D) 0.14 mg/kg (Jack and Colburn, 1982). In each study, data for only one individual was illustrated.
Figure 3-8. Predicted (solid lines) vs. observed concentrations (Mean + SD) of diazepam (squares) and DMD (triangles) plasma levels following one 10-mg diazepam im autoinjector administered into the midanterolateral thigh. A) simulations and data for the first 1.4 h, B) same simulations and data extending out 240 h. Data taken from Lehmann and Wannarka (2008).

Table 3-5. Human $V_{\text{max}}$ Values Optimized to Experimental Data

<table>
<thead>
<tr>
<th>Data Source</th>
<th>$V_{\text{max}1}$ (mg/kg/h)</th>
<th>$V_{\text{max}2}$ (mg/kg/h)</th>
<th>$V_{\text{max}3}$ (mg/kg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jack and Colburn, 1983</td>
<td>5.0</td>
<td>0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Ochs et al., 1985</td>
<td>4.0</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Klotz et al., 1976</td>
<td>3.8</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Greenblatt and Divoll, 1983</td>
<td>4.0</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Lehmann and Wannarka, 2008</td>
<td>2.0</td>
<td>0.5</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Average ± SD</strong></td>
<td><strong>3.8 ±1.09</strong></td>
<td><strong>0.18 ±0.18</strong></td>
<td><strong>0.31 ±0.22</strong></td>
</tr>
</tbody>
</table>

Using the human values from Tables 3-1, and 3-3, which includes the average $V_{\text{max}}$ values complied in Table 3-5, plasma and tissue concentrations in man are predicted and shown in Figure 3-9.
Conclusions

A PBPK model was developed, which predicts diazepam concentrations in multiple tissues, including the drug’s target tissue, the brain. Diazepam was chosen as a suitable anticonvulsant to model as it remains the primary therapeutic used to treat status epilepticus. Some basic assumptions used in the model include: 1) all tissue densities were equivalent with water (i.e., 1.0 g/ml), 2) instant equilibrium occurs between tissue and blood in flow-limited compartments, 3) 100% of the parent compound, diazepam, is eliminated via metabolism in the liver, 4) tissue concentrations, \( (C_T) \)s, include amounts and volumes of capillary beds, extracellular fluid and possible deep pools, and 5) tissue uptake is largely influenced by the plasma \( FF \).

Interspecies and individual differences, particularly in plasma binding and metabolism did not allow for direct extrapolation of the model from rat to human by simply applying human-specific physiological parameters (i.e., blood flows and tissue volumes). This is typical of highly lipophilic compounds, which tend to bind more readily to proteins. The unbound fraction of diazepam in plasma is much lower in humans than in rats (i.e., 0.016-0.03 vs. 0.14). In addition, diazepam metabolism differs between species and within species. Individual metabolism rates are influenced by genetic factors, coexisting disorders (particularly chronic liver disorders and advanced coronary failure), and drug interactions (especially those involving induction or inhibition of liver enzymes). These phenotypic differences demonstrate the utility of kinetic investigations and \textit{in vitro} studies on drug metabolism at clinically relevant substrate concentrations, when evaluating novel therapeutics. Considering the wide physiological variations in the drug’s metabolism, model simulations matched experimental data well.

The major metabolite of diazepam, DMD, was modeled, as it has been reported to extend diazepam’s half-life and have a major impact upon its pharmacodynamics. However, in both the rat and human, DMD does not exceed diazepam concentrations in brain or plasma, respectively,
at earlier time points, which are most important in regards to acute dosing following SE onset. This is in contrast to concentration profiles of diazepam and DMD in mouse and guinea pig brains, in which DMD levels exceed diazepam following a single iv injection (Marcucci et al., 1971). In rats and humans, however, DMD levels may exceed those of diazepam following repeated dosing, as large amounts would be stored in fat and slowly released (Marcucci et al., 1979). Hence, the capacity to simulate metabolite concentrations becomes more importance when extrapolating acute diazepam data from mice, guinea pigs or other species which metabolize the compounds more extensively; or when modeling diazepam exposures in any species involving multiple doses over a prolonged period.

It is well-known that individuals metabolize diazepam differently. Reasons for these differences may be attributed to genetic factors, age or even normal variations in physiology across the general population. Because metabolism is the primary elimination route for diazepam, the metabolic constants used are sensitive parameters. Further improved upon via sensitivity analysis to identify which parameters have the greatest impact upon kinetics. Then, based upon a priori information regarding the sensitive parameters, those with the most uncertainty can be identified for further study. Variability analysis would also enhance the model’s reliability by predicting a range of physiological reasonable concentrations that reflects the population diversity.

Lastly, plasma, brain and other tissues concentrations predicted from the proposed PBPK model were validated against data from studies, in which diazepam was administered alone in normal animals. When diazepam is administered following the onset of seizures, higher brain concentrations may be expected due to seizure disruption of the blood brain barrier (BBB) and subsequent increased permeability. However, Capacio et al (2001) has compared diazepam concentrations in plasma and particular brain regions [cerebellum, cortex, brain stem and cerebral spinal fluid (CSF)] in rats that had undergone seizures with rats who had not, and found significantly higher levels in the plasma of animals that seized, but no significant difference in
concentrations of the selected brain regions or CSF. Given the high lipophilicity of diazepam
(log $k_{ow} \approx 2.8$), the BBB may not be much of a hindrance to its uptake. Therefore, using a model
in which brain permeability was parameterized using data from animals dosed with diazepam
alone, to validate brain concentrations achieved during SE appears appropriate.

In the following chapter, the PBPK model is linked with the GABA$_A$ receptor trafficking model,
developed in Chapter 2, and used to predict receptor occupancy, which is then correlated with
efficacy and pharmacoresistance with increasing time from seizure onset.
Chapter 4. Linking Diazepam PBPK with PD, based upon GABA\textsubscript{A} Receptor Trafficking

In this chapter, the cellular model of GABA\textsubscript{A} receptor trafficking (Chapter 2) and the PBPK model for diazepam (from Chapter 3) are linked to characterize the degree of receptor occupancy required to terminate seizures. The occupancy of GABA\textsubscript{A} receptors by BZs has been found to be a good correlate, or dose metric for a number of different BZ effects (Greenblatt and Sethy, 1990; Ito et al., 1993; Facklam et al., 1992). Theoretically, by accounting for the concentration of diazepam reaching the GABA\textsubscript{A} receptor (the “biophase” concentration) as well as the rapidly diminishing levels of that receptor during SE, we may investigate whether the level of receptor occupancy remains a consistent dose metric for the anticonvulsant effect of diazepam when given either before or after the onset of SE \textit{in vivo}.

Predicting Receptor Occupancy by Diazepam Before and After Onset of SE

Experimental Data

To examine the impact that the rapidly changing GABA\textsubscript{A} receptor expression during SE, plays in the pharmacoresistance to diazepam \textit{in vivo}, we use the dose response data from two studies, which evaluated the drug’s anticonvulsant efficacy when given either before (“seizure prevention/survival”) or after induction of SE (“seizure termination/reversal”). Seizure prevention/survival studies have limited applicability to modeling pharmacoresistance because the anticonvulsant is delivered before seizure-induced changes in the receptor surface expression occur. However, such studies do provide an estimate of receptor occupancy required for full protection when an anticonvulsant is administered at time zero or slightly before seizure onset (during normal conditions). Below is a brief description of both studies.
Igari et al. (1985) measured time courses of anticonvulsant efficacy and receptor occupancy in brain when seizures were induced at different times after iv administration of diazepam. Rats were administered 1.2 mg/kg diazepam iv at time zero, then challenged with 100 mg/kg ip of pentylenetetrazol at 3, 10, 30, 60, 90, 120, 150, 180 and 240 min post diazepam administration and monitored for behavioral seizures. Pentylenetetrazol is a GABA_A antagonist, used to induce seizures. In addition, rats which received only diazepam, were sacrificed at the same time points of the pentylenetetrazol administrations in the other (seizure-induced) group of rats. Whole brains (minus cerebellum, brain stem and olfactory bulbs) were harvested for ex vivo determination of the apparent number of diazepam occupied receptors, using ^3^H-diazepam (2.7 nM) and unlabeled diazepam (3 μM) binding to the P_2 synaptosomal fraction. Among the pentylenetetrazol challenged animals, all vehicle pretreated rats exhibited all stages of seizures (based on Bastian et al., 1959) followed by death. On the other hand only those that received the pentylenetetrazol challenge three min post diazepam were fully protected. The anticonvulsant efficacy decreased with time (as circulating diazepam diminished), with no protection seen 240 min post diazepam.

Kapur and Macdonald (1997) measured the anticonvulsant effect from various iv doses of diazepam, administered either 10 or 45 minutes after the induction of SE by pilocarpine. Status epilepticus was induced in rats by ip injection of lithium chloride at 3 mEq/kg followed by an ip injection of pilocarpine at 50 mg/kg, 16-24 hr later. Three stages of behavioral seizures, as classified by Racine et al. (1972), were induced in all rats within one to five minutes of the pilocarpine injection. Diazepam was then administered at increasing doses either 10 or 45 min post pilocarpine and the percent of rats that were seizure free within 5 min were counted, as shown in Figure 4-1.
Figure 4-1. Efficacy of Diazepam in controlling brief (10 min) or prolonged (45 min) pilocarpine induced seizures in rats. Seizures were induced in 70–150 gm rats by ip injection of LiCl at 3 mEq/kg followed 16–24 hr later by ip injection of pilocarpine at 50 mg/kg. Behavioral seizures started within 1–5 min in all rats. Diazepam was administered ip 10 min (black squares, n =14) or 45 min (red squares, n = 12) after pilocarpine injection. The percent of rats that were seizure free within 5 min of diazepam are plotted against the log of the diazepam dose (Figure recreated from Kapur and Macdonald, 1997).

Predicting Receptor Occupancy

Fractional receptor occupancy (FO) can be calculated as a function of the drug’s concentration and affinity for a receptor as shown:

\[
Fractional\ Occupancy = \frac{C_{Br(unbound)}}{C_{Br(unbound)} + K_{d(in\ vivo)}}
\]  
Equation (4.1)

where: \(C_{Br}\) represents the unbound concentration of the drug reaching the receptor and \(K_{d(in\ vivo)}\) is the equilibrium dissociation constant, or the brain concentration \(C_{Br}\) required to occupy half of the receptors (FO = 0.5).

It is believed that only free drug in the interstitial spaces of the brain is available to interact with most CNS receptors (Watson et al., 2009). Therefore, in an attempt to better correlate receptor occupancy with pharmacodynamics, it is essential to predict unbound brain concentrations that are physiologically relevant. Thus equation 4.1 becomes:

\[
FO = \frac{C_{Br(unbound)}}{C_{Br(unbound)} + K_{d(unbound)}}
\]  
Equation (4.2)
As described in Chapter 3, the PBPK model predicts whole brain concentrations, which were validated against measured data. Steady state concentrations of free (unbound) diazepam in brain and blood have been measured using microdialysis probes in rats, receiving an acute steady state infusion (Dubey et al., 1989) and in dogs, administered diazepam 3 times a day (every 8 h) and stabilized at 27 mg/kg/d for 18 weeks (Wala et al., 1991). The ratio of free fractions (unbound) in microdialysis perfusate of brain and plasma of rats was 0.95 ± 0.17. In diazepam dependent dogs, the free brain/plasma concentration ratio was 0.982 ± 0.039. These studies suggest that the unbound fractions in the brain are close to the unbound fraction in plasma. Therefore, considering the free drug hypothesis, it appears reasonable to consider the free drug concentration in plasma \( C_{Br\,\text{unbound}} \) as a surrogate for (equal to) free brain concentration \( C_{Br\,\text{unbound}} \) especially for lipophilic drugs like diazepam, which passively partition across the BBB.

Under this assumption, since \( PB_r = C_{Br}/C_{V\,\text{unbound}} \) is the brain (total) to plasma (unbound) concentration ratio, the unbound concentration in the brain, \( C_{Br\,(\text{unbound})} \), can be calculated as:

\[
C_{Br\,(\text{unbound})} = \frac{C_{Br\,}}{PB_r}
\]  

Equation (4.3)

Substituting for \( C_{Br\,} \) from equation (4.3) into equation (4.2) (and multiplying top and bottom by \( PB_r \)) gives:

\[
FO = \frac{C_{Br\,}}{C_{Br\,} + PB_r K_{d\,(\text{unbound})}}
\]  

Equation (4.4)

So, comparing equation (4.1) and (4.4) gives:

\[
K_{d\,(\text{unbound})} = \frac{K_{d\,(in\,\text{vivo})}}{PB_r}
\]  

Equation (4.5)

This approach was used by Igari et al., (1985) in their investigation into the relation among diazepam time course and receptor occupancy, mentioned above. Therefore, the apparent \( K_{d\,(in\,\text{vivo})} \) (which was calculated to be 226 ng/ml, based on measured brain concentrations vs. anticonvulsant effect), and the whole brain to plasma (unbound) ratio, \( PB_r \) of 7.03, which was
previously determined in a pharmacokinetic study by the same authors (see Table 3-2), an apparent $K_d$ \( (\text{unbound}) \) of 0.033 mg/L (= \( \frac{226}{7.03} \) ng/ml) is derived.

However, this “unbound” in vivo dissociation constant value is still nearly 50 times higher than dissociation constants measured in vitro for specific binding of diazepam to the P2 synaptosomal fraction of different brain regions, $K_d$ \( (\text{in vitro}) \), which range from 2.3 nM (6.56x10\(^{-4}\) mg/L) to 9.57 nM (2.73x10\(^{-3}\) mg/L)(Arendt et al. 1987; Braestrup and Squires., 1977; Igari et al., 1985; Mohler and Okada, 1977; Nutt and Minchin, 1983, Wu and Phillis, 1986). The most obvious explanation for the discrepancy between the $K_d$ \( (\text{in vitro}) \) and the “effective half saturation constant”, $K_d$ \( (\text{unbound}) \), are the different conditions for assaying receptor function and binding characteristics. The in vivo “unbound” value is derived from a functional study performed under physiological conditions, whereas in vitro binding studies are performed under conditions established artificially to obtain the P2 fraction for optimizing the ligand binding measurement. The presence of proteins (in vivo) that bind to the receptor may be partially responsible. It has been suggested that the high affinity seen in vitro reflects a desensitized state of the receptor (Edgar and Schwartz, 1992). Because we cannot actually determine how much drug is reaching the synaptic region in vivo, the $K_d$ \( (\text{unbound}) \) is used to calculate receptor occupancy from the dose response studies described above.

Igari et al. reported a very high correlation (\( r=0.977 \)) between receptor occupancy and anticonvulsant effect. Therefore, the magnitude of the physiological response (or seizure termination) should be proportional to the amount of diazepam bound to the GABA\(_A\) receptor. Because the surface expression of GABA\(_A\) receptors is quickly diminishing with seizure duration, we combine the decreasing number of receptors with the fractional occupancy of the remaining receptors (equation 4.2), to determine the percent of original receptor occupancy of the original receptor number (FOORN):

\[
\text{FOORN} = \frac{R_{St}}{R_{So}} \times \frac{C_{Br(\text{unbound})}}{C_{Br(\text{unbound})^+K_d(\text{unbound})}} \times 100
\]

Equation (4.6)
where $R_s$ and $R_{s0}$ are the levels of surface GABA$_A$ receptors at time, $t$, and at time zero, respectively. This equation asserts that as the number of original receptors declines, a higher concentration of ligand is required to achieve the same occupancy. It is also assumed that the legend’s on and off rates are fast enough so that the amounts of free ligand and ligand-specific binding sites are in instant equilibrium.

Because the metabolite, DMD, is also centrally active and shares similar affinity for the GABA$_A$ receptor, the model was written to include DMD’s possible contribution to receptor occupancy. While levels of diazepam and DMD can be measured in the rat brain, it is not possible to determine the relative contributions of diazepam and DMD to net receptor occupancy based on the 	extit{ex vivo} techniques. Therefore comparison of net occupancy values (attributable to diazepam plus DMD) with predicted brain concentrations requires normalization of DMD concentration values (mg/L) based on the relative molecular weights of diazepam and DMD (284.7 and 270.7, respectively) and relative 	extit{in vitro} benzodiazepine receptor dissociation constants taken from the same study (9.57 and 5.58 nM, respectively) (Arendt et al. 1987) as follows:

“Brain DZ equivalents of DMD (unbound)” = \( \left( C_{\text{B}_{\text{DMD}}} \times \frac{284.7}{270.7} \times \frac{9.57}{5.58} \right) / P\text{B}_{\text{DMD}} \) Equation (4.4)

where: $C_{\text{B}_{\text{DMD}}}$ is the brain concentration of DMD and $P\text{B}_{\text{DMD}}$ represents the ratio of whole brain DMD concentration to plasma (unbound) DMD concentration, which is approximately 8.0 (see Table 3-3). The unbound “brain equivalents” are then accounted for by their addition to the unbound brain concentrations of diazepam in Equation 4.3.

**Results**

Based on the approach described, time courses of free brain concentrations were simulated from the doses used in the two studies described above and linked with the simulated time-dependant changes in the GABA$_A$ receptor numbers during SE. In Igari et al. (1985) 	extit{ex vivo}
measurements of receptor occupancy were performed at 5 time points following the administration of 1.2 mg/kg diazepam. Figure 4-2 illustrates the model predictions of brain concentration and receptor occupancy from that study, used here for model validation. It is important to remember that in these simulations, diazepam was administered in the presence of no seizures, therefore the basal or “original” number of receptors was available for occupancy.

Figure 4-2. Model simulations of brain concentration and receptor occupancy time courses following 1.2 mg/kg diazepam in rats. On left, lines (black and red) and symbols (squares and triangles) indicate model simulations and measured diazepam and DMD concentrations, respectively. The simulated brain concentrations are used to predict receptor occupancy (on right shown as receptor occupancy(line) vs. ex vivo measurements(squares) (Igari et al., 1985) (n=3 rats).

Igari reported that only those animals administered diazepam 3 min before the pentylenetetrazol challenge remain 100% seizure free. Therefore, based upon the ex vivo receptor measurement taken at 3 min (Figure 4-2 B), close to 37% receptor occupancy was achieved, which resulted in complete prevention of behavioral seizures. At 10 min post diazepam, the measured receptor occupancy was approximately 25% and animals had experienced twitching. Therefore, the actual level of receptor occupancy required for complete protection, when diazepam is given immediately before onset of seizures may actually be somewhere between 25 and 37%.
Next, predictions of receptor occupancy when diazepam is used in a “seizure reversal” scenario, were simulated based on the experiments performed by Kapur and Macdonald (1997). These researchers induced stage 5 seizures in all rats by pilocarpine iv. Rats were then treated with increasing doses of diazepam at either 10 or 45 min post seizure onset (see Figure 4-3). The percent of rats observed to be seizure free within 5 min of diazepam was counted as the response. Therefore, we simulate GABA\textsubscript{A} receptor trafficking, using the SE parameters established in Chapter 2, to establish the change in surface receptor levels at the time when the anticonvulsant response was actually measured (15 and 50 min post SE onset). For comparison with Igari’s study, these endpoints were also simulated for conditions where diazepam is given at time zero of seizure onset (when 100% of receptors are available). In addition, the dose 1.2 mg/kg was also added to these predictions, as it was the lowest dose shown by Igari when diazepam was administered 3 minutes prior to seizure induction.) Simultaneously, simulations of brain concentrations 5 min after each dose of diazepam listed in Figure 4-3, including 1.2 mg/kg, were performed. The 5 min timepoint represents the time when the “effect” or percent seizure free were counted. Finally, receptor occupancies were predicted from these simulations and illustrated in Figures 4-4.

![Figure 4-3. Experimental design of Kapur and Macdonald (1997). Seizures were induced at time zero and either 10 or 45 min later, increases doses (shown) were administered and animals were observed for 5 min for signs of seizures.](image-url)
Figure 4-4. Model predicted brain concentrations five minutes post iv injection of 1.2, 2.0, 7.5, 10, 20, 30, 50 and 100 mg/kg diazepam in rats vs. changing percent receptor occupancy when the diazepam doses are delivered either 0, 10 or 45 minutes post onset of SE. The black dotted line shows indicates the predicted brain concentrations at 37% receptor occupancy, the reported occupancy for seizure prevention when diazepam is delivered at time zero of seizure initiation (based on Igari et al.1985). Red circles indicate the predicted brain concentrations and occupancy levels from diazepam doses reported by Kapur and Macdonald (1997) to reverse seizure activity, when delivered 10 and 45 minutes into SE. The blue, red and green dashed lines indicate the changing maximum percent of original number of available receptors at times 0, 10 and 45 min post onset of SE. Arrows indicate the brain concentrations required to for 55% occupancy.

Receptor occupancy was also simulated for humans, by using the human physiological and metabolic parameters listed in Chapter 3. Parameters driving GABA\textsubscript{A} receptor trafficking were assumed to be the same as those derived from rat hippocampal cultures under SE conditions. In addition, the same $K_d$ \textit{unbound (in vivo)} was used. GABA\textsubscript{A} receptor dissociation constants of various BZs in rat and man have been determined by \textit{in vitro} experiments using brain tissue homogenate by Okada (1980). The values of the dissociation constant of each BZ in human brain were almost the same as that in rat brain, suggesting that any species difference in the receptor binding affinity of BZs is very small. The resulting predictions in human are shown in Figure 4-5.
Figure 4-5. Model predicted brain concentrations five minutes post iv injection of 1.2, 2.0, 7.5, 10, 20, 30, 50 and 100 mg/kg diazepam in humans vs. changing percent receptor occupancy when the diazepam doses are delivered either 0, 10 or 45 minutes post onset of SE. The two arrows indicate the brain concentrations required to achieve 55% receptor occupancy when diazepam is administered either 10 or 45 min post onset of SE.

Table 4-1. Percent Receptor Occupancy & Brain Concentration (mg/kg) Required for Maximum Anticonvulsant Effect

<table>
<thead>
<tr>
<th>Time after seizure onset</th>
<th>% Occupancy for Max Effect</th>
<th>[Brain] Required for EC_{100%}</th>
<th>[Brain] Required for EC_{100%}</th>
<th>HumanEC_{100%}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rat Human</td>
<td>Rat</td>
<td>Rat</td>
</tr>
<tr>
<td>0 min</td>
<td>37%</td>
<td>1.2</td>
<td>&gt;0.5</td>
<td>&gt;0.42</td>
</tr>
<tr>
<td>10 min</td>
<td>55%</td>
<td>10.0</td>
<td>2.9</td>
<td>0.29</td>
</tr>
<tr>
<td>45 min</td>
<td>55%</td>
<td>85</td>
<td>11.0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Conclusions

Relating fractional receptor occupancy to efficacy is an indirect but reliable way to assess relative intrinsic efficacy of anticonvulsants. The model approach demonstrated here suggests that the reported level of GABA\textsubscript{A} receptor occupancy required for preventing seizure onset...
(approximately 37%) is insufficient to arrest on-going seizures. Approximately 55% occupancy of the original number of receptors is predicted to correspond with the lowest successful diazepam dose, found to be 100% successful for seizure reversal when administered 10 min into SE in Kapur et al.’s study (Figures, 4-4 and 4-1). Interestingly at 45 minutes into SE, 55% occupancy also corresponds with the dose observed to result in 100% seizure free rats (100 mg/kg) (see Figure 4-1). These results suggest that while receptor occupancy may not be a consistent biomarker for both seizure prevention and seizure reversal, it does appear to be a consistent biomarker for seizure reversal. The brain concentrations required to achieved 55% occupancy (the “EC100”) increases dramatically between 10 and 45 minutes post SE onset, requiring almost a 9 and 5 fold increase for the rat and human, respectively.

Fifty five percent occupancy for seizure termination appears reasonable, when compared with other measured values from pharmacological studies on the effects of diazepam and other BZs, administered alone. Facklam et al. (1992) demonstrated that 30-50% GABA<sub>A</sub> receptor occupancy by diazepam in vivo results in sedative effects. They also demonstrated that in vitro 35% occupancy enhanced chloride current by 25%. Sixty percent occupancy by the BZ, flumazenil, has been shown to induce unconsciousness (Amrein et al., 1988).

Assuming diazepam binding with each individual GABA<sub>A</sub> receptor results in the same Cl<sup>-</sup> influx, one may logically assume that the same number of occupied receptors (percent of original receptors) would be needed to achieve the same results. The predicted need for an 18% increase in receptor occupancy between seizure prevention and seizure reversal reflects more than a diminishing number of available GABA<sub>A</sub> receptors. This is suggested by the model, given total saturation of available receptors was not achieved at the doses experimentally determined to be successful at 10 min post SE onset. Likewise at 45 min, only the highest dose begins to approach receptor saturation. In addition to reduced GABA<sub>A</sub> receptors, changes in the expression of other receptors that influence membrane potential also appear to be responsible for the increase in therapeutic threshold. While BZ-sensitive GABA<sub>A</sub> receptor numbers are declining, NDMA and
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are increasing at excitatory synapses, aggravating the loss of inhibitory GABAergic signals, by making the membrane more excitable (Naylor, 2010). AMPA antagonists have demonstrated effectiveness in arresting SE when administered 1 h after exposure to the NA, soman (Figueiredo et al., 2011). It is estimated that NMDA mEPSCs increase in amplitude 23% during SE, with an estimated 38% increase in the number of postsynaptic NMDA receptors at 1 h by mean-variance analysis (Naylor, 2010). Non-NMDA receptors also show an increase in number at synaptic sites, with SE (19% amplitude increase with estimated 22% increase in postsynaptic receptor numbers; p < 0.001) (Prybylowski et al., 2002). Therefore, the surface accumulation of AMPA and NMDA receptors further upsets a diminishing GABA_A receptor inhibitory mechanism for ongoing seizure activity and a vicious cycle ensues. Early loss of synaptic inhibition through receptor internalization and/or desensitization upsets a balance with excitation and explains the pharmacoresistance to BZs.

Accounting for the metabolite DMD did not significantly impact receptor occupancy predictions from these acute simulations in either rat or human. This is because the metabolite concentrations achieved did not exceed those of the parent compound in the acute scenarios simulated here. At the early time points when diazepam efficacy was assessed, desmethyldiazepam concentrations were close to an order of magnitude lower than those of diazepam (Figure 4-2). However, the inclusion of this major metabolite would be of relevance if simulating longer term treatment of SE or epilepsy, or possibly in cases of repeated dosing to arrest seizures. The longer half life of DMD would result in its build up in fat and brain. Therefore, the time-course of net receptor occupancy in brain would be longer than would be attributed to diazepam alone. The diazepam metabolites can play an important role in net receptor occupancy and overall pharmacological activity.
Chapter 5. Discussion

This dissertation presents a computational approach, combining \textit{in vitro} and \textit{in vivo} data, for examining the rapid loss of diazepam’s efficacy as an anticonvulsant, with increasing duration of SE. A number of recent studies indicate that the pharmacoresistance seen during SE can to a large degree be attributed to rapid internalization of BZ-sensitive GABA\(_A\) receptors (Naylor \textit{et al.}, 2005; Goodkin \textit{et al.}, 2005; Feng \textit{et al.}, 2008). To investigate the roles that GABA\(_A\) receptor dynamics together with diazepam’s pharmacokinetics play in this time-dependent pharmacoresistance PBPK/PD modeling is used. Physiologically-based pharmacokinetic/pharmacodynamic modeling allows for the integration of various data sets in a mechanistically meaningful way. More importantly, the physiological and biochemical basis of the model’s parameterization allows for extrapolation across species, doses and dose routes. Therefore, the approach demonstrated here is especially well suited for evaluating the efficacy of anticonvulsants, since controlled clinical trials cannot be readily performed for ethical reasons. Human data tends to be anecdotal and extremely variable from case to case, with seizure cause and time of onset being uncontrolled variables.

The PBPK portion of this modeling effort predicts the changing concentrations of diazepam and its major metabolite, DMD, in the brain and several other tissues in rats and humans. The simulated concentrations were validated against measured tissue kinetic data in the rat as well as plasma data in humans. The brain concentration kinetics or “biophase” kinetics is an important determinant in the time course and intensity of the drug’s CNS effects. The PD components predict the temporal pattern of the drug’s pharmacological effect. In this case, the changing surface expression of the targeted BZ-sensitive GABA\(_A\) receptors, both constitutive and during on-going seizures, is simultaneously linked with PBPK simulations of brain diazepam.
concentrations to predict \textit{in vivo} \text{GABA}_A \text{ receptor occupancy} when the drug is administered at different times following the onset of SE.

Equal doses (in mg/kg) of diazepam result in higher circulating levels in human plasma than that of rats, due to a greater extent of binding to plasma proteins and reduced metabolic clearance in humans. However, the increase in plasma binding also results in lower concentration in brain and other tissues in humans. While, the major metabolite DMD is centrally active, it does not significantly contribute to receptor occupancy in the acute dosing scenarios predicted here. The simulations here predict receptor occupancy resulting only a few minutes after diazepam administration. During these early time points, DMD concentrations are nearly an order of magnitude lower than those of diazepam in both rats and humans. If the model is used in the future for simulating receptor occupancy from repeated doses of diazepam, the metabolite is expected to contribute significantly, as concentrations build up. Also if the model is used to simulate dosing in individuals who are poor metabolizers, DMD may exceed diazepam levels in a relatively short period. To account for such individuals, as well as variations in the general public, future iterations should include variability analysis of the model’s most sensitive parameters. Variability analysis would also enhance the model’s usefulness by predicting a range of physiologically reasonable concentrations that reflects the population diversity.

To account for the changing surface expression of BZ-sensitive \text{GABA}_A receptors during SE, a simplified model which treats the dendritic spine as a single cell was developed. The trafficking of the receptors between the postsynaptic and the other regions of the dendritic spine were described by rate constants. Receptor trafficking is a complex process involving multiple proteins and processes. Therefore, for simplicity, each rate constant represents a composite of such processes, optimized to produce results consistent with the experimental data used in this analysis. Overall, measured trafficking rates observed across a number of different \textit{in vitro} SE models and our SS analysis of the model’s parameters (which was based on measured receptor densities) were consistent, instilling confidence in the model’s final parameters. Minor
differences in rates seen between studies likely arose from differences in cell types (neuronal vs. recombinant cells), labeling techniques or the effects of the label itself on the receptor. An example of such a difference was the exocytosis rate, $k_{ex}$, observed between neuronal and recombinant cell types. Receptor internalization and exocytosis measured in neuronal cultures (Joshi and Kapur, 2009) were consistent with the SS analysis, whereas the $k_{ex}$ was nearly double in transfected HEK cells. The difference may well be due to the lack of synaptic clustering and a lower surface receptor density in non-neuronal cultures. Therefore, while neuronal and recombinant cells share similar rates of constitutive endocytosis, recombinant cells may display a higher rate of exocytosis in an attempt to maintain stable surface levels. For this reason, the parameters established from neuronal cultures were used in the final PD model, as they more accurately reflect in vivo dynamics.

Together, the trafficking model and the studies used to parameterize the receptor trafficking model demonstrate that the surface stability of GABA_A receptors can be dynamically and specifically regulated, enabling neurons to modulate cell surface receptor number upon the appropriate cues, such as activity. The kinetics of the model depends upon various composite trafficking parameters that could be targets of second messenger pathways activated during seizures. The model was used to explore the consequence of targeting different parameters to see how these parameter changes can reproduce a wide range of experimental data. To simulate SE conditions, increases in the rates depicting endocytosis, $k_{in}$, and the release from scaffolding proteins, $k_a$, appeared sufficient to describe in vitro SE models in cultured neurons, using high K⁺ or low Mg²⁺. The increased level of internalization seen from the in vitro data at 1 h agreed with ex vivo measurements from pilocarpine challenged rats. Therefore, there is confidence in the receptor trafficking model’s parameterization for depicting changes in GABA_A receptor levels in vivo.

Why there is a rapid "down regulation" of GABA_A receptors at the synapse during SE is indeterminate, being contrary to protective homeostatic mechanisms. However, the ability of
neurons to rapidly modulate GABA$_A$ receptor density at inhibitory synapses upon excitatory activity by regulating synaptic clustering and endocytic processes may have evolved as an important mechanism for learning. At the onset of long term potentiation (LTP), excitatory postsynaptic potentials (EPSPs) are more likely to elicit an action potential in the postsynaptic cells if there is a reduction in IPSPs (Wang and Stelzer, 1996). Bannai et al., (2009) hypothesized that activity-dependent neuronal disinhibition may be an underlying mechanism for the induction of LTP, stating that “lateral diffusion, regulated through receptor-scaffold interactions, provides a simple mechanism for rapid and reversible activity dependent modulation of synaptic strength. During SE, the antihomeostatic excitation-inhibition regulation may be defective, resulting in exaggerated loss of inhibition.” Novel pharmacological targets for SE as well as an improved understanding of the mechanisms behind learning and memory may be elucidated from quantitative studies on the details controlling receptor diffusion dynamics.

Future expansion of the receptor trafficking portion of the model may include the BZ-insensitive receptors, as more data becomes available. The BZ-insensitive GABA$_A$ receptor subtypes, containing the δ subunit, are not rapidly internalized during SE and actually demonstrate a trend toward increased expression at the synapse (Joshi and Kapur, 2009). Drugs that bind to these subtypes, such as neurosteroids, could represent potential therapeutics that could be used in combination with, or instead of standard BZs.

The model may also be expanded to explore altered receptor kinetics resulting from chronic exposure to BZs, the phenomenon known as tolerance. Investigations in both primary neuronal and cell cultures and in vivo have demonstrated concentration- and time-dependent reductions in the efficacy of GABA to enhance inhibition with chronic BZ exposure (Wong et al., 1994), involving either post-translational modification of GABA$_A$ receptor proteins or changes in subunit stoichiometry.

It has been reported that approximately 37% receptor occupancy is required to prevent onset of behavioral seizures in rats when diazepam is administered minutes before a
chemoconvulsant (Igari et al., 1985). In mice, full protection against pentylenetetrazol-induced seizures has been demonstrated by approximately 30% occupancy (Paul et al., 1978). These studies, however, reflect idealized scenarios in which the therapeutic is given before, or at the same time as the onset of seizures (seizure prevention rather than seizure reversal). Realistically, the scenario is much different. Epidemiological studies have shown that time to seizure treatment in the U.S. varies broadly with only about 41% of all patients receiving their first antiepileptic drug within 30 minutes (Pellock et al., 2004). Our model predicts a required 55% occupancy of the original number of receptors for termination of on-going behavioral seizures. This value appears to be a consistent requirement after the onset of SE, as it delineates the percent of receptor occupancy achieved at the lowest doses identified as successful in terminating seizures at both 10 and 45 min into SE, in Kapur and Macdonald’s 1997 study. Although, this degree of receptor occupancy is achieved at a higher degree of saturation of the fewer remaining receptors at 45 min than 10 min, necessitating a correspondingly higher brain concentration (see Figure 4-4). The model also suggest that if SE continues for much longer than 45 min, no dose of diazepam will be able to fully terminate behavioral seizures because there will not be enough receptors available to achieve 55% occupancy. The GABA_A trafficking model predicts a 45% loss in surface receptors at 1h into SE. Based upon measured receptor occupancy levels for various effects of diazepam, 55% occupancy lies somewhere between levels reported to cause sedation (30-50% occupancy) (Facklam et al.,1992b) and unconsciousness (60% occupancy) (Amrein et al., 1988). In principle the model could be used to optimize BZ dosing regimens (determining the adequate dose depending on time since seizure onset).

The reason for an 18% increase in the therapeutic threshold for receptor occupancy from prevention to reversal modes is likely to be attributed to more than just a loss of GABA_A receptors. There is also an increase in excitatory transmitters and receptors (Naylor, 2010). NDMA and AMPA receptors are increasing at excitatory synapses, aggravating the loss of inhibitory tone associated with GABAergic losses. These rapid receptor changes indicate that
several therapeutic options for treating SE exist. A combination of agents that block either AMPA or NMDA receptors and enhance GABA_A receptors may provide the most efficacious route to restore inhibitory tone to seizing hippocampal networks. Agents that target the BZ-insensitive extrasynaptic GABA_A receptor may also be effective, given these are not rapidly internalized during SE. Drugs that target scaffolding proteins in a way that might enhance the stability of GABA_A receptors at the synapse, could possibly restore inhibitory tone. Therefore, many possibilities for novel therapeutics exist. However, much caution must be taken in the search for suitable agents that alter the expression or functionality of neuroreceptors, especially given that the receptors’ abilities to rapidly modulate neuronal messages is critical for normal brain functioning.

The modeling approach demonstrated here provides a potential means of extrapolating dose response and optimizing the therapeutic windows of similar-acting drugs. This model specifically, can be expanded many ways, possibly offering insights into a larger collection of GABA_A receptor trafficking data. The ODE model could also be modified to explore the trafficking of different receptors and the pharmacodynamic interactions of drugs or chemicals that target them. For example, the model could be extended to describe the simultaneous trafficking of AMPA and NMDA receptors during SE and ultimately used to identify a superior therapeutic “cocktail”, which targets each of these receptors in a quantitatively optimal manner. Such a model also would have applications for exploring situations, unrelated to seizures, such as synaptic plasticity, learning and memory. Finally, a future endeavor for this type of modeling may be to relate it to ion transport, providing a link to hyperpolarization and depolarization models.
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