University of Alberta

Neurochemical and neuroprotective aspects of phenelzine and its active metabolite β-phenylethylidenehydrazine

by

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Abstract

Phenelzine (PLZ) is a monoamine oxidase (MAO) inhibitor that also inhibits the activity of GABA-transaminase (GABA-T), causing significant and long-lasting increases in brain GABA levels. Inhibition of MAO prior to PLZ administration has been shown to prevent the GABAergic effects of the drug, strongly suggesting that a metabolite of PLZ formed by the action of MAO is responsible for the GABAergic effects. While PLZ has been used clinically for decades for its antidepressant and antipanic effects, it has more recently been shown to be neuroprotective in an animal model of ischemia. The aim of the experiments described in this thesis was to identify the active metabolite of PLZ, and to determine the neurochemical mechanisms by which PLZ and this metabolite exert their neuroprotective effects (with a particular focus on degenerative mechanisms observed in cerebral ischemia and Alzheimer's disease (AD)). The development of an analytical assay for β-phenylethylidenehydrazine (PEH) was a major breakthrough in this project and permitted the positive identification of this compound as the active metabolite of PLZ. Further experiments demonstrated that PLZ and PEH could be neuroprotective in cerebral ischemia and AD not only by reducing excitotoxicity via increased GABAergic transmission, but also by (a) increasing brain ornithine, which could potentially lead to a decrease in glutamate synthesis and/or a decrease in polyamines (whose metabolism produces toxic aldehydes); (b) inhibiting the activity of human semicarbazide-sensitive amine oxidase (SSAO), an enzyme whose activity is

increased in AD producing excessive amounts of the toxic aldehyde formaldehyde (FA); (c) by sequestering FA *in vitro*, forming a non-reactive hydrazone product. Since PEH appears to mediate or share the neurochemical effects of PLZ, two propargylated analogs of PEH were synthesized and tested for their potential as PEH prodrugs. Surprisingly these analogs were not particularly effective prodrugs *in vivo*, but they possessed an interesting neurochemical properties on their own (the ability to elevate brain levels of glycine), and warrant further investigation as potential antipsychotic agents. Together, these results suggest that PLZ and its active metabolite, PEH, should be further investigated for their neuroprotective potential in cerebral ischemia and in AD.

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List of Abbreviations

α-KG	α-ketoglutarate
μL	microlitre
μM	micromolar
°C	degrees Celsius
3-AP	3-aminopropanal
AD	Alzheimer's disease
ALA	alanine
ALA-T	alanine transaminase
ANOVA	analysis of variance
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARG	arginine
ATP	adenosine triphosphate
CNS	central nervous system
CSF	cerebral spinal fluid
DA	dopamine
ddH_20	double-distilled water
diPrPEH	dipropargyl-PEH
DMSO	dimethylsulfoxide
DOPAC	3,4-dihydroxyphenylacetatic acid
ECD	electrochemical detection
EC	electron capture detection
FA	formaldehyde
g	gram
GAD	glutamic acid decarboxylase
GABA	γ-aminobutyric acid

GABA-T	GABA-transaminase
GAT	GABA transporter
GC	gas chromatography
GLU	glutamate
HCl	hydrochloric acid
5-HIAA	5-hydroxyindole-3-acetic acid
4-HNE	4-hydroxynonenal
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine; serotonin
HVA	homovanillic acid
HYD	hydralazine
IBLC	*N*-isobutyryl-L-cysteine
i.p.	intraperitoneal(ly)
kg	kilogram
L	litre
M	molar
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
mg	milligram
mL	milliliter
mM	millimolar
mOD	change in optical density
mRNA	messenger ribonucleic acid
MS	mass spectrometry
N	normal
NaEDTA	sodium ethylenediaminetetraacetic acid
NE	norepinephrine

ng	nanogram
nM	nanomolar
nm	nanometer
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
ODC	ornithine decarboxylase
OPA	*o*-phthaldialdehyde
ORN	ornithine
ORN-T	ornithine transaminase
PAA	phenylacetic acid
PAR	pargyline
PBS	phosphate-buffered saline
PEA	β-phenylethylamine
PEH	β-phenylethylidenehydrazine
PD	Parkinson's disease
PFBA	pentafluorobenzaldehyde
PFBC	pentafluorobenzoyl chloride
PFBSC	pentafluorobenzenesulfonyl chloride
PLP	pyridoxal 5'-phosphate
PLZ	phenelzine
PrPEH	propargyl-PEH
ROS	reactive oxygen species
SEM	standard error of the mean
SSAO	semicarbazide-sensitive amine oxidase
SSRI	selective serotonin reuptake inhibitor
TCP	tranylcypromine

TOA	tri-n-octylamine
UV	ultraviolet
V	volts

Chemical name	Supplier
acetic acid (glacial)	Fisher Scientific Canada
acetonitrile, HPLC grade	Fisher Scientific Canada
4-aminoantipyrine	Sigma-Aldrich Canada
γ-aminobutyric acid (GABA)	Sigma-Aldrich Canada
^{3}H-γ-aminobutyric acid (GABA)	Perkin Elmer
aminoethylisothioluronium bromide	Sigma-Aldrich Canada
cyclohexane	Fisher Scientific Canada
3,4-dihydroxyphenylacetic acid (DOPAC)	Sigma-Aldrich Canada
dopamine (DA)	Sigma-Aldrich Canada
D-serine (D-SER)	Sigma-Aldrich Canada
2,4-dichlorophenol	Sigma-Aldrich Canada
dimethylsulfoxide (DMSO)	Fisher Scientific Canada
ethyl acetate	Fisher Scientific Canada
formaldehyde	Sigma-Aldrich Canada
glutathione	Sigma-Aldrich Canada
HEPES buffer	Fisher Scientific Canada
homovanillic acid (HVA)	Sigma-Aldrich Canada
horseradish peroxidase	Sigma-Aldrich Canada
hydralazine (HYD)	Sigma-Aldrich Canada
hydrochloric acid (HCl)	Fisher Scientific Canada
5-hydroxyindoleacetic acid (5-HIAA)	Sigma-Aldrich Canada
5-hydroxytryptamine (5-HT)	Sigma-Aldrich Canada
5-hydroxy[G-^{3}H]tryptamine creatinine sulfate	Perkin Elmer
isobutyryl-L-cysteine (IBLC)	Sigma-Aldrich Canada
o-phthaldialdehyde	Pierce Chemicals
methanol, HPLC grade	Fisher Scientific Canada
2-methylbutane	Fisher Scientific Canada
α-ketoglutarate	Sigma-Aldrich Canada
L-alanine (ALA)	Sigma-Aldrich Canada
L-arginine (ARG)	Sigma-Aldrich Canada
L-glutamate	Sigma-Aldrich Canada
L-glutamine	Sigma-Aldrich Canada
L-glycine	Sigma-Aldrich Canada
L- serine (L-SER)	Sigma-Aldrich Canada
L-taurine	Sigma-Aldrich Canada

methylamine (MA)	Sigma-Aldrich Canada
N-bis-(2-propynyl)-phenylethylidenehydrazine (diPrPEH)	Dr. E. Knaus (U. of Alberta)
N-mono-(2-propynyl)-phenylethylidenehydrazine (PrPEH)	Dr. E. Knaus (U. of Alberta)
nicotinamide adenine dinucleotide (NAD)	Sigma-Aldrich Canada
(-)-noradrenaline (NA) hydrochloride	Sigma-Aldrich Canada
ornithine (ORN) dihydrochloride	Sigma-Aldrich Canada
pentafluorobenzaldehyde (PFBA)	Sigma-Aldrich Canada
pentafluorobenzenesulfonyl chloride (PFBSC)	Sigma-Aldrich Canada
pentafluorobenzoyl chloride (PFBC)	Sigma-Aldrich Canada
perchloric acid	Fisher Scientific Canada
phenelzine (PLZ) sulfate	Sigma-Aldrich Canada
(β-phenyl[1-^{14}C]ethylamine chloride	Perkin Elmer
phenylethylidenehydrazine (PEH)	Dr. E. Knaus (U. of Alberta)
phosphoric acid	Fisher Scientific Canada
potassium carbonate (K_2CO_3)	Fisher Scientific Canada
potassium chloride	Fisher Scientific Canada
potassium phosphate	Fisher Scientific Canada
pyridoxal phosphate (PLP)	Sigma-Aldrich Canada
scintillation fluid (ReadySafe™)	Fisher Scientific Canada
sodium bicarbonate	Fisher Scientific Canada
sodium chloride	Fisher Scientific Canada
sodium ethylinediaminetetraacetic acid (NaEDTA)	Fisher Scientific Canada
sodium hydroxide	Fisher Scientific Canada
sodium octyl sulfate (SOS)	Sigma-Aldrich Canada
sodium phosphate (monobasic, anhydrous)	Fisher Scientific Canada
tetrahydrofuran	Fisher Scientific Canada
toluene	Fisher Scientific Canada
tranylcypromine (TCP) hydrochloride	Sigma-Aldrich Canada
tri-n-octylamine (TOA)	Sigma-Aldrich Canada
triton X-100	Fisher Scientific Canada

CHAPTER 1.

GENERAL INTRODUCTION

1.1 FOREWORD

The importance of neuroprotection and neurorescue in the prevention and treatment of neurological and psychiatric disorders is a critical area of research. While the etiologies of most psychiatric conditions are not completely understood, a neurodegenerative component appears to be present in many disorders; examples include the neurodegeneration observed in Alzheimers's Disease (AD) and Parkinson's disease (PD). External insults may also result in psychiatric complications involving neurodegeneration, as is often observed in traumatic brain injury and cerebral ischemia (stroke). Identification and counteraction of the initial events preceding the toxic cascades could limit or even prevent neurodegeneration, and in this way may lead to successful therapeutic interventions.

Phenelzine (β-phenylethylhydrazine; PLZ) is a monoamine oxidase (MAO) inhibitor that is used clinically for its antidepressant and anxiolytic effects and has more recently been shown to be neuroprotective in a rodent model of cerebral ischemia. PLZ differs from other MAO inhibitors in that it also causes marked increases in brain levels of the amino acid neurotransmitter γ-aminobutyric acid (GABA) by inhibiting the activity of its catabolic enzyme GABA-transaminase (GABA-T). Importantly, evidence suggests that some of the neurochemical effects of PLZ, including those on brain GABA levels, may be mediated by the formation of an active metabolite, proposed by some researchers to be β-phenylethylidenehydrazine (PEH). PEH possesses many of the same neurochemical properties as PLZ, and has also been shown to be neuroprotective in ischemia. The main foci of this thesis was to establish that PEH is indeed a metabolite of PLZ and to further understand the neurochemical actions of PLZ and PEH that may contribute to their neuroprotective effects.

1.2 GABA

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS). It is found ubiquitously throughout the brain and spinal cord, and, with the exception of the retina, is found only in trace amounts in other tissues (Erdo and Wolff 1990; Cooper *et al.* 2003). Brain levels of GABA, like those of glutamate (the major excitatory neurotransmitter in the CNS) are markedly higher than levels of the amine neurotransmitters (Cooper *et al.* 2003).

GABA is synthesized *in vivo* in GABAergic nerve terminals by a metabolic pathway known as the GABA shunt, shown in Figure 1-1. Intraneuronal glutamate is metabolized to GABA by glutamic acid decarboxylase (GAD), and is packaged into vesicles for release into the synapse. Released GABA is rapidly inactivated by reuptake by GABA transporters (GATs), Na^+- and Cl^--dependent transporters that take up GABA into either presynaptic nerve terminals or surrounding glial cells (Olsen and Betz 2006). Three distinct GATs have been identified, and it is thought that GAT-1 mediates the reuptake of GABA into presynaptic neurons and that GAT-2 and GAT-3 mediate GABA uptake into glial cells (Borden 1996). GABA that is taken into the presynaptic neuron is either repackaged into vesicles for re-release, or is degraded to succinic semialdehyde by GABA-T. Succinic semialdehyde is subsequently metabolized to succinic acid by succinic semialdehyde dehydrogenase, and taken into the Krebs cycle. α-Ketoglutarate from the Krebs cycle is metabolized to glutamate, which is then metabolized to GABA, completing the cycle of the GABA shunt. GABA that is taken into glia is not available for immediate re-release, and is instead degraded by GABA-T in a similar metabolic pathway to that in neurons. Due to the absence of GAD in glial cells, however, glutamate generated from the Krebs cycle cannot be metabolized to GABA, and is instead metabolized to glutamine by glutamine synthetase. Glutamine is transported back into neurons, where it is

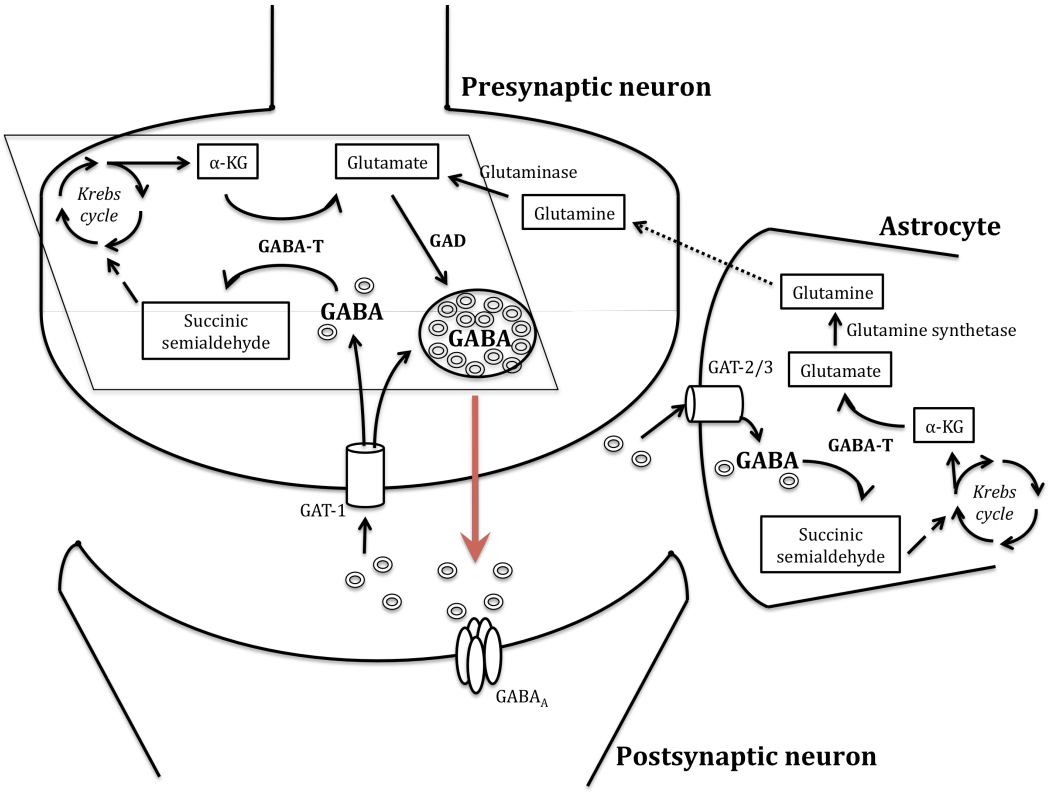

Figure 1-1. Schematic diagram of the GABA shunt. α-KG: α-ketoglutarate; GAT: GABA transporter; GABA-T: GABA-transaminase; GAD: glutamic acid decarboxylase.

converted to glutamate by glutaminase and cycled back into the GABA shunt (Brambilla *et al.* 2003).

GABA-T mediates the transamination reaction in which α-ketoglutarate acts as an amine acceptor in the generation of glutamate, and GABA acts as an amine donor in the generation of succinic semialdehyde. GABA-T is a mitochondrial enzyme that is ubiquitously distributed throughout the body, and is found in both neurons and glia (Kugler 1993; Cooper *et al.* 2003). Interestingly, studies have suggested that a very small amount of GABA-T exists in nerve endings, suggesting that the majority of GABA taken into the presynaptic neuron is repackaged for release, and that the majority of GABA degradation occurs at some extraneuronal site (Cooper *et al.* 2003). Both GABA-T and GAD require pyridoxal-5'-phosphate (PLP) as a co-factor, although GABA-T has a higher affinity for PLP than does GAD (Cooper *et al.* 2003). Unlike GABA-T, GAD has only been located in GABA-synthesizing neurons (Kugler 1993). Two forms of GAD have been identified, GAD_{65} and GAD_{67}, which differ in molecular weights, cellular localizations and affinities for PLP (Erlander *et al.* 1991).

1.3 MONOAMINE OXIDASE

Monoamine oxidase (MAO) is a flavin-containing enzyme that catalyzes the oxidative deamination of endogenous and exogenous amines and is located primarily in the outer mitochondrial membrane. Deamination of monoamines by MAO leads to the formation of their corresponding aldehydes (which are then metabolized to carboxylic acids by aldehyde dehydrogenase), hydrogen peroxide (H_2O_2), and either ammonia (in primary amine metabolism) or a substituted amine (in secondary amine metabolism). MAO is believed to play a protective role by deactivating exogenous amines, by terminating the actions of amine neurotransmitters, and by regulating intracellular amine stores (Youdim *et al.* 2006).

Two distinct isoforms of MAO exist. The isoenzymes were originally distinguished by their respective substrate and inhibitor sensitivities, and have since been shown to exhibit distinct amino acid sequences and tissue distributions. MAO-A primarily metabolizes epinephrine, norepinephrine (NE), and serotonin (5-HT), whereas MAO-B preferentially metabolizes β-phenylethylamine (PEA) (Fowler and Tipton 1984). Dopamine (DA) is thought to be metabolized by both MAO isoforms (O'Carroll *et al.* 1983), and although inhibition of both MAO-A and -B is necessary to increase brain DA levels (Green *et al.* 1977), it has been reported that DA degradation is mediated primarily by MAO-A in the rodent brain and by MAO-B in the primate brain (Johnston 1968). In terms of substrate specificity, MAO-A is selectively inhibited by low doses of clorgyline (Johnston 1968), while MAO-B is inhibited by low doses of *l*-deprenyl (Knoll and Magyar 1972). Most tissues in the body express both MAO isoforms, with the exception of the placenta, which expresses primarily MAO-A (Weyler and Salach 1985), and platelets and lymphocytes, which express only MAO-B (Bond and Cundall 1977; Donnelly and Murphy 1977). In the human brain MAO activity is highest in the basal ganglia and the hypothalamus and lowest in the cerebellum and neocortex (O'Carroll *et al.* 1983), and positron-emission tomography studies with radiolabelled irreversible inhibitors of MAO-A and -B have demonstrated that the isoenzymes are distributed unevenly throughout brain regions (Fowler *et al.* 1987).

Changes in brain MAO activity in normal aging and pathological conditions have been studied. The general consensus is that MAO-B activity increases with age in both rodents and humans, while reports on MAO-A have been conflicting, with studies demonstrating unchanged (Gottfries *et al.* 1975; Benedetti and Keane 1980; Fowler *et al.* 1980; Leung *et al.* 1981; Arai and Kinemuchi 1988; Fowler *et al.* 1997) or increased MAO-A activity in aged brains (Shih 1979). Increased MAO-B activity has also been consistently observed in many brain regions of patients suffering from AD compared to

age-matched controls, whereas MAO-A activity was reported unchanged or increased (Adolfsson *et al.* 1980; Jossan *et al.* 1991; Sherif *et al.* 1992; Saura *et al.* 1994). Interestingly, high intracellular Ca^{2+} levels have been shown to increase MAO-A activity (Egashira *et al.* 2003; Samantaray *et al.* 2003), and it has been suggested by Cao and colleagues (2007) that age- and neurodegenerative disease-related increases in Ca^{2+}-may lead to increases in MAO-A activity. Platelet MAO-B activity is significantly higher in AD, dementia and PD compared to control values (Adolfsson *et al.* 1980; Bongioanni *et al.* 1997; Zhou *et al.* 2001). Increased MAO activity may contribute to the neurodegeneration observed in these disorders, due to the increased production of toxic by-products such as phenylacetaldehyde and H_2O_2 (Strolin Benedetti and Dostert 1989; Cao *et al.* 2007).

1.4 MONOAMINE OXIDASE INHIBITORS

The discovery of MAO inhibitors (MAOIs) happened serendipitously in the early 1950s when iproniazid, a hydrazine ($R_1N–NR_2$) derivative developed to treat tuberculosis, was observed to exert antidepressant effects in a number of patients (Fox and Gibas 1953). Its mood-elevating properties were later attributed to MAO inhibition, and other MAOIs were developed for the treatment of depression. Today, a wide range of MAOIs is available to treat many psychiatric and neurological conditions in addition to depression, including anxiety disorders, AD and PD (Bortolato *et al.* 2008).

MAOIs are generally classified into several categories. The reversible MAOIs transiently inhibit MAO, while MAOIs that irreversibly bind to the enzyme or its cofactors, so-called “suicide inhibitors,” are known as irreversible MAOIs. Further, non-selective MAOIs inhibit both MAO isoforms, whereas selective MAOIs inhibit only one isoform (although most selective MAOIs inhibit both isoforms at high doses). Isoenzyme selectivity of MAOIs determines their therapeutic and neurochemical outcomes; for example, the antidepressant actions of MAOIs are dependent solely upon inhibition of

MAO-A, since antidepressant effects are mediated by increases in brain levels of 5-HT and NE. Selective MAO-B inhibitors are generally not antidepressants (although transdermally administered l-deprenyl has now been reported to have antidepressant effects (Lee and Chen 2007)), but may slow the progression of PD in the early stages of the illness, most likely due to the inhibition of DA metabolism (Bortolato *et al.* 2008). MAO-B inhibition may also be effective in reducing the oxidative stress-induced pathological changes observed in AD, counteracting the increased MAO-B activity found in the brains of these patients (Zhou *et al.* 2001) and possibly decreasing the generation of reactive oxygen species (ROS) from increased H_2O_2 production (Bortolato *et al.* 2008). These data are discussed in greater detail in Section 1.7.3.3.

While MAOIs are effective in treating a number of pathological conditions, they are not without side effects, which can include drowsiness, dizziness and weight gain. A potentially severe hypertensive condition, referred to as the "cheese effect", can occur when MAO-A is irreversibly inhibited. Tyramine and other sympathomimetic amines which are usually metabolized by MAO-A in the gut are instead able to enter the circulation and stimulate the release of NE, causing headaches and even hypertensive crisis, coma, and death (Youdim and Finberg 1987). Tyramine is found in foods such as aged cheeses and wines, and thus strict dietary restrictions are imposed on individuals taking irreversible MAOIs. This condition can be avoided by the use of reversible MAOIs (e.g. moclobemide), since tyramine is able to displace the inhibitor in peripheral systems and can be metabolized subsequently. Because of this potentially fatal side effect, MAOIs have generally not been used as first-line treatments.

1.5 PHENELZINE

PLZ is a potent irreversible, non-selective MAOI. It is used clinically for the treatment of a number of psychiatric disorders, including major depression (McGrath *et al.* 1986), atypical depression (Paykel *et al.* 1982;

Quitkin *et al.* 1989; Quitkin *et al.* 1990), panic disorder (Sheehan *et al.* 1980; Buigues and Vallejo 1987), social anxiety disorder (Liebowitz *et al.* 1988) and post-traumatic stress disorder (Davidson *et al.* 1987). It has also been shown to significantly reduce neuronal loss in a rodent model of transient forebrain ischemia (Wood *et al.* 2006b).

Given its potent inhibitory actions on MAO, it is not surprising that PLZ increases brain levels of the classical monoamine neurotransmitters (McKim *et al.* 1983; Baker *et al.* 1984; McKenna *et al.* 1991; Griebel *et al.* 1998) and trace amines (phenylethylamine (PEA), tyramine and tryptamine) (Philips and Boulton 1979). PLZ and other hydrazines are thought to inhibit MAO via the rapid tight binding of a diazene metabolite to the flavin cofactor of the enzyme (flavin adenine dinucleotide, or FAD), therefore reducing the bioavailability of (and sterically hindering) the active cofactor and reducing enzyme activity (Holt *et al.* 2004). Remarkably, PLZ also markedly increases brain levels of the amino acids GABA (Popov and Matthies 1969; Perry and Hansen 1973; Baker *et al.* 1991; McKenna *et al.* 1991; McManus *et al.* 1992; Paslawski *et al.* 1995; Todd and Baker 1995; Parent *et al.* 1999, 2000; Yang and Shen 2005) and alanine (ALA) (Wong *et al.* 1990; Tanay *et al.* 2001; Yang and Shen 2005), and using *in vivo* microdialysis in rodents, PLZ was demonstrated to increase extracellular levels of both GABA and ALA in the striatum (Tanay *et al.* 2001; Parent *et al.* 2002). Increased brain levels of GABA and ALA are thought to be primarily the result of PLZ-induced inhibition of their respective catabolic enzymes, GABA-T (Popov and Matthies 1969; McKenna *et al.* 1991; McManus *et al.* 1992) and ALA-transaminase (ALA-T) (Paslawski 1998; Tanay *et al.* 2001). Both GABA-T and ALA-T require PLP as a cofactor, and PLZ has been shown to inhibit a number of other PLP-dependent enzymes (Dyck and Dewar 1986; Yu and Boulton 1992; Holt *et al.* 2004). PLZ is thought to bind to PLP, forming a hydrazone ($R_1C{=}N{-}NR_2$) adduct and therefore reducing the availability of the active cofactor (thus reducing enzyme activity) (Holt *et al.* 2004). Chronic PLZ

treatment has been reported to reduce plasma PLP in humans by forming a pyridoxalhydrazone complex (Malcolm *et al.* 1994), supporting this postulation (although results from Lydiard and colleagues (1989) did not agree with this finding of reduced plasma PLP).

While administration of PLZ to rodents increases brain GABA levels up to 3-4 times control values (Baker *et al.* 1991), GABA-T activity is not inhibited *in vivo* by more than 50% even at doses as high as 60 mg/kg (Popov and Matthies 1969), suggesting that other, as-of-yet unidentified mechanisms may also be involved in PLZ's GABAergic effect. Importantly, administration of PLZ resulted in a transient but significant decrease in brain levels of glutamine and glutamate (Paslawski *et al.* 1995; Paslawski 1998; Yang and Shen 2005), a decrease in glutamate-glutamine cycling flux between neurons and glia (Yang and Shen 2005), and a decrease in KCl-evoked glutamate release (Michael-Titus *et al.* 2000). Together these results suggest that PLZ may cause a transient decrease in glutamatergic transmission. Chronic PLZ administration mildly inhibits GAD activity, although the inhibition of GABA-T is much stronger (McKenna *et al.* 1994), and mRNA levels encoding GAD_{65}, GAD_{67} or GABA-T are not altered by chronic PLZ treatment (Lai *et al.* 1998). The possibility that PLZ may reverse the activity of the GATs, thus exporting GABA from the presynaptic neuron rather than mediating its uptake, has also been suggested (Duffy *et al.* 2004).

It should be noted that while studies consistently report that PLZ causes transient decreases in whole brain glutamine levels, the effects of PLZ on glutamate are much less robust, with some (Yang and Shen 2005), but not all (Parent *et al.* 2000) studies reporting a decrease in whole brain glutamate levels. As such, an effect of PLZ on glutamatergic transmission should be interpreted with caution. In this thesis, PLZ- induced decreases in glutamatergic transmission are included in the discussions of PLZ's neurochemical effects since a reduction in glutamate activity is supported by some studies, but it should be kept in mind that not all studies support this conclusion and further investigations are warranted to determine why these

inconsistencies are observed. In this regard, it is of interest that colleagues in the Neurochemical Research Unit have recently found that PLZ decreases glutamate release from astrocytes (Song, Baker and Todd, personal communication).

1.6 β-PHENYLETHYLIDENEHYDRAZINE (PEH)

PLZ is intriguing in that it not only inhibits MAO, but is also a substrate for MAO (Clineschmidt and Horita 1969b, 1969a). This has been substantiated by studies showing that in humans 6-8 weeks of chronic treatment is required to achieve steady-state plasma levels (Mallinger and Smith 1991), and by the fact that inhibition of MAO prior to PLZ administration in rats abolishes a number of neurochemical effects attributed to PLZ, including the inhibition of GABA-T activity and the elevation of brain GABA (Popov and Matthies 1969; Todd and Baker 1995, 2008). Clineschmidt and Horita (1969b, 1969a) proposed that the active metabolite generated from PLZ by MAO was phenylacetic acid, and while it has been since confirmed that phenylacetic acid is indeed a major metabolite of PLZ in humans (Robinson *et al.* 1985), it is not believed to be formed by the actions of MAO and it does not inhibit GABA-T (Baker, unpublished observations), suggesting that another active metabolite likely mediates the GABAergic effects observed after PLZ administration.

In 1972 it was suggested by Tipton and Spires that PEH was the active metabolite of PLZ, and while *in vitro* studies supported this theory (Tipton 1972), it was not confirmed that PEH was formed *in vivo* from PLZ. PEH was later synthesized and administered to rodents, and was shown to inhibit GABA-T and elevate brain GABA in rodents to a similar degree to that observed with PLZ (Paslawski *et al.* 2001). Furthermore, like PLZ, PEH elevated whole brain ALA levels, transiently decreased whole brain glutamine levels and increased extracellular GABA in the striatum (Parent *et al.* 2002). It is currently believed that a number of PLZ's neurochemical

effects, including those mentioned here, may be mediated by the formation of PEH. Despite the similarities in the neurochemical actions of PLZ and PEH, one important difference is that, unlike PLZ, PEH has only weak inhibitory effects on MAO (Paslawski *et al.* 2001), suggesting that PEH could be an interesting therapeutic alternative to PLZ in some disorders since the "cheese effect" would be unlikely to occur. Indeed, PEH itself was shown to reduce epileptic activity in rat hippocampal slices (Duffy *et al.* 2004) and to possess neuroprotective effects in transient forebrain ischemia in the gerbil (Tanay *et al.* 2002). The chemical structure of PEH and proposed metabolism of PLZ to PEH are shown in Figure 1-2.

1.7 THERAPEUTIC MECHANISMS OF ACTION OF PLZ

1.7.1 Antidepressant mechanisms of action of PLZ

Aside from the potent MAO-inhibiting action of PLZ, which undeniably confers antidepressant effects, the GABAergic properties of PLZ also likely contribute to its antidepressant actions. Preclinical studies have shown that depressive-like behaviour in rats is prevented or reversed with GABA or with drugs that facilitate GABAergic transmission (such as muscimol or 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP)), and the inhibition of depressive-like behaviour by antidepressant drugs can be prevented with $GABA_A$ receptor antagonists (reviewed in Tunnicliff and Malatynska 2003). Clinical studies have revealed decreases in plasma (Petty *et al.* 1990; Petty 1994), cerebral spinal fluid (CSF) (Gold *et al.* 1980; Gerner *et al.* 1984) and brain (Sanacora *et al.* 1999) levels of GABA in depressed patients compared to healthy controls. Chronic treatment with selective serotonin reuptake inhibitors (SSRIs) (Sanacora *et al.* 2002) and repeated electroconvulsive therapy (Sanacora *et al.* 2003) increase brain GABA levels in depressed patients. Together, these data support a therapeutic role for GABA facilitation in depression, and the elevation in brain GABA by PLZ may contribute to its successful use as an antidepressant agent.

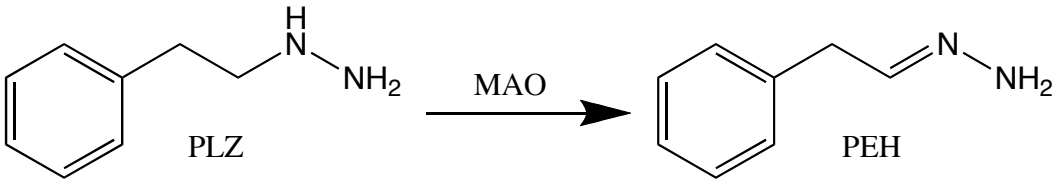

Figure 1-2. The chemical structure and proposed formation of PEH from PLZ

Studies suggest that PLZ may also transiently decrease glutamatergic transmission, although as mentioned earlier, further studies are warranted to clarify conflicting results. Recently the role of the glutamatergic system in mood disorders has been of great interest, and a wealth of evidence suggests that glutamate may be dysregulated in major depression (Sanacora *et al.* 2008). Brain and plasma glutamate levels have been shown to be elevated in patients suffering from depression compared to controls (Mitani *et al.* 2006; Hashimoto *et al.* 2007), and glutamate receptor pathology in major depression has been reported (Nudmamud-Thanoi and Reynolds 2004). Individuals with depression may also exhibit a significant reduction in glial cell number and density in various brain regions (Cotter *et al.* 2001; Bowley *et al.* 2002), and given that glial uptake is the primary mechanism of glutamate clearance from the synapse, neuronal hyperexcitability may result from insufficient clearance of glutamate from the synapse. Additionally, glutamate receptor antagonists have been shown to elicit antidepressant-like effects in animal models of depression (Trullas and Skolnick 1990) and rapid therapeutic effects in depressed patients (Berman *et al.* 2000; Zarate *et al.* 2006). From these findings it is reasonable to suggest that a decrease in glutamatergic transmission induced by PLZ could also contribute to the antidepressant properties of the drug.

1.7.2 Anxiolytic and antipanic mechanisms of action of PLZ

While 5-HT and NE are known to play a role in the pathophysiology of anxiety, a wealth of evidence also indicates that decreased GABAergic transmission is related to anxiety disorders. Preclinical and clinical studies have demonstrated the anxiogenic effects of reduced GABAergic function and the anxiolytic effects of increased GABAergic transmission (Dorrow *et al.* 1983; Sherif and Oreland 1995). In fact, the majority of pharmacological treatments currently prescribed for anxiety disorders, including

benzodiazepines, SSRIs, venlafaxine and PLZ, have been shown to facilitate the GABAergic system *in vivo* (Sanacora *et al.* 2002; Streeter *et al.* 2005) although not all of these drugs were considered in the past to be GABAergic agents. Abnormalities in the GABAergic systems of individuals suffering from anxiety disorders have also been reported. Individuals suffering from social anxiety disorder were shown to have reduced GABA levels in the thalamus compared to healthy volunteers (although whole brain GABA levels were comparable in both groups) (Pollack *et al.* 2008), and patients with panic disorder have been shown to exhibit reduced GABA levels in the occipital cortex (Goddard *et al.* 2001), reduced sensitivity to $GABA_A$ receptor agonists (Roy-Byrne *et al.* 1996) and to have fewer benzodiazepine-type binding sites (on the $GABA_A$ receptor) in the insular cortex (Cameron *et al.* 2007) compared to healthy controls. PLZ is effective for the treatment of social anxiety disorder and panic disorder (Sheehan *et al.* 1980; Buigues and Vallejo 1987; Liebowitz *et al.* 1988), and the GABAergic effects of PLZ likely contribute to its anxiolytic and antipanic effects. Consistent with this, PLZ effectively reduced anxiety-like behaviour in rodents (assessed using the elevated-plus maze) only at doses that elevated brain levels of GABA (Paslawski *et al.* 1996).

Since GABA appears to be an important modulator of anxiety, it is not surprising that the excitatory glutamatergic system (with which it is in a delicate balance) is also implicated in anxiety-related disorders. The majority of evidence for a glutamatergic influence on anxiety comes from animal studies, where alteration of the glutamate system has been reported to alter anxiety-like behaviour; the NMDA antagonists ketamine and MK-801 have been shown to reduce anxiety-like behaviour in rodents (Sharma and Kulkarni 1993; Engin *et al.* 2009), for example, and the metabotropic glutamate receptor agonist LY354740 (which reduces the presynaptic release of glutamate) has been reported to prevent lactate-induced panic attacks in rats (Shekhar and Keim 2000). Clinically, individuals with social anxiety disorder were shown to have elevated glutamate levels in the

anterior cingulate cortex (but not the occipital cortex) (Phan *et al.* 2005) and higher whole brain and thalamus levels of glutamate and glutamine (Pollack *et al.* 2008) compared to healthy controls. Together with the increase in brain GABA, the modest decrease in glutamatergic transmission induced by PLZ may contribute to its anxiolytic and antipanic effects.

1.7.3 Neuroprotective mechanisms of action of PLZ

Aside from its well-established antidepressant and antipanic effects, PLZ has more recently been shown to significantly reduce neuronal loss in a rodent model of cerebral ischemia (Wood *et al.* 2006b). This is an important finding that could potentially not only lead to a reduction in the disability that so often occurs following stroke in humans, but also provide insight into novel therapeutic interventions for other neurodegenerative conditions. As such, it is important to understand the mechanisms by which PLZ exerts its neuroprotective effects. The potential mechanisms by which PLZ may be neuroprotective are reviewed here, with particular emphasis on two neurological conditions possessing degenerative components, cerebral ischemia and AD.

The death of brain tissue resulting from oxygen depletion due to a blockage of blood flow to the brain, and the clinical symptoms associated with this tissue death, is collectively referred to as cerebral ischemia (Green *et al.* 2003). Focal cerebral ischemia refers to a blockage in blood flow to a specific region of the brain (which occurs when a blood vessel is occluded, for example), whereas global ischemia refers to a complete blockage in blood flow (which occurs in cardiac arrest, for example). While the central core of the tissue injury is considered to be irreparably damaged, the surrounding tissue, or penumbra, undergoes delayed cell death, and it is believed that rapid intervention can prevent (or at least reduce) the degree of cell loss in this region. Neuroprotective drugs for the treatment of ischemia focus on rescuing brain cells in the penumbra (Green *et al.* 2000; 2003).

AD is a chronic neurodegenerative disorder characterized clinically by cognitive deficits and behavioural and psychological symptoms, including loss of memory, judgment and reasoning, difficulty with day-to-day tasks, and changes in communication abilities. A definitive diagnosis of AD can only be confirmed histopathologically by the presence of neuritic plaques and neurofibrillary tangles. Neuritic plaques are extracellular deposits of aggregated proteins such as β-amyloid (Aβ) (Ma *et al.* 1994) and are toxic to neurons, triggering oxidative stress and apoptosis (Loo *et al.* 1993). Neurofibrillary tangles are intracellular protein aggregates, composed mainly of hyperphosphorylated microtubule-associated proteins such as *tau* (Goedert *et al.* 1989).

1.7.3.1 Phenelzine elevates brain GABA

Phenelzine's ability to produce rapid and long-lasting increases in brain levels of GABA is an important pharmacological property that may be relevant to the neuroprotective properties of the drug. Stimulation of the GABAergic system may counteract excitotoxicity (nerve cell death resulting from the excessive activity of glutamate and other excitatory amino acids and their receptors), which is thought to be an important mechanism of degeneration in a number of neurological and psychiatric conditions, including ischemia and AD.

1.7.3.1.1 Relevance of elevated GABA to cerebral ischemia

While the mechanisms of neurotoxicity in ischemia are complex and overlapping and are still the focus of much research, excitotoxicity is believed to play a significant role in ischemic cell death. In ischemia, restriction of blood flow causes a disruption of neuronal homeostasis; eventual cessation of ATP production results in the failure of Na^+/K^+ ATPase and Ca^{2+} ATPase, for example, leading to the passive influx of Na^+, K^+ and Ca^{2+} ions and subsequent membrane depolarization and excessive neurotransmitter

(notably glutamate) release (Juurlink and Sweeney 1997; Lo *et al.* 2003). The actions of ATP- and Na^+/K^+ -dependent glutamate uptake transporters are also reversed in the event of ATP depletion, further contributing to the excessive glutamate concentrations in the extracellular space. Overstimulation of glutamate ion channel receptors (N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors) exacerbates the influx of Ca^{2+} and membrane depolarization, aggravating excitotoxicity. Excitotoxic cell death appears to be mediated in part by necrosis, which is stimulated by Ca^{2+} influx. Excessive Ca^{2+} influx mediates other deleterious effects, including the production of ROS which leads to oxidative stress, and the initiation of intracellular cascades that promote apoptosis. The remarkable increase in brain glutamate levels following ischemia has been demonstrated in many studies (Kanthan *et al.* 1995; Juurlink and Sweeney 1997; Yang *et al.* 2001; Iqbal *et al.* 2002; Lo *et al.* 2003), and a reduction in glutamatergic activity and/or neurotransmission has been shown to be neuroprotective in this context.

Importantly, an increase in brain GABA is observed concomitant to the increase in glutamate in ischemia; however, the increase in GABA is much more transient than that of glutamate, and many studies have confirmed that the initial increase in brain GABA is followed by a longer-lasting decrease in brain levels of this inhibitory neurotransmitter (Baldwin *et al.* 1993; Shuaib *et al.* 1994; Mainprize *et al.* 1995; Zeng *et al.* 2007). While both vesicular and non-vesicular GABA release may be temporarily increased following an ischemic event (possibly a compensatory measure to minimize excitotoxic damage), GABA synthesis may actually be decreased, reflecting an overall, and more long-lasting, decrease in GABAergic function (Green *et al.* 2000; Schwartz-Bloom and Sah 2001). Indeed, it has been reported that GABA synthesis is reduced in the mouse neocortex following ischemia (Green *et al.* 1992). Moreover, GABA-T expression was shown to be increased in the gerbil hippocampus several days after ischemia-reperfusion, possibly reflecting an increase in the degradation of GABA (Kang *et al.* 2000). This functional

decrease in GABAergic activity likely exacerbates the neuronal damage induced by excitotoxicity in the long term, since the opposing actions of the GABAergic system on the hyperactive glutamatergic system are reduced (Green *et al.* 2000).

While antagonism of glutamate NMDA receptors reduces cell loss in both *in vitro* and *in vivo* models of excitotoxicity, increasing GABAergic transmission also counteracts excitotoxic damage, and may do so clinically with fewer adverse side-effects. Preclinical studies have shown that GABAergic agents, including tiagabine and vigabatrin (gamma-vinyl GABA) can reduce the extent of ischemia-mediated neuronal damage *in vivo* and *in vitro* (Shuaib *et al.* 1992; Shuaib *et al.* 1996; Iqbal *et al.* 2002; Costa *et al.* 2004). Presumably, increased GABAergic transmission attenuates excitotoxicity (a) by inhibiting presynaptic neurons, thus reducing the release of glutamate, and (b) by hyperpolarizing the postsynaptic membrane, reducing cell excitability. Since PLZ inhibits GABA-T and rapidly and markedly elevates brain GABA levels, it is not surprising that PLZ has been shown to reduce neuronal damage in animal models of ischemia (Wood *et al.* 2006b).

1.7.3.1.2 Relevance of elevated GABA to Alzheimer's disease

Excitotoxicity has also been shown to play a significant role in the neurodegeneration observed in AD. Abnormal cellular machinery for glutamate uptake has been reported in AD brains, leading to high synaptic glutamate levels and excitotoxicity. For example, a reduction of glutamate uptake sites has been reported in AD brains compared to control brains (Cowburn *et al.* 1988; Scott *et al.* 1995; Li *et al.* 1997; Liang *et al.* 2002) and aberrant neuronal expression of glutamate uptake sites has been associated with *tau* protein accumulation (Scott *et al.* 2002; Thai 2002). In addition to excitotoxic necrosis, apoptosis and accumulation of ROS, elevated intracellular Ca^{2+} in AD brains may activate Ca^{2+}-sensitive protein kinases,

which in turn lead to the hyperphosphorylation of proteins such as *tau* (Mattson *et al.* 1992).

GABAergic deficits have been reported in AD, although the data are conflicting and complicated by variables such as illness severity and post-mortem handling of brain tissue (Lanctot *et al.* 2004). Despite the contradictory clinical evidence for the effectiveness of GABAergic drugs in treating of chronic degenerative disorders such as AD (Lanctot *et al.* 2004), GABAergic drugs may protect against chronic degeneration by counteracting the damaging effects of excitotoxicity. PLZ's potential as a treatment for the long-term neurodegeneration observed in chronic conditions such as AD should be considered. Importantly, facilitation of GABAergic transmission has been shown to produce neuroprotective effects both *in vivo* and *in vitro* against β-amyloid mediated toxicity (Gu *et al.* 2003; Lin and Jun-Tian 2004; Louzada *et al.* 2004; Lee *et al.* 2005; Marcade *et al.* 2008), suggesting that modulation of GABAergic activity by PLZ could be neuroprotective in AD via other mechanisms in addition to attenuating glutamate-mediated excitotoxicity.

1.7.3.2 Phenelzine binds to some reactive aldehydes

The neurotoxic role of reactive aldehydes in ischemia and neurodegenerative disorders has been the focus of much research in recent years. Aldehydes such as 3-aminopropanal (3-AP), acrolein, phenylacetaldehyde and formaldehyde (FA), for example, are produced in small amounts in normal metabolic reactions; metabolism of the polyamines spermidine and spermine, via the actions of polyamine oxidase, produces putrescine (another polyamine) and 3-AP and acrolein as by-products (Seiler 2000). The deamination of PEA to phenylacetaldehyde is catalyzed by MAO-B, and the metabolism of methylamine (MA) and aminoacetone, via the action of semicarbazide-sensitive amine oxidase (SSAO), produces FA and methylglyoxal, respectively (Matyus *et al.* 2004). Other aldehydes, such as

acrolein, 4-hydroxynonenal (4-HNE) and malondialdehyde, are generated as products of lipid peroxidation (oxidative damage to lipids by ROS) (Esterbauer *et al.* 1991; Uchida *et al.* 1998), and high aldehyde concentrations (free and/or protein-bound) are considered to be biological markers of oxidative stress (Tomitori *et al.* 2005).

Enzymes are present in the cytosol to metabolize the aldehydes, since accumulation of these highly reactive molecules often leads to cytotoxicity. Free aldehydes rapidly bind to amino acids, proteins, nucleic acids and lipids, forming irreversible adducts that can cause inhibition of protein, RNA and DNA synthesis, and can interfere with the functioning of enzymes, membrane transporters and cell membranes (including a disruption in Ca^{2+} homeostasis) (Esterbauer *et al.* 1991; Lovell *et al.* 2001). Acrolein was shown to induce apoptosis via direct mitochondrial toxicity (Picklo and Montine 2001), whereas 3-AP has been shown to enter into lysosomes, causing lysosomal leakage or rupture and resulting in mitochondrial damage and the activation of apoptotic cascades (and often cellular necrosis as well) (Ivanova *et al.* 1998; Li *et al.* 2003; Yu *et al.* 2003; Yu *et al.* 2004). FA causes lipid peroxidation, which in turn stimulates the production of ROS and other toxic aldehydes (Teng *et al.* 2001), and also produces hydroxyl radicals in the presence of H_2O_2 under basic conditions (Lichszteld and Kruk 1977). Further, several aldehydes have been shown to deplete levels of the endogenous antioxidant glutathione, exacerbating oxidative damage (White and Rees 1984; Horton *et al.* 1997).

While antioxidant treatment would theoretically counteract the actions of ROS and therefore reduce lipid peroxidation and the generation of the aldehyde byproducts, antioxidants have not been effective in preventing aldehyde-mediated cytotoxicity experimentally (Wood *et al.* 2006a) or clinically (Gilgun-Sherki *et al.* 2002). Alternatively, an effective method for reducing aldehyde-mediated toxicity is by binding, or "sequestering," the aldehydes, producing a non-reactive and non-toxic product. This concept involves the scavenging of aldehydes by a drug that binds rapidly and tightly

to the aldehyde, resulting in the formation of an inert product and thus reducing the reactive "aldehyde load." Many studies have demonstrated the success of aldehyde sequestration in reducing aldehyde-mediated toxicity; for example, *N*-benzylhydroxylamine, cyclohexylhydroxylamine and *t*-butylhydroxylamine were shown to sequester 3-AP, presumably forming inert oximes, and to decrease aldehyde-mediated neurodegeneration *in vitro* (Wood *et al.* 2006a). Aminoguanidine sequesters FA *in vitro* and *in vivo* (Kazachkov *et al.* 2007). Acrolein and 3-AP have been shown to be sequestered by hydralazine, dihydralazine and PLZ, producing inert hydrazones (Burcham *et al.* 2002; Wood *et al.* 2006b), and PLZ reduced 3-AP mediated neuronal loss in an *in vivo* model of cerebral ischemia (Wood *et al.* 2006b). PLZ was also shown to sequester 4-HNE *in vitro* (Galvani *et al.* 2008). The free hydrazine group of PLZ binds the aldehyde to produce an inert hydrazone molecule, and thus PLZ would be expected to effectively sequester other reactive aldehydes as well, reducing aldehyde-mediated toxicity.

1.7.3.2.1 Relevance of aldehyde sequestration to cerebral ischemia

The neurotoxic potential of aldehydes in ischemia is a relatively new topic of research, but it is apparent that reactive aldehydes play a major role in ischemic brain damage. Polyamine synthesis and metabolism are markedly increased in the ischemic brain, likely resulting, at least in part, from an increase in the activity of ornithine decarboxylase (ODC), the enzyme that metabolizes the amino acid ornithine (ORN) to putrescine (Paschen *et al.* 1987). Dysregulation of the polyamine system results in the overproduction of 3-AP and acrolein to levels that are toxic to neurons and glia (Ivanova *et al.* 1998). Indeed, inhibition of ODC or of polyamine oxidase (the enzyme that generates 3-AP) significantly reduced the infarct volume in ischemia (Kindy *et al.* 1994; Dogan *et al.* 1999). Sequestration of reactive aldehydes with PLZ was also shown to reduce the degree of neuronal damage in ischemic brains (Wood *et al.* 2006b).

It should be noted that elevated levels of other reactive aldehydes have been observed in animals and humans following ischemia; increased brain levels of 4-HNE were observed in animals following focal cerebral ischemia (Matsuda *et al.* 2009), and increased plasma levels of acrolein were reported in stroke patients (Tomitori *et al.* 2005). However, the authors of these studies suggested that the presence of the aldehydes represented biochemical markers of oxidative stress and of diagnosis of stroke rather than commenting on the possible toxic consequences of increased aldehyde levels. Further research will clarify the role of these aldehydes in ischemic brain damage.

1.7.3.2.2 Relevance of aldehyde sequestration to Alzheimer's disease

Many lines of evidence suggest that oxidative damage plays a role in AD-related neurodegeneration. High levels of free aldehydes and/or protein adducts formed by acrolein, 4-HNE, malondialdehyde and methylglyoxal (all products of lipid peroxidation) have been detected in AD brains, often colocalized with neurofibrillary tangles (Sayre *et al.* 1997; Markesbery and Lovell 1998; Calingasan *et al.* 1999; Lovell *et al.* 2001), and it is likely that elevated levels of these aldehydes both result from and contribute to the oxidative damage observed. Indeed, elevated acrolein levels have been suggested to contribute to the mitochondrial dysfunction in AD (Pocernich and Butterfield 2003), and several aldehydes, including FA, were shown to induce Aβ aggregation and fibrillogenesis *in vitro* (Chen *et al.* 2006). FA was also shown to form irreversible adducts with Aβ proteins, producing amyloid-like complexes (Gubisne-Haberle *et al.* 2004), and to induce polymerization of tau both i*n vitro* and *in vivo* (Nie *et al.* 2007). Importantly, the expression of SSAO, the enzyme that metabolizes MA to FA, has been reported to be increased in AD brains compared to control brains (Ferrer *et al.* 2002), and SSAO-mediated deamination has been suggested to play a role in the pathogenesis of AD (Yu 2001). Importantly, sequestration of FA with

aminoguanidine was shown to prevent FA-induced Aβ aggregation both *in vivo* and *in vitro* (Kazachkov *et al.* 2007), suggesting that drugs able to sequester FA may be of significant value in reducing AD-related pathology and neurotoxicity. While aminoguanidine itself is not useful clinically due to its harmful side effects, these results highlight the importance of identifying other aldehyde-sequestering drugs able to protect against FA-mediated (and other aldehdye-mediated) AD pathology.

1.7.3.3 Phenelzine inhibits MAO and SSAO activity

Increased MAO-B activity has been reported in aged individuals and in several neurodegenerative disorders (Fowler *et al.* 1980; Jossan *et al.* 1991), and increased intracellular Ca^{2+} (which has been observed in AD and other neurodegenerative diseases) has been shown to contribute to increased MAO-A activity (Cao *et al.* 2007) (although findings regarding changes in MAO-A activity in AD and other degenerative disorders is conflicting). The toxic products of MAO-catalyzed reactions (which include the reactive aldehyde phenylacetaldehyde and H_2O_2) likely contribute to the neurodegeneration observed in these individuals. PLZ and other MAOIs would be expected to provide neuroprotection by reducing the production of these toxic products, particularly in conditions where MAO activity is increased. The cytotoxic effects of reactive aldehydes were discussed in Section 1.7.3.2. H_2O_2 is a major ROS that can be converted to toxic hydroxyl free radicals in the presence of transition metal ions (such as Fe^{2+}), leading to oxidative stress (Cantoni *et al.* 1989). Indeed, evidence for oxidative stress in the brains of individuals with chronic neurodegenerative diseases is well documented. Moreover, the therapeutic effects of *l*-deprenyl and rasagiline, selective MAO-B inhibitors currently used for the treatment of PD and AD, have been linked to reductions in oxidative stress (Bortolato *et al.* 2008).

PLZ also inhibits the activity of SSAO (Lizcano *et al.* 1996), a copper- and quinone-containing enzyme that, like MAO, catalyzes the oxidative

deamination of amines to produce their respective aldehydes, as well as ammonia and H_2O_2. SSAO is located primarily on the outer membrane of vascular endothelial cells, smooth muscle cells and adipose cells, and is also found circulating in the blood. In the brain it is located solely in the cerebral vasculature (Lewinsohn 1981; Zuo and Yu 1993). SSAO deaminates MA and aminoacetone (endogenous amines) to produce FA and methylglyoxal (respectively) (Matyus *et al.* 2004). SSAO also metabolizes xenobiotic amines, and benzylamine has been shown to be one of the best known substrates for SSAO (Matyus *et al.* 2004).

Inhibition of SSAO would reduce the production of the toxic reaction products FA, methylglyoxal and H_2O_2. It is important to note that while PLZ has been shown to inhibit bovine lung SSAO (Lizcano *et al.* 1996), to our knowledge it has not been demonstrated that PLZ inhibits human SSAO. Since differences in SSAO inhibitor efficacy have been observed between species, it is important to confirm the ability of PLZ to inhibit human SSAO to provide insight for clinical applications of SSAO inhibitors (Matyus *et al.* 2004).

1.7.3.3.1 Relevance to Alzheimer's disease

As previously mentioned, platelet and brain MAO-B activity have been reported increased in neurodegenerative disorders including AD (Adolfsson *et al.* 1980; Jossan *et al.* 1991; Sherif *et al.* 1992; Saura *et al.* 1994), and phenylacetaldehyde and H_2O_2 produced from MAO-mediated reactions may contribute to the neurodegeneration in these disorders. MAO inhibition could potentially reduce this effect. Endogenous deamination products of SSAO such as FA and, to a lesser extent H_2O_2, are also cytotoxic. The damaging role of FA in the pathology of AD was discussed in Section 1.7.3.2.2. Interestingly, the activity and expression of SSAO is reportedly elevated in AD serum and brains (respectively) (Ferrer *et al.* 2002; del Mar Hernandez *et al.* 2005), suggesting that inhibition of SSAO could potentially lead to neuroprotective effects by reducing the formation of toxic products.

1.7.3.4 Phenelzine decreases body temperature

PLZ decreases body temperature by several degrees Celsius in laboratory animals (Sowa 2006). The beneficial effects of hypothermia in terms of neuroprotection are well documented, and in clinical populations it has been shown that reduction of brain temperature after an ischemic insult or traumatic brain injury results in a significant improvement in outcome. The mechanism(s) of hypothermic neuroprotection have been studied in laboratory animals, and it has been shown that induction of hypothermia greatly reduces ischemia-induced glutamate release (Mitani and Kataoka 1991; Illievich *et al.* 1994; Winfree *et al.* 1996), decreases cerebral metabolism (thus decreasing the need for ATP and oxygen), and suppresses the production of ROS (Kil *et al.* 1996). Other GABAergic agents, including diazepine, tiagabine, muscimol and even GABA itself, have also been shown to reduce body temperature, and the hypothermic effects of diazepam and tiagabine were shown to contribute to their neuroprotective effects (Iqbal *et al.* 2002; Davies *et al.* 2004; Frosini *et al.* 2004).

The hypothermic effects of PLZ were mentioned here solely in the interest of completing the discussion on PLZ's putative mechanisms of neuroprotection. The experiments described in this thesis do not further explore PLZ's hypothermic effects.

1.8 THESIS OVERVIEW

The need for novel neuroprotective drugs is urgent. While many putative neuroprotective agents have produced promising results in animal studies, the efficacy of such agents has been disappointing in clinical populations, due to peripheral toxicities and/or unfavourable side effects. The studies described in this thesis contribute to a (slowly) growing body of literature suggesting that the currently prescribed antidepressant and antipanic drug PLZ may provide effective neuroprotective properties for toxicity observed in neurodegenerative conditions such as cerebral ischemia and AD. The

studies described here also support previous suggestions that an active metabolite of PLZ, namely PEH, mediates a number of PLZ's neurochemical effects, and that PEH warrants investigation as a putative neuroprotective agent in its own right.

Specifically, this thesis addressed the following questions:

1. Is PLZ indeed metabolized *in vivo* by MAO to its "putative active metabolite," PEH? (Chapter 2)

2. Does *in vivo* formation of PEH mediate PLZ-induced changes in whole brain amino acid levels? (Chapter 2)

3. Does inhibition of MAO *in vitro* prior to PLZ application abolish the effects on GABA-T as it does *in vivo*? (Chapter 3)

4. Do PLZ and/or PEH affect whole brain levels of ORN, an amino acid metabolically related to GABA, glutamate, and the polyamines (all of which are implicated in neurodegenerative and neurotoxic processes in ischemia and AD)? (Chapter 4)

5. Do PLZ and/or PEH sequester the reactive aldehyde FA *in vitro*? (Chapter 5)

6. Do PLZ and PEH inhibit the activity of human SSAO (which exhibits increased activity in AD brains and metabolizes MA to produce FA) *in vitro*? (Chapter 5)

7. Do PLZ and/or PEH increase brain levels of MA, which is a substrate for SSAO and the precursor to FA (which may be related to the pathophysiology of AD)? (Chapter 5)

8. Can PEH prodrugs be produced by the chemical addition of one or two propargyl functional groups onto the PEH molecule? (Chapter 6)

Results from the studies described in this thesis lend further support to the suggestions that PLZ could effectively reduce neurodegeneration in several neurological and psychiatric conditions. The results presented here also reveal that PEH is an active metabolite of PLZ formed *in vivo* by MAO,

and provide strong evidence for a possible therapeutic role of PEH in neurodegenerative conditions.

1.9 BASIC PRINCIPLES OF CHROMATOGRAPHY AND COMPOUND DETECTION RELEVANT TO THIS THESIS

Chromatography is a technique used to separate, identify and/or analyze complex mixtures. Two types of chromatography, namely gas chromatography (GC) and high performance liquid chromatography (HPLC), were used in this thesis. Mass spectrometry (MS) and electron capture detection (ECD) were combined with GC, and fluorescence detection and electrochemical detection (EC) were combined with HPLC.

1.9.1 Gas chromatography (GC)

In GC, a small amount of sample is introduced into the instrument, vaporized and injected onto the head of an analytical column. The mobile phase is an inert gas (such as helium or nitrogen) that continually flows through the system, and carries the sample through the analytical column (which contains the stationary phase, a very high boiling point liquid) to the detector. Separation is achieved in the column via the differential interactions of the compounds with the stationary phase coated on the inside of the column (Skoog *et al.* 1996a). Compounds in a mixture are separated in the column by their different affinities for the stationary phase; compounds interacting with the stationary phase with greater affinity remain in the column longer (that is, have longer retention times) than those having low affinities to the stationary phase. In other words, compounds eluting rapidly (those having a short retention times) do not interact with the stationary phase as strongly as compounds having a longer retention time. This principle is shown in Figure 1-3.

Analytes eluting from the column too closely together can be further separated by preprogramming changes in the temperature of the oven, which houses the analytical column. Increasing the temperature changes the nature

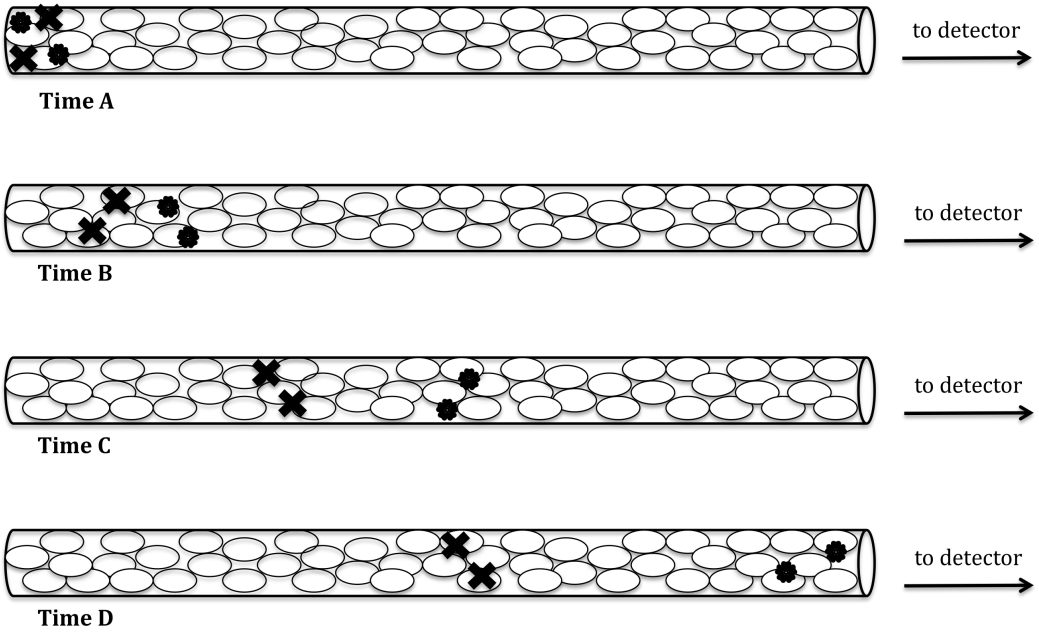

Figure 1-3. A simplified diagram illustrating compound separation using chromatography. In this figure, mobile phase (either a gas or a liquid) continually flows through the column (shown above), which is lined (in the case of the most frequently used GC columns) or packed (in the case of HPLC) with stationary phase particles. Compounds ✖ and ❁ are separated because ✖ interacts more strongly with the stationary phase, and therefore spends more time in equilibrium with the stationary phase (and less time being carried by the mobile phase). In GC, compounds having a lower affinity for the stationary phase will be carried more rapidly by the mobile phase to the detector. In HPLC, compound ❁ may interact preferentially with the mobile phase, and therefore would spend more time in equilibrium with the mobile phase and thus is carried more rapidly through the column and to the detector.

of the interactions between the compounds and the stationary phase, and changing the oven temperature program (for example, changing the rate of temperature increase, or setting the temperature to change in a series of steps) changes the retention times of compounds relative to each other. GC can only be used for the analysis of compounds that are volatile; therefore the compounds must be heat-stable and the sample must be prepared in a medium that can be vaporized. Derivatization, the process in which an analyte is reacted with another compound (the derivatizing agent) to produce a derivative of greater stability, volatility, or sensitivity for the detector, is often an integral part of the assay procedure.

Electron capture detection (ECD) is used for the detection of electron-absorbing compounds in a gas by measuring the decrease in the number of free electrons in the detector. The electron source of the detector is a radioactive isotope with continuous beta decay (such as ^{63}Ni), and the disruption of the baseline current by the absorption of electrons by analytes is recorded as a peak in the spectrum (Dybowski and Kaiser 2002). GC-EC was used in this thesis to measure brain levels of PEH following derivatization (Chapter 2).

1.9.2 High performance liquid chromatography (HPLC)

In HPLC, a sample is introduced into the liquid mobile phase, which flows through an analytical column that is packed with solid stationary phase. Compound separation is achieved in a similar manner as that in GC, but analytes can also interact with the mobile phase (whereas in GC the mobile phase is inert and serves only to carry the analyte through the column) (Skoog *et al.* 1996b). One or more mobile phases (which typically consist of aqueous buffers and/or organic solvents) can be used to achieve analyte separation; isocratic flow refers to the flow of a single mobile phase, while a

gradient elution combines two (or more) mobile phases in a preprogrammed manner to alter compound retention times.

Unlike GC, samples analyzed using HPLC do not need to be volatile, since they remain in liquid phase throughout the separation procedure. HPLC is useful for many biological molecules, which often have high boiling points and/or that are thermally unstable. Furthermore, since the mobile phase is generally (at least in part) aqueous, an extraction step is often not needed in the assay procedure.

Fluorescence detection was used with HPLC in this thesis to measure amino acids (Chapter 2 and 3). The use of fluorescence detectors is based on the principle that many organic compounds absorb energy (light) from the ultraviolet (UV) spectrum and emit the energy at a longer wavelength (typically in the visible spectrum). The amount of light emitted is directly proportional to the concentration of the fluorescent species. Fluorescence detectors emit UV energy (at a set wavelength) onto the sample, and the amount of fluorescence emitted (at a set wavelength) by the sample is recorded in arbitrary fluorescence units (Baker *et al.* 1985). Compounds are often derivatized with a fluorescent agent to improve sensitivity (as is the case with amino acids, which are commonly derivatized with a fluorescent agent such as *o*-phthaldialdehyde).

Electrochemical detection (EC) was used with HPLC in this thesis to measure brain levels of biogenic amines and of PEH analogs (Chapter 6). In EC, species are generally oxidized (but may also be reduced) at the surface of an electrode, which has an applied constant potential (Odontiadis and Rauw 2007). The change in current occurring in response to the oxidation of the compound as it passes by the electrode is measured, and is proportional to the concentration of analyte present.

1.9.3 Mass Spectrometry (MS)

MS is a powerful analytical technique that can be used to identify and quantify compounds, and can also provide information about chemical

structures of compounds. While there are many types of MS analyzers, a quadrupole mass filter MS was used for the experiments in this thesis, and will be described here; a review of the other MS types can be found elsewhere (Sloley *et al.* 2007). The quadrupole mass filter consists of four parallel voltage-carrying rods ("the quadrupole"). Compounds are separated in the column of the GC and, upon entering the MS analyzer, are bombarded with a high-energy electron beam, resulting in the formation of high-energy molecular ions (the original compound minus one electron). The molecular ions generally fragment into smaller, more stable ion derivatives, and enter into the quadrupole from one end. The quadrupole produces both an electrical field and a radiofrequency at right angles, which causes the ions to oscillate in a pattern specific to the ratio of the ion's mass (m) to charge (z; which, in the experiments here, is almost always +1) ratio (Silverstein and Webster 1998). The MS can be set to detect a range of m/z ratios, where the frequencies of the electrical field and radiofrequency are varied. The MS can also be set to detect compounds with a known m/z ratio; in this case, the electrical fields and radiofrequency applied by the quadrupole are such that only the particles with the specifically set m/z ratio travel the entire length of the quadrupole without striking the poles, whereas other compounds exhibit unstable oscillations and thus are lost in the quadrupole and are not detected. The sensitivity for compounds of interest is greatly increased in the latter case (Silverstein and Webster 1998). In this thesis, GC-MS was used to measure the sequestration of FA by PLZ and PEH (Chapter 5), and to confirm the structures of the PEH analogs (Chapter 6)

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CHAPTER 2.

β-PHENYLETHYLIDENEHYDRAZINE (PEH) IS AN ACTIVE METABOLITE OF PHENELZINE (PLZ)

2.1 INTRODUCTION

Phenelzine (PLZ) is a potent, irreversible nonselective inhibitor of monoamine oxidase (MAO). PLZ has been used clinically for several decades to treat depression and anxiety disorders (panic disorder and social anxiety disorder), and was recently shown to possess neuroprotective effects in a rodent model of cerebral ischemia (Wood *et al.* 2006). Studies in the late 1960's revealed that PLZ possessed several interesting neurochemical properties in addition to its MAO-inhibiting effects; Clineschmidt and Horita (1969a, 1969b) demonstrated that PLZ was not only an inhibitor of MAO, but was also a substrate for the enzyme, and Popov and Matthies (1969) reported that PLZ dose-dependently increased brain GABA levels in rodents, an effect resulting from the inhibition of GABA-transaminase (GABA-T) . Inhibition of MAO prior to PLZ administration inhibited this GABAergic effect (Popov and Matthies 1969), which further supported the proposal that PLZ was also a substrate for MAO. The GABA-T-inhibiting properties of PLZ and the subsequent elevation in brain GABA have since been well established (Perry and Hansen 1973; Baker *et al.* 1991; McKenna *et al.* 1991; McManus *et al.* 1992; Paslawski *et al.* 1995; Todd and Baker 1995; Parent *et al.* 1999, 2000; Yang and Shen 2005; Todd and Baker 2008).

The metabolism of PLZ is, to date, far from completely understood, and is reviewed elsewhere (Baker *et al.* 1999). While β-phenylethylamine (PEA) and phenylacetic acid have been shown to be metabolites of PLZ (Baker *et al.* 1982; Dyck *et al.* 1985; Robinson *et al.* 1985), β-phenylethylidenehydrazine (PEH) also received early attention as a putative oxidation metabolite of PLZ. Although it was not definitively shown *in vivo* to be a metabolite of PLZ, *in vitro* studies suggested that PEH could be the immediate product formed from PLZ by the action of MAO (Tipton and Spires 1972; Patek and Hellerman 1974; Yu and Tipton 1989). PEH was shown to exhibit weak and transient inhibitory effects on MAO (Chessin *et al.* 1959; Patek and Hellerman 1974), although its effects on GABA were not examined in early studies.

Direct administration of PEH to rats results in an elevation of brain GABA and an inhibition of GABA-T comparable to that observed after administration of an equimolar dose of PLZ (Paslawski *et al.* 2001; MacKenzie *et al.* 2008a), and studies have confirmed that inhibition of MAO prior to PLZ administration prevents the increase in GABA (Paslawski *et al.* 1995; Todd and Baker 1995, 2008). Both PLZ and PEH increase brain levels of alanine (ALA) and ornithine (ORN) (Tanay *et al.* 2001; MacKenzie *et al.* 2008a; MacKenzie *et al.* 2008b; Todd and Baker 2008) and cause a transient reduction in brain levels of glutamine (Paslawski *et al.* 1995; Paslawski *et al.* 1998; Parent *et al.* 2000; Paslawski *et al.* 2001; Yang and Shen 2005); these effects with PLZ are also abolished in PLZ-treated animals when MAO is inhibited prior to PLZ administration (Paslawski *et al.* 1995; MacKenzie *et al.* 2008b; Todd and Baker 2008). Furthermore, both PLZ and PEH cause increases in extracellular GABA levels in the caudate-putamen in rats (Parent *et al.* 2002).

Despite the wealth of data supporting a role for PEH in the GABAergic effects (and possibly in some other neurochemical effects) of PLZ, definite evidence for the *in vivo* formation of this putative metabolite has, until now, been lacking due to the lack of availability of an analytical assay for PEH. It would be of great value to demonstrate irrefutably that PEH is the active metabolite mediating the GABAergic effects of PLZ, particularly given that the clinical use of PLZ is limited due to the fact that it is a strong irreversible inhibitor of MAO-A, and thus prone to a severe food-drug interaction with tyramine-containing foods (the so-called "cheese-effect") (Youdim and Finberg 1987). The elevation in brain GABA likely contributes to the antidepressant, anxiolytic and neuroprotective effects of PLZ, and since PEH does not appreciably affect MAO activity, it could potentially be used clinically for its GABAergic properties without the risk of hypertensive crisis.

The primary goal of the experiments described in this chapter was to demonstrate that PLZ is metabolized *in vivo* to PEH by the action of MAO. A rapid and sensitive method for the quantification of PEH in brain and liver

was developed and applied to tissues from rats treated with PEH or PLZ. To confirm that MAO was catalyzing the oxidation of PLZ to PEH, MAO was inhibited with the nonselective MAO inhibitor tranylcypromine (TCP) in another group of rats prior to PLZ administration. PEH was detected in the brains and livers of animals treated with PLZ, whereas no PEH was detected in PLZ-treated animals that had been pretreated with TCP, demonstrating clearly that PEH is a metabolite of the commonly-used antidepressant, antipanic and neuroprotective drug PLZ, and is formed by the action of MAO.

The second goal of the experiments described here was to further characterize the role of PEH formation in the changes in brain amino acid levels induced by PLZ administration. Whole brain amino acid levels were measured in animals where PLZ was oxidized *in vivo* to PEH or where the oxidation was prevented with TCP pretreatment. Amino acids were also measured in PEH-treated animals pretreated with either vehicle or TCP. Results demonstrated that all the changes in amino acid levels by PLZ were dependent on PEH formation, further supporting the postulation that PEH is an active metabolite of PLZ, and that it warrants further investigation as a therapeutic agent in its own right.

2.2 METHODS

2.2.1 Animals

For the experiments described in this chapter, as well as in Chapters 4, 5, and 6, male Sprague-Dawley rats weighing 200-400g were used. Animals were pair-housed in polycarbonate cages with free access to food (Purina Rat Chow) and water, and were maintained on a 12-hour light-dark cycle (lights on at 0700). All procedures carried out in this thesis were approved by the University of Alberta Biosciences Animal Policy and Welfare Committee and were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 Drug administration

PLZ sulfate and TCP hydrochloride were dissolved in distilled water and PEH was dissolved in corn oil. All drugs were injected intraperitoneally (i.p.), and doses reported are based on free base weights of the drugs. For the time study, rats were injected with PLZ or PEH (30 mg/kg) or their corresponding vehicle solutions (saline or corn oil, respectively) and were killed by decapitation 30 minutes, 60 minutes, 3 hours or 6 hours following drug injection. For the MAO-inhibition study and the measurement of amino acid levels, animals were pretreated with either TCP (1 mg/kg) or vehicle (saline), 1 hour later were treated with PLZ (30 mg/kg), PEH (30 mg/kg) or vehicle, and were killed by decapitation 3 hours later. Brains and livers were rapidly removed, flash-frozen in 2-methylbutane on dry ice, and stored at -80˚C until the time of PEH and amino acid analysis.

2.2.3 *Ex vivo* determination of PEH

Partially thawed tissues were homogenized in 8 volumes of ice-cold methanol, and homogenates were centrifuged for 10 minutes (4°C, 13,000 g). A portion of the supernatant (500 μl) was transferred to a glass tube and reacted with 10 μl pentafluorobenzaldehyde (PFBA). Samples were incubated in the dark at room temperature for 30 minutes, then dried under vacuum at ambient temperature and reconstituted in 200 μl cyclohexane, and after a brief centrifugation samples were injected into the gas chromatography (GC) system. The derivatization reaction of PEH with PFBA is shown in Figure 2-1. Standard curves were prepared by adding known concentrations of PEH to tubes containing naïve tissue homogenate, and were carried out simultaneously with each assay run.

Derivatized PEH was measured using an Agilent 6890 Series GC system equipped with a micro-electron capture detector (EC; Agilent) attached to a HP-5MS column (with a length of 30 m, an internal diameter of 0.250 mm, and a film thickness of 0.25 μm). The initial oven temperature was set to

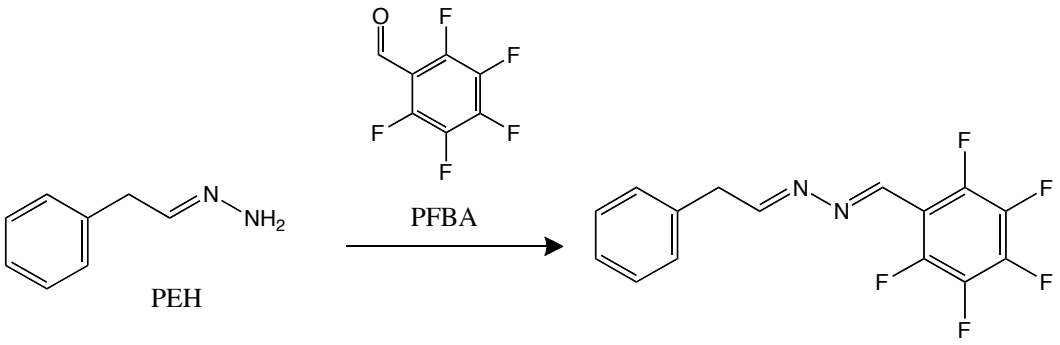

Figure 2-1. Derivatization reaction of PEH with PFBA to form a fluorinated hydrazone derivative.

80°C and was increased at 15°C/minute until a final temperature of 295°C was reached. The oven was held at 295°C for 15 minutes before returning to the initial conditions. The temperature of the injector was set at 250°C. Helium was used as the carrier gas, with argon-methane (10-90) gas used as the makeup gas at the detector.

2.2.4 *Ex vivo* determination of whole brain amino acid levels

Brain levels of the amino acids GABA, ALA, glutamate, glycine, L- and D-serine, taurine, glutamine, arginine (ARG) and aspartate were determined using a modification of a procedure previously described by Grant *et al* (2006) for the quantification of amino acids using high pressure liquid chromatography (HPLC) combined with fluorescence detection, following derivatization with fluoraldehyde reagent [*o*-phthaldialdehyde (OPA)] and isobutyryl-L-cysteine (IBLC). A 100 µl portion of brain homogenate (homogenized in 5 volumes of ice-cold ddH_2O) was added to 400 µl ice-cold methanol and centrifuged for 4 minutes at 13,000g, 4°C. The supernatants were further diluted 2-fold in ice-cold water and transferred to HPLC vials. A portion of the supernatant (5µl) was reacted with a mixture of OPA/IBLC (5µl) in the injection loop of a Waters Alliance 2690XE system for 90 seconds before injection onto the analytical column. The OPA/IBLC mixture was prepared by dissolving 1 mg OPA and 2 mg IBLC in 100 µl methanol and 900 µl 0.2M sodium borate. Samples were held at 4°C prior to derivatization. Chromatographic separation was achieved using a Waters Alliance 2695 separations module with a Symmetry C_{18} 5µm (4.6 x 150mm) column connected to a Symmetry C_{18} guard column, all held at 30°C. Mobile phase "A" consisted of 85% 0.04M sodium phosphate buffer and 15% methanol, with the pH adjusted to 6.2 with phosphoric acid. Mobile phase "B" consisted of 53.4% 0.04M sodium phosphate buffer, 44.2% methanol and 2.4% tetrahydrofuran, with the pH adjusted to 6.2 with sodium hydroxide. Both mobile phases were filtered and degassed under vacuum using 0.2 µm filters.

Initial mobile phase concentration was 85% mobile phase "A" and 15% mobile phase "B", and this composition was programmed to gradually increase over 35 minutes to reach 100% mobile phase "B," which was maintained for 10 minutes and then returned to initial conditions. The total run time was 60 minutes, and flow rate was maintained at 0.5ml/minute. Fluorescence emitted by the thioalkyl derivatives of the amino acids was detected using a Shimadzu RF10A fluorescence detector, set to an excitation wavelength of 344 nm and an emission wavelength of 443 nm. Data were collected and analyzed using the Empower Pro software package (Waters). A calibration curve consisting of varying amounts of the amino acids of interest was prepared and run simultaneously with each assay run.

2.3 RESULTS

2.3.1 PEH assay development

The derivatization and extraction of amines and hydrazines with PFBA has been reported previously (Mozayani *et al.* 1987; Riggs *et al.* 2008). In the method described here PEH was reacted with PFBA at room temperature, resulting in a fluorinated hydrazone derivative that remained stable in cyclohexane for at least 24 hours. The structure of the derivative was confirmed using combined GC-mass spectrometry (MS).

The assay was both sensitive and reproducible. In the brain, the limit of detection and of quantitation for PEH was 10 ng/g and 20 ng/g, respectively. Inter- and intra-assay variability in brain tissue, determined using 400 pg PEH (on column), were 8.12% and 11.09%, respectively. Standard curves for PEH in both brain and liver homogenates were linear up to 1000 ng/g PEH, with r^2 values consistently greater than 0.98. The retention time for PEH was approximately 11.2 minutes. A chromatogram comparing a high standard (1000 ng) and a blank standard (0 ng) is shown in Figure 2-2, and a chromatogram comparing a PEH-treated brain and a vehicle-treated brain is shown in Figure 2-3.

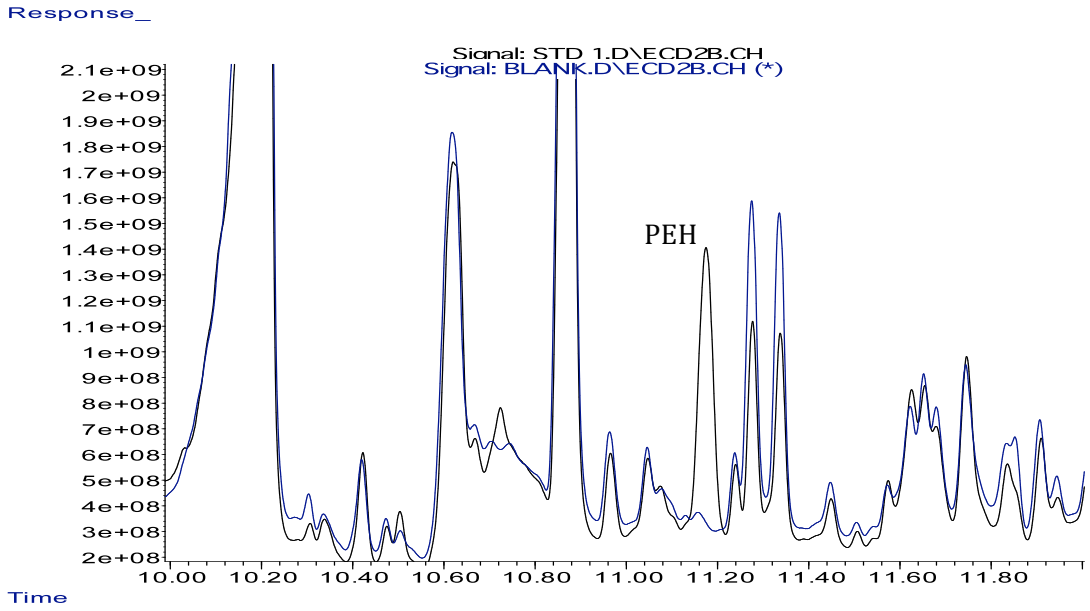

Figure 2-2. Chromatogram comparing a high standard (1000 ng/g PEH) and a non-spiked brain (0 ng/g PEH).

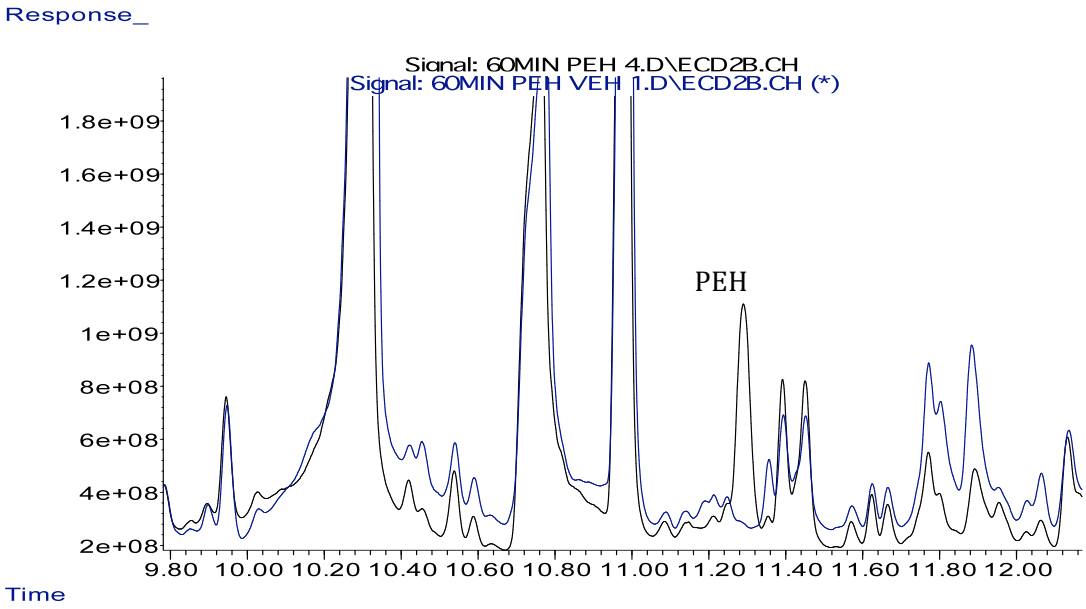

Figure 2-3 Chromatogram comparing extracts from a PEH-treated brain and a vehicle-treated brain.

2.3.2 PEH levels in brains and livers of PEH- and PLZ-treated animals

Results from PEH-treated animals indicated that PEH crossed the blood-brain barrier; brains that were collected 30 minutes after a single i.p. injection of PEH contained 4250 ± 1160 ng/g brain tissue (mean ± SEM). High concentrations of the drug were also found in the livers of these animals (5972 ± 1387 ng/g). PEH levels decreased with time in the brains and livers of PEH-treated animals, and at 6 hours following PEH administration (the last timepoint tested) PEH levels were very low in both brains and livers (139.0 ng/g and 336.2 ng/g, respectively).

Animals acutely treated with PLZ also exhibited high amounts of PEH in both brains and livers, clearly demonstrating that PLZ is metabolized *in vivo* to PEH. Interestingly, PEH levels were higher in tissues of animals treated with PLZ than in animals treated with an equimolar dose of PEH itself; brains and livers collected 30 minutes following PLZ administration contained 5719 ± 491 ng/g and 7018 ± 304 ng/g tissue (respectively). PEH levels decreased with time in the brains and livers of PLZ-treated animals; 6 hours following drug administration, PEH levels were 408 ± 62 ng/g in brains and 887 ± 212 ng/g in livers. These data are shown in Figures 2-4 and 2-5.

2.3.3 Effect of prior MAO inhibition on PEH formation in brain

Given that PEH was presumed to be formed by the action of MAO on PLZ, inhibition of MAO prior to PLZ administration was expected to inhibit the formation of PEH. Indeed, prior inhibition of MAO with TCP (using a dose previously shown to inhibit MAO by over 90%) completely prevented the formation of PEH in brains. As expected, administration of TCP prior to PEH injection did not affect brain levels of PEH. These data are shown in Figure 2-6.

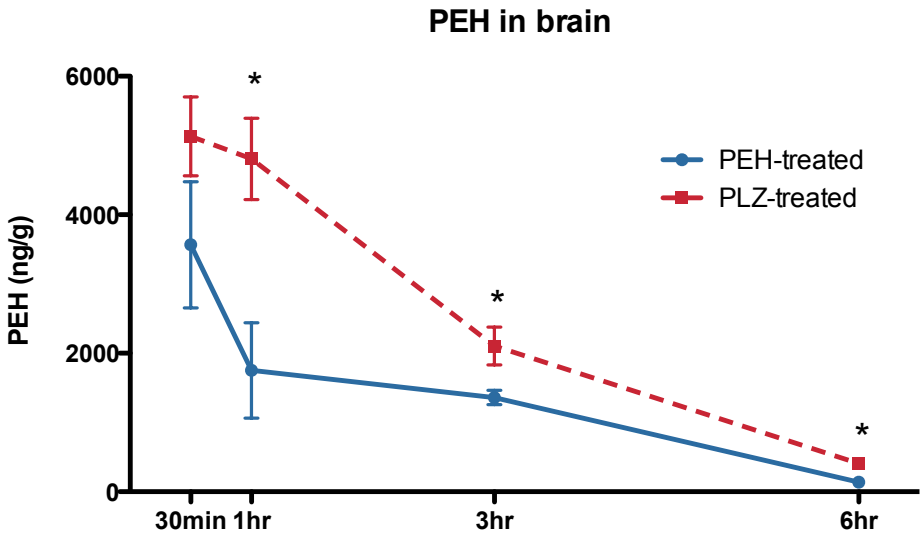

Figure 2-4. Brain levels of PEH from animals treated acutely with PEH (30 mg/kg; circles, solid line) or PLZ (30 mg/kg; squares, dashed line) at 30 minutes, 1 hour, 3 hours and 6 hours after drug administration. n = 4-6 for each group. Results are expressed as mean PEH value (ng/g brain tissue) ± SEM. * denotes a significant difference ($p < 0.05$) between drug treatments at the timepoint indicated.

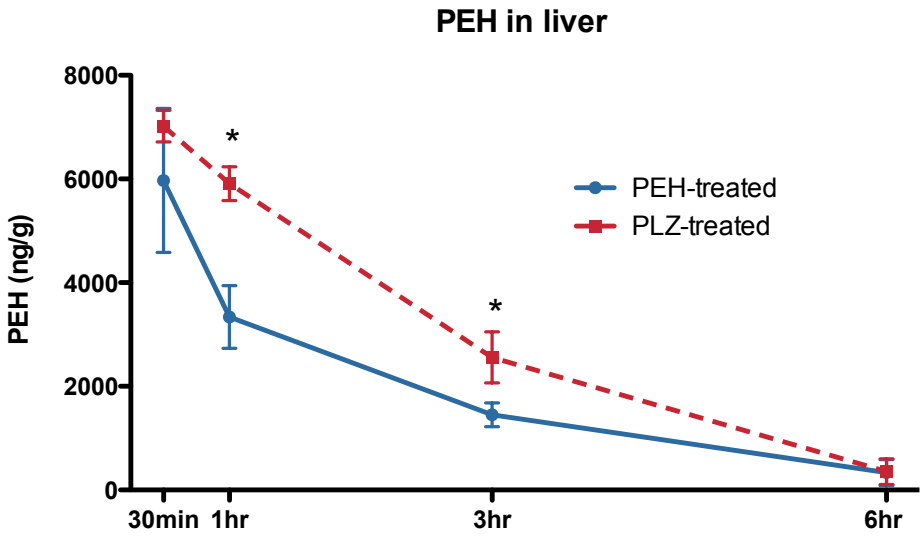

Figure 2-5. Liver levels of PEH from animals treated acutely with PEH (30 mg/kg; circles, solid line) or PLZ (30 mg/kg; squares, dashed line) at 30 minutes, 1 hour, 3 hours and 6 hours after drug administration. n = 4-5 for each group. Results are expressed as mean PEH value (ng/g liver tissue) ± SEM. * denotes a significant difference ($p < 0.05$) between drug treatments at the timepoint indicated.

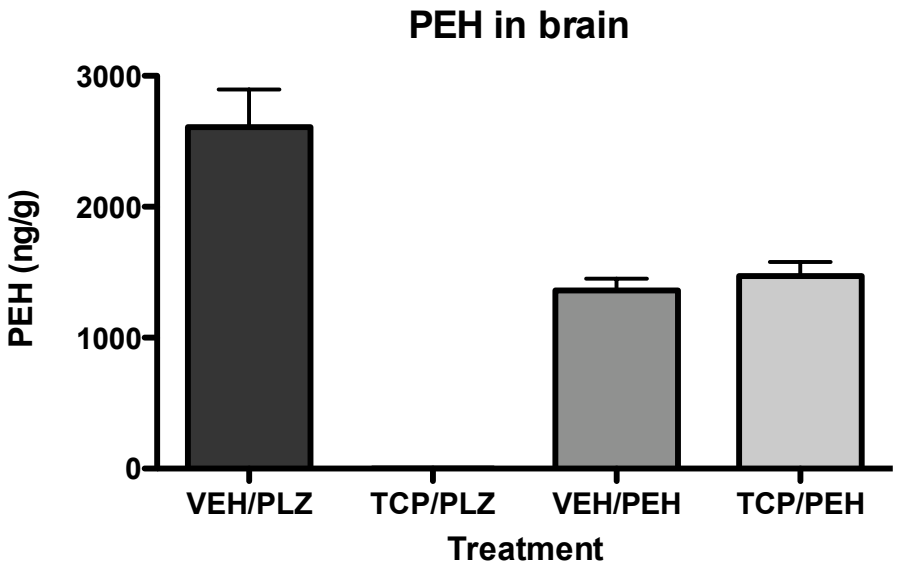

Figure 2-6. Effect of TCP pretreatment on PEH levels in PLZ- and PEH-treated brains (30 mg/kg; 3 hours). n = 4-5 for each group. Results are expressed as mean ng/g brain tissue ± SEM. PEH was not detected in TCP/PLZ-treated brains.

2.3.4 Effect of prior MAO inhibition on amino acid levels in brain

Control amino acid levels (from VEH/VEH-treated animals) are shown in Table 2-1. These values are consistent with previously reported values (Perry 1982). Amino acid values from the TCP controls (i.e. TCP/VEH) did not differ significantly from control (VEH/VEH) values (data not shown).

Amino acid levels were measured in PLZ-treated brains where PLZ was metabolized to PEH (VEH/PLZ) and where metabolism to PEH was prevented with prior administration of TCP (TCP/PLZ). Confirming previous findings, GABA and ALA were significantly elevated in VEH/PLZ treated brains (reaching 393% and 419% of control, respectively) whereas inhibition of PEH formation with TCP abolished the increase in these amino acids. Also confirming previous studies, a decrease in brain glutamine was observed with VEH/PLZ, reducing brain glutamine to 55% of control. In brains where PEH formation was inhibited by TCP pretreatment, glutamine levels were not different from vehicle-treated controls. Furthermore, brain ARG levels were significantly increased in VEH/PLZ treated brains (reaching 140% of control), and inhibition of PEH formation prevented this effect. Brain levels of aspartate, glutamate, L-serine, D-serine and taurine were not significantly altered by either VEH/PLZ or TCP/PLZ treatment. While VEH/PLZ treatment did not alter brain glycine levels, TCP/PLZ treatment caused a small but significant increase in brain glycine levels. These results are shown in Figure 2-7.

Whole brain amino acid levels were also measured in the brains of PEH-treated animals pretreated with either vehicle or TCP. Comparable increases in brain GABA, ALA and ARG to those observed with VEH/PLZ treatment were observed, increasing levels to 337%, 327% and 125% of control, respectively (although the increase in ARG did not reach statistical significance in VEH/PEH-treated brains). A decrease in brain glutamine similar to that seen in the VEH/PLZ group was also observed in the VEH/PEH-treated brains (reducing glutamine to 56% of control).

	Control amino acid values (μg/g) n = *5*
Aspartate	426.2 ± 20.0
Glutamate	1818.1 ± 136.11
L-serine	61.8 ± 4.2
D-serine	17.7 ± 2.5
Glutamine	968.2 ± 73.1
Glycine	73.8 ± 1.9
Arginine	17.4 ± 1.9
Taurine	763.7 ± 23.7
ALA	67.3 ± 7.1
GABA	245.1 ± 6.6

Table 2-1. Whole brain amino acid levels for control (vehicle-treated) brains. Values, expressed in ng/g brain tissue, are the mean ± SEM for the corresponding controls of both PLZ- and PEH-treated animals.

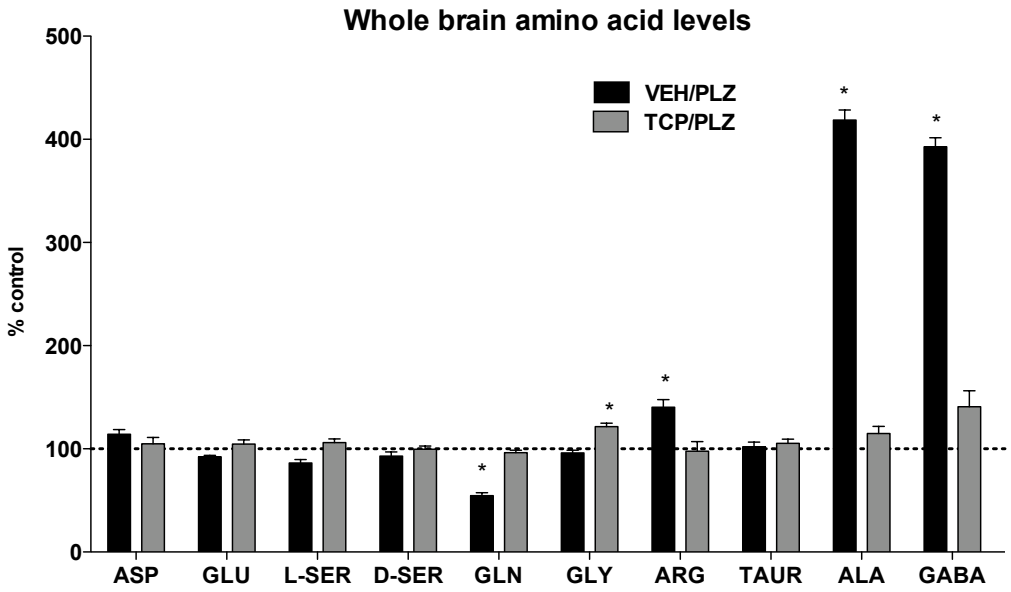

Figure 2-7. Whole brain amino acid values in PLZ-treated brains (30 mg/kg; 3 hours) pretreated with vehicle (VEH/PLZ) or TCP (TCP/PLZ). n = 5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls (VEH/VEH).

Additionally, glutamate and D-serine were significantly reduced in VEH/PEH-treated brains, decreasing to 86% and 80% of control, respectively. As expected, TCP pretreatment of PEH-treated animals did not alter amino acids compared to VEH/PEH values. Whole brain aspartate, L-serine, glycine and taurine levels in PEH-treated brains were not significantly different from controls. These data are shown in Figure 2-8.

2.4 DISCUSSION

The results presented here provide, for the first time, solid evidence that the antidepressant and antipanic drug PLZ is metabolized *in vivo* to an active metabolite, PEH. The formation of this metabolite appears to be essential for some of PLZ's neurochemical effects, some of which (particularly the elevation in brain GABA levels) might mediate some therapeutic properties of PLZ. These results suggest that PEH may be useful as a GABAergic therapeutic agent in its own right. As mentioned previously, it has the advantage over PLZ in that diet would not have to be controlled for fear of the "cheese effect."

2.4.1 PLZ is metabolized to PEH *in vivo*

The most significant finding of the experiments described here is the unequivocal demonstration that PEH is formed *in vivo* from PLZ by the action of MAO. While it has been hypothesized for some time that PEH is the active metabolite of PLZ, definitive evidence for the formation of PEH has been lacking because attempts to develop an analytical assay for PEH have until now been unsuccessful. Derivatives of PEH studied thus far appear to be unstable under aqueous conditions, and PEH itself appears rapidly broken down in the presence of heat. In the method described here, homogenization of brain and liver tissue in methanol and derivatization with PFBA at room temperature ensured stability.

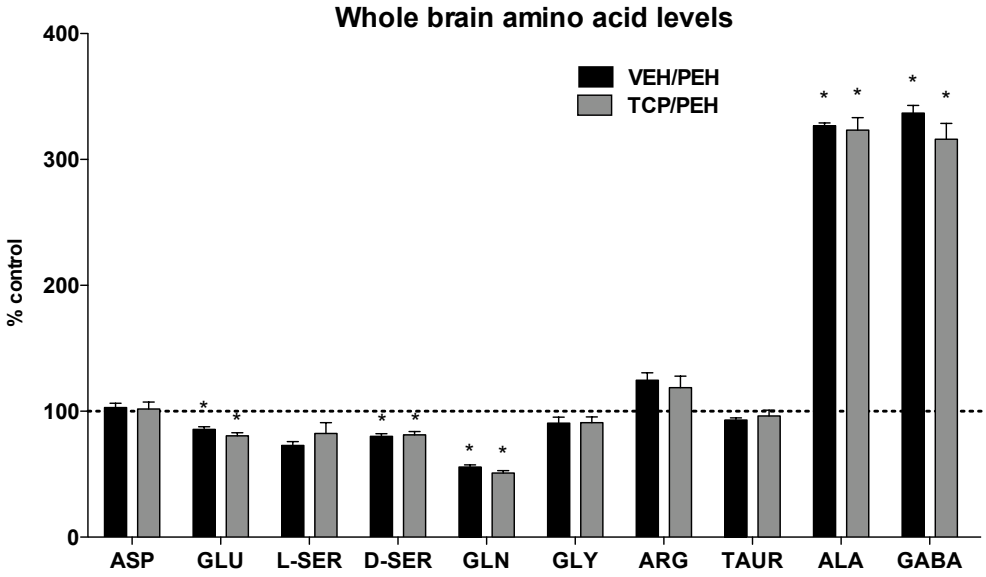

Figure 2-8. Whole brain amino acid values in PEH-treated brains (30 mg/kg; 3 hours) pretreated with vehicle (VEH/PEH) or TCP (TCP/PEH). n = 5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls (VEH/VEH).

Application of this procedure to tissues of rodents treated with PLZ revealed that the formation of PEH from PLZ occurred rapidly; 30 minutes after PLZ administration large amounts of PEH were present both in the brain and liver. Six hours after PLZ administration, most of the PEH was metabolized and/or cleared from the brain and liver. Recently it was suggested that PEH may be an intermediate in the metabolism of PLZ to phenylacetic acid (Binda *et al.* 2008). Further studies are necessary to confirm or refute this theory, and these are now underway in the Neurochemical Research Unit; preliminary findings indicate that administration of PEH to rats does result in marked increases in brain levels of phenylacetic acid (Banasch, MacKenzie, Rauw and Baker, unpublished data observations). Importantly, inhibition of MAO with TCP prior to PLZ administration abolished the formation of PEH, confirming speculations that MAO catalyzes the metabolism of PLZ to PEH. Given previous work demonstrating that selective inhibition of either MAO-A or -B prior to PLZ administration both lead to significant reduction of the GABA-T inhibitory effect and elevation of brain GABA by PLZ (Todd and Baker 1995), it is likely that both MAO isoforms are involved in the oxidation of PLZ to PEH.

2.4.2 PEH formation mediates a number of PLZ's neurochemical effects

The results reported here confirm previous speculation that PEH formation mediates the acute GABAergic effects of PLZ. Given that inhibition of MAO prior to PLZ administration prevents the elevation of brain GABA (Todd and Baker 1995, 2008) and prevents the formation of PEH, together with the finding that PEH itself inhibits GABA-T and elevates brain GABA (Paslawski *et al.* 2001; MacKenzie *et al.* 2008a), it seems evident that PEH is the GABAergic compound rather than PLZ. Interestingly, while PEH levels are very high early after PEH or PLZ administration, the GABAergic effects of both drugs peak later; the elevation in brain GABA is maximal at approximately 3-6 hours following PLZ or PEH administration (Baker *et al.* 1991; Paslawski *et al.* 2001; MacKenzie *et al.* 2008a). The inhibition of GABA-

T by PEH also appears to be quite long-lasting, given that a single administration of PLZ at 30 mg/kg (the dose used here) produced an increase in brain GABA that was still significantly greater than control GABA levels 24 hours later (Parent *et al.* 2000). Hydrazines such as PLZ and PEH are thought to inactivate GABA-T via the covalent binding of their hydrazine moiety to pyridoxal-5'-phosphate (PLP) (Yamada *et al.* 1993; Lightcap *et al.* 1995; Lightcap and Silverman 1996). Consistent with this, decreased plasma levels of the active form of vitamin B6 (which is PLP) have been reported in patients taking PLZ (Malcolm *et al.* 1994) (but see Lydiard *et al.* 1989, who did not report any changes in plasma PLP after PLZ administration).

PEH also mediates other neurochemical effects of PLZ aside from those on the GABAergic system. Confirming previous findings (Todd and Baker 2008), PEH appears to mediate the marked increase in brain ALA observed following PLZ administration. This is not surprising since ALA is metabolized by the PLP-dependent enzyme ALA-T, and PEH likely inhibits the activity of ALA-T as it does GABA-T. Also consistent with previous work (Paslawski *et al.* 1995), the transient decrease in glutamine induced by PLZ appears to be mediated by PEH formation, since inhibition of PEH formation completely reversed the decrease in glutamine observed, and direct administration of PEH itself produced a decrease in brain glutamine. Glutamine is a precursor and a metabolite of the excitatory amino acid glutamate, and a functional decrease in glutamatergic transmission may be reflected here by the decrease in whole brain glutamine levels, given that it has been shown *in vivo* using $^{1}H/[^{13}C]$-MRS that a decrease in brain glutamine induced by PLZ was accompanied by a decrease in glutamine-glutamate cycling flux between neurons and glia (Yang and Shen 2005), and that chronic PLZ administration decreased the KCl-evoked release of glutamate from cortical brain slices (Michael-Titus *et al.* 2000). PEH itself decreased whole brain glutamate levels in the present study, an effect that was also observed with PLZ by Yang and Chen (2005), but this decrease seems to be transient and inconsistent (Parent *et al.* 2000; MacKenzie *et al.* 2008a). The decrease in D-serine

induced by PEH administration would also be expected to contribute to a reduction in glutamatergic transmission, given the role of this amino acid as a potent co-agonist at the glycine binding site of the glutamate NMDA receptor (Miller 2004; Mustafa *et al.* 2004; Schell 2004). It is known that D-serine is formed from L-serine by a PLP-dependent amino acid racemase (Yoshimura and Goto 2008), and it is conceivable that PEH inhibits this enzyme; studies on this possibility are now underway in the Neurochemical Research Unit.

Finally, PLZ increased brain levels of ARG, an effect that was also prevented by inhibition of PEH formation. To our knowledge, this is the first study to report an alteration in brain ARG by PLZ. PEH itself also appeared to modestly increase brain ARG, although this latter effect did not reach statistical significance. Administration of PLZ results in greater PEH levels in the brain than direct administration of PEH itself, however, which could explain why the increase in brain ARG induced by PEH was not as marked as that observed following PLZ administration. Furthermore, ARG was significantly increased 6 hours after administration of PEH at the same dose used here (Chapter 6). Further dose- and time- response studies are warranted to further clarify this interesting effect of PLZ and PEH on brain ARG levels, particularly since ARG is a precursor of nitric oxide (NO), an important neuromodulator in the brain and cardiovascular system. Interestingly, plasma levels of ARG and metabolites of NO were recently reported to be low in depressed patients and in preliminary studies the antidepressant paroxetine increased levels of ARG (Chrapko *et al.* 2006; Hess and Le Melledo, unpublished).

2.4.3 PEH may be an effective therapeutic agent in and of itself

PEH could be an effective therapeutic agent in its own right. High concentrations of PEH were measured in the brain 30 minutes after its administration, indicating that it crosses the blood-brain barrier readily. PEH has minimal effects on MAO activity (Paslawski *et al.* 2001; MacKenzie *et al.* 2008a), and therefore the potentially fatal food-drug interaction that occurs

with PLZ and certain foodstuffs would not be a problem. Importantly however, like PLZ, PEH increases brain GABA levels , an effect that could be important in a number of neuropsychiatric disorders.

Due to its GABA enhancing properties, PEH could potentially be useful for the treatment of conditions that exhibit deficits in GABAergic transmission, such as depression, anxiety, epilepsy, or ischemia. Accumulating evidence suggests that a decrease in GABAergic function may be associated with depression, and that restoration of GABAergic transmission is associated with amelioration of symptoms (Petty 1995; Brambilla *et al.* 2003; Sanacora *et al.* 2003; Bhagwagar *et al.* 2004; Sanacora *et al.* 2005). Interestingly, the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and norfluoxetine were shown to be effective in treating depressive-like symptoms in rodents at doses that were insufficient to inhibit serotonin reuptake but that increased brain levels of allopregnanolone, a neurosteroid that positively modulates $GABA_A$ receptor activity (Pinna *et al.* 2006). Interestingly, the SSRIs have also been shown using magnetic resonance spectroscopy techniques to increase levels of GABA in some brain areas (Sanacora *et al.* 2002). These results suggest that facilitation of GABAergic transmission alone could potentially be sufficient to produce antidepressant effects, supporting the idea that PEH itself could be an effective antidepressant agent.

The role of increased GABAergic transmission in the treatment of anxiety disorders is well established, suggesting that PEH itself could also be effective in the treatment of anxiety-related disorders. The currently-used pharmacological treatments for anxiety disorders (which include benzodiazepines and SSRIs) involve a facilitation of GABAergic transmission, and the ongoing search for novel anxiolytic agents has focused on compounds that increase GABAergic function (Rupprecht *et al.* 2006). Taking into account the fact that PLZ has been shown to exert anxiolytic effects in rodents on the elevated-plus maze only at doses that increase brain GABA levels (Paslawski *et al.* 1996) and that the elevation in brain GABA induced

by PLZ is mediated by PEH formation (current results described in this thesis), it is likely that PEH itself would be an effective anxiolytic agent.

Given that many currently-used anticonvulsant drugs are GABAergic agents (Gibbs and McNamara 2006), PEH could also be an effective candidate for the management of epilepsy. Consistent with this, PEH was shown to reduce epileptiform activity in hippocampal neuronal *in vitro* (Duffy *et al.* 2004). Moreover, PLZ was recently reported to be more potent than vigabatrin (a currently prescribed anticonvulsant drug that inhibits GABA-T) at elevating brain GABA levels (Todd and Baker 2008), further supporting the conjecture that PEH should be an effective anticonvulsant agent (since from the results presented here it is clear that the GABA-elevating properties of PLZ are due to PEH formation).

Finally, PEH might be an interesting therapeutic option for the prevention of neuronal damage observed following cerebral ischemia given that a significant decrease in the brain GABA/glutamate ratio is observed during the time where excitotoxicity is believed to mediate ischemic neuronal death (Shuaib *et al.* 1997; Yang *et al.* 2001), PEH-mediated increases in GABA may counteract the excitotoxicity and result in neuroprotective actions. PEH administration has indeed been shown to reduce ischemic neuronal damage in a gerbil model of global ischemia (Tanay *et al.* 2002), as have other GABA-enhancing agents. This potential therapeutic effect of PEH also warrants further investigation.

Assuming that PEH does in fact decrease glutamatergic function, this effect could also lead to a number of therapeutic outcomes directly, as well as indirectly from decreased opposition of GABAergic function. Accumulating evidence suggests that glutamatergic dysregulation plays a role in the pathophysiology of depression (reviewed in Sanacora *et al.* 2008) and anxiety disorders (reviewed in Bergink *et al.* 2004), and the reduction of glutamatergic neurotransmission is being pursued in the development of novel pharmacological agents for the treatment of both depressive and anxiety disorders. Moreover, a decrease in the neurotransmission of

excitotoxic glutamate by elevated brain GABA could contribute to the neuroprotection by PEH observed after cerebral ischemia in rodents, since excitotoxicity is known to play a significant role in ischemic neuronal death (Juurlink and Sweeney 1997).

ARG is metabolized to a number of molecules important for cell signaling, including ORN (which can be further metabolized to glutamate or to polyamines), nitric oxide (NO), and agmatine (Wu and Morris 1998) (which may function as a neuromodulator or a neurotransmitter during physiological stress, and may possess therapeutic effects (Halaris and Plietz 2007)). Changes in brain levels of ARG could, therefore, have a number of important downstream consequences. Changes in NO production, for example, may be relevant for the antidepressant effects of PLZ, since clinical studies have suggested that patients with depression may exhibit a deficiency in NO production, and the SSRI paroxetine was shown to normalize NO metabolite levels in such patients (Chrapko *et al.* 2004; Chrapko *et al.* 2006). Alternatively, agmatine has been shown to possess antidepressant, anxiolytic and neuroprotective effects in rodents (Gilad *et al.* 1996; Olmos *et al.* 1999; Zomkowski *et al.* 2002; Aricioglu and Altunbas 2003), and thus alterations in the ARG-agmatine pathway could also lead to clinical effects. This is the first report to demonstrate that PLZ increases brain ARG, and the mechanism of this effect warrants further investigation.

2.4.4 Future considerations

A common theme throughout this chapter has been that PEH warrants consideration as a therapeutic agent. Now that an analytical assay is available for the measurement of PEH, many important questions can be addressed. One critical question that remains is whether PEH continues to be produced during chronic PLZ treatment. It seems logical that chronic PLZ treatment would result in the complete inhibition of MAO, thus abolishing the formation of PEH. However, brain GABA levels in rats remain elevated during chronic PLZ treatment (McManus *et al.* 1992; McKenna *et al.* 1994; Baker *et al.*,

unpublished), and given that PLZ itself does not increase brain GABA, it seems reasonable to suggest that PEH somehow continues to be produced. Chronic PLZ administration to rats may not produce complete (100%) inhibition of both MAO-A and -B (Urichuk *et al.* 2000), and it is possible that a small amount of MAO remaining active may be sufficient to generate enough PEH to produce its GABAergic (and other) neurochemical effects. Alternatively, it is possible that other enzyme systems may contribute to the oxidation of PLZ. This matter, along with many others, unquestionably warrants exploration.

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CHAPTER 3.

IN VITRO INHIBITION OF GABA-TRANSAMINASE BY PLZ AND PEH

3.1 INTRODUCTION

The monoamine oxidase inhibitor (MAOI) phenelzine (PLZ) is metabolized to β-phenylethylidenehydrazine (PEH) *in vivo* (Chapter 2). The formation of PEH was shown to be mediated by the action of MAO, given that it was not detected in the brains of PLZ-treated rodents that were pretreated with the nonselective MAOI tranylcypromine (TCP; Chapter 2). Inhibition of MAO prior to PLZ administration has also been shown to completely abolish the well-established inhibitory effects of acute PLZ administration on the activity of GABA-transaminase (GABA-T), preventing the marked increase in brain GABA normally evoked by PLZ (Popov and Matthies 1969; Perry and Hansen 1973; Todd and Baker 1995, 2008). These data, together with the finding that PEH itself inhibits GABA-T and causes increases in brain GABA (Paslawski *et al.* 2001), suggest that the GABA-elevating properties of acute PLZ administration are mediated by PEH.

PLZ and PEH not only inhibit GABA-T activity *in vivo*, but also do so *in vitro*. It is reasonable to assume that, as in the *in vivo* situation, the formation of PEH mediates the PLZ-induced GABA-T inhibition *in vitro* and that inhibition of MAO *in vitro* prior to PLZ application would prevent the formation of PEH and thus prevent the inhibition of GABA-T. An important difference between the two experimental conditions, however, is that the GABA-T inhibition induced by PLZ and PEH *in vivo* does not exceed approximately 50%, even at high drug doses (Popov and Matthies 1969; unpublished observations). Conversely, both PLZ and PEH inhibit GABA-T in a concentration-dependent manner *in vitro*, and almost complete GABA-T inhibition can be achieved ((Yu and Boulton 1991); unpublished observations). The reason for this discrepancy is not known.

The study described in this chapter was undertaken to determine whether inhibiting MAO *in vitro* would, in turn, prevent the GABA-T inhibition normally observed after application of PLZ (as is observed *ex vivo*). It was hypothesized that, despite differences in the degree of GABA-T

inhibition induced by PLZ and PEH *in vivo* and *in vitro*, that inhibition of MAO would nevertheless abolish the GABA-T inhibition normally produced by PLZ. PEH-induced GABA-T inhibition (and the effect of prior inhibition of MAO) was also assayed, with the assumption that prior inhibition of MAO would not affect PEH-mediated GABA-T inhibition.

3.2 METHODS

A flowchart summary of the methods for this chapter is shown in Figure 3-1. Since the aim of the studies described here was to establish whether *in vitro* inhibition of MAO prior to PLZ application abolishes the GABA-T inhibiting effects, preliminary experiments were first conducted to determine the minimum concentrations of two irreversible nonselective MAO inhibitors, TCP and pargyline (PAR) required to inhibit MAO-A and -B by 100%, along with concentrations of PLZ and PEH that inhibited GABA-T activity by 50%. MAO was inhibited *in vitro* prior to PLZ or PEH application, and GABA-T activity was subsequently determined. The details are described below.

3.2.1 *In vitro* determination of MAO inhibition by TCP and PAR

The minimum concentration of the MAO inhibitors TCP and PAR required to completely inhibit MAO was determined. A range of drug concentrations (10^{-3}M to 10^{-6}M) was applied to naïve brain homogenates and the lowest drug concentrations of TCP and PAR that inhibited both MAO-A and -B by 100% were used in subsequent experiments.

MAO activity was determined in triplicate using a modified protocol described by Lyles and Callingham (1982). Naïve brain homogenate (in 5 volumes of ddH_20) was further diluted by a factor of 9 in 0.2M KH_2PO_4 (pH 7.8). In borosilicate glass culture tubes on ice, 25µl of homogenate and 25µl of the appropriate drug concentration of TCP or PAR prepared in KH_2PO_4 (or

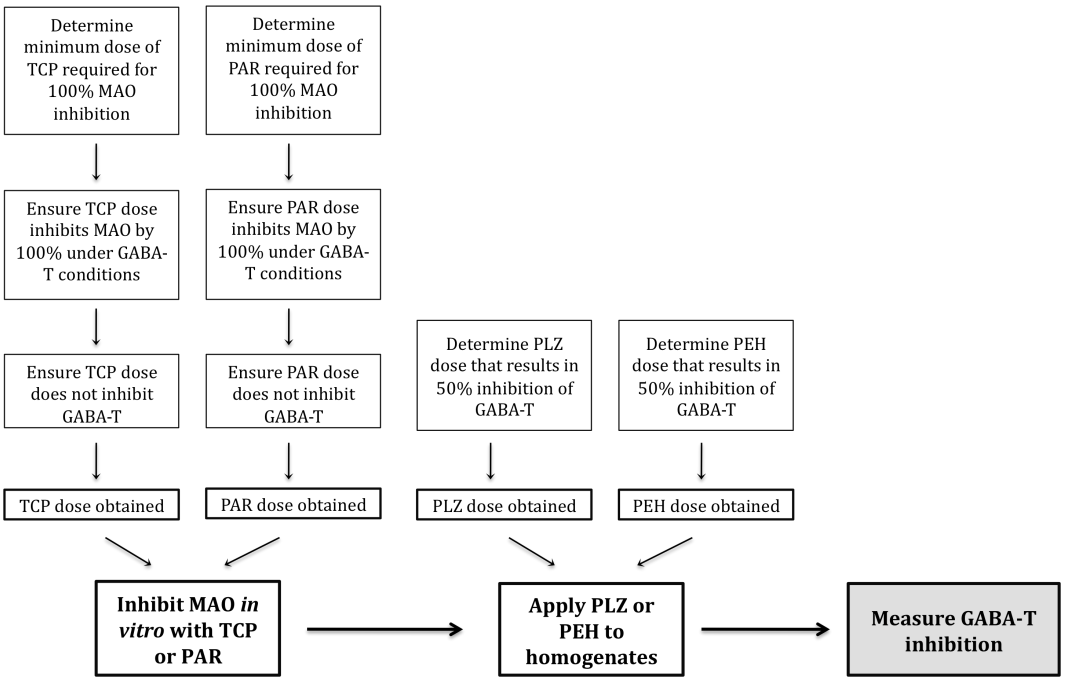

Figure 3-1. Flowchart summary of the procedures in Chapter 3.

KH_2PO_4 alone for controls) were added. To blanks, 10 μl 3M hydrochloric acid was also added to prevent the reaction from proceeding. Following a 10 minute incubation at 37°C, tubes were placed on ice and the appropriate radiolabelled substrate was added. Tubes were flushed with O_2, stoppered quickly and incubated again for 10 minutes at 37°C. Tubes were subsequently placed on ice and 10μl 3M HCl were added to the rest of the tubes. After the addition of 1 ml ethyl acetate/toluene (1:1 v/v, water-saturated), tubes were vortexed briefly and centrifuged at low speed (470 g) for 30 seconds. A portion of the organic layer (700 μl) was transferred into a scintillation vial containing 4 ml scintillation fluid (Beckman Coulter ReadySafe© liquid scintillation cocktail) and 50 μl glacial acetic acid, and radioactivity (dpm) was counted using a liquid scintillation counter (Beckman LS 7500).

Radiolabelled 5-HT (5-hydroxy[G-^{3}H]tryptamine creatinine sulfate) and PEA (β-phenyl[1-^{14}C]ethylamine chloride) were used as substrates for MAO-A and MAO-B, respectively. Due to the high specific activity of the stock radiolabelled substrates, stocks were diluted with their respective unlabelled amines, giving final dilutions of 50 mM, 1 μCi/μmol for 5-HT and 10 mM, 1 μCi/μmol for PEA. These diluted stocks were further diluted in 0.2M potassium phosphate (1/100) for the assay.

3.2.2 *In vitro* determination of MAO inhibition by TCP and PAR under GABA-T assay conditions

Given that the ultimate goal of the experiments described here was to determine the effects of prior MAO inhibition on the ability of PLZ and PEH to inhibit GABA-T, it was necessary that MAO be inhibited *in vitro* by TCP or PAR under the GABA-T assay conditions rather than the optimized MAO assay conditions. The differences in the conditions of the assays are subtle,

but to ensure that the selected concentrations of TCP and PAR completely inhibited MAO under the GABA-T assay conditions (as they did under the MAO conditions), MAO activity was measured using the buffers, incubation media and volumes used in the GABA-T assay.

In borosilicate glass culture tubes on ice, 10 μl naïve brain homogenate homogenized in 5 volumes ice-cold ddH_20 and further diluted by a factor of 2 in GABA-T homogenizing medium (described in Section 3.2.3), 15 μl GABA-T incubation medium and 5μl of the appropriate drug concentration prepared in incubation medium (or incubation medium alone for controls) were mixed. 3M HCl (5μl) was added to blank tubes. Tubes were incubated for 10 minutes at 37°C, followed by the addition of 30 μl of the appropriate MAO radiolabelled substrate to each tube. After a second incubation (10 minutes, 37°C), tubes were placed on ice and 5 μl HCl (3M) was added to the rest of the tubes. Ethyl acetate/toluene (1:1 v/v, water-saturated; 600 μl) were added to each tube, and after vortexing and centrifuging, 300μl of the top layer were added to 4ml of scintillation fluid. Radioactivity (dpm) was counted in a scintillation counter.

3.2.3 *In vitro* determination of GABA-T inhibition by TCP and PAR

To ensure that the inhibition of GABA-T by PLZ or PEH was not affected by prior administration of TCP or PAR, the selected concentrations of TCP and PAR (Section 3.2.2) were tested for their potential to inhibit GABA-T activity. GABA-T activity was measured in triplicate using a modified method described by Sterri and Fonnum (1978). Naïve rat brains homogenized in 5 volumes of ice-cold ddH_2O were further diluted by a factor of 2 in homogenizing medium (described below). In microfuge tubes on ice, 10 μl of tissue homogenate (or 10 μl of ddH_20 for blanks) were added to 15 μl of incubation medium and 5 μl of TCP or PAR prepared in incubation medium (or incubation medium alone for controls). Tubes were incubated at 37°C for 10 minutes, and then returned to the ice bath where 2ul of ddH_20 was added.

After a second 10 minute incubation at 37°C, tubes were placed on ice for 5 minutes to ensure the contents of the tubes were completely cooled, and 5 μl of ^{3}H-mixture were added to each tube. After a 30 minute incubation at 37°C, the reaction was terminated by placing the microfuge tubes on ice and adding 100 μl of the previously-prepared liquid anion exchanger tri-n-octylamine (TOA; preparation described below). The tubes were capped, vortexed and centrifuged at 4°C for 2 minutes at 13,000 g. Following centrifugation, 35μl of the organic layer were retained and added to scintillation vials containing 4ml of scintillation fluid. Radioactivity (dpm) was counted for 3 minutes using a liquid scintillation counter.

The homogenizing medium was composed of 40% volume/volume (v/v) glycerol, 2% v/v Triton X-100, 2 mM glutathione, 2 μM pyridoxal phosphate, 2 mM sodium ethylenediaminetetraacetic acid (NaEDTA) and 10 mM K_2HPO_4 in ddH_2O. The pH was adjusted to 7.4 using glacial acetic acid. The incubation medium was composed of 263 μM Tris buffer, 5 mM α-ketoglutarate, 1 mM NAD and 1 mM (AET). The ^{3}H-mixture was composed of 8 μl ^{3}H-GABA (8μCi), and 1.4 mM non-radiolabelled GABA in 232 μl ddH_20. The TOA was prepared as follows: 2.2 ml of TOA combined with 22.8 ml of ethyl acetate was added to 49.2 ml of ddH_2O and 850 μl phosphoric acid. The resultant mixture was shaken for 4 minutes and centrifuged at 13000 g for 10 minutes, and the organic layer was retained.

3.2.4 Determination of PLZ and PEH concentrations resulting in 50% GABA-T inhibition

Concentration-inhibition curves were generated for PLZ and PEH to determine the concentration of each drug that would inhibit GABA-T by 50%. GABA-T activity was determined using the same procedure described above, except that for the first incubation the tissue homogenate was incubated with 20 μl incubation medium, and a second 10-minute incubation was added, where 2 μl of PLZ or PEH solutions (or 2 μl of H_2O or DMSO for controls,

respectively) were added to the homogenate and incubation medium. The rest of the procedure was carried out as described above. From the curves generated, the concentrations of each drug required to inhibit GABA-T by 50% were calculated. Sigmoidal concentration-response curves were fitted to data obtained using GraphPad Prism® software.

3.2.5 Determination of GABA-T activity following MAO inhibition by TCP or PAR and subsequent application of PLZ or PEH

After the appropriate drug concentrations of TCP, PAR, PLZ and PEH were determined, these concentrations were applied to the final *in vitro* procedure. The inhibition of GABA-T was determined in the manner described in Section 3.2.3, the first incubation including homogenate, incubation medium and either TCP, PAR or vehicle (VEH; incubation medium), and the second incubation involving the addition of 2μl PLZ, PEH or VEH (H_2O or DMSO, respectively).

3.3 RESULTS

3.3.1 Selection of appropriate TCP and PAR concentrations

TCP and PAR at concentrations of 10^{-3}M to 10^{-6}M were tested for their abilities to completely inhibit MAO-A and -B. Both TCP and PAR required a minimum concentration of 10^{-5}M to inhibit MAO by 100%. Under the GABA-T assay conditions, 10^{-5}M TCP also inhibited MAO by 100% (98% and 101% inhibition of MAO-A and -B, respectively); however 10^{-5}M PAR inhibited MAO-A and -B by 77% and 100% of control, respectively, and therefore a greater PAR concentration was selected to ensure the complete inhibition of MAO under GABA-T conditions. At 10^{-4}M, PAR inhibited MAO-A and -B by 99% and 101% (respectively) under GABA-T conditions. TCP (10^{-4}M and 10^{-5}M) and PAR (10^{-4}M) were subsequently used to inhibit the formation of PEH; two doses of TCP were used to ensure that complete MAO inhibition, and not some unknown drug interaction, was mediating the results. Alone,

TCP and PAR at the selected concentrations did not significantly inhibit GABA-T activity (data not shown).

3.3.2 Determination of PLZ and PEH concentrations resulting in 50% GABA-T inhibition

Concentration-inhibition curves for PLZ and PEH were generated by plotting the logarithm (log) of the PLZ or PEH concentration on the x-axis versus the % GABA-T inhibition on the y-axis. These curves are shown in Figure 3-2.

To determine the drug concentrations that inhibited GABA-T by 50%, a simple calculation for EC_{50} value was not appropriate because, by definition, the EC_{50} refers to the concentration of drug that evokes a response halfway between the baseline (bottom of the curve) and the maximum response (top of the curve) (Kenakin 2004). Since the top of the curves for PLZ and PEH did not reach 100% inhibition, the EC_{50} value would give a drug concentration inhibiting GABA-T by 50% of the inhibitor potential, but corresponding to an absolute GABA-T inhibition less than 50%. Therefore, the concentrations of PLZ and PEH required to inhibit enzyme activity by 50% were determined by first calculating the percentage of the maximum GABA-T inhibition possible by each drug corresponding to 50% absolute GABA-T inhibition, using Equation 3.1 (where *F* represents the percentage of possible inhibition for PLZ or PEH corresponding to 50% absolute inhibition of GABA-T).

$$50\% \text{ absolute inhibition} = F = (50 / (\text{span}) \times 100) \qquad \text{[Equation 3.1]}$$

For PLZ, for example, the maximum point on the curve was 87.92 and the minimum point was -0.645 (and the span of the curve was 88.56) therefore PLZ inhibited GABA-T by 50% at 56.46% of PLZ's maximum GABA-T inhibition:

$$50\% \text{ inhibition } (F) = (50 / 88.56) \times 100 = 56.46$$

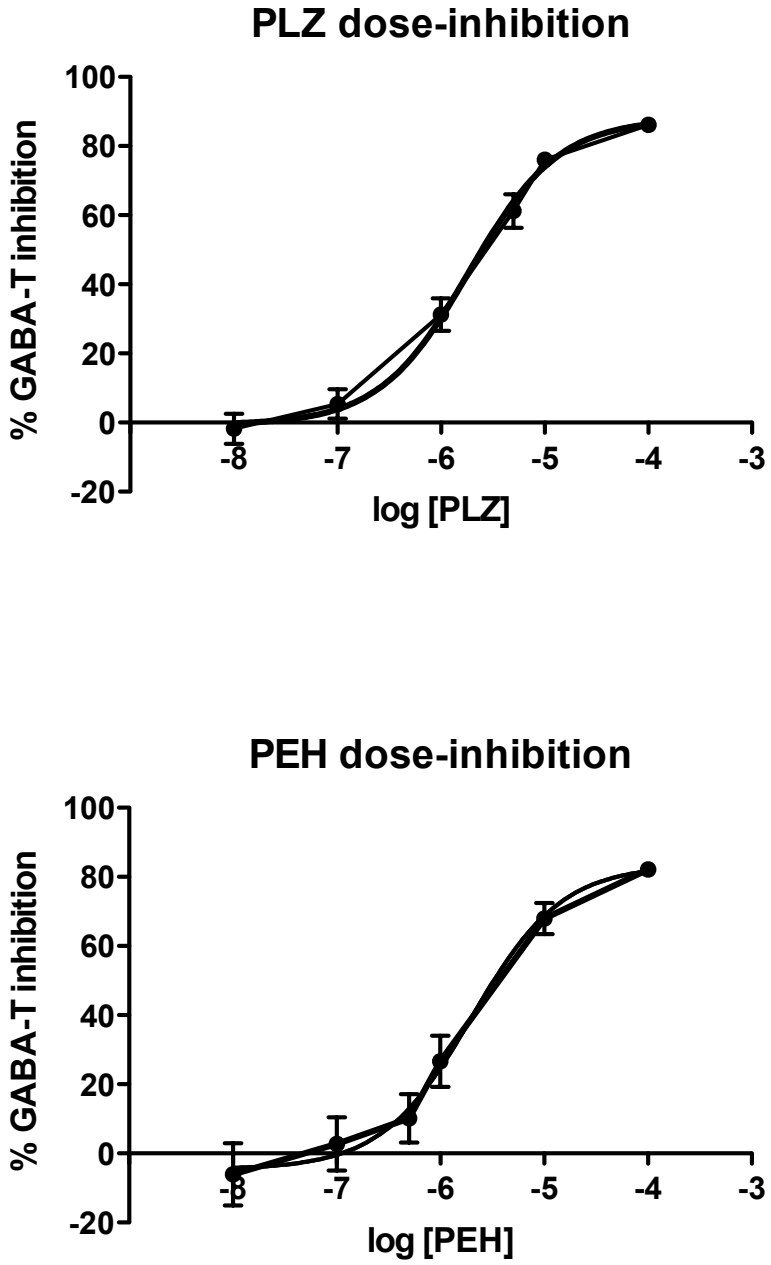

Figure 3-2. Concentration-inhibition curves for PLZ (top) and PEH (bottom) for GABA-T *in vitro*. Each point represents mean ± SEM (n = 4-5).

Rather than calculating the EC_{50} for PLZ, the $EC_{56.46}$ (that is, the concentration that inhibits GABA-T by 56.46% of PLZ's potential inhibition) was calculated using Equations 3.2 and 3.3.

$$\log EC_{50} = \log EC_F - (1/\text{HillSlope}) \times \log (F/(100-F)) \qquad \text{[Equation 3.2]}$$

$$Y = \text{bottom} + (\text{top-bottom}) / (1+10^{(\log EC\ 50 - X) \times \text{HillSlope}}) \qquad \text{[Equation 3.3]}$$

In Equations 3.2 and 3.3, *F* is the percent of the maximal inhibition of PLZ or PEH corresponding to an absolute GABA-T inhibition of 50%, Y is the response (% inhibition) and X is the logarithm of the drug concentration.

Under the assay conditions used in these experiments, it was determined using the formulas above that PLZ and PEH at concentrations of 2.4×10^{-6} M and 2.6×10^{-6} M (respectively) were required to inhibit GABA-T activity by 50%. These concentrations were applied to subsequent GABA-T inhibition studies.

3.3.3 Inhibition of GABA-T by PLZ and PEH after inhibition of MAO with TCP and PAR

GABA-T inhibition was measured in VEH-pretreated and MAOI-pretreated homogenates after the application of either PLZ or PEH. As expected, application of PLZ or PEH to homogenates pretreated with VEH resulted in approximately 50% GABA-T inhibition.

When PLZ was applied to TCP- or PAR-pretreated homogenates, the inhibition of GABA-T was significantly reduced compared to the inhibition observed when the drugs were applied to VEH-treated (control) homogenates, but surprisingly was not completely abolished. In homogenates where MAO was inhibited by TCP at 10^{-4}M or 10^{-5}M and by PAR at 10^{-4}M, GABA-T inhibition by PLZ was reduced to 25%, 23% and 24% inhibition, respectively. These data are shown in Figure 3-3. As expected,

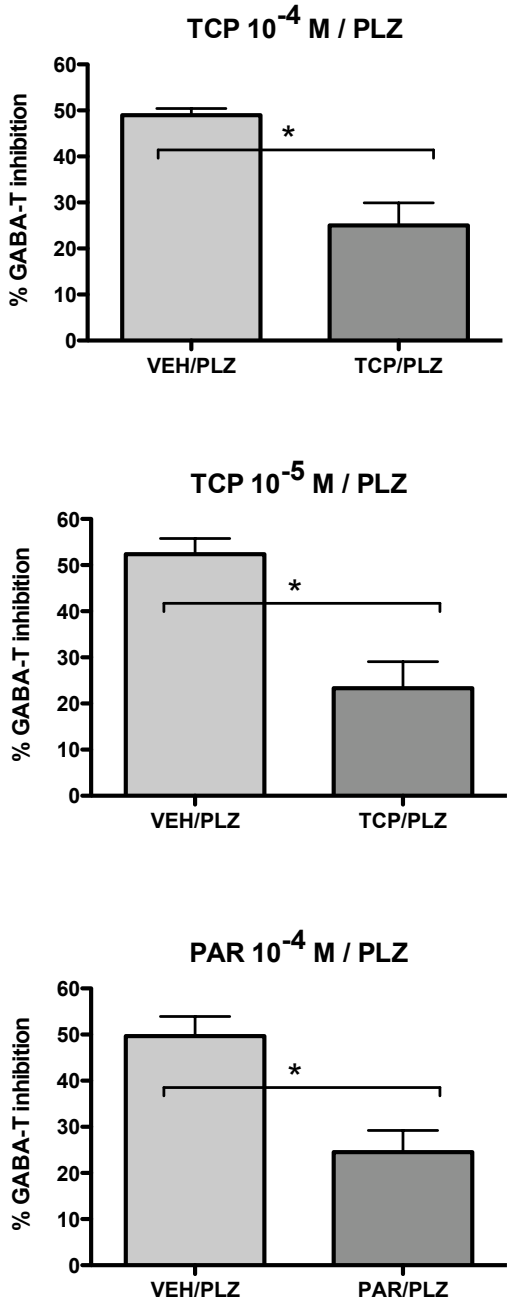

Figure 3-3. Inhibition of GABA-T by PLZ in brain homogenates pretreated with either VEH, TCP (10^{-4}M or 10^{-5}M) or PAR (10^{-4}M). * denotes significant differences ($p < 0.05$) between designated groups. n = 4-5 for each group.

MAOI/VEH groups did not produce significant inhibition of GABA-T (data not shown).

Application of PEH to TCP- or PAR-pretreated homogenates did not markedly affect the degree of GABA-T inhibition compared to that observed in VEH/PEH-treated homogenates. This was expected, since inhibition of MAO does not affect PEH metabolism. PAR-pretreatment did not decrease the GABA-T inhibition produced by PEH, and in fact there was a very small, but significant increase. These data are shown in Figure 3-4. MAOI/VEH groups did not produce significant inhibition of GABA-T (data not shown).

3.4 DISCUSSION

The results obtained here were unexpected, but provide some interesting insight into the interactions of PLZ and PEH with GABA-T, and suggest that PEH may not mediate all of PLZ's GABAergic effects.

The inhibitory effect of PLZ on GABA-T has been demonstrated both *ex vivo* and *in vitro*, although a greater degree of inhibition can be attained *in vitro.* Importantly, PLZ is metabolized to PEH *in vivo* by the action of MAO, and inhibition of MAO completely inhibits the formation of PEH and completely abolishes the ability of PLZ to inhibit GABA-T after acute PLZ administration (Chapter 2; Todd and Baker 2008). These data, together with the findings that PEH itself also inhibits GABA-T *ex vivo* and *in vitro* to a similar extent in both experimental conditions as PLZ itself suggest that the formation of PEH mediates the GABA-T inhibiting properties of PLZ. The goal of the experiments described here was to determine whether inhibition of MAO *in vitro* would abolish the inhibitory effect of PLZ on GABA-T, as is observed *ex vivo*.

PLZ metabolism to PEH has been prevented *in vivo* by completely inhibiting the activities of MAO-A and -B with TCP prior to PLZ administration (see Chapter 2). TCP and another irreversible, non-hydrazine MAOI, PAR, were used in the present studies to inhibit MAO *in vitro* prior to PLZ application. Non-hydrazine MAOIs were selected since many hydrazine-

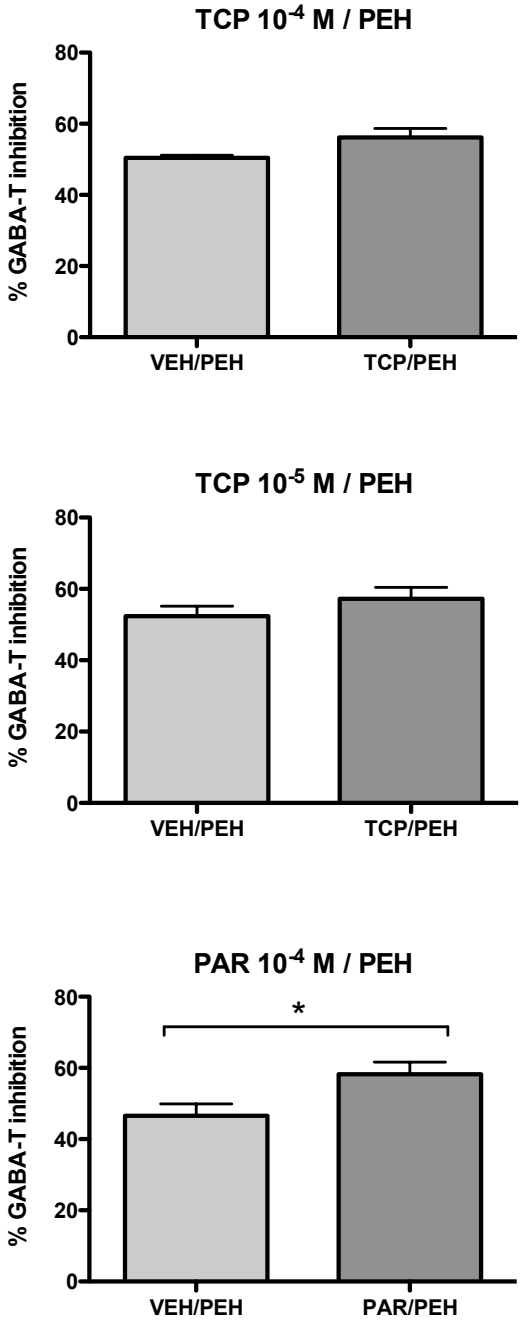

Figure 3-4. Inhibition of GABA-T by PEH in brain homogenates pretreated with either VEH, TCP (10^{-4}M or 10^{-5}M) or PAR (10^{-5}M). * denotes significant differences between designated groups ($p < 0.05$). n = 4-5 for each group.

containing compounds have been shown to inhibit pyridoxal-5'-phosphate (PLP)-dependent enzymes (McCormick and Snell 1961), including GABA-T (Perry *et al.* 1981; Lightcap *et al.* 1995). It was anticipated that the complete inhibition of MAO *in vitro* by these drugs would prevent the formation of PEH from PLZ, and thus prevent the inhibition of GABA-T.

Pretreatment of brain homogenates with either PAR or TCP resulted in a significant decrease in PLZ-induced GABA-T inhibition compared to VEH/PLZ-treated homogenates, although curiously the inhibition was not completely abolished. This result was unexpected, since *in vivo* data suggests that PEH formation mediates PLZ-induced GABA-T inhibition. Interestingly, the degree of the reduction in GABA-T inhibition appeared to be consistent across all MAOI pretreatment conditions; inhibition of GABA-T in the MAOI/PLZ conditions were approximately half of that elicited by the VEH/PLZ conditions (that is, inhibition was reduced from roughly 50% inhibition in the VEH/PLZ conditions to roughly 25% inhibition in the MAOI/PLZ conditions). This consistency implies that the decrease in GABA-T inhibition observed here (and in previous *in vivo* studies) is only partly a result of MAO inhibition rather than some unknown interaction between PLZ and TCP. Together, these data would suggest that either (a) a small amount of PEH was formed in the current studies despite the complete inhibition of MAO prior to PLZ administration, or (b) that PLZ itself inhibits GABA-T *in vitro*, despite the fact that it appears not to do so after acute administration *in vivo*. Inhibition of MAO prior to PEH application did not affect GABA-T activity in TCP-treated homogenates, although PAR/PEH-treated homogenates exhibited significantly greater GABA-T activity than PAR/VEH-treated controls. While PAR has been shown previously to increase brain GABA levels (Schatz and Lal 1971), this GABAergic mechanism of action was not attributed to GABA-T inhibition, and the concentration of PAR used in the present experiments was shown to have no significant effect on GABA-T activity (data not shown). It is possible that the significant difference

observed in the PAR/PEH-treated homogenates was an artifact, and this issue warrants further investigation.

It was assumed in these experiments that the complete inhibition of MAO by TCP and PAR prevented the formation of PEH. Because PEH derivatives appear to be unstable under aqueous conditions (unpublished observations), and since the assay for the determination of PEH requires that the assay medium be methanol (Chapter 2) rather than water, as is the case in the current study, it was not possible to confirm experimentally that PEH formation was completely abolished (or that it even occurred) in MAO-inhibited homogenates. However, it can be assumed with great certainty that the formation of PEH does not mediate the GABA-T inhibition observed in the MAOI/PLZ conditions here; the present data confirm that MAO was inhibited by 100% by both TCP and PAR even under the GABA-T assay conditions, and even if a minute amount of active enzyme was present to oxidize PLZ to PEH, it is unlikely that sufficient PEH would be produced to inhibit GABA-T by 25%, which was consistently observed in the MAOI/PLZ conditions.

The other, and more plausible, explanation for the current results is that PLZ itself possesses some inhibitory effect on GABA-T, despite the fact that it does not appear to mediate *in vivo* GABA-T inhibition after acute drug administration. The exact mechanism of PLZ and PEH-induced GABA-T inhibition is not known. The inhibitory effects of hydrazine-containing compounds on PLP-dependent enzymes are well-established (McCormick and Snell 1961), and PLZ and PEH would not be expected to behave any differently than other hydrazines in this regard. However, studies in this area of interest are far from clear. Hydrazines are believed to inhibit PLP-dependent enzymes, including GABA-T, at least in part by binding to PLP to generate a hydrazone, reducing the availability of the active cofactor and thus reducing enzyme activity (Lightcap *et al.* 1995; Holt *et al.* 2004). Whether hydrazines bind to free or to enzyme-bound PLP has been considered, but is not known (Uchida and O'Brien 1964). It has also been suggested that the PLZ-PLP hydrazone may irreversibly inhibit GABA-T (Yoo *et al.* 1996),

although these findings have been questioned (Holt, personal communication). Furthermore, while Malcolm and colleagues (1994) reported that chronic PLZ treatment reduced plasma vitamin B6 levels in humans, Lydiard and coworkers (1989) found no change in plasma PLP after chronic PLZ treatment. Clearly more studies in this area are warranted.

Based on the present findings, our previous speculation that PEH mediates PLZ-induced GABA-T inhibition should perhaps be modified. PLZ itself may not directly inhibit GABA-T *in vivo* after a single administration, but could potentially play a more prominent role in the inhibition observed after chronic PLZ administration when MAO should be almost completely inhibited. In rodents treated with PLZ for 28 days, GABA-T activity was significantly reduced compared to vehicle-treated animals (McManus *et al.* 1992; McKenna *et al.* 1994), and given that MAO would be expected to be almost (if not completely) inhibited at this time, and thus the presence of PEH negligible, it is reasonable to suggest that PLZ exerts inhibitory actions on GABA-T after chronic administration. If a small amount of MAO were remaining active, some PEH would also be generated, which could contribute to the GABA-T inhibition. It is clear that PEH plays a role in the acute GABAergic effects of PLZ *in vivo*, and the data presented here confirm that PEH also contributes to the PLZ-induced GABA-T inhibition *in vitro*, since the abolition of PEH in the MAOI/PLZ conditions produced a consistent, but not a complete, decrease in GABA-T inhibition.

It is unclear why PLZ would mediate inhibition of GABA-T activity *in vitro* and also in a chronic (but not an acute) state *in vivo*. Interestingly, however, some hydrazines have been shown to be slow-binding inhibitors of GABA-T (Lightcap and Silverman 1996), and while PLZ may not cause significant inhibition of GABA-T after an acute administration because it is rapidly cleared and/or metabolized before sufficient amounts can bind and inhibit GABA-T, higher and more stable PLZ levels (as would be present over the course of chronic PLZ administration) may be able to inhibit enzyme activity. Brain levels of PEH are much higher after a single PLZ administration than

brain levels of PLZ itself, and this could explain why PEH appears to mediate the rapid GABA-T inhibition observed after a single PLZ administration *in vivo*, and why inhibition of these high PEH levels prevents the acute GABA-T inhibiting effects of PLZ. Further studies are necessary to confirm this hypothesis.

It also remains unclear why PLZ and PEH do not inhibit GABA-T activity *in vivo* to a greater extent than 50%. Interestingly, similar findings have been reported with the GABA-T inhibitor vigabatrin (γ-vinyl-GABA); even at very high doses (1600 mg/kg), vigabatrin was not able to inhibit GABA-T activity by more than approximately 60% *ex vivo* in the rodent brain, but inhibited the enzyme completely in brain and in some peripheral tissues *in vitro* (Bolton *et al.* 1989; Jacob *et al.* 1990; Valdizan and Armijo 1992). This finding with vigabatrin is the same as we found with PLZ and PEH when comparing *ex vivo* and *in vitro* results and it should be remembered that the milieu in which the enzymes and drugs are operating are quite different *ex vivo* and *in vitro*. In the studies with vigabatrin it was suggested that the ceiling effect of GABA-T inhibition observed with vigabatrin *ex vivo* may be due to the fact that the increased brain GABA levels resulting from enzyme inhibition (a) compete with vigabatrin for the active site on the enzyme, reducing accessibility of the inhibitor to the enzyme; and/or (b) shift the equilibrium between pyridoxal and pyridoxamine forms of the enzyme to the pyridoxamine form, against which vigabatrin is ineffectual (Bolton *et al.* 1989). No evidence suggests that PLZ inhibits GABA-T by binding to the enzyme at the same site as GABA does, and, as mentioned earlier, the mechanisms of PLZ- and PEH-induced GABA-T inhibition are not understood well enough to comment on whether they may inhibit one form of the enzyme preferentially over another. GABA-T is found inside both neurons and glia, and the possibility that PLZ enters into these cells through the GABA-transporter (GAT) has not been explored. Interestingly, GABA and ALA have been shown to inhibit GABA-transporter (GAT) activity (Borden 1996),

and it is plausible that once these amino acid levels reach high concentrations, GAT activity is inhibited, preventing PLZ access to intracellular GABA-T (and thus limiting GABA-T inhibition by PLZ *in vivo*) (Mousseau, personal communication). This possibility could explain why PLZ inhibits GABA-T activity by up to 100% *in vitro*, since neurons and glia are ruptured during homogenization. Studies are undoubtedly warranted to investigate this possibility.

The results presented here suggest that while PEH may play an integral role in the rapid GABAergic effects of PLZ, it may be less important in the chronic effects of the drug, since it appears that PLZ indeed possesses some GABA-T inhibiting potential on its own. It is now clear that the role of PEH in the acute and the chronic GABAergic effects of PLZ need to be considered separately, a distinction that, until now, has not been highlighted. We may now be able to consider the role of PEH on the GABAergic effects of PLZ from a more accurate viewpoint.

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CHAPTER 4.

PLZ AND PEH INCREASE RAT WHOLE BRAIN ORNITHINE LEVELS

* A version of this chapter has been published. MacKenzie E.M., Grant S.L., Baker G.B., Wood P.L. (2008) Phenelzine causes an increase in brain ornithine that is prevented by prior monoamine oxidase inhibition. Neurochem Res 33(3) 430-6.

4.1 INTRODUCTION

Phenelzine (PLZ) is a potent, irreversible inhibitor of monoamine oxidase (MAO)-A and –B (McKenna *et al.* 1991; Parent *et al.* 2000). It is effective in treating the symptoms of depression, panic disorder, and social anxiety disorder, and its antidepressant, antipanic and anxiolytic properties have been demonstrated in rodents (Caille *et al.* 1996; Paslawski *et al.* 1996; Griebel *et al.* 1998). It is also receiving increasing attention for its neuroprotective/neurorescue properties as demonstrated by its ability to reduce neuronal loss in a transient forbrain ischemia model in gerbils (Wood *et al.* 2006).

PLZ has a number of important neurochemical actions. While it is not surprising, given its potent inhibitory effects on MAO, that PLZ increases brain levels of the monoamine neurotransmitters (McKim *et al.* 1983; Baker *et al.* 1984; McKenna *et al.* 1991; Griebel *et al.* 1998), it has also been shown to increase brain levels of the amino acids GABA (Popov and Matthies 1969; Perry and Hansen 1973; Baker *et al.* 1991; McKenna *et al.* 1991; McManus *et al.* 1992; Paslawski *et al.* 1995; Todd and Baker 1995; Parent *et al.* 1999, 2000; Yang and Shen 2005) and alanine (ALA) (Wong *et al.* 1990; Tanay *et al.* 2001; Yang and Shen 2005). It has been demonstrated, using *in vivo* microdialysis, that PLZ can increase extracellular GABA and ALA levels in the striatum (Tanay *et al.* 2001; Parent *et al.* 2002). Increased brain levels of GABA and ALA are thought to be primarily the result of PLZ-induced inhibition of their respective catabolic enzymes, GABA-transaminase (GABA-T) (Popov and Matthies 1969; McKenna *et al.* 1991; McManus *et al.* 1992) and ALA-transaminase (ALA-T) (Tanay *et al.* 2001), although other, as-of-yet unknown factors may also be involved (Todd and Baker 1995). The increased GABA levels in brain may contribute to the neuroprotective effects of PLZ, since other GABAergic drugs have been demonstrated to be neuroprotective (Chen *et al.* 2000; Schwartz-Bloom and Sah 2001; Iqbal *et al.* 2002). In the case of these other drugs, it has been proposed that their increased GABAergic actions counteract the excitatory effects of increased glutamate

observed in ischemia. PLZ has also been shown to reduce glutamatergic transmission (as evidenced by a decrease in glutamine-glutamate cycling and a reduction in KCl-evoked glutamate release from cortical slices) (Michael-Titus *et al.* 2000; Yang and Shen 2005) and to sequester reactive aldehydes (Wood *et al.* 2006), both of which could also be contributing to neuroprotection.

PLZ is not only an inhibitor of MAO, but is also a substrate for this enzyme (Clineschmidt and Horita 1969b, 1969a; Tipton and Spires 1972; Yu and Tipton 1989). Interestingly, studies have shown that the GABA-elevating effect of PLZ can be inhibited by prior inhibition of MAO (Popov and Matthies 1969; Todd and Baker 1995), suggesting strongly that a metabolite formed by the action of MAO on PLZ plays an important role in this neurochemical effect. We have now identified this metabolite of PLZ as β-phenylethylidenehydrazine (PEH), and administration of the MAO inhibitor tranylcypromine (TCP) prior to PLZ administration completely inhibits PEH formation (Chapter 2) and prevents both the inhibition of GABA-T activity (Popov and Matthies 1969) and the elevations in brain GABA produced by PLZ (Popov and Matthies 1969; Todd and Baker 1995), suggesting strongly that PEH formation mediates the GABAergic effects of acute PLZ administration. PEH formation likely mediates other neurochemical effects of PLZ as well.

In a recent preliminary study, we found that ornithine (ORN) levels are also increased in rat brain following PLZ administration. ORN is a non-essential amino acid that is synthesized from arginine (ARG) via arginase as part of the urea cycle, and is metabolized to both glutamate via ornithine transaminase (ORN-T) and to polyamines (putrescine, spermidine and spermine) via ornithine decarboxylase (ODC) (Seiler 2000). While the highest concentrations of ORN are found in the liver, drug-induced changes in brain levels of ORN may provide information relevant to the pharmacological treatment of psychiatric illness, given the close

relationships between ORN, GABA and glutamate (the latter two amino acid neurotransmitters being strongly linked to a number of psychiatric conditions) and between ORN and the polyamines (whose metabolism produces reactive aldehydes, which are found in toxic levels in some neurological conditions). These relationships are shown in Figure 4-1.

The present study was conducted to determine the dose-response and time-response effect of PLZ administration on rat brain concentrations of ORN. Furthermore, to determine whether the formation of PEH was contributing to the actions of PLZ on brain ORN, MAO-mediated PLZ metabolism was inhibited by pre-treatment with the non-selective MAO inhibitor TCP. Changes in brain ORN produced by PEH itself were also investigated.

4.2 METHODS

4.2.1 Drug administration

PLZ sulfate and TCP HCl were dissolved in distilled water and PEH was dissolved in corn oil, and all drugs were injected intraperitoneally (i.p.) for the studies described. All doses mentioned are based on free base weight of the drugs. For the PLZ dose-response study, rats were injected with vehicle (physiological saline) or PLZ (7.5, 15, or 30 mg/kg) and were killed by decapitation 3 hours after injection. For the PLZ time-response study, rats were injected with vehicle or PLZ (15 mg/kg) and were killed by decapitation 1, 3, 6, 12, or 24 hours after injection. In the PEH study, rats were injected with vehicle (corn oil) or PEH (15 mg/kg) and brains were collected 3 hours after drug administration. For the MAO-inhibition study, animals were pretreated either with vehicle or TCP (1mg/kg; previous studies in our laboratories have shown that this produces greater than 90% inhibition of MAO (Todd and Baker 1995)), and 1 hour later were treated either with vehicle, PLZ (15mg/kg) or PEH (15 mg/kg). Animals were decapitated 3 hours following vehicle or PLZ injection. Brains were rapidly removed and

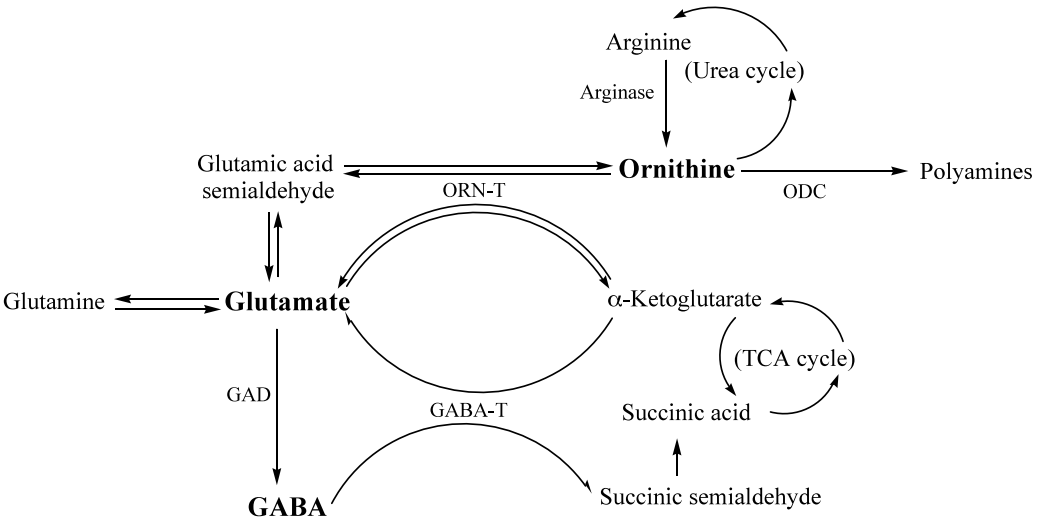

Figure 4-1. Metabolic relationship between ornithine, glutamate, GABA, arginine and the polyamines. ORN-T: ornithine transaminase; ODC: ornithine decarboxylase; TCA: tricarboxylic acid; GABA-T: GABA-transaminase

flash-frozen in 2-methylbutane on dry ice, and stored at -80˚C until the time of ORN analysis.

4.2.2 Determination of ornithine levels

Partially-thawed brains were halved along the midline, and half brains were homogenized in 5 volumes of ice-cold water. ORN levels were determined using HPLC combined with fluorescence detection, following derivatization with fluoraldehyde reagent (o-phthaldialdehyde (OPA) with mercaptoethanol), using a method previously described for the quantification of amino acids (Parent *et al.* 2001). Briefly, protein precipitation was achieved by adding a small amount of homogenate (100 µl) to ice-cold methanol (300µl). Following centrifugation (13000 g, 4˚C), the supernatant was transferred to HPLC inserts, and maintained at 4˚C in the autosampler. A portion of the supernatant (5µl) was reacted with OPA (5 µl) in the injection loop of a Waters Alliance 2690XE system for 1.5 minutes before injection onto the analytical column (Waters Symmetry C18 3.5 µm (4.6 x 150 mm), connected to a uBondapak C18 precolumn insert (10µm, 125Å)), held at 30˚C. A Waters 474 fluorescence detector was set to an excitation wavelength of 260 nm and an emission wavelength of 455 nm. Data were collected and analyzed using the Empower Pro software package (Waters).

4.2.3 Statistical Analysis

Data were analyzed by analysis of variance (ANOVA), followed by the Newman-Keuls test. Statistical significance was established using a probability value of <0.05.

4.3 RESULTS

Whole brain ORN levels for vehicle-treated animals were 1.10 ± 0.07 µg/g tissue. This value was determined using control values for the dose-response

study, the time-response study and the MAO-inhibition studies (N=44), and is consistent with literature values for brain ORN in the rat (Perry 1982).

4.3.1 Dose-response study

PLZ dose-dependently increased whole brain levels of ORN. ORN levels were significantly increased following 7.5 mg/kg, 15 mg/kg and 30 mg/kg PLZ, reaching 238 ± 27%, 445 ± 45% and 812 ± 77% of control values, respectively, 3 hours after drug injection. These results are shown in Figure 4-2.

4.3.2 Time-response study

The administration of PLZ (15mg/kg) resulted in a significant increase in brain ORN concentrations at all time intervals tested. The effect of a single PLZ injection on ORN was long-lasting, as ORN levels were still significantly elevated 24 hours after PLZ injection (321 ± 42% of control values). The maximum increase in brain ORN was observed 6 and 12 hours following PLZ injection (652 ± 67% and 649 ± 27% of vehicle-treated brains, respectively). These results are shown in Figure 4-3.

4.3.3. MAO inhibition study

As shown in Figure 4-4, the PLZ-induced increase in brain ORN was abolished by inhibiting the activity of MAO. This dose of TCP has been shown previously to inhibit MAO activity by greater than 90% 1 hour after injection (Todd and Baker 1995). Animals that received a PLZ injection following a vehicle injection had significantly increased ORN levels compared to all other treatment groups, with ORN values reaching 369 ± 62% of control values. Brain levels of ORN were at control values in the animals pretreated with TCP prior to PLZ administration, suggesting that a metabolite of PLZ formed by the action of MAO was likely responsible for the increase in ORN observed. No significant difference in ORN was observed between TCP/VEH and VEH/VEH treatment groups ($p>0.05$).

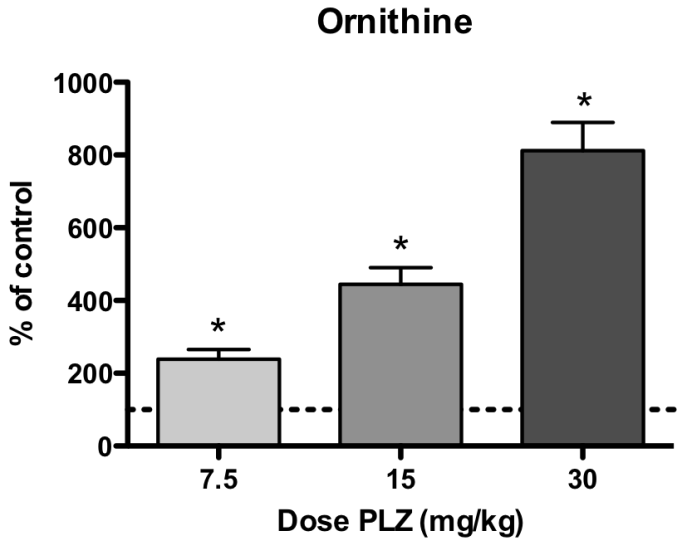

Figure 4-2. Dose-dependent effects of PLZ on rat brain ORN levels 3 hours after drug administration. n = 6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

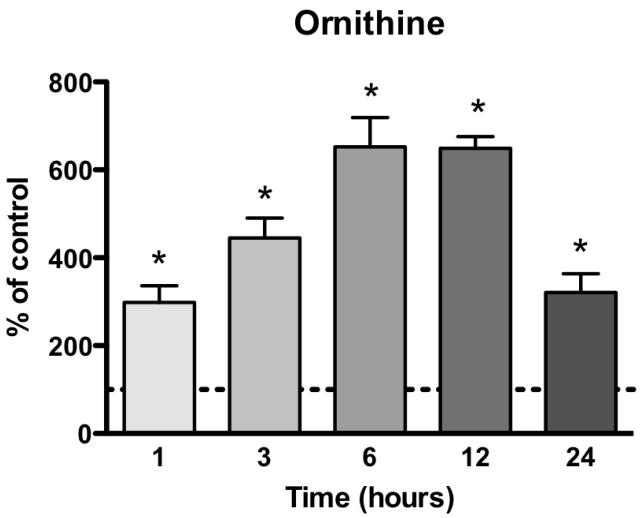

Figure 4-3. Time-dependent effects of PLZ (15 mg/kg) on rat brain ORN levels. n = 6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

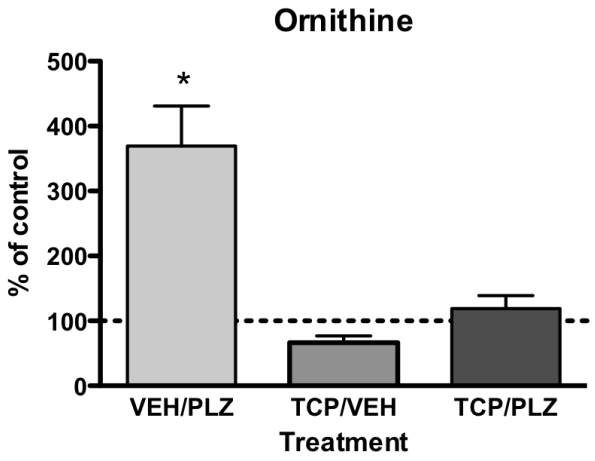

Figure 4-4. Effects of PLZ (15 mg/kg) on rat whole brain ornithine levels with and without prior inhibition of MAO by TCP. n = 5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

To determine if PEH could be the active metabolite of PLZ that leads to the increase in brain ORN, PEH was directly administered to animals pretreated with either vehicle or TCP. PEH alone (observed in the VEH/PEH treatment group) caused a significant increase in brain ORN levels, reaching 678 ± 53% of control values 3 hours after drug injection. As expected, pretreatment with TCP did not affect the PEH-induced increase in brain ORN; no significant difference was observed between the VEH/PEH and TCP/PEH groups ($p>0.05$). These results are shown in Figure 4-5.

4.4 DISCUSSION

The results of the present study demonstrate that a single administration of the antidepressant/antipanic drug PLZ to rats results in a dose- and time-dependent increase in whole brain concentrations of ORN. The increase in ORN was abolished by inhibition of MAO prior to PLZ administration and was significant following direct administration of PEH, indicating that PEH formation is most likely responsible for PLZ's effects on brain ORN. These data contribute to our knowledge of the neurochemical properties of PLZ, and support previous suggestions that the active metabolite of PLZ produced by the action of MAO is responsible for a number of these neurochemical effects.

PLZ markedly increased brain ORN levels at all doses and time-points tested. The precise mechanism by which PLZ increases brain ORN levels remains unclear, but likely involves changes in the activity of the catabolic enzymes ORN-T and/or ODC. It is likely that PLZ inhibits ORN-T, given that PLZ also inhibits the activity of GABA-T and ALA-T, two pyridoxal 5'-phosphate (PLP)-dependent transaminase enzymes. Binding of the hydrazine moiety of PLZ to this cofactor has been suggested to form an inactive hydrazone complex, thus inhibiting enzyme activity and increasing substrate levels (Yu and Boulton 1992). ORN-T also requires PLP as a cofactor, and thus its activity may be inhibited (and ORN levels increased) by PLZ in a

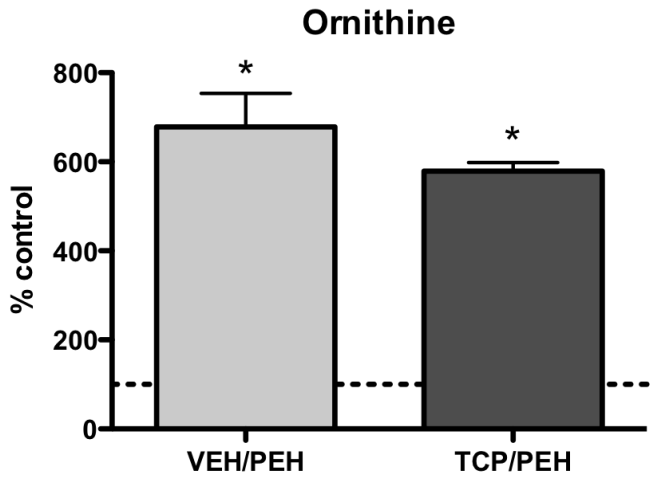

Figure 4-5. Effect of PEH with and without prior inhibition of MAO by TCP. n = 3 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

similar manner as that of GABA-T and ALA-T. This is supported by the findings that GABA-T and ORN-T are closely related in terms of structure and activity (Seiler 2000), and that all reported GABA-T inhibitors, except for ethanolamine-O-sulfate and vigabatrin, are also inhibitors of ORN-T (Jung and Seiler 1978; Jung *et al.* 1984; Seiler 2000). Furthermore, selective inhibition of ORN-T, which is heavily concentrated in nerve terminals (Wong *et al.* 1982), leads to accumulation of synaptic ORN (Daune and Seiler 1988) and drastically elevates ORN levels in the rodent brain (Daune *et al.* 1988; Seiler *et al.* 1989). It should be noted that aside from this direct effect of PLZ, ORN-T may also be indirectly inhibited by PLZ, as GABA itself is a competitive inhibitor of ORN-T, acting as part of a negative-feedback mechanism to regulate ORN-T activity *in vivo* (Yoneda *et al.* 1982). PLZ elevates brain GABA within 1 hour of PLZ administration (Baker *et al.* 1991), and the substantial and long-lasting elevation in GABA may contribute to ORN-T inhibition, and thus to the elevation of brain ORN observed here.

Inhibition of ODC activity by PLZ may also contribute to the increase in brain ORN observed. However, inhibition of ODC alone is not likely to result in the increase in brain ORN observed in the present study, as the ODC pathway is not a significant means of ORN catabolism in the rodent brain (Raina *et al.* 1976). In accordance with this, it was shown that selective inhibition of ODC in mice did not alter brain ORN levels, whereas inhibition of ORN-T alone or together with ODC resulted in a significant increase in brain ORN (Seiler *et al.* 1989). In more recent experiments, we have also observed that PLZ increases brain levels of ARG, a precursor to ORN. It is possible that some of this increased ARG may be converted to ORN. It is clear that further experiments are warranted to determine the effects of PLZ on the activities of ORN-T and ODC, and also on the conversion of ARG to ORN.

Inhibition of MAO prior to PLZ administration prevented the increase in brain ORN levels, suggesting that a metabolite of PLZ is responsible for the increase in ORN observed. This is consistent with previous studies that have

shown that blockade of PLZ metabolism by MAO inhibition prevents the GABA-T inhibiting action of PLZ and its elevation of brain GABA (Todd and Baker 1995). PEH has now been shown to be the metabolite of PLZ formed *in vivo* by the action of MAO (see Chapter 2), and given that it, too, possesses a hydrazine moiety and elevates brain GABA by inhibiting the activity of its catabolic transaminase enzyme, it is likely that this metabolite mediates the increase in brain ORN induced by PLZ in a similar manner. These findings add to the growing body of evidence suggesting that PEH formation plays an important role in some of the neurochemical effects of PLZ.

Interestingly, although PLZ itself is an unsubstituted hydrazine, it appears that its metabolism to PEH is necessary for its GABA-T- (and probably ORN-T-) inhibiting effects. Similarly, the unsubstituted hydrazine isoniazid does not inhibit GABA-T itself, but is metabolized to hydrazine, which has been shown to inhibit GABA-T (Perry *et al.* 1981) and increase brain levels of GABA (Popov and Matthies 1969; Perry *et al.* 1981; Matsuyama *et al.* 1983); chronic treatment with hydrazine also increases ORN concentrations in rat plasma (Perry *et al.* 1981). While it is unclear why some hydrazine drugs affect the activity of these transaminase enzymes and others do not, it is likely that a free hydrazine is required for at least some of these effects, given that substituted hydrazine MAO inhibitors do not increase brain GABA or ALA levels (McKenna *et al.* 1991; Yamada *et al.* 1993).

Understanding the neurochemical effects of PLZ is important for establishing the mechanisms by which PLZ exerts its clinical effects. In addition to its MAO-inhibiting properties, it is feasible that the GABA-elevating action of PLZ may contribute to its antidepressant effects, given that plasma and CSF GABA levels have reported to be significantly reduced in depressed patients compared to healthy controls (reviewed in (Petty 1995)), and that positive modulators of the $GABA_A$ receptor (including the 3α-reduced neuroactive steroids) have been reported to possess antidepressant effects (Uzunova *et al.* 2006). Interestingly, SSRI antidepressants have also

now been demonstrated, using magnetic resonance spectroscopy techniques, to increase brain levels of GABA in humans *in vivo* (Sanacora *et al.* 2002; Bhagwagar *et al.* 2004). Drugs that facilitate GABAergic transmission, such as tiagabine, vigabatrin, pregabalin and benzodiazepines, also possess anxiolytic and antipanic properties (Zwanzger and Rupprecht 2005; Tassone *et al.* 2007), and a study with rodents suggests that the anxiolytic effect of PLZ in the elevated-plus maze is related to its GABA-elevating effect (Paslawski *et al.* 1996).

Recent attention has been focused on the neuroprotective/neurorescue properties of MAO inhibitors (Youdim and Weinstock 2002; Sowa *et al.* 2004; Youdim *et al.* 2006; Baker *et al.* 2007). The GABA-elevating action of PLZ, which would presumably counteract the excitotoxic effects of glutamate observed in the transient forebrain ischemia in the gerbil (Shuaib and Kanthan 1997; Green *et al.* 2000; Schwartz-Bloom and Sah 2001; Yang *et al.* 2001; Wang and Shuaib 2005), has been proposed as one mechanism by which this drug is neuroprotective in this animal model (Sowa *et al.* 2004). PLZ has also been shown to reduce glutamatergic neurotransmission in the brain (Michael-Titus *et al.* 2000; Yang and Shen 2005), and to sequester the toxic aldehydes formed as by-products of increased polyamine metabolism resulting from ischemic insult (Wood *et al.* 2006). Furthermore, altered ODC activity and polyamine metabolism have been reported in individuals suffering from stroke (Tomitori *et al.* 2005) and some neurodegenerative diseases (Bernstein and Muller 1995; Morrison *et al.* 1998), possibly contributing to the increased brain, plasma and cerebral spinal fluid concentrations of reactive aldehydes observed in some of these patients (Zarkovic 2003; Tomitori *et al.* 2005).

The PLZ-induced accumulation of brain ORN observed in the present study may be an indicator of decreased formation of glutamate and/or polyamines, which may contribute to neuroprotection (decreased glutamate resulting in reduced excitotoxicity and decreased polyamine metabolism resulting in decreased formation of toxic aldehydes such as 3-aminopropanal

(3-AP) and acrolein and reactive oxygen species (ROS)). PEH formation likely mediates these effects, given that PEH is formed *in vivo* from PLZ in large amounts, and has been shown to elevate brain GABA (Paslawski *et al.* 2001) and ORN, and exerts neuroprotective effects in the transient forebrain ischemia model (Tanay *et al.* 2002). At the present time, decreased formation of glutamate seems unlikely since in short- and long-term (2 weeks) studies (Paslawski *et al.* 1995; Baker *et al*, unpublished; Chapter 2 of this thesis), any decreases in brain levels of glutamate are very short-lived . However, levels of glutamine, a major metabolite of glutamate, are decreased more dramatically (Paslawski *et al.* 1995; Baker et al, unpublished; Chapter 2 of this thesis), although this effect is not as long-lasting as the effect on ORN. It is also of interest that Sowa (Sowa 2006), in a chronic study of global cerebral ischemia in gerbils, found 4-fluoroPEH reduced the markedly increased frontal cortex levels of glutamate back to control levels.

Importantly, the "cheese effect," a pressor effect that is sometimes observed with irreversible inhibitors of MAO-A such as PLZ when foods containing sympathomimetic amines such as tyramine are ingested, would be unlikely to occur with PEH, given that PEH administration to rats does not significantly inhibit MAO activity (Paslawski *et al.* 2001). This suggests that PEH may be an important therapeutic alternative to PLZ in some disorders, since this analogue appears to retain its putative neuroprotective properties, i.e. increases whole brain GABA and ORN, yet would not require the strict dietary restrictions imposed by the cheese effect.

In summary, the present experiments demonstrate that a single administration of PLZ resulted in a dramatic and long-lasting increase in brain levels of ORN, and that inhibition of PLZ metabolism (by inhibiting MAO) abolishes this effect on brain ORN. These results, together with previous findings that inhibition of MAO prior to PLZ administration prevents the *in vivo* formation of PEH and the present findings that PEH itself increases brain ORN, indicate that PEH formation is likely responsible for the effect of PLZ on brain ORN. This is consistent with previous suggestions that

an active metabolite of PLZ may be responsible for a number of the neurochemical and clinical effects of the drug, and underscore the need for further studies to determine the effects of PLZ and its metabolite on the enzymes related to ORN metabolism.

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CHAPTER 5.

PLZ AND PEH INHIBIT SEMICARBAZIDE-SENSITIVE AMINE OXIDASE (SSAO) AND SEQUESTER ITS TOXIC PRODUCT, FORMALDEHYDE (FA)

5.1 INTRODUCTION

The antidepressant/antipanic drug phenelzine (PLZ) is neuroprotective in an animal model of cerebral ischemia (Wood *et al.* 2006). The mechanisms by which PLZ exerts it neuroprotective effects are beginning to be elucidated, and PLZ-induced increases in brain GABA levels (apparently resulting from inhibition of GABA-transaminase (GABA-T)) and its potential for decreasing glutamatergic transmission are probable avenues by which neuroprotection is achieved. PLZ causes marked increases in brain ornithine (ORN) (MacKenzie *et al.* 2008), which may reflect a decrease in polyamine synthesis, and a concurrent decrease in reactive aldehydes that are produced as by-products. Like some other hydrazine drugs, PLZ has also been shown to react with the reactive aldehydes 3-aminopropanal (3-AP) and acrolein, reducing their toxic effects by forming a nonreactive hydrazone product (Wood *et al.* 2006; Galvani *et al.* 2008). Reactive aldehydes, such as 3-AP, acrolein, 4-hydroxynonenal (4-HNE) and formaldehyde (FA) are produced in normal metabolic reactions or as products of lipid peroxidation. These aldehydes are extremely reactive, and form irreversible adducts with proteins and single-stranded DNA, resulting in apoptosis, necrosis, and exacerbation of lipid peroxidation (Esterbauer *et al.* 1991; Yu *et al.* 2003; Yu *et al.* 2004; Ellis 2007). Reducing the "aldehyde load" by sequestering these highly reactive molecules has been shown to be neuroprotective in a number of *in vitro* and *in vivo* studies (Burcham *et al.* 2002; Webster *et al.* 2005; Wood *et al.* 2006; Galvani *et al.* 2008).

PLZ is a monoamine oxidase (MAO) inhibitor as well as a substrate for MAO (Clineschmidt and Horita 1969a; 1969b), as it has been shown to be readily oxidized by MAO to form the active metabolite β-phenylethylidenehydrazine (PEH; Chapter 2). PEH itself warrants investigation as a therapeutic agent, given that it shares a number of PLZ's neurochemical effects (including the marked increases in brain GABA and ORN and the decreases in brain glutamine) but lacks the ability to

significantly inhibit the activity of MAO (Paslawski *et al.* 2001; MacKenzie *et al.* 2008); the latter property would preclude any of the side effects common to irreversible MAO inhibitors such as PLZ. PEH is also a hydrazine-containing compound and, thus, it is reasonable to presume that it could also sequester reactive aldehydes.

While the inhibitory effects (or lack thereof) of PLZ and PEH on the activities of MAO and GABA-T are well documented, the inhibitory effects of PLZ on the activity of semicarbazide-sensitive amine oxidase (SSAO) are less well characterized; those of PEH are not known at all. SSAO is an amine oxidase that is distinguished from MAO in the chemical nature of its cofactors and in the specificity of inhibitors and substrates (although some overlap exists in the latter regard) (Matyus *et al.* 2004). SSAO is a copper- and quinone-containing amine oxidase that catalyzes the deamination of amines to produce their respective aldehydes, as well as ammonia and hydrogen peroxide (H_2O_2). The trace amine methylamine (MA) is an endogenous substrate for SSAO, for example, and is oxidized to produce the reactive aldehyde formaldehyde (FA), as well as ammonia and H_2O_2. SSAO is located primarily in vascular endothelial cells, smooth muscle cells, adipose cells and in the plasma, and in the brain it is located solely in the cerebral vasculature (Lewinsohn 1981; Zuo and Yu 1993). Importantly, the activity and/or expression of SSAO has been reported to be increased in many pathological conditions, including diabetes mellitus, atherosclerosis, cerebral infarction, congestive heart failure and, most relevant to this thesis, Alzheimer's disease (AD) (Boomsma *et al.* 1995; Boomsma *et al.* 1997; Ferrer *et al.* 2002; Karadi *et al.* 2002; del Mar Hernandez *et al.* 2005; Jiang *et al.* 2008). Increased SSAO leads to an increase in the formation of FA, whose toxic properties are well established. Importantly, it has been suggested by many studies that FA may contribute to the pathophysiology of many of the disorders mentioned above, including AD (Gubisne-Haberle *et al.* 2004; Chen *et al.* 2006; Chen *et al.* 2007; Kazachkov *et al.* 2007; Nie *et al.* 2007).

PLZ has been shown to inhibit the activity of bovine lung SSAO (Lizcano *et al.* 1996), and it is believed that enzyme inactivation occurs via the binding of PLZ to the quinone cofactor of SSAO (Holt *et al.* 2004). The clinical relevance of SSAO inhibition in bovine lung must be interpreted with caution, as differences in substrate and inhibitor affinities for SSAO have been reported between species. To our knowledge, evidence for the inhibition of human SSAO by PLZ not been reported.

The aim of the experiments described here was to determine the putative neuroprotective mechanisms of PLZ and PEH resulting from their interactions with SSAO and its toxic product, FA. More specifically, the ability of PLZ and PEH to inhibit human SSAO and to sequester FA, the product of SSAO-mediated oxidation of MA, was determined *in vitro*. The effects of PLZ and PEH on whole brain MA levels were also measured *ex vivo*. Results from these studies strongly suggest that both PLZ and PEH could be potentially useful in the treatment of AD-related neurotoxicity, as well as in the treatment of other conditions that are mediated by increased activities of SSAO and/or increased concentrations of the toxic aldehyde FA.

5.2 METHODS

5.2.1 Inhibition of human SSAO by PLZ and PEH *in vitro*

The inhibition of human SSAO by PLZ and PEH was measured *in vitro* using a modification of the assay procedure described by Holt and Palcic (2006). The assay is based on the principle that H_2O_2 is a substrate for horseradish peroxidase, and in the presence of another peroxidase substrate, 4-aminoantipyrine, and 2,4-dichlorophenol, a quinoneimine dye is stoichiometrically produced. For the assay conducted here, H_2O_2 generated as a product of MA oxidation by SSAO was reacted with the chromogen (i.e. the mixture containing peroxidase, 4-aminoantipyrine, and 2,4-dichlorophenol) to produce the dye. This reaction is shown in Figure 5-1. Absorbance was measured using a SPECTRAmax 190 platereader (Molecular Devices) at a wavelength of 510nm.

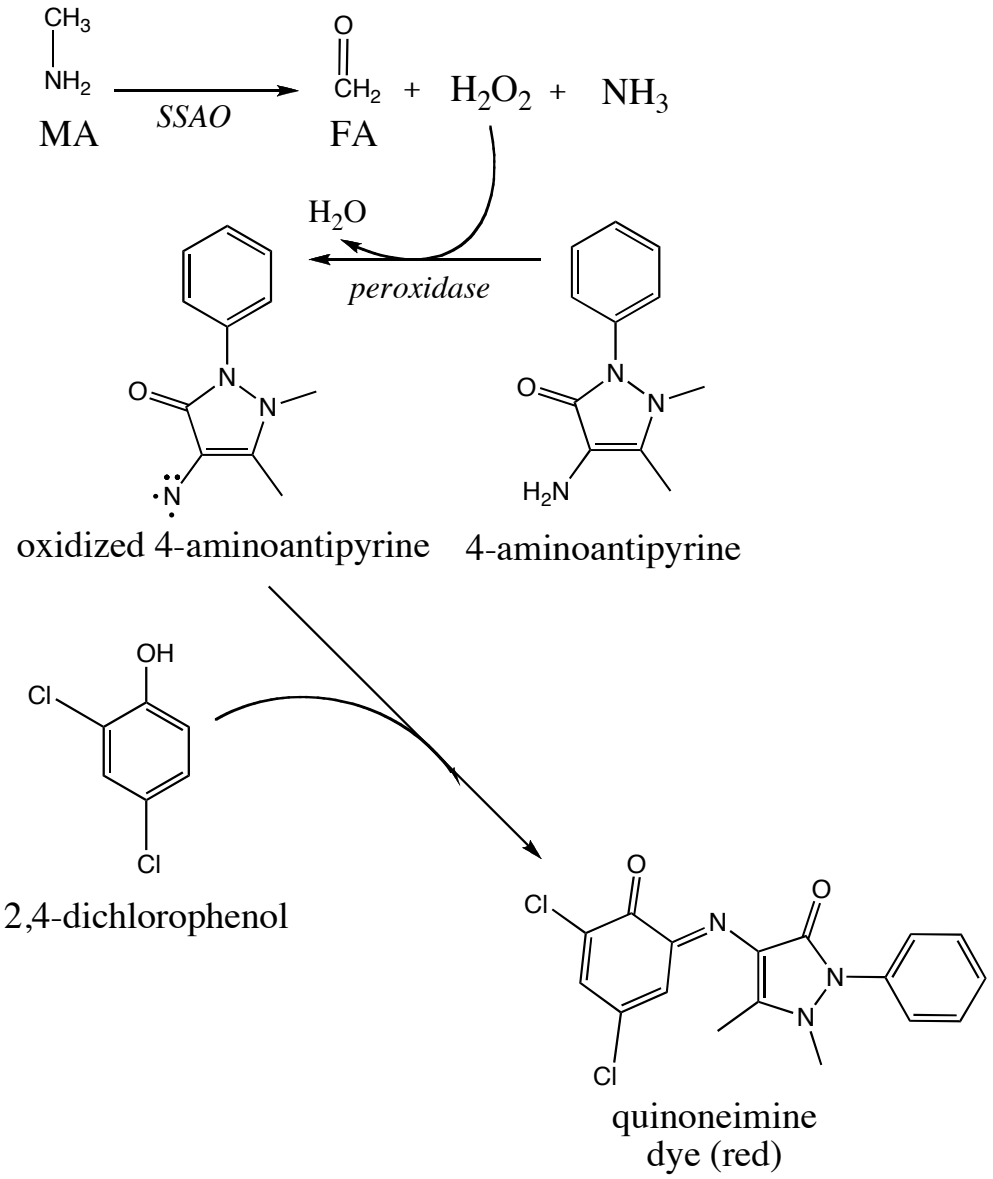

Figure 5-1. An illustration of the horseradish peroxidase-coupled reaction producing a quinoneimine dye that is produced as a result of the oxidation of MA to FA by SSAO (adapted from Holt and Palcic, 2006).

Stock solutions of PLZ and PEH (prepared in ddH_20 and DMSO, respectively) were diluted to a series of concentrations (10^{-11}M to 10^{-7}M). PEH dilutions contained a final DMSO concentration of 1%. Pure soluble human SSAO purified from CHO cells over-expressing the human enzyme (obtained from Dr. A. Holt, Department of Pharmacology, University of Alberta), was prepared in physiological HEPES buffer to a final concentration of 20 nM. Chromogen was prepared by mixing 4-aminoantipyrine (500 uM), 2,4-dichlorophenol (1 mM) and horseradish peroxidase (4 U/ml) in physiological HEPES, and was combined with MA (prepared in ddH_2O; 5 mM final concentration).

In triplicate, 75 μl of inhibitor (PLZ or PEH) was pipetted into individual wells of a clear 96-well microplate and warmed at 37°C for approximately 5 minutes in the chamber incubator of the platereader. This warming step was conducted to prevent any lag period in the inhibition of enzyme activity by PLZ or PEH upon enzyme addition. The SSAO-inhibition reactions were initiated by the addition of 75μl SSAO (in physiological HEPES) to each well (5 nM final concentration). Following a 60-minute incubation at 37°C, 150 μl of MA/chromogen mixture were added to each well, and the microplate was returned to the plate reader for 30 minutes, where the initial velocities of the change in optical density (mOD) were measured. Curves were fitted by nonlinear regression of a four parameter logistic equation using Graphpad Prism®.

5.2.2 Sequestration of FA by PLZ and PEH *in vitro*

The abilities of PLZ and PEH to sequester FA were tested *in vitro* by observing the appearance of a hydrazine-FA hydrazone peak and the disappearance of the FA peak using GC-MS, using a procedure modified from Wood and colleagues (2006). The formation of PLZ-FA and PEH-FA hydrazones are shown in Figure 5-2.

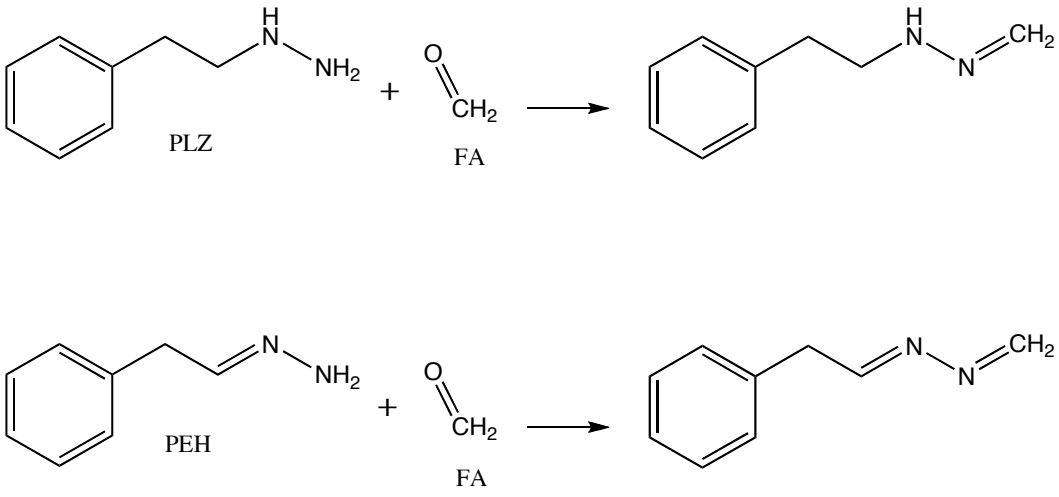

Figure 5-2. Formation of hydrazone compounds by the sequestration of FA by PLZ (top) and PEH (bottom).

5.2.2.1 Sequestration of FA by PLZ

FA (2mM) and/or PLZ (4mM) were added to glass test tubes, and the final reaction volume was adjusted to 200μl with phosphate-buffered saline (PBS) pH = 7.2. PBS alone (200μl) was added to blanks. Tubes were incubated at room temperature in the dark for 15 minutes in order for PLZ-FA hydrazone formation to proceed in appropriate samples. All tubes were then basified with 25% potassium carbonate (K_2CO_3; 100μl) prior to the addition of 2ml of toluene containing 2μl pentafluorobenzoyl chloride (PFBC), and were shaken vigorously for 5 minutes. After centrifugation (3 minutes, 1000 g), the solvent (top) layer and the aqueous (bottom) layer were separated. A portion of the solvent layer (1.75ml), containing the PFBC-derivatized hydrazone, was transferred to a new glass vial and taken to dryness under vacuum. The residue was taken up in 200μl cyclohexane and transferred to GC vials, and 1μl was injected into the GC system. Meanwhile, a portion of the aqueous layer (150 μl), containing the remaining (unsequestered) FA, was transferred to a new glass vial, and hydralazine (HYD; 4mM) was added. These tubes were incubated at room temperature in the dark for 5 minutes, at which time FA was derivatized with HYD. Toluene (2ml) was subsequently added to each tube, and after vigorously shaking (5 minutes) and centrifuging (3 minutes), a portion (1.75 ml) of the solvent layer, now containing the HYD-derivatized FA, was transferred to new glass tubes, and dried under vacuum. Following reconstitution in 100 μl cyclohexane, the solution was transferred to GC vials. The derivatization reactions of the FA-PLZ hydrazone with PFBC and of FA with HYD are shown in Figure 5-3.

5.2.2.2 Sequestration of FA by PEH

The procedure for the sequestration of FA (2mM) by PEH (4mM) was carried out in the same manner as the sequestration of FA by PLZ, except that

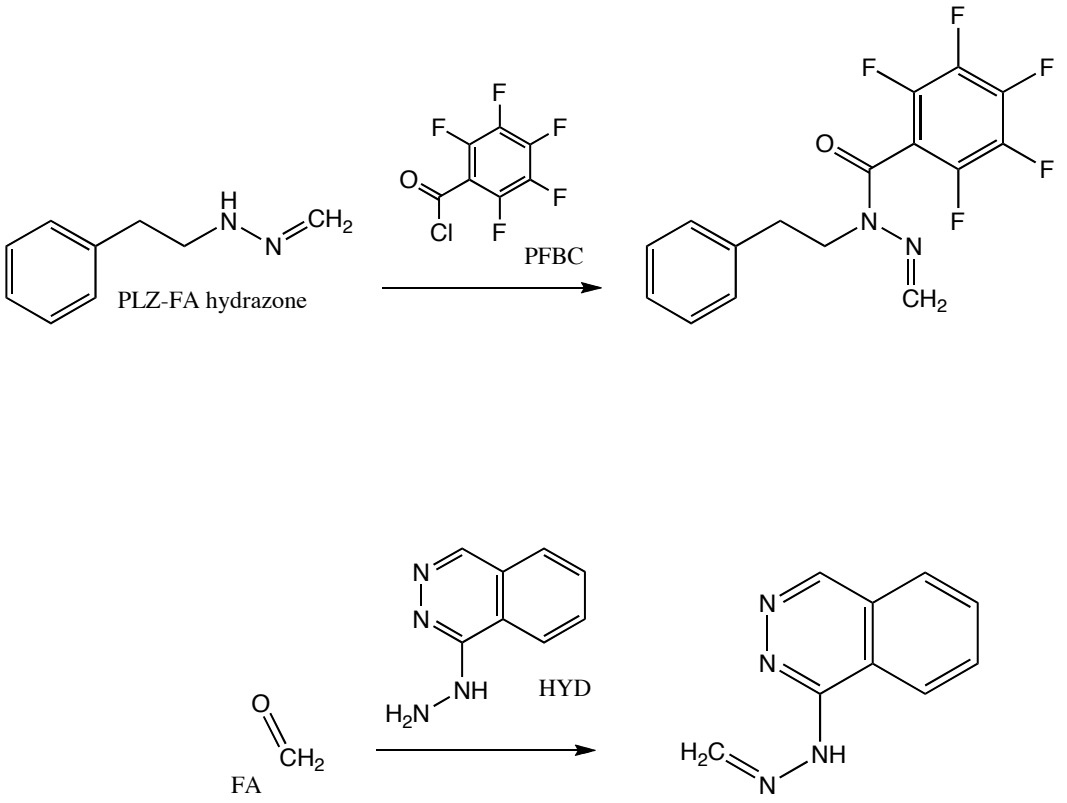

Figure 5-3. Derivatization of the sequestration product of PLZ and FA with PFBC (top scheme). Unsequestered FA was derivatized with HYD (bottom scheme).

the FA-PEH hydrazone was not derivatized by PFBC; instead, the FA-PEH hydrazone was extracted with 2ml toluene only, and the underivatized hydrazone (Figure 5-2) was measured on the GC-MS. The derivatization of FA was carried out as described in Section 5.2.2.1.

5.2.2.3 GC-MS analysis of PLZ-FA and PEH-FA hydrazones and of FA

Hydrazone formation and FA disappearance were measured using an Agilent 6890 Series GC System equipped with an Agilent 5973 Network MS. The column was an Agilent HP-5MS 30mm x 250mm with a 0.25um film thickness. The oven temperature program was set to an initial temperature of 80°C and held for 2 minutes, followed by an increase of 15°C/minute until 295°C was reached. The temperature was held at 295°C for 5 minutes. A total ion scan using mass spectrometry was performed to measure PLZ-FA and PEH-FA hydrazones and FA.

5.2.3 *Ex vivo* analysis of whole brain MA

Male Sprague-Dawley rats were injected i.p. with either PLZ (30 mg/kg), PEH (30 mg/kg), or vehicle (saline or corn oil, respectively), and were killed by decapitation 3 hours after drug injection. Brains were extracted and rapidly frozen in 2-methylbutane on dry ice and then transferred to vials and stored at -80°C.

For MA analysis, partially-thawed brains were halved and homogenized in 5 volumes of ice-cold 0.1N perchloric acid ($HClO_4$) and centrifuged for 5 minutes (13,000g). A portion of the supernatant (1ml) was basified with 200μl 25% K_2CO_3, and 3ml of a solution (ethyl acetate (EA) : acetonitrile, 9:1 v/v) containing 50μl pentafluorobenzoylsulfonyl chloride (PFBSC) was added. Tubes were shaken for 10 minutes, centrifuged for 5 minutes, and the solvent (top) layer was transferred to new glass tubes and dried under vacuum. Samples were reconstituted in 200μl cyclohexane and 1μl was

injected onto the analytical column of the gas chromatograph (GC) equipped with a mass spectrometer (MS). Standard curves were prepared by adding a known concentrations of MA (0-200 ng) to 0.1N $HClO_4$, and were carried out with each sample run. The derivatization of MA with PFBSC is shown in Figure 5-4.

The GC-MS was programmed to scan for m/z = 261 between 5.3 and 8.0 minutes to measure MA. The oven temperature was set initially to hold for 2 minutes at 100°C, then to increase at 8°C/minute until 295°C was reached. The temperature was held at 295°C for 2 minutes until the end of the sample run.

5.3 RESULTS

5.3.1 Inhibition of human SSAO by PLZ and PEH

The inhibitory effects of PLZ and PEH on human SSAO were assessed by measuring the initial rates of change in optical density resulting from the peroxidase-coupled formation of the quinoneimine dye (Holt and Palcic 2006). The results obtained here are, to my knowledge, the first to demonstrate that PLZ inhibits human SSAO, and are undoubtedly the first to demonstrate that PEH, the active metabolite of PLZ, also inhibits the activity of the human enzyme. Both PLZ and PEH were relatively potent inhibitors of the enzyme, with IC_{50} values of 4.2 nM and 5.9 nM, respectively. These data are shown in Figure 5-5.

5.3.2 Sequestration of FA by PLZ and PEH *in vitro*

Results from this study clearly demonstrate that both PLZ and PEH bind to FA, forming hydrazone complexes. The formation of the hydrazones was accompanied by significant reductions in the amount of FA present, indicating that both PLZ and PEH successfully sequestered the reactive aldehyde *in vitro*.

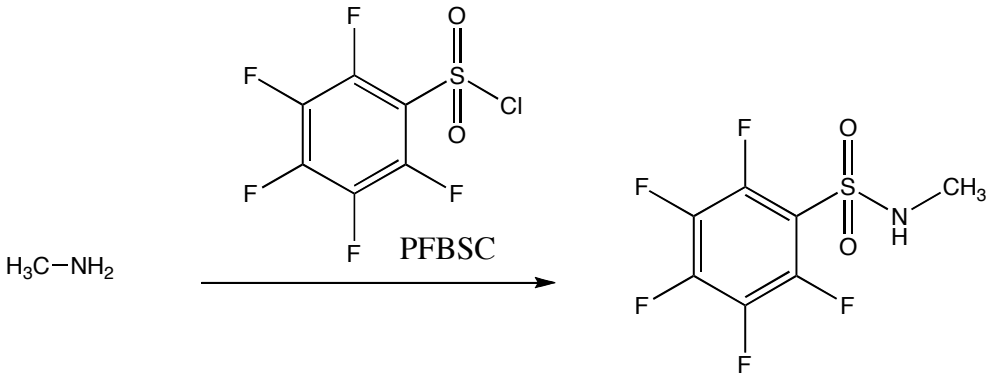

Figure 5-4. Derivatization reaction of MA with PFBSC.

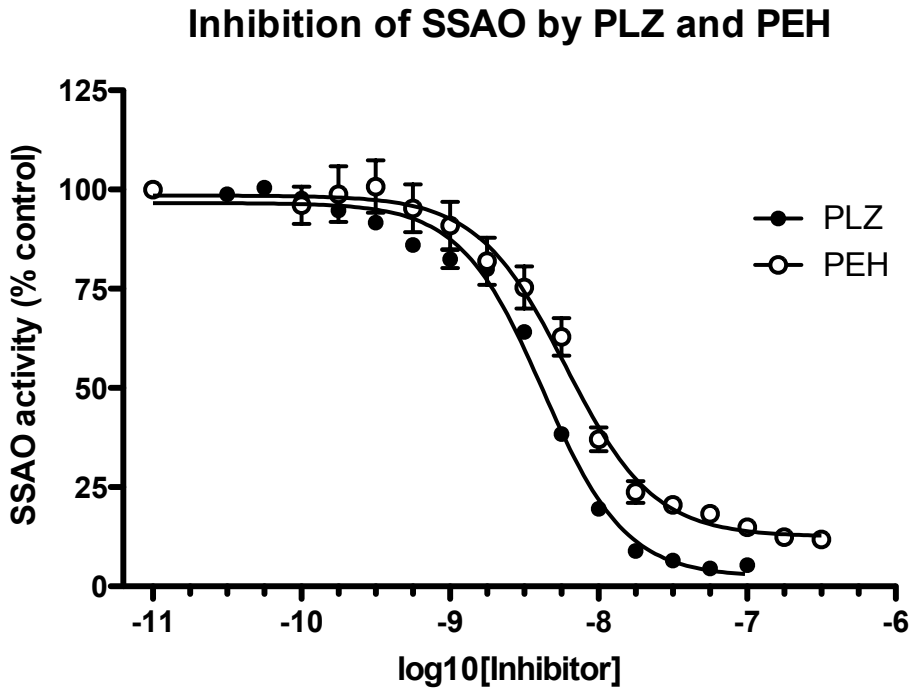

Figure 5-5. Effects of PLZ (dark circles) and PEH (open circles) on MA oxidation by hSSAO. Values are expressed as % control SSAO activity (mean ± SEM of 3 replicates)

5.3.2.1. Sequestration of FA by PLZ

A prominent peak (with a retention time of 13.06 minutes) corresponding to a PFBC-derivatized PLZ-FA hydrazone was present in samples containing both FA and PLZ, but was not present in samples containing FA alone, PLZ alone, or blanks (Figure 5-6). The mass spectrum of the peak confirmed the identity of the hydrazone, and its fragmentation pattern is shown in Figure 5-7. A prominent peak with a retention time of 12.8 minutes was shown to be FA derivatized by HYD; this peak was large in samples containing FA alone, markedly reduced in samples containing both FA and PLZ, and barely visible in samples containing PLZ alone and blanks (Figure 5-8). The mass spectrum confirmed the identity of FA derivatized with hydralazine; a strong parent ion (m/z=172) was observed with little fragmentation (data not shown). The FA peak in samples containing both FA and PLZ was reduced by 72% compared to samples containing FA alone, demonstrating the ability of PLZ to sequester FA *in vitro*. These data are shown in Figure 5-11.

5.3.2.2 Sequestration of FA by PEH

The underivatized FA-PEH hydrazone peak was detected at a retention time of 8.6 minutes. This peak was present in samples containing both FA and PEH, but not in samples containing FA alone, PEH alone or blanks (Figure 5-9). The mass spectrum confirmed the identity of the hydrazone, and its fragmentation pattern is shown in Figure 5-10. The FA peak in samples containing both FA and PEH was reduced by 48% compared to samples containing FA alone, confirming that PEH was also able to sequester FA *in vitro*. These data, with those of PLZ, are shown in Figure 5-11.

5.3.3 *Ex vivo* analysis of whole brain MA

The mass spectrum of the MA-PFBSC derivative confirmed its identity, and the fragmentation pattern is shown in Figure 5-12. Standard curves for

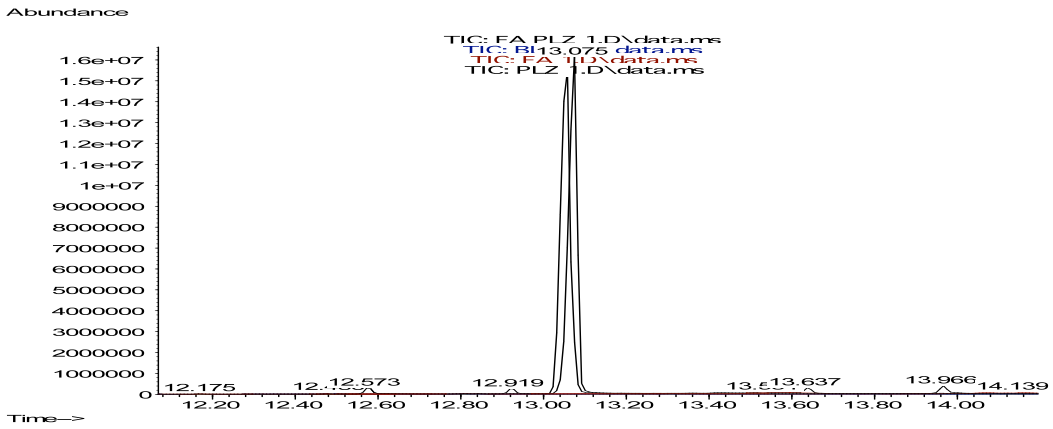

Figure 5-6. Overlaid chromatograms of PLZ alone, FA alone, PLZ-FA (hydrazone) and blank. While the hydrazone peak (the first of the two close peaks) and a PLZ peak (the second of the two peaks) are very close together, the mass spectrum of both peaks confirmed their identities. FA alone and blank chromatograms are not evident here because they do not contain any peaks in this region of the chromatogram.

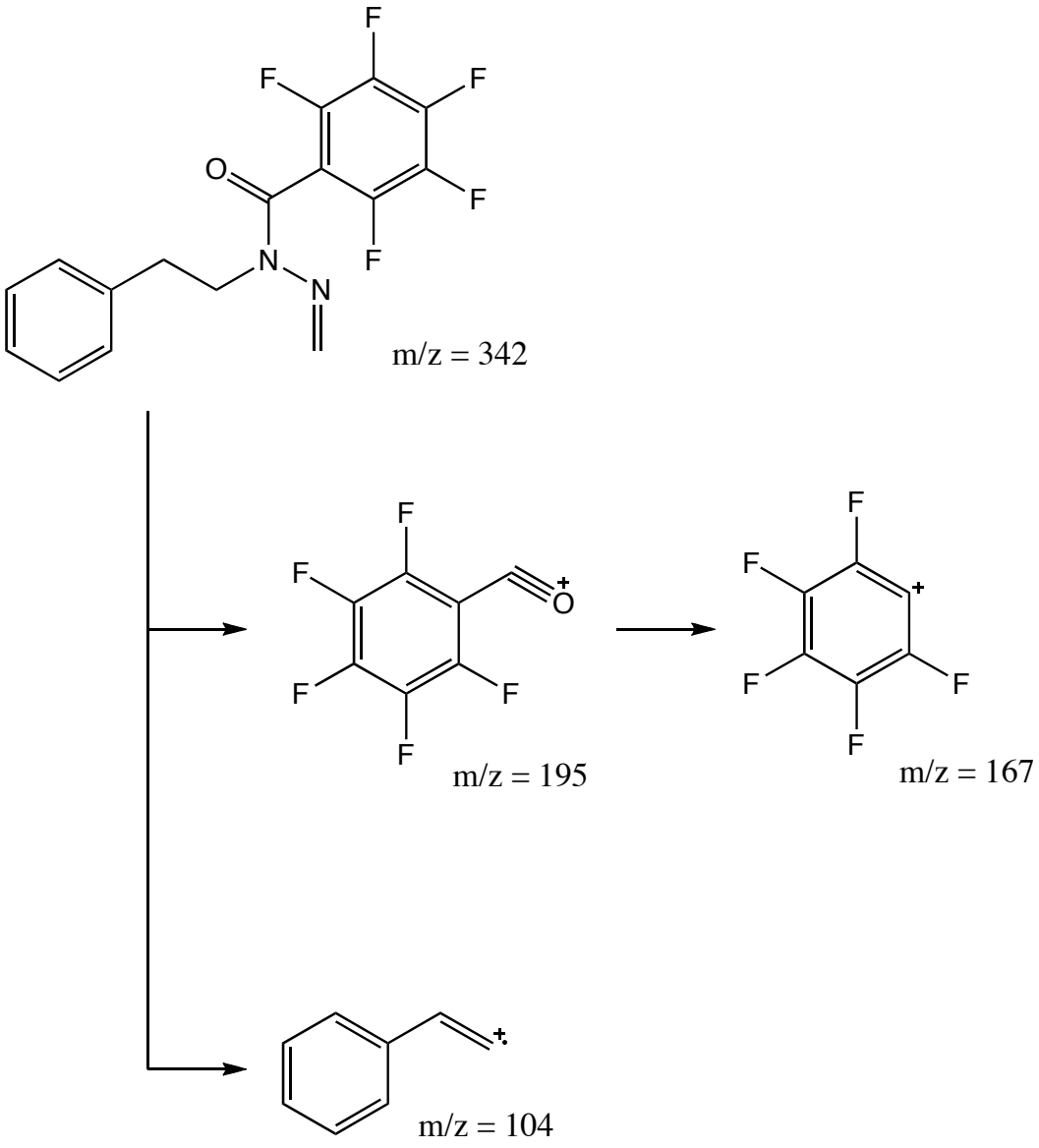

Figure 5-7. Mass fragmentation of the derivatized hydrazone formed from PLZ and FA.

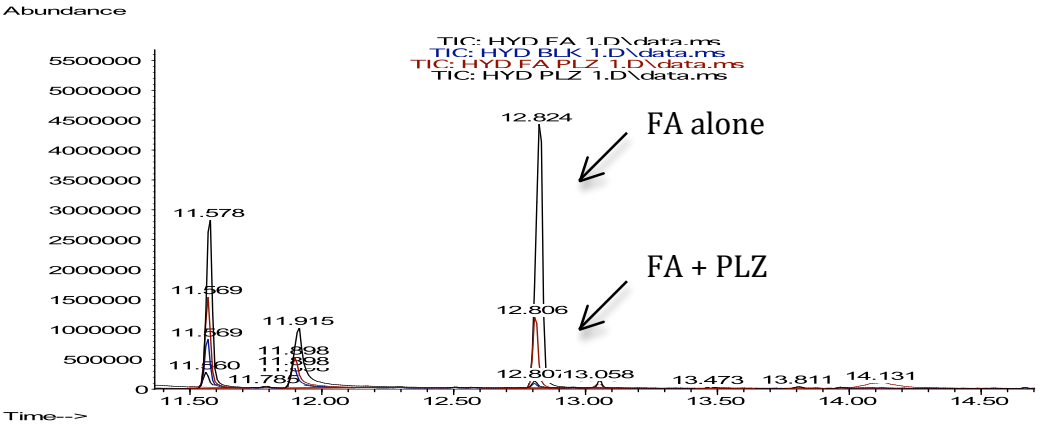

Figure 5-8. A sample chromatogram demonstrating the prominent FA peak at 12.8 minutes. Notably, the sample containing FA alone has a much bigger FA peak than the sample containing FA and PLZ together, suggesting that PLZ has sequestered free FA.

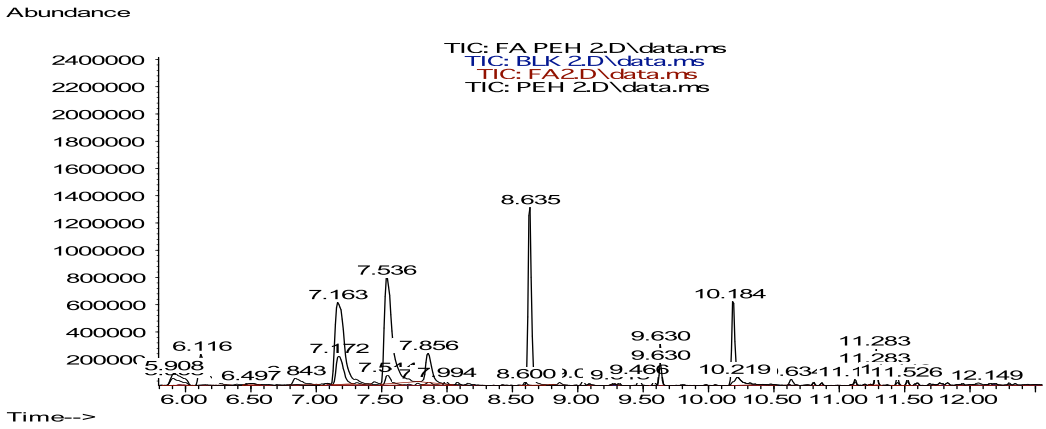

Figure 5-9. Chromatogram demonstrating the formation of a PEH-FA hydrazone at 8.6 minutes. Chromatograms of PEH alone, FA alone and the blank are also overlaid above, but are not evident because the PEH-FA hydrazone is not present in these samples.

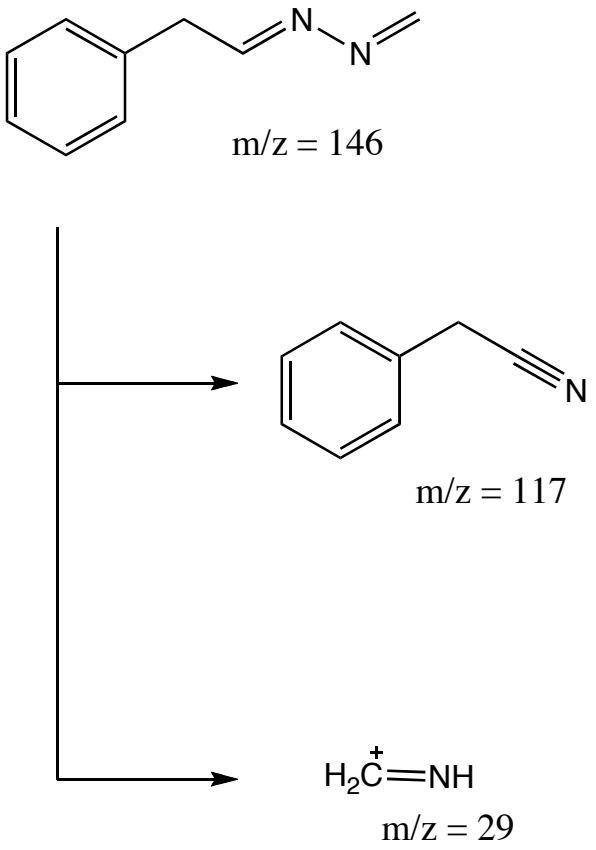

Figure 5-10. Mass fragmentation of the hydrazone formed by the reaction of PEH with FA.

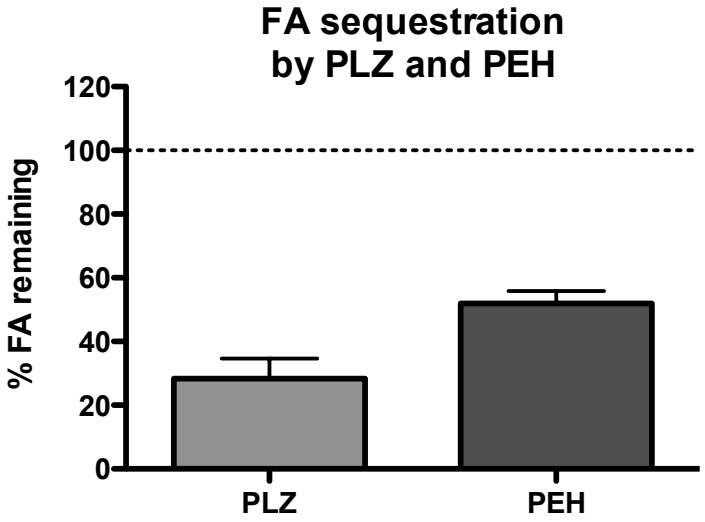

Figure 5-11. Free FA levels following incubation (15 minutes) with PLZ or PEH. Data represents the percentage of FA remaining relative to samples containing FA alone (mean ± SEM), n = 5-7 for each group.

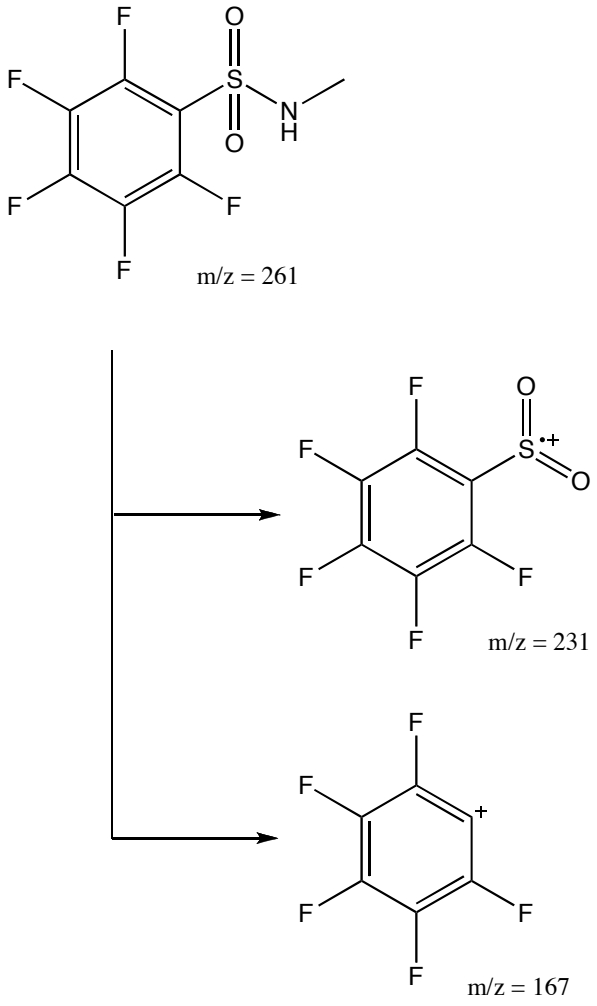

Figure 5-12. Fragmentation pattern of MA derivatized with PFBSC

MA were linear up to 400 ng/g, and were reproducible between runs. Whole brain MA values in vehicle-treated brains were 326 ± 68 ng/g (mean ± SEM).

Since PLZ and PEH inhibit SSAO, it was hypothesized that i.p. administration of these drugs would increase brain levels of the SSAO substrate MA. Indeed, 3 hours after drug administration, PLZ and PEH elevated brain MA levels to 214% and 192% of control, respectively. These data are shown in Figure 5-13.

5.4 DISCUSSION

The data presented in this chapter suggest novel mechanisms by which PLZ and PEH may exert neuroprotective actions via their effects on the SSAO system. PLZ and PEH inhibited the activity of human SSAO *in vitro*, an effect that was expected to reduce the oxidation of MA to the reactive aldehyde FA. Indeed, administration of PLZ and PEH to rodents significantly increased brain MA levels, indicating that SSAO inhibition causes accumulation of substrate and provides further evidence that FA formation would be reduced. Additionally, PLZ and PEH were able to sequester FA *in vitro*, resulting in the formation hydrazone complexes and a reduction in the concentration of free FA. Taken together, these data suggest that treatment with either PLZ or PEH would likely reduce FA-mediated neurotoxicity, not only because FA synthesis would be greatly reduced due to the inhibition of its synthetic enzyme, but also because FA is sequestered by these drugs. These data have important implications for the treatment of AD, since the activity and expression of SSAO have been reported to be increased in the brains of these patients (Ferrer *et al.* 2002; del Mar Hernandez *et al.* 2005), presumably leading to increases in levels of the toxic aldehyde FA, which has been shown to directly contribute to some aspects of the neuropathology of AD (discussed below).

To our knowledge, this is the first study to show that PLZ and PEH inhibit the activity of human SSAO. This is an extremely important finding, since

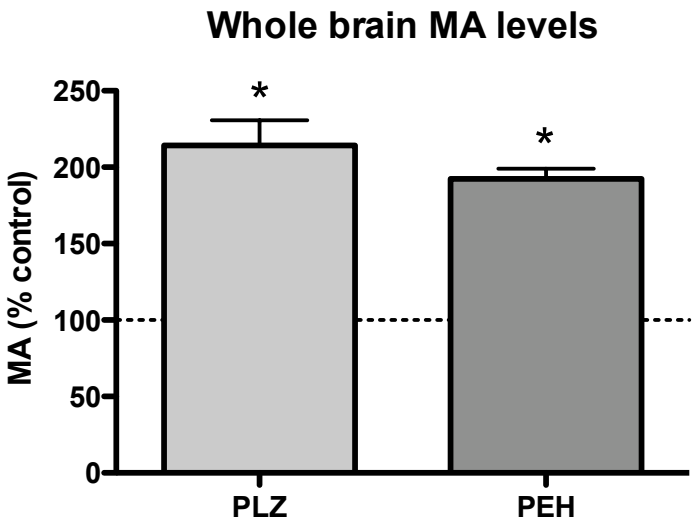

Figure 5-13. Whole brain MA levels 3 hours following PLZ (30 mg/kg) or PEH (30 mg/kg) administration. Values are expressed as mean ± SEM (% control). * denotes significantly different ($p < 0.05$) compared to vehicle controls.

inhibitor sensitivity varies between species, limiting the predictive value of animal studies for clinical applications. PLZ has been shown to inhibit bovine lung SSAO (Lizcano *et al.* 1996), but the current findings that human SSAO is also potently inhibited by PLZ provide a solid basis for evidence supporting putative neuroprotective actions of PLZ in a clinical setting. PEH also inhibited human SSAO, further supporting the notion that PEH should be investigated as a novel neuroprotective agent. While the inhibitor potencies of PLZ and PEH for rat SSAO relative to human SSAO are not known, the finding that PLZ and PEH caused marked elevations in rat brain MA (as measured *ex vivo*) confirms that inhibition of SSAO *in vivo* results in substrate accumulation, and it is probable that the formation of FA is reduced.

Both PLZ and PEH also sequestered FA *in vitro*. This result was not unexpected, since PLZ (and other hydrazines) have been shown to sequester other aldehydes, including 3-AP, acrolein and 4-HNE (Wood *et al.* 2006; Galvani *et al.* 2008). The decrease in FA concentration was greater with PLZ than with PEH at 15 minutes suggesting that PLZ may sequester the aldehyde more readily than PEH, but comprehensive time studies should now be conducted.

The abilities of PLZ and PEH to decrease the accumulation of reactive FA, by inhibiting FA synthesis and by sequestering FA produced from MA, is an extremely important result in terms of their putative neuroprotective actions. A number of pathological conditions, including AD, have been suggested to be mediated and/or exacerbated, at least in part, by an increase in SSAO activity and the consequent neurochemical sequelae (Ferrer *et al.* 2002; del Mar Hernandez *et al.* 2005). Increased expression of SSAO has been shown to be colocalized with β-amyloid (Aβ) deposits, which are major constituents of neuritic plaques in AD brains (Ferrer *et al.* 2002), supporting a role for this enzyme in the pathology of AD. Evidence from many studies has also suggested that FA is involved in the pathogenesis of AD; for example, FA was shown to form irreversible adducts with Aβ proteins *in vivo*,

producing amyloid-like complexes (Gubisne-Haberle *et al.* 2004). FA also induced Aβ aggregation and fibrillogenesis *in vitro* (Chen *et al.* 2006; Chen *et al.* 2007), and induced polymerization of tau protein (a major constituent of neurofibrillary tangles) both *in vitro* and *in vivo* (Nie *et al.* 2007). Aggregation of Aβ proteins was shown to be prevented by the sequestration of FA with aminoguanidine (Kazachkov *et al.* 2007), and the sequestration of FA with PLZ and PEH observed here could be expected to produce a similar result.

It should be noted that the reactive aldehydes acrolein and 4-HNE, products of lipid peroxidation, have also been shown to be increased in AD brains (Lovell *et al.* 1997; Markesbery and Lovell 1998; Lovell *et al.* 2001; Williams *et al.* 2006). In addition to the toxic effects of FA in the AD brain discussed above, reactive aldehydes also exert more general cytotoxic effects, resulting in apoptosis and necrosis and exacerbation of lipid peroxidation. Given that previous studies have shown that PLZ successfully sequesters 3-AP and acrolein and the present findings that PLZ (and PEH) also sequesters FA, it is reasonable to expect that PLZ and PEH would also sequester the excess aldehydes in AD brains in addition to FA, reducing the aldehyde load and thus contributing to neuroprotection.

Hydrazines are among the most powerful inhibitors of SSAO and have been reported to sequester various reactive aldehydes, but many of these drugs possess toxic effects or severe side effects (Matyus *et al.* 2004). For example, aminoguanidine has been shown to sequester FA *in vivo* and *in vitro*, but its clinical utility is limited due to potentially dangerous side effects (Kazachkov *et al.* 2007), including gastrointestinal symptoms, abnormalities in liver function, anti-nuclear associated lupus-like illness, and rarely anti-neutrophil cytoplasmic antibody-associated vasculitis (Freedman *et al.* 1999). While the toxicity of PEH remains to be determined in the human population, it shows no apparent toxicity in rodents. PLZ is well tolerated if the appropriate dietary precautions are taken, and has been available clinically for over 50 years. Therefore, PLZ and PEH could be important

therapeutic tools in reducing SSAO/FA-mediated neurotoxicity in AD and in other pathological conditions. Importantly, AD shares a number of pathological features and risk factors with other vascular disorders (such as atherosclerosis and diabetes mellitus) (Messier 2003), and SSAO pathology may quite possibly be a common link between these disorders (Yu 2001). While PLZ and PEH would not provide a "cure" for AD or other diseases related to abnormalities in the SSAO pathway, maintaining drug levels could reduce the toxic effects of excessive aldehydes, reducing neurotoxicity and presumably the behavioural and psychological symptoms associated with these diseases.

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CHAPTER 6.

EVALUATION OF MONO- AND DI-PROPARGYL ANALOGS OF PEH AS PEH PRODRUGS

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6.1 INTRODUCTION

β-Phenylethylidenehydrazine (PEH) is a metabolite of the antidepressant/ antipanic/neuroprotective drug phenelzine (PLZ) and is formed by the action of monoamine oxidase (MAO) on PLZ (Chapter 2). PEH shares a number of neurochemical properties with PLZ, including (a) the inhibition of GABA-transaminase (GABA-T) activity (Paslawski *et al.* 2001); (b) the ability to increase brain levels of GABA (Paslawski *et al.* 2001; Parent *et al.* 2002), ALA (Chapter 2) and ornithine (ORN) (MacKenzie *et al.* 2008); and (c) the ability to transiently decrease brain levels of glutamine (Paslawski *et al.* 2001).

Interestingly, inhibition of MAO prior to PLZ administration inhibits the formation of PEH (Chapter 2) and prevents some of these effects, such as inhibition of GABA-T and elevation of GABA and ornithine (Popov and Matthies 1969; Todd and Baker 1995; MacKenzie *et al.* 2008). PEH formation may also contribute to some of the therapeutic effects of PLZ. For example, the anxiolytic properties of PLZ have been shown to be related to the drug's facilitatory effect on GABAergic transmission (Paslawski *et al.* 1996), and given that the increase in GABA appears to be dependent upon PEH formation, it is reasonable to suggest that PEH may mediate the anxiolytic effects of PLZ. Furthermore, PLZ and PEH have both been shown to be neuroprotective in animal models of global ischemia (Tanay *et al.* 2002; Wood *et al.* 2006), and while the mechanism(s) for this have not been elucidated, the ability of PLZ and PEH to increase brain GABA (Popov and Matthies 1969; Baker *et al.* 1991; McKenna *et al.* 1991; McManus *et al.* 1992; Paslawski *et al.* 1995; Parent *et al.* 1999, 2000), to potentially reduce glutamatergic transmission (Michael-Titus *et al.* 2000; Yang and Shen 2005), and to sequester reactive aldehydes (Chapter 5 of this thesis; Wood *et al.* 2006) probably contribute to neuroprotection. Importantly, PEH differs from PLZ in that it does not appreciably inhibit MAO activity (Paslawski *et al.* 2001). Given the strict dietary restrictions that are necessary for individuals

taking PLZ due to potentially dangerous interactions between the drug and tyramine-rich foods, PEH may be a useful alternative for conditions thought to involve GABAergic dysfunction and in which PLZ is effective (e.g. depression, social anxiety disorder, panic disorder) but is not used as a first-line drug because of this adverse effect. Certainly, PEH should be further investigated as a therapeutic drug in and of itself.

In the experiments described in this chapter, two analogs of PEH, namely *N*-mono-(2-propynyl)-PEH (PrPEH) and *N*-bis-(2-propynyl)-PEH (diPrPEH), were synthesized as potential prodrugs of PEH and compared to PEH with regard to their ability to inhibit GABA-T and MAO-A and -B *ex vivo*. Metabolic removal of *N*-propynyl (*N*-propargyl) group(s) has been shown to occur readily in a number of arylalkylamine drug molecules (Durden *et al.* 1975; Pirisino *et al.* 1978; Reynolds *et al.* 1978; Philips 1981; Weli and Lindeke 1985, 1986; Yoshida *et al.* 1986; Heinonen *et al.* 1989; Kalasz *et al.* 1990; Shin 1997; Bach *et al.* 2000; Am *et al.* 2004; Rittenbach *et al.* 2007). Moreover, effective prodrugs have been produced by the addition of one or two propynyl groups to the biogenic amine β-phenylethylamine (PEA), increasing brain, liver and plasma levels of PEA when compared to administration of the non-propargylated form of the amine and prolonging its clearance from the body (Rao *et al.* 1987a, 1987b). Since PEH appears to possess a number of potentially beneficial neurochemical properties, it was of interest to see if the drugs synthesized here are effective prodrugs. The development of an analytical assay for PrPEH and diPrPEH demonstrated that these drugs were able to penetrate the blood-brain barrier, and analysis for PEH in PrPEH- and diPrPEH-treated brains was used to determine their ability to act as prodrugs of PEH.

6. 2 METHODS

6.2.1 Synthesis of PrPEH and diPrPEH

PrPEH and diPrPEH were synthesized by Dr. E.E. Knaus, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. A mixture of 1-

(2-propynyl)hydrazine (compound **1** in Figure 6.1) and 1,1-bis-(2-propynyl)hydrazine (**2**) was condensed with phenylacetaldehyde (**3**) in methanol at 25°C, as illustrated in Figure 6-1. The products, *N*-mono-(2-propynyl) (PrPEH; **4**) and *N*-bis-(2-propynyl) (diPrPEH; **5**), were separated using silica gel flash chromatography, and the structures were confirmed using high-resolution nuclear magnetic resonance (NMR) spectroscopy (MacKenzie *et al.* 2008). The products also yielded single peaks using HPLC under a variety of conditions (see Section 6.3.5 for details) and their purity was further confirmed using HPLC-MS.

6.2.2 Drug injections

For the time study, rats were injected intraperitoneally (i.p) with equimolar amounts of PEH (30 mg/kg), PrPEH (38.5 mg/kg) or diPrPEH (47 mg/kg) or vehicle (corn oil), and were decapitated 1, 3, 6 or 12 hours after drug injection. For the dose study, rats were injected with low and high doses of PrPEH (19.25 mg/kg and 77 mg/kg) or diPrPEH (23 mg/kg and 94 mg/kg), and brains were collected 3 hours after drug injection, flash-frozen in 2-methylbutane on solid carbon dioxide, and stored at -80°C. Partially-thawed brains were homogenized in 5 volumes of ice-cold ddH_2O, and stored in aliquots at -80°C for use in subsequent analyses (GABA-T, MAO-A and MAO-B activities, and brain levels of amino acids, biogenic amines and the drugs) after addition to the appropriate assay medium.

6.2.3 *Ex vivo* analysis of GABA-T activity

GABA-T activity was measured in triplicate using a modification of the radiochemical procedure of Sterri and Fonnum (1978). Rat brains

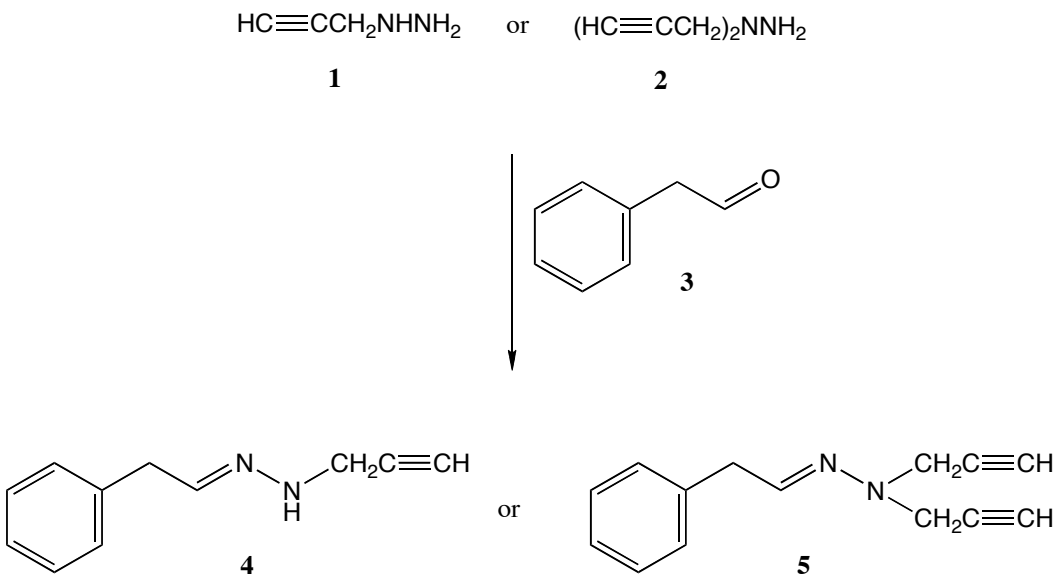

Figure 6-1. Synthesis of PrPEH (**4**) and diPrPEH (**5**).

(homogenized in 5 volumes ice-cold ddH_2O) were further diluted by a factor of 2 in homogenizing medium. Tissue homogenate (10 μl) and 20 μl of incubation medium were added to microfuge tubes on ice, which were then incubated at 37°C for 30 minutes. ddH_2O (10 μl) was added instead of tissue homogenate for blanks. The reaction was terminated by placing the microfuge tubes on ice and adding 100 μl of the previously-prepared liquid anion exchanger tri-n-octylamine (TOA) (preparation described below). Tubes were capped, vortexed and centrifuged at 4°C for 2 minutes at 13000 g. Following centrifugation, 35 μl of the organic layer was drawn off and added to scintillation vials containing 4ml of scintillation fluid (Beckman Coulter ReadySafe™ liquid scintillation cocktail). Radioactivity (dpm) was counted for 3 minutes using a liquid scintillation counter (Beckman LS 7500).

The homogenizing medium was composed of 40% volume/volume (v/v) glycerol, 2% v/v Triton X-100, 2 mM glutathione, 2 μM pyridoxal phosphate, 2 mM sodium ethylenediaminetetraacetic acid (NaEDTA) and 10 mM K_2HPO_4 in ddH_2O. The pH was adjusted to 7.4 using glacial acetic acid. The incubation medium was composed of 1 μl of ^{3}H-GABA (1 μCi), 248 mM Tris, 5 mM non-radiolabelled GABA, 5 mM α-ketoglutarate, 1mM NAD and 1 mM aminoethylisothioluronium bromide in ddH_2O. The TOA was prepared as follows: 2.2 ml of TOA combined with 22.8 ml of ethyl acetate was added to 49.2 ml of ddH_2O and 850 μl phosphoric acid. The resultant mixture was shaken for 4 minutes and centrifuged at 834 g for 10 minutes, and the organic layer was retained.

6.2.4 *Ex vivo* analysis of MAO activity

MAO activity was determined using a modified protocol described by Lyles and Callingham (1982). Brain homogenates in 5 volumes ddH_2O were further diluted by a factor of 16 in 0.2M potassium phosphate buffer (adjusted to pH 7.8). In borosilicate glass culture tubes on ice, 50μl tissue homogenate and 50μl of appropriate radiolabelled substrate were mixed. To

the blanks, 10 μl 3M hydrochloric acid (HCl) was also added to prevent the reaction from proceeding. Tubes were flushed with O_2, stoppered quickly and incubated in a water bath at 37°C for 10 minutes. Tubes were then placed on ice and 10 μl 3M HCl were added to the rest of the tubes. After the addition of 1 ml ethyl acetate/toluene (1:1 v/v, water-saturated) (1 ml), tubes were vortexed briefly and centrifuged at low speed (470 g) for 30 seconds. A portion (700 μl) of the organic layer was transferred into a scintillation vial containing 4 ml scintillation fluid and 50 μl glacial acetic acid, and radioactivity (dpm) was counted for 3 minutes using a scintillation counter.

Radiolabelled 5-HT (5-hydroxy[G-^{3}H]tryptamine creatinine sulfate) and PEA (β-phenyl[1-^{14}C]ethylamine chloride) were used as substrates for MAO-A and MAO-B, respectively. Due to the high specific activity of the stock radiolabelled substrates, stocks were diluted with their respective unlabelled amines, giving final dilutions of 50 mM, 1 μCi/μmol for 5-HT and 10 mM, 1 μCi/μmol for PEA. These diluted stocks were further diluted in 0.2M potassium phosphate (1/100) for the assay.

6.2.5 *Ex vivo* analysis of amino acid levels

Brain levels of the amino acids GABA, alanine, glutamate, glycine, L- and D-serine, taurine, glutamine and arginine (ARG) were determined using a modified procedure previously described by Grant *et al* (2006) for the quantification of amino acids using HPLC combined with fluorescence detection, following derivatization with fluoraldehyde reagent [o-phthaldialdehyde (OPA)] and isobutyryl-L-cysteine (IBLC). The procedure is described in Section 2.2.

6.2.6 *Ex vivo* analysis of biogenic amines

Brain levels of the biogenic amines noradrenaline (NA), dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin (5-HT) and its metabolite 5-

hydroxyindoleacetic acid (5-HIAA) were measured using a procedure described by Baker *et al* (1987). Tissue homogenates (in 5 volumes of ddH_2O) were further diluted by a factor of 10 in ice-cold 1N perchloric acid containing 10mg EDTA/100ml and 0.05 mM ascorbic acid, and samples were centrifuged for 4 minutes at 13,000g at 4°C. The supernatant was transferred to HPLC vials and injected onto the analytical column, an Atlantis dC_{18} 3µm (3.0 x 100mm) column connected to an Atlantis dC_{18} guard column. The mobile phase consisting of 55mM $NaH_2PO_4 \cdot H_2O$, 0.85mM sodium octyl sulfate, 0.37mM NaEDTA and 8% acetonitrile was filtered through a 0.2µm filter after the pH was adjusted to 2.9 with phosphoric acid. The mobile phase was pumped through the system at a flow rate of 0.3 ml/minute. The voltage on the ECD was set to 0.75V.

6.2.7 Analysis of PrPEH, diPrPEH and PEH levels in brain

The procedure for measuring PrPEH and diPrPEH levels in drug-treated brains was the same as for the biogenic amines assay with the following exceptions: (1) after diluting the brain samples in 1N perchloric acid, the samples were centrifuged for 5 minutes at 15000g and 4°C; and (2) the pH of the mobile phase was adjusted to 3.7. The structures of the PEH analogs were confirmed using GC-mass spectrometry (MS). The procedure for the determination of brain PEH levels was described in Section 2.2.

6.2.8 Analysis of PEH levels in brain

Brain levels of PEH were measured using the second half of the treated brains. The procedure for the analysis of PEH was described Section 2.2.2.

6.2.8 Statistics

Data were analyzed by analysis of variance (ANOVA), followed by the Newman-Keuls test. Statistical significance was established using a probability value of <0.05.

6.3 RESULTS

6.3.1 *Ex vivo* analysis of GABA-T activity

In the time study, PEH significantly reduced GABA-T activity at all time points tested; activity was reduced to 75% of controls at 1 hour post-injection, 66% of controls at 3 and 6 hours post-injection, and activity remained inhibited 12 hours after PEH injection (70% of control). The degree of GABA-T inhibition by PEH at this dose is consistent with previous data (Paslawski *et al.* 2001). PrPEH and diPrPEH also decreased GABA-T activity at 1, 3 and 6 hours following drug administration, although the inhibitory effect was significantly weaker than that observed following PEH administration at 6 hours for PrPEH and 3 and 6 hours for diPrPEH (PrPEH inhibited GABA-T to 73%, 76% and 77% of control at 1, 3 and 6 hours respectively, and diPrPEH inhibited GABA-T to 86% of control at 1, 3 and 6 hours after injection). GABA-T activity was not inhibited at 12 hours following PrPEH and diPrPEH injection. These results are shown in Figure 6-2.

In the dose study, PrPEH significantly inhibited GABA-T at both the low dose (19.25 mg/kg) and the high dose (77 mg/kg), reducing enzyme activity to 78% and 65% of control, respectively. diPrPEH also inhibited GABA-T at the low dose (23.5 mg/kg) and high dose (94 mg/kg), where GABA-T activity was reduced to 80% and 65% of control, respectively. These results are shown in Figure 6-3.

6.3.2 *Ex vivo* analysis of MAO activity

PEH has been shown previously to have only a weak, transient effect on MAO-A and -B activity *ex vivo* (Paslawski *et al.* 2001), and in the time study, PEH did not alter the activity of either MAO-A or -B in brain tissue taken from

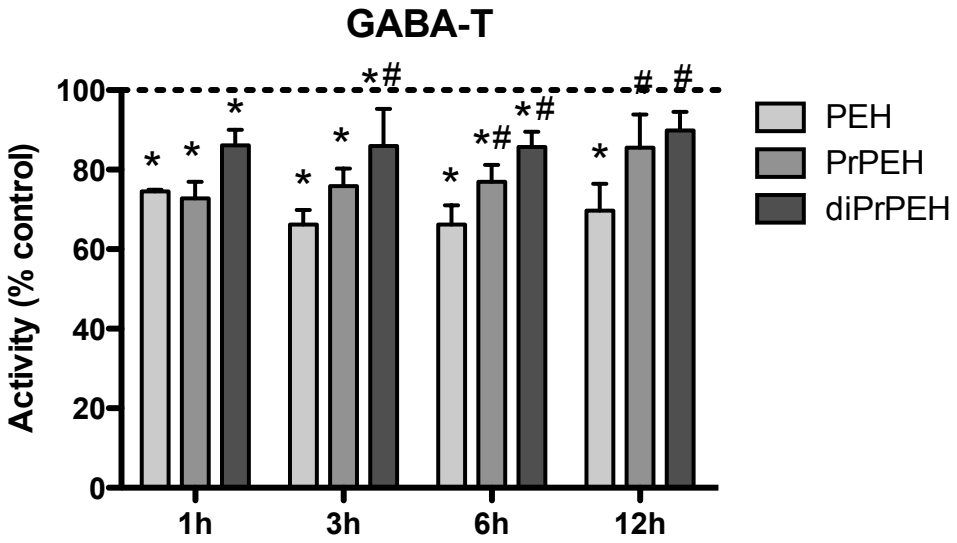

Figure 6-2. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on GABA-T activity *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH; # denotes $p \leq 0.05$ compared to PEH.

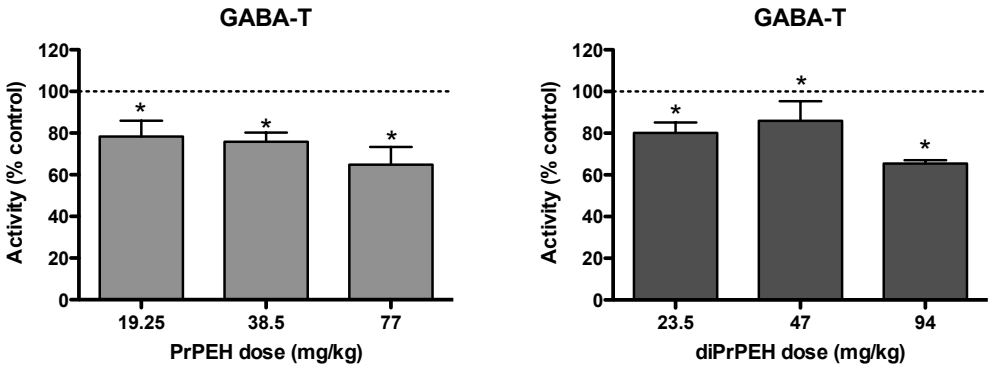

Figure 6-3. Dose-dependence study of PrPEH (left) and diPrPEH (right) on the activity of GABA-T at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % of control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

PEH-treated rats. PrPEH had a weak, transient effect on MAO-A, significantly reducing activity at 3 and 6 hours following drug administration to 78% and 70% of control values, respectively, and diPrPEH did not significantly alter MAO-A activity at any time point tested. In contrast, both PrPEH and diPrPEH had relatively marked inhibitory effects on MAO-B activity, significantly reducing MAO-B activity at all time points tested (except for diPrPEH at 1 hour, which did not reach statistical significance). These effects were particularly pronounced at 6 and 12 hours following drug injection, where MAO-B activity was reduced by PrPEH to 24% and 29% of control at 6 and 12 hours (respectively) and by diPrPEH to 58% and 25% of control at 6 and 12 hours (respectively).

In the dose study, PrPEH inhibited both MAO-A and -B at the high dose (77.0 mg/kg), inhibiting MAO-A to 64% of control and MAO-B to 38% of control; PrPEH at the low dose (19.25 mg/kg) did not significantly inhibit either MAO isoforms. diPrPEH significantly inhibited MAO-B at the high dose, reducing the enzyme activity to 67% of control; MAO-B was not inhibited by the low dose of diPrPEH, and MAO-A was not inhibited by either drug dose. These results are shown in Figures 6-4 to 6-7.

6.3.3 *Ex vivo* analysis of amino acid levels

Consistent with our previous findings (Paslawski *et al.* 2001), the *ex vivo* time studies demonstrated that PEH significantly increased brain GABA and ALA levels at all timepoints (except for ALA at 1 hour) following drug administration. Elevations in brain GABA and ALA were greatest at 3 hours following PEH injection, reaching 418% and 489% of control values, respectively. PEH did not significantly alter brain glutamate or glycine, nor did it affect brain levels of D-serine or L-serine at any timepoint relative to controls. A transient decrease in brain glutamine was observed at 3 hours following PEH injection, where glutamine was decreased to 63% of control values; this effect on glutamine is consistent with previous findings

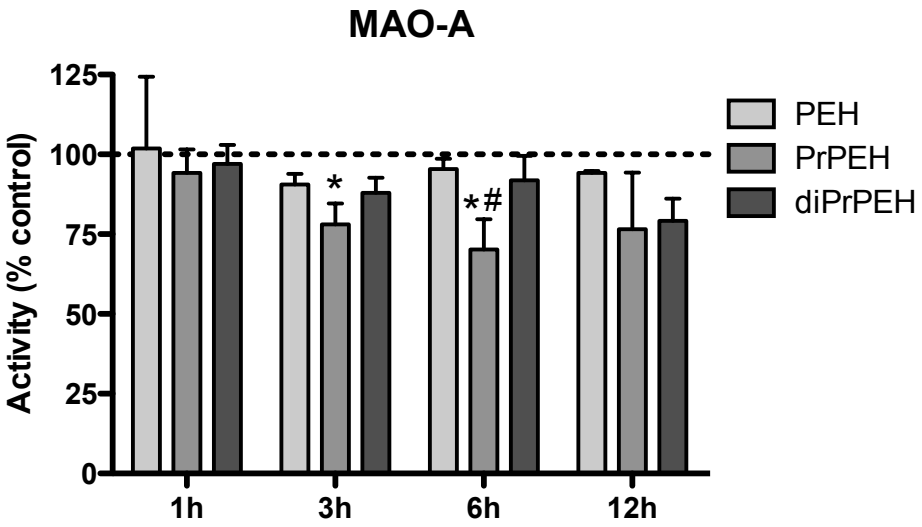

Figure 6-4. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on MAO-A activity *ex vivo*. n = 3-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

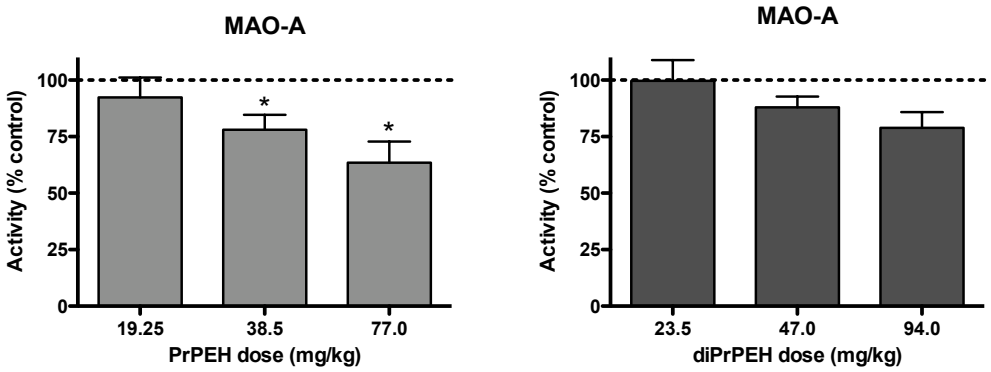

Figure 6-5. Dose-dependence study of PrPEH (left) and diPrPEH (right) on the activity of MAO-A at 3 hours *ex vivo*. n = 3-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

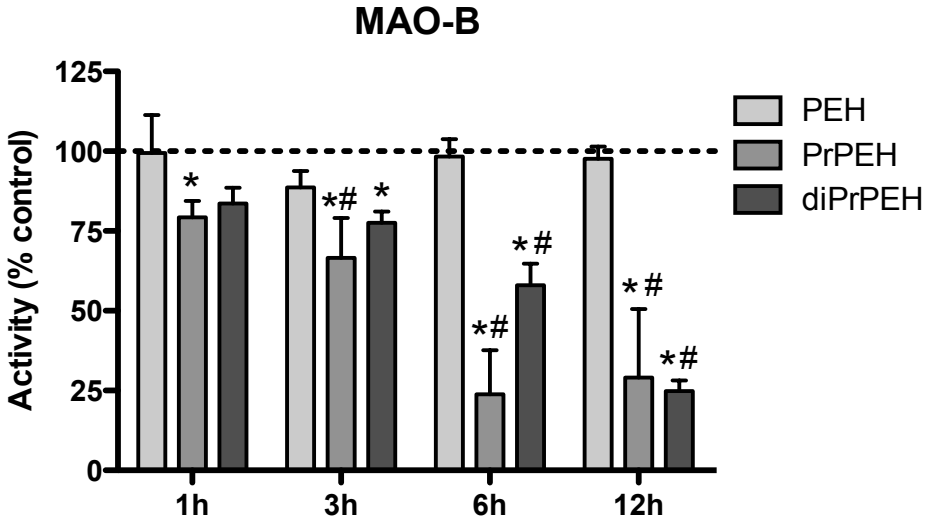

Figure 6-6. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on MAO-B activity *ex vivo*. n = 3-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

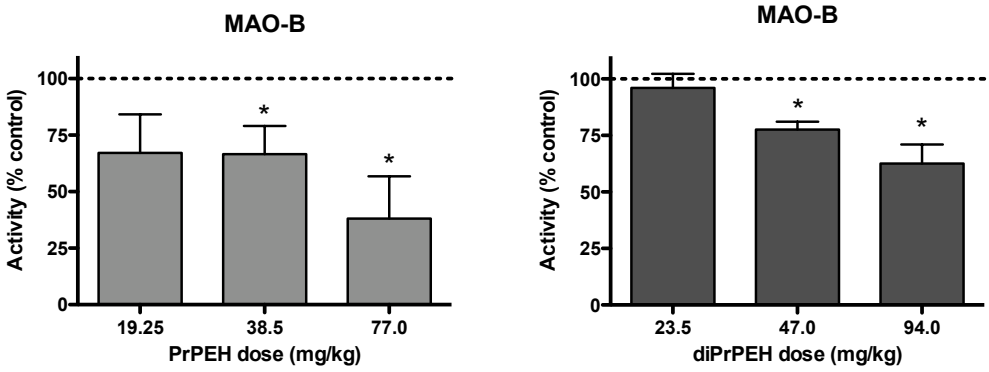

Figure 6-7. Dose-dependence study of PrPEH (left) and diPrPEH (right) on the activity of MAO-B at 3 hours *ex vivo*. n = 3-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

(Paslawski 1998). PEH also significantly increased brain taurine at 6 hours post-injection (increasing taurine to 126% of control).

Neither PrPEH nor diPrPEH significantly altered GABA, ALA or glutamate levels at any time point tested. Further, high and low doses of PrPEH (77 mg/kg and 19.25 mg/kg, respectively) and diPrPEH (94 mg/kg and 23.5 mg/kg, respectively) at 3 hours did not affect the brain levels of these amino acids (Figures 6-8 to 6-13). However, interestingly PrPEH and diPrPEH caused marked increases in brain glycine levels; PrPEH significantly increased glycine levels at 1, 3 and 6 hours post-injection, peaking at 216% of control at 6 hours, and diPrPEH increased glycine levels at 1, 3, 6 and 12 hours post-injection, peaking at 248% of control at 12 hours. In the dose study, the high dose of PrPEH at 3 hours increased brain glycine levels to 171% of control (compared to the increases elicited by the low and middle doses, increasing glycine by 143% and 138% of control, respectively). There did not appear to be any dose-dependent effects of diPrPEH on the elevation in brain glycine, with the low, middle and high dose elevating brain levels by 152%, 139% and 154%, respectively. These data are shown in Figures 6-8 to 6-15.

In the time study, PrPEH did not affect brain levels of L- or D-serine or glutamine at any timepoint, and taurine was significantly increased only at 6 hours post-injection (increasing taurine levels to 127% of control). diPrPEH significantly decreased L-serine at 1 and 3 hours following drug injection, decreasing values to 63% and 67% of control, respectively, but did not affect the levels of D-serine , taurine or glutamine at any of the timepoints tested. Results from the dose study revealed that administration of the high and low doses of both PrPEH and diPrPEH significantly decreased brain levels of L-serine, but no other changes were observed in brain levels of D-serine, taurine or glutamine with either analog. These results are shown in Figures 6-16 to 6-23.

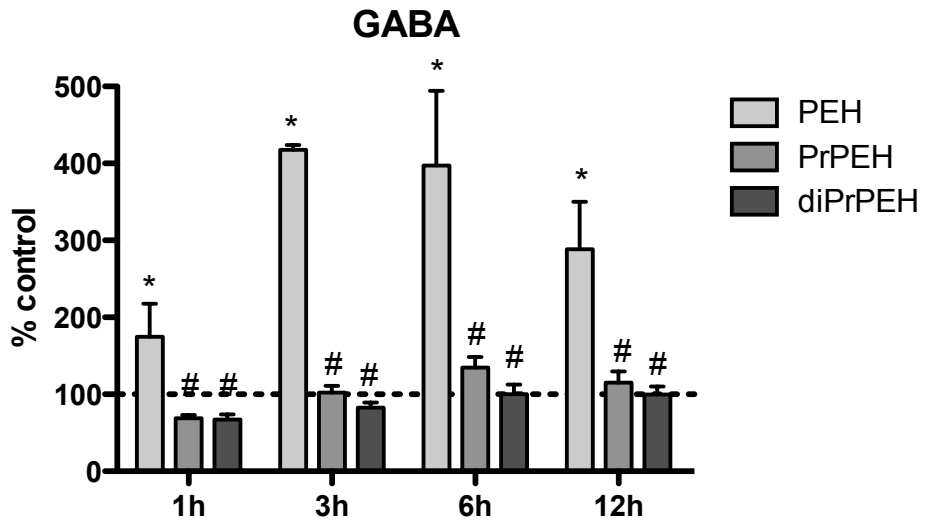

Figure 6-8. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain levels of GABA in rats. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

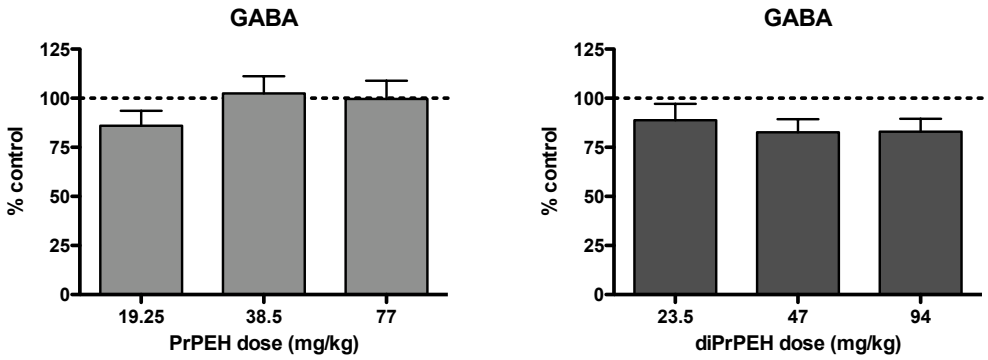

Figure 6-9. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain GABA levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

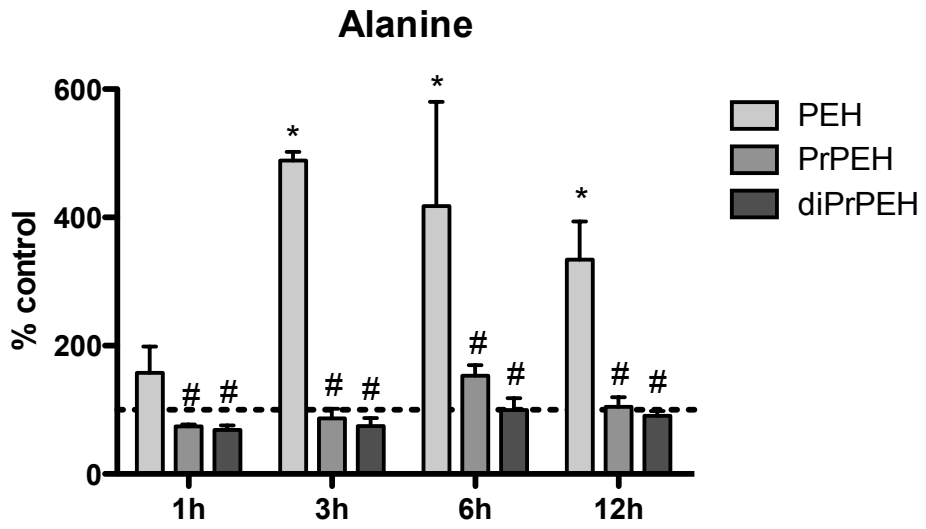

Figure 6-10. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain levels of ALA in rats. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

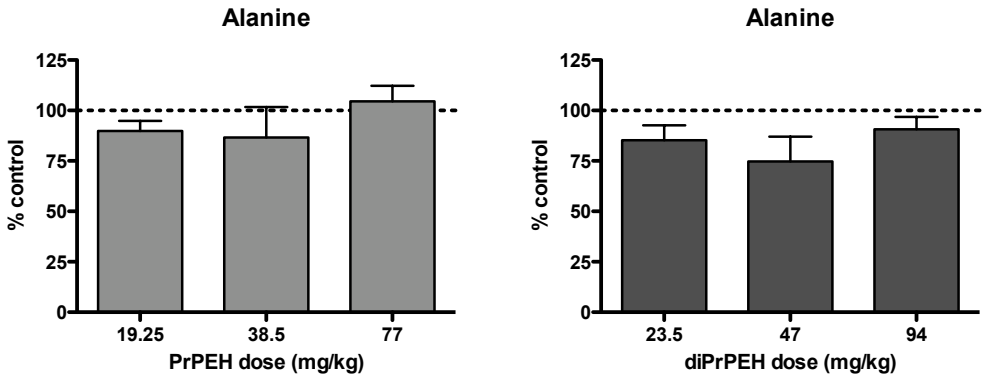

Figure 6-11. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain ALA levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

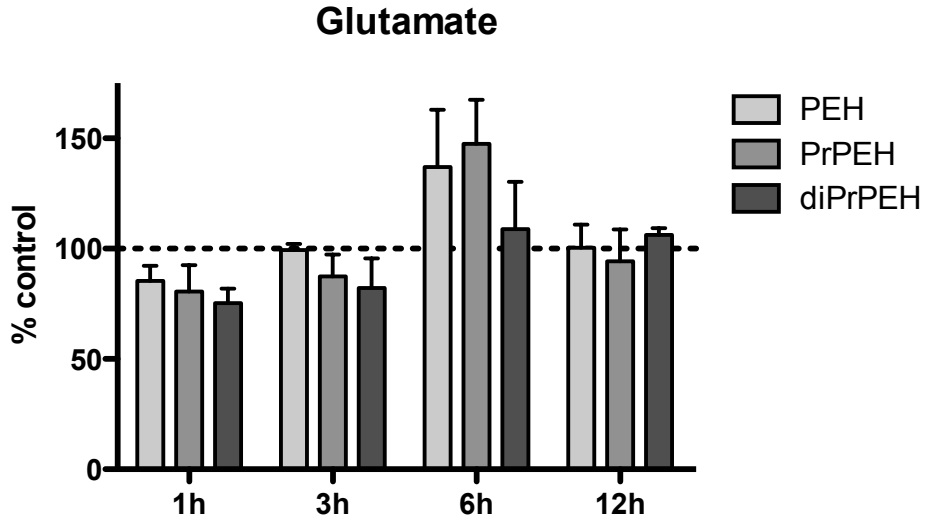

Figure 6-12. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain levels of glutamate in rats. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

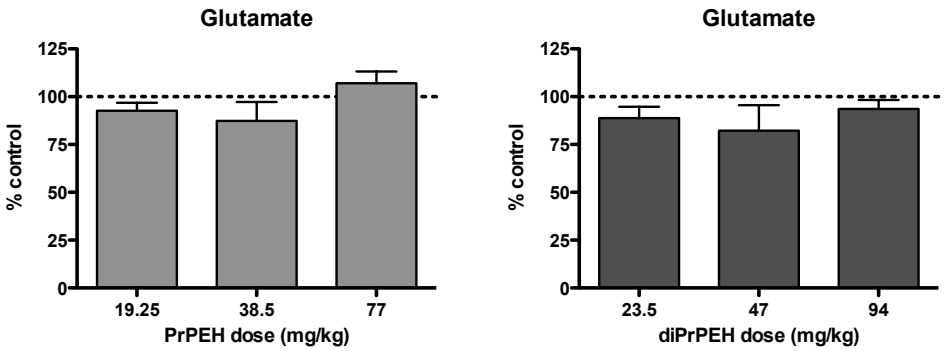

Figure 6-13. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain glutamate levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

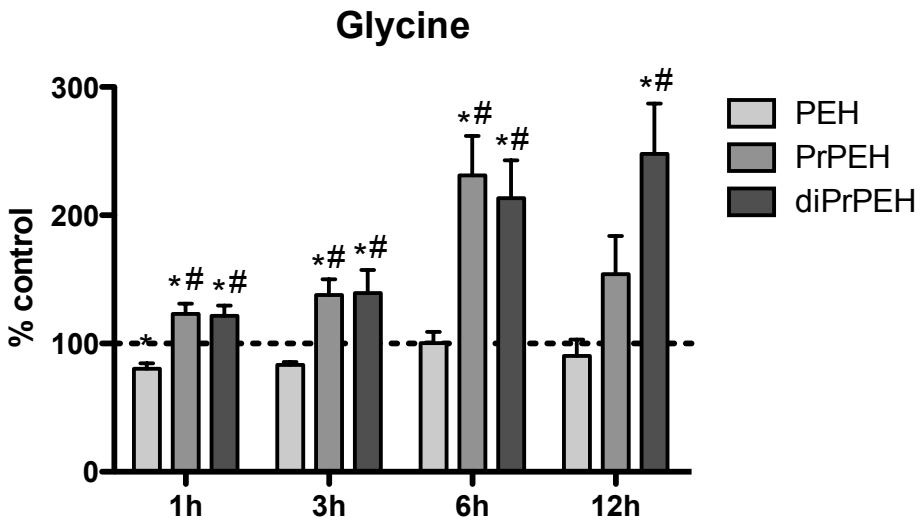

Figure 6-14. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain glycine levels. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes p 0.05 compared to PEH.

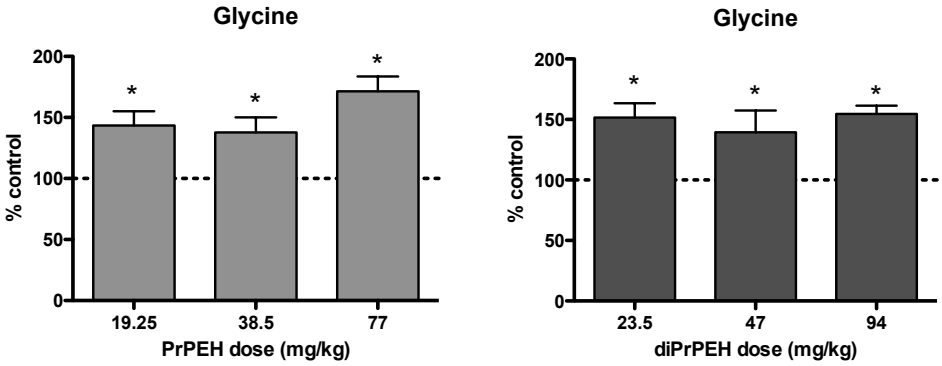

Figure 6-15. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain glycine levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

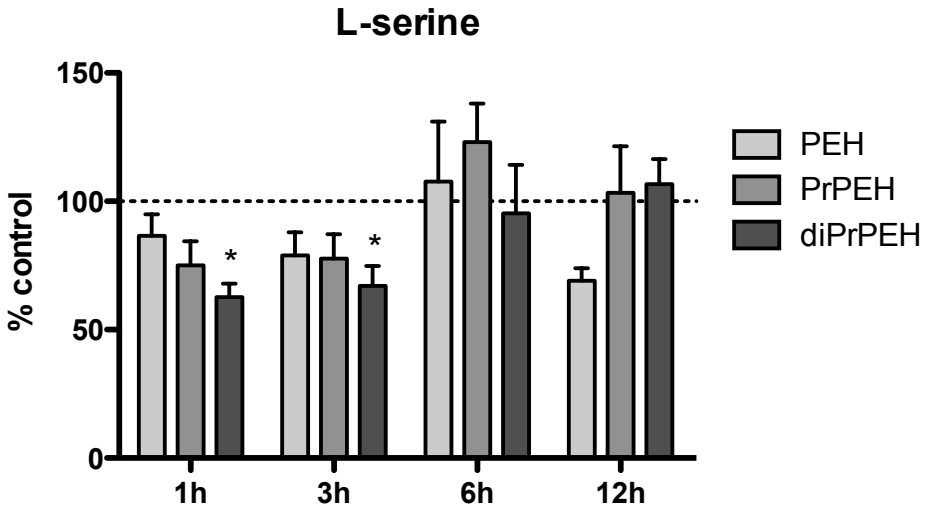

Figure 6-16. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain levels of L-serine in rats. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

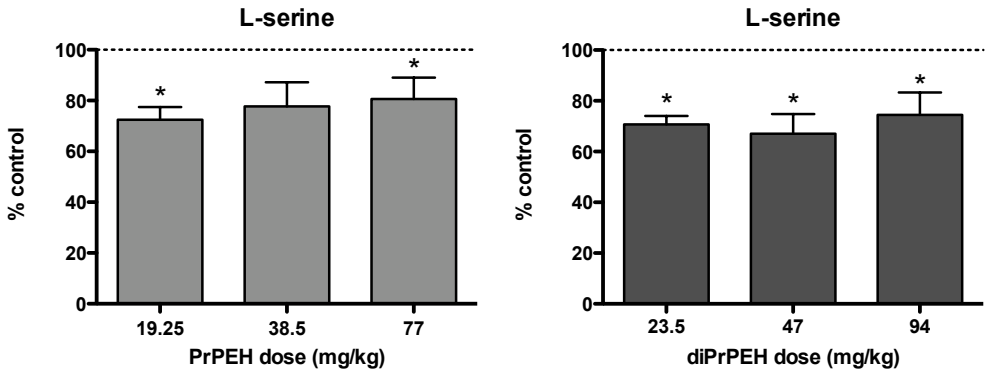

Figure 6-17. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain L-serine levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

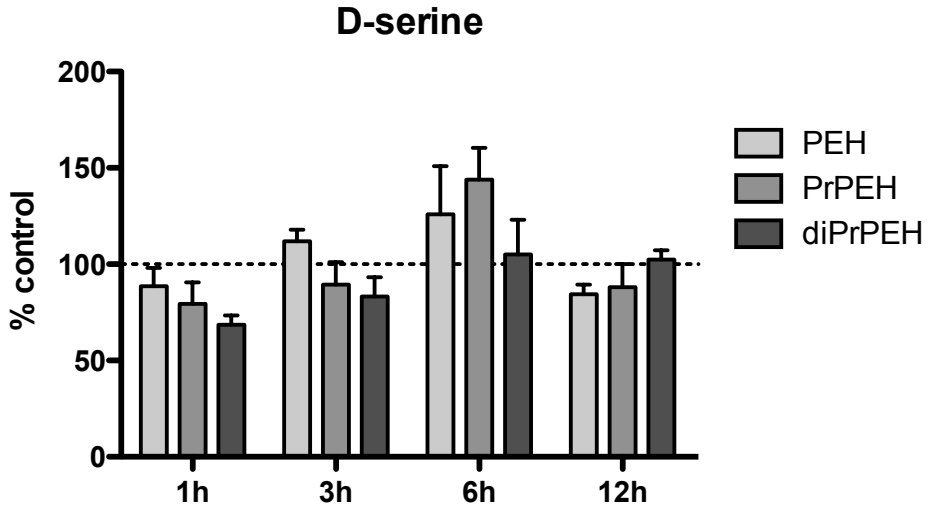

Figure 6-18. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain levels of D-serine in rats. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

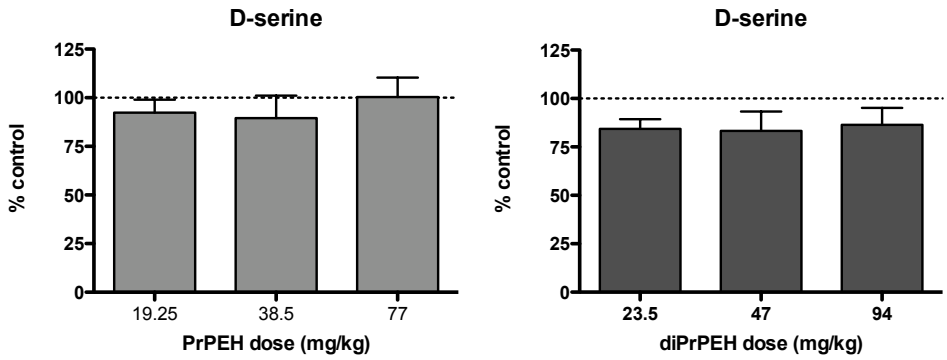

Figure 6-19. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain D-serine levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

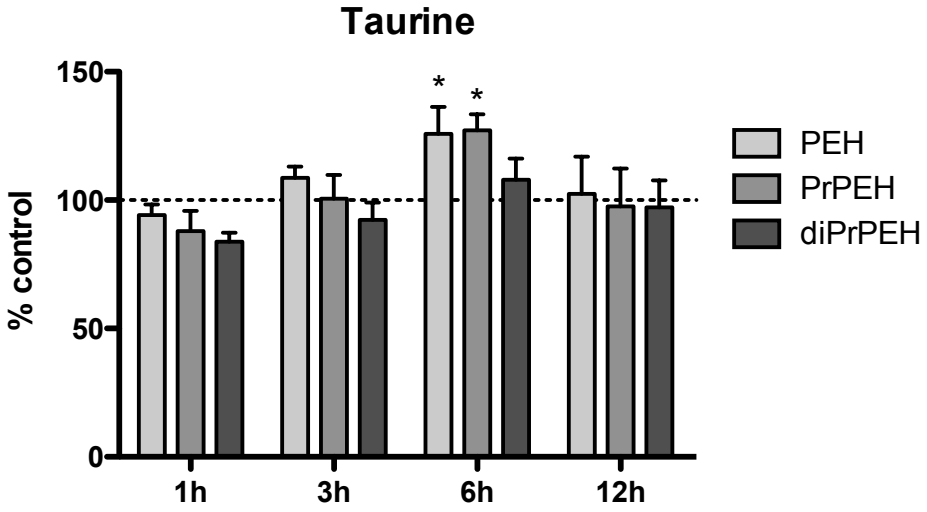

Figure 6-20. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain taurine levels. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

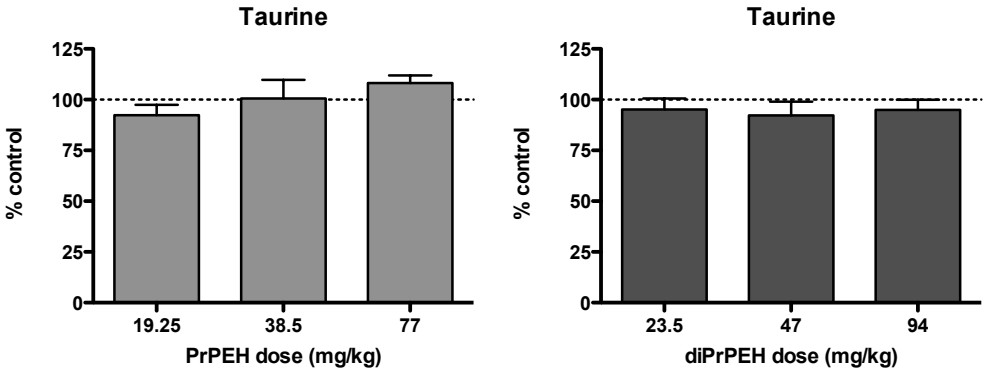

Figure 6-21. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain taurine levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

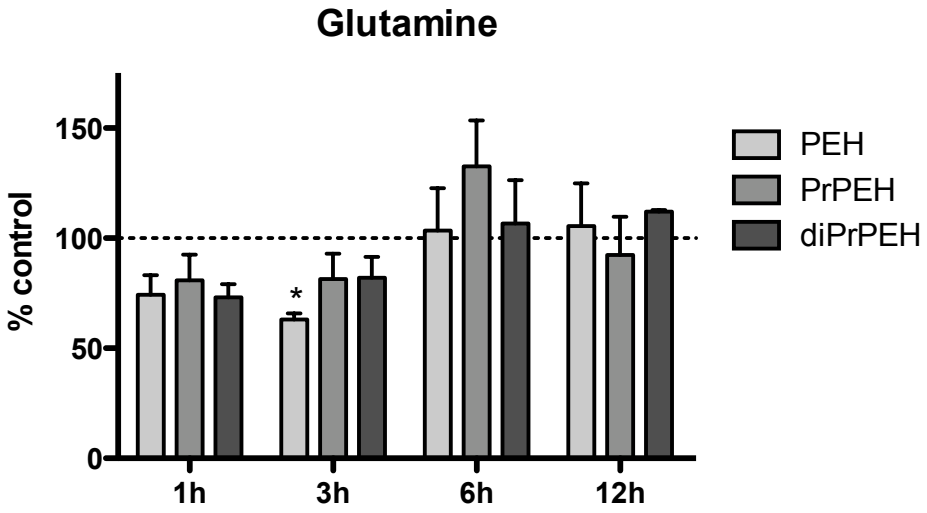

Figure 6-22. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain glutamine levels. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

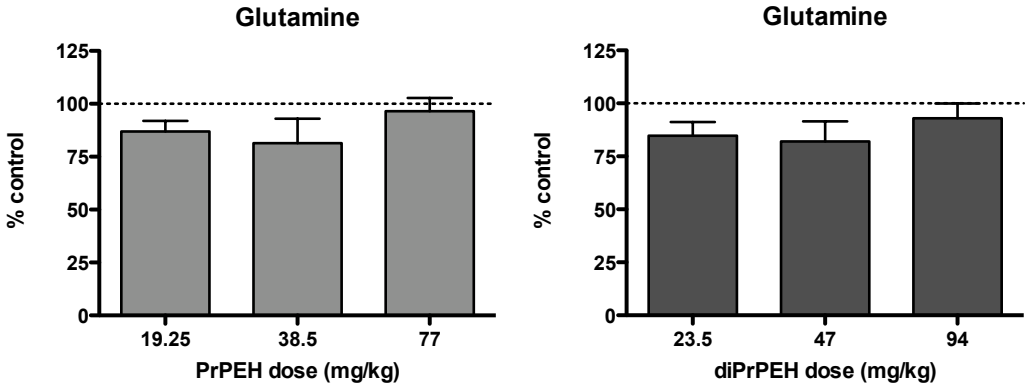

Figure 6-23. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain glutamine levels at 3 hours *ex vivo*. n = 4-6 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

6.3.4 *Ex vivo* analysis of biogenic amines

Whole brain biogenic amine levels for the time and dose studies for all vehicle-treated animals (controls) are shown in Table 6-1. Biogenic amine values are expressed as means ± SEM. The brain levels obtained are consistent with previously reported values (Baker *et al.* 1987).

As expected from the lack of effect on MAO, PEH had relatively minor effects on brain levels of biogenic amines. With the exception of an increase in 5-HT 1 hour after drug injection and in DA 12 hours after drug injection, PEH did not significantly change the levels of any of the amines tested at any timepoint. PrPEH and diPrPEH also exerted modest changes in the levels of biogenic amines; both drugs increased 5-HT 6 hours after drug administration, and increased DA and NA levels 12 hours after drug administration. DOPAC was significantly decreased by PrPEH at 3, 6 and 12 hours after injection and by diPrPEH at all timepoints tested. These results are shown in Figures 6-24 to 6-35.

6.3.5 Assay development and validation for PEH analogs

An analytical assay using HPLC-ECD was developed for the determination of PrPEH and diPrPEH levels in drug-treated brains. The sensitivity for both PrPEH and diPrPEH increased as the pH of the mobile phase increased (pH range tested was 2.9-3.7). Drug sensitivity also increased as the ECD voltage was increased, but at voltages higher than 0.8V the baseline was too unstable, and therefore the voltage was set to 0.75V for the assay. A small contaminant peak was present underneath the peak for PrPEH, and attempts to remove the contaminant peak (which included basification and subsequent acidification of the samples to remove any biogenic amines, and diluting the brain homogenates (a) in higher concentrations of perchloric acid; (b) in perchloric acid in methanol or acetonitrile; or (c) in perchloric acid without EDTA and ascorbic acid) were not successful. Attempts to increase the retention time of the PrPEH peak to

	Biogenic amine control values (ng/g) n = *34-35*
NA	284 ± 7.0
DA	484.6 ± 25.7
DOPAC	109.5 ± 6.2
HVA	58.7 ± 2.7
5-HT	256.2 ± 13.9
5HIAA	218.3 ± 25.6

Table 6-1. Whole brain biogenic amine levels for control (vehicle-treated) brains. Values, expressed in ng/g brain tissue, are the means ± SEM for both time study and the dose study combined.

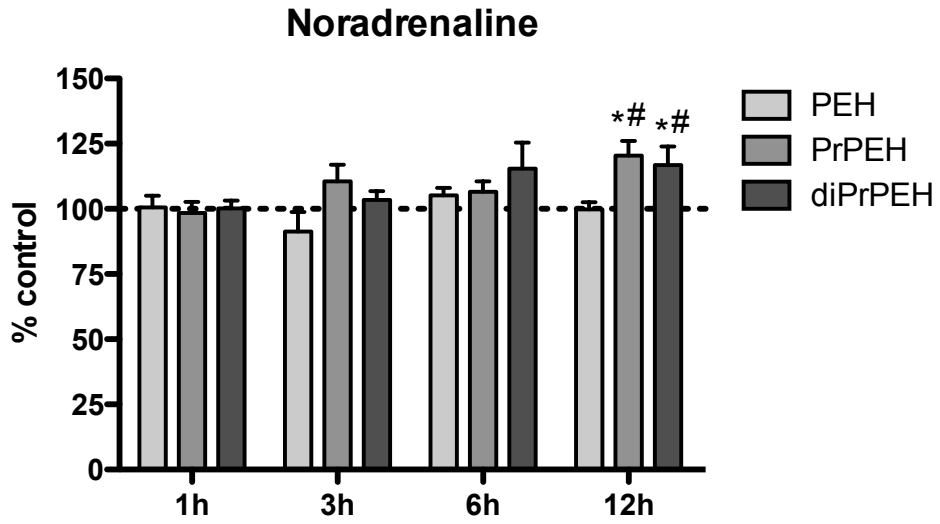

Figure 6-24. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain NA levels. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

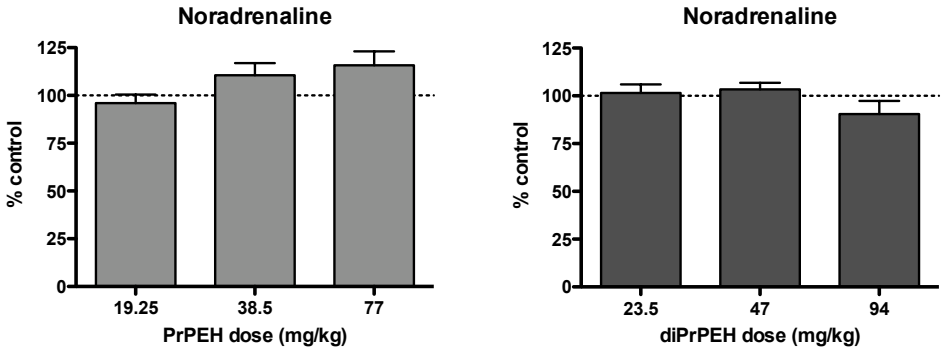

Figure 6-25. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain NA levels at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

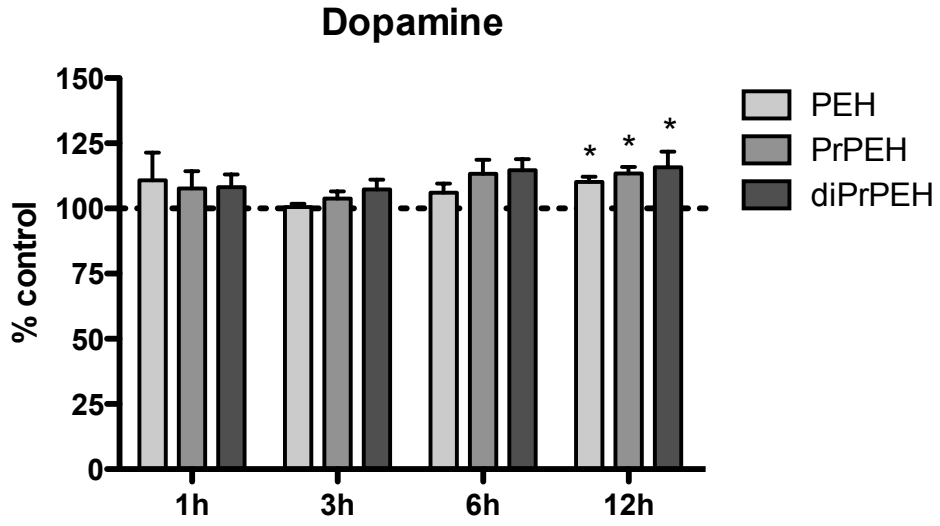

Figure 6-26. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain DA levels. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

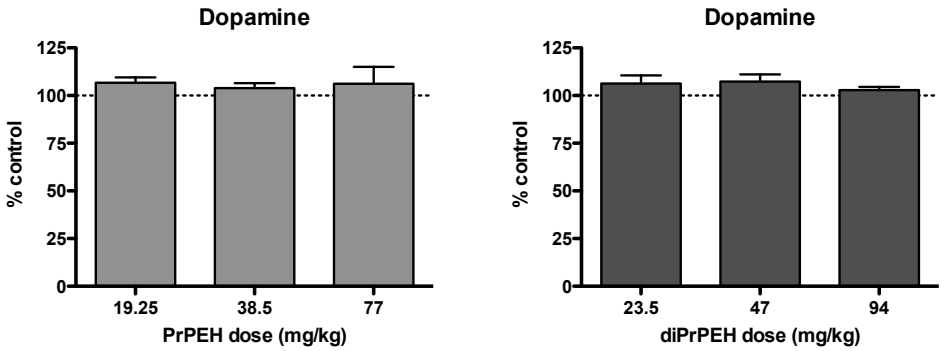

Figure 6-27. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain DA levels at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

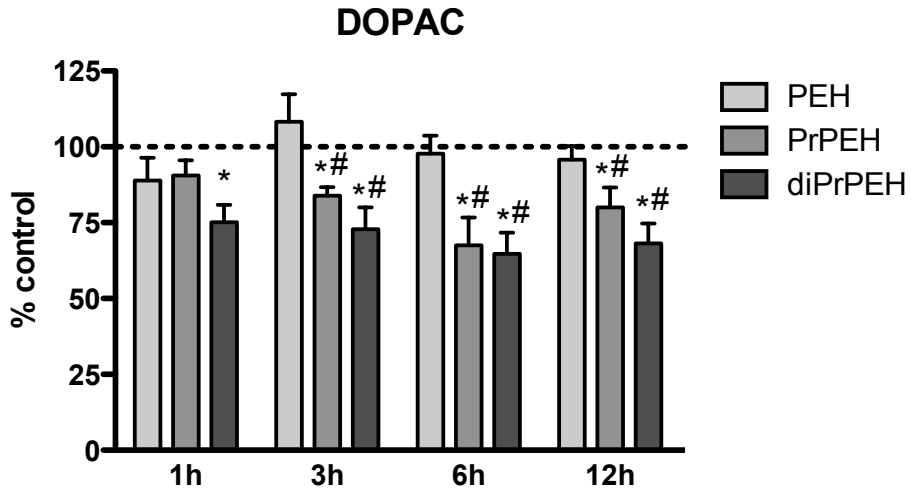

Figure 6-28. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain DOPAC levels. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

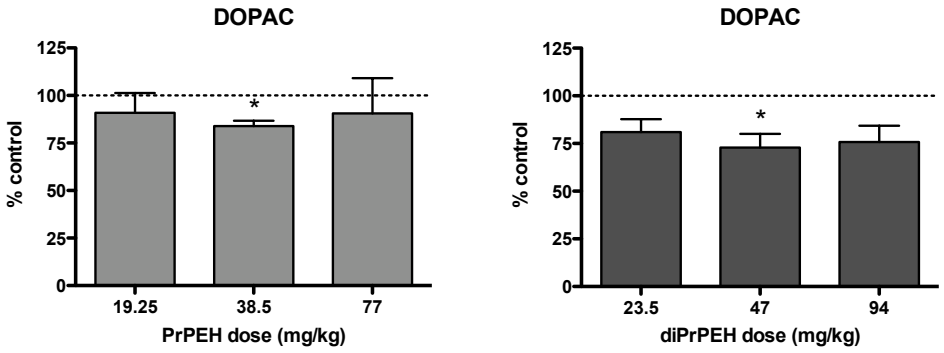

Figure 6-29. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain DOPAC levels at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

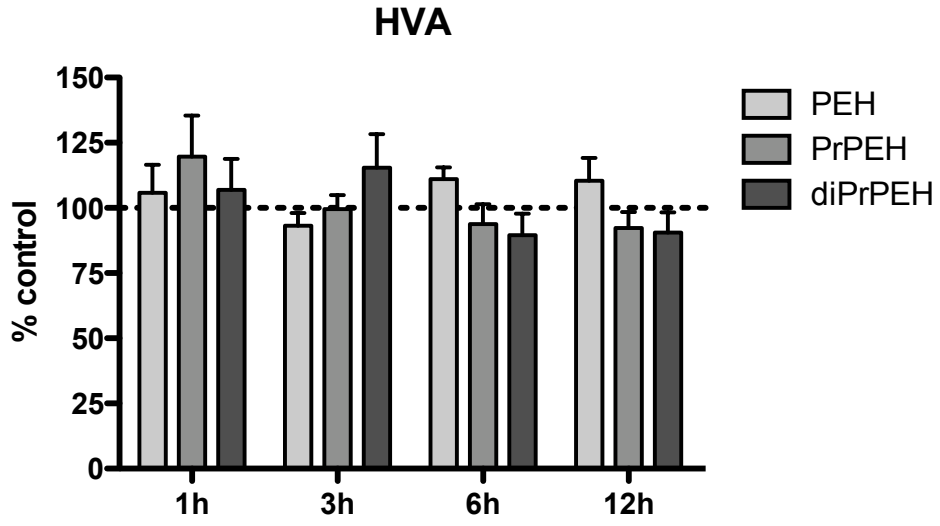

Figure 6-30. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain HVA levels. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

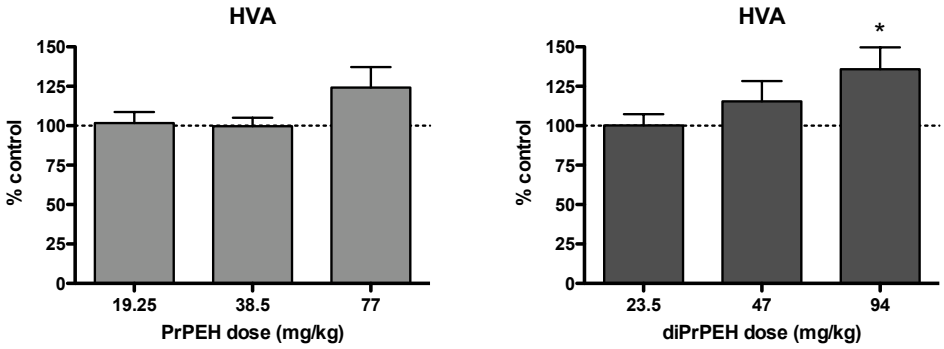

Figure 6-31. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain NA levels at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

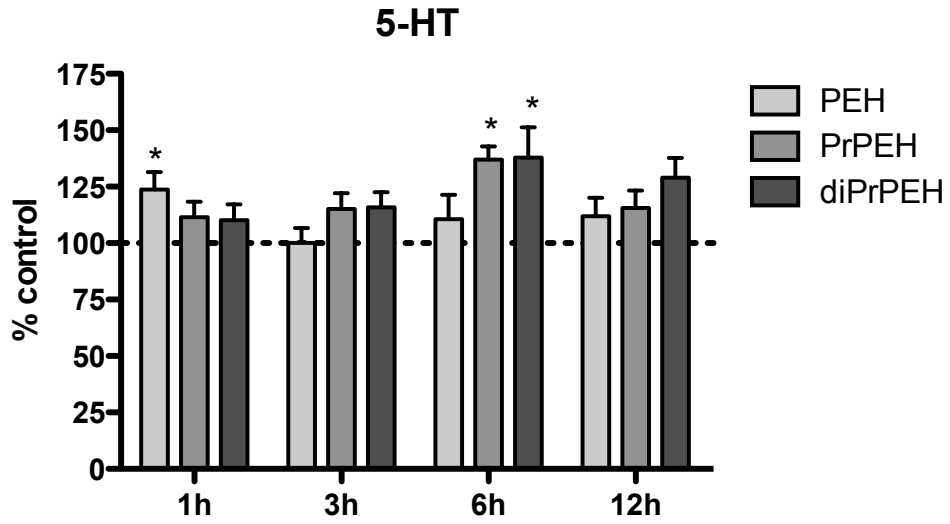

Figure 6-32. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain 5-HT levels. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

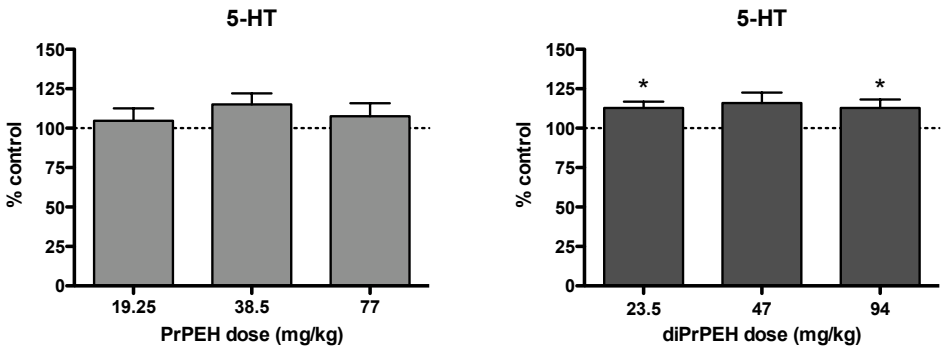

Figure 6-33. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain 5-HT levels at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

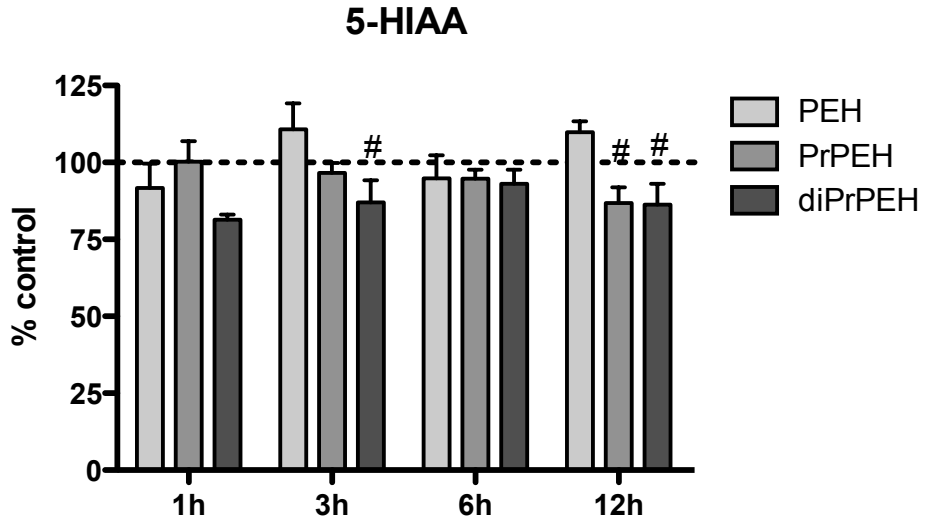

Figure 6-34. Time-dependence study of PEH (30 mg/kg), PrPEH (38.5 mg/kg) and diPrPEH (47 mg/kg) on brain 5-HIAA levels. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls; # denotes $p < 0.05$ compared to PEH.

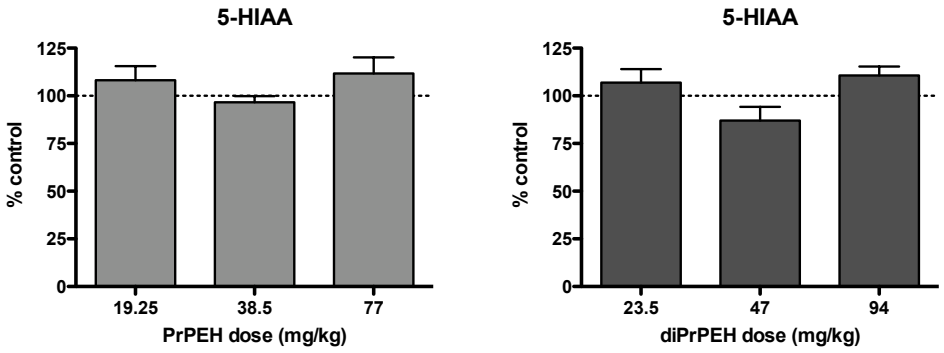

Figure 6-35. Dose-dependence study of PrPEH (left) and diPrPEH (right) on brain 5-HIAA levels at 3 hours *ex vivo*. n = 4-5 for each group. Results are expressed as mean % control ± SEM. * denotes $p < 0.05$ compared to vehicle controls.

move it away from the solvent front were also unsuccessful; increasing the concentration of acetonitrile or sodium octyl sulfate in the mobile phase slightly increased the retention time of diPrPEH but did not have significant effects on the PrPEH retention time. The contaminant peak was small relative to the lowest standard on the standard curve, but was evident in the 0 ng (blank) standard, which accounted for the contaminant peak in the quantitation of PrPEH in brain samples.

The retention times for PrPEH and diPrPEH were approximately 5.5 minutes and 14 minutes, respectively. The limit of detection for PrPEH was 250 pg (on column) and the limit of quantitation was 375 pg (on column); the limits of detection and of quantitation for diPrPEH were 156 pg (on column). Assay recovery, intra-assay variability and inter-assay variability were determined using 2 ng (on column) PrPEH and 17.5 ng (on column) diPrPEH. Recoveries for PrPEH and diPrPEH were 87% and 64%, respectively, and within-run coefficients of variation were 3.4% and 3.1%, respectively. Structures of the PrPEH and diPrPEH were confirmed using GC-MS, and the fragmentation patterns are shown in Figures 6-36 and 6-37.

Calibration curves for the PEH analogs were produced using naïve rat brain tissue, and were run in parallel with each sample set. Curves were generated by plotting the peak height of PrPEH or diPrPEH on the y-axis versus the concentration of each compound on the x-axis. The curves were linear up to 2.67 ng of PrPEH and 20 ng of diPrPEH on column (i.e. 400 ng/g tissue and 2500 ng/g tissue, respectively). Typical calibration curves for PrPEH and diPrPEH are shown in Figure 6-38, and chromatograms of a high standard and blank are shown in Figure 6-39, and of a diPrPEH-treated and vehicle-treated brain in Figure 6-40.

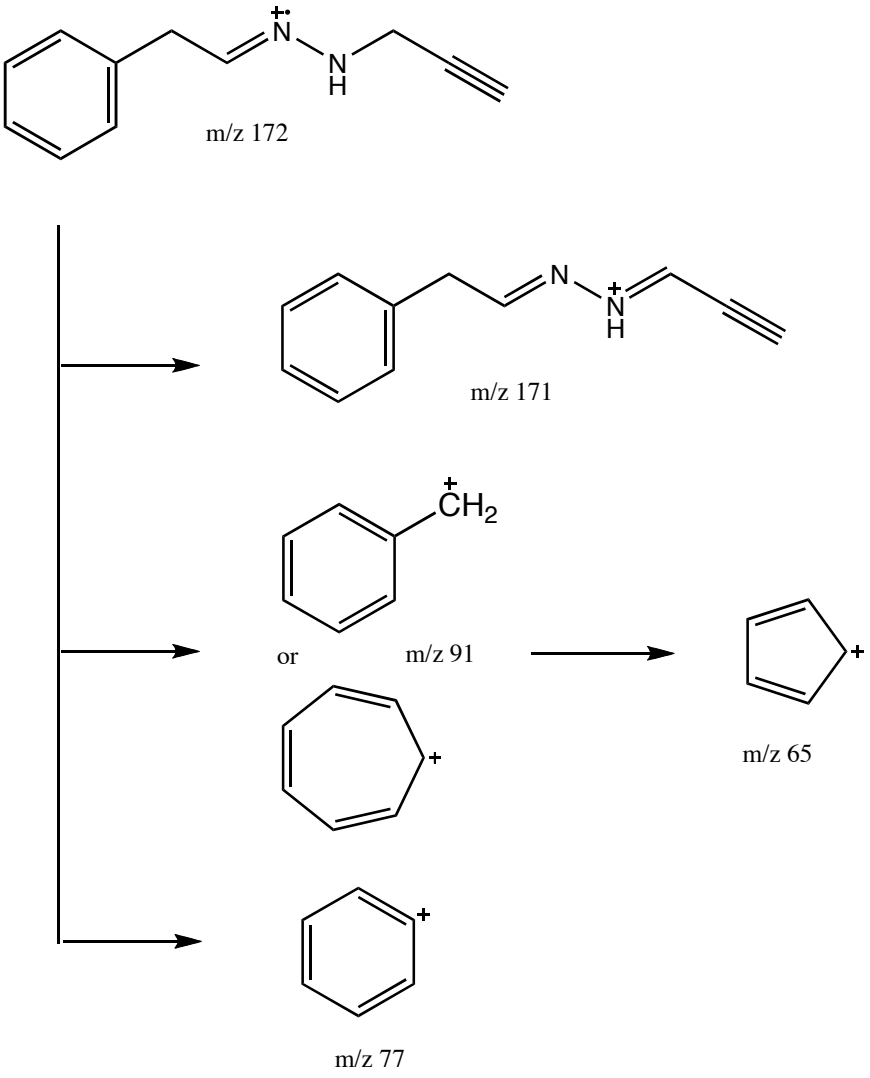

Figure 6-36. Mass fragmentation pattern of PrPEH.

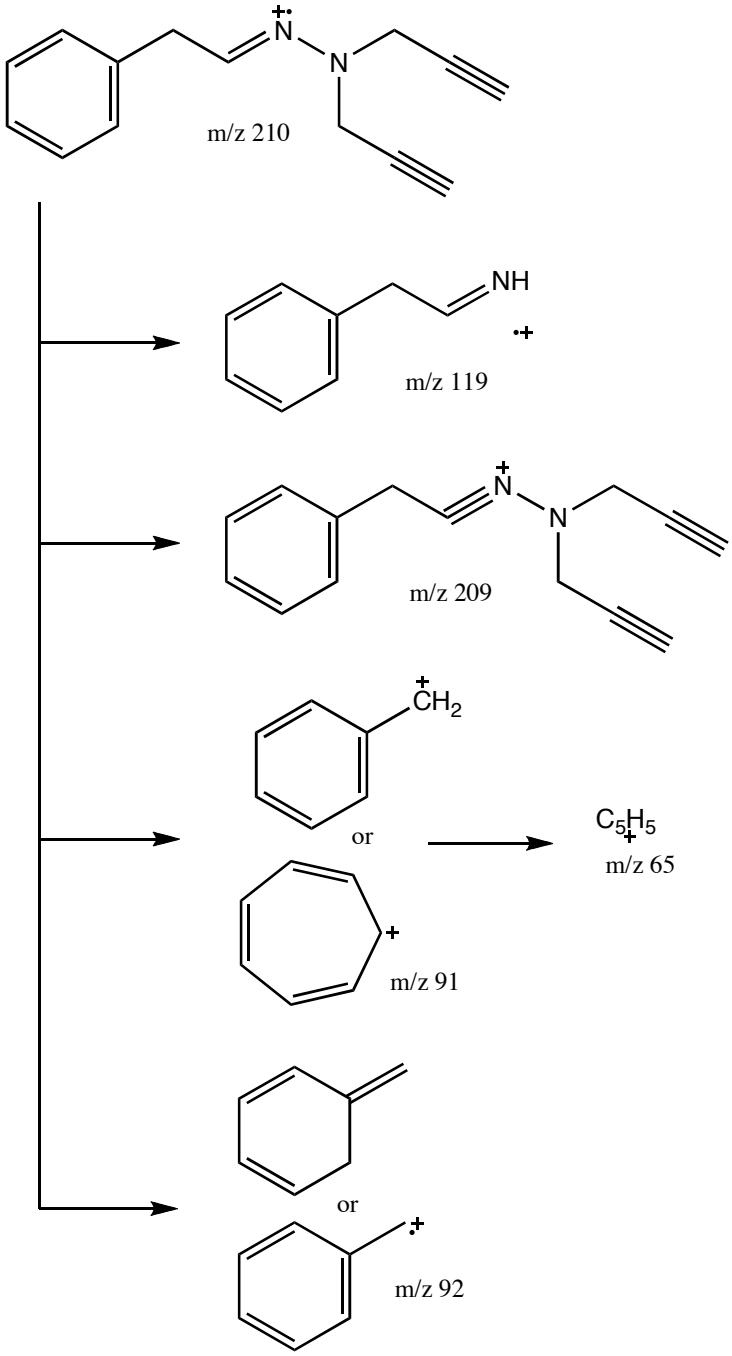

Figure 6-37. Mass fragmentation pattern of diPrPEH.

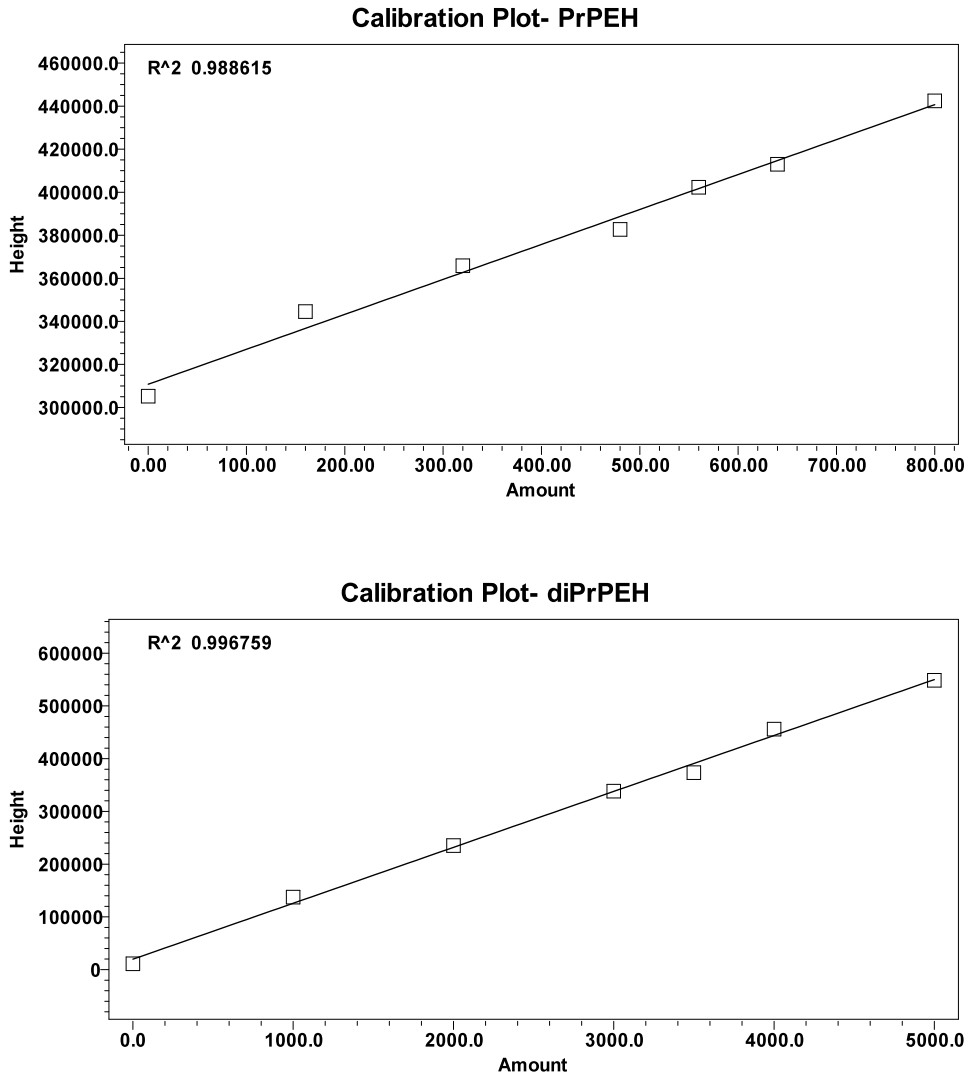

Figure 6-38. Calibration curves for PrPEH (top) and diPrPEH (bottom).

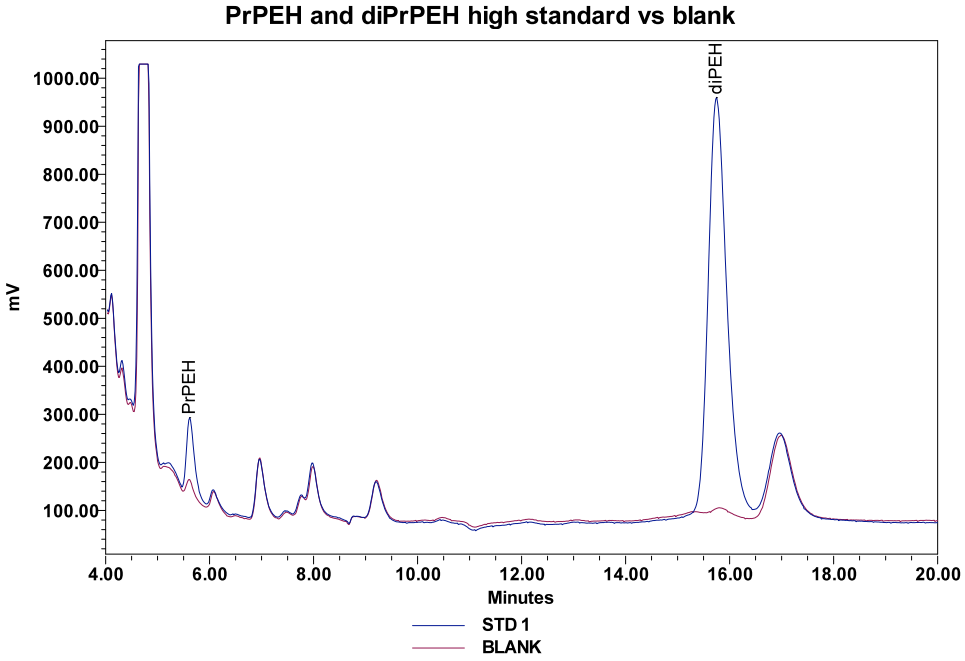

Figure 6-39. Overlaid chromatograms of a high standard of PrPEH and diPrPEH (STD 1; upper trace) and a blank standard (BLANK; lower trace).

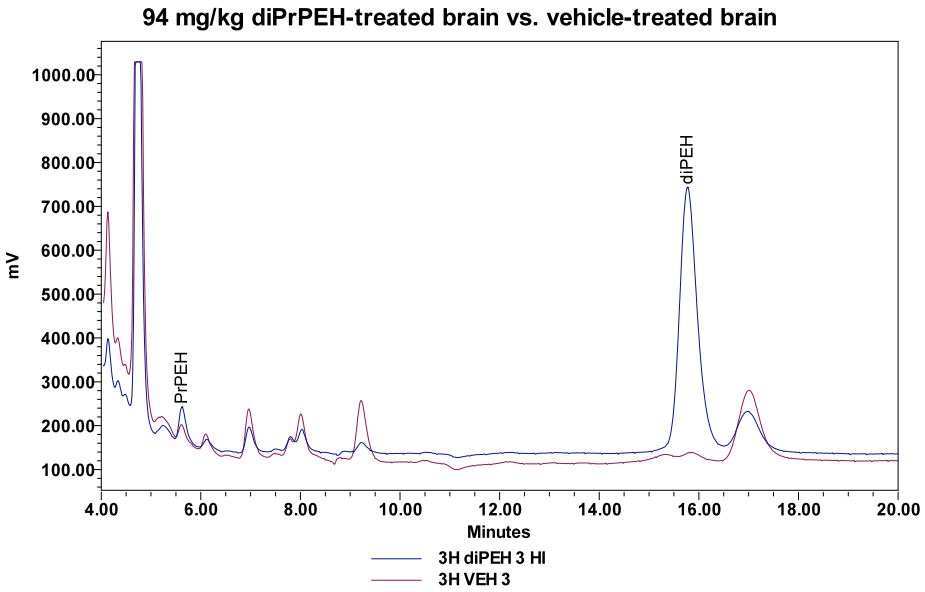

Figure 6-40. Overlaid chromatograms of a 94 mg/kg diPrPEH-treated brain at 3 hours (3H diPrPEH 3 HI; upper trace) and a control (vehicle-treated) brain (3H VEH 3; lower trace).

6.3.6 Brain PrPEH and diPrPEH levels

PrPEH was detected in the brains of animals treated with PrPEH (38.5 mg/kg) 1, 3 and 6 hours after drug administration (Figure 6-41), and diPrPEH was detected in the brains of animals treated with diPrPEH (47 mg/kg) at all timepoints tested (Figure 6-42). Drug concentrations were highest 1 hour after drug administration, and brain levels of diPrPEH were much higher than PrPEH levels in the respective drug-treated brains (1778 ng PrPEH/g brain tissue in PrPEH-treated brains, compared to 11.2 μg diPrPEH/g in diPrPEH-treated brains 1 hour after drug administration). At 12 hours after drug injection, 855 ng/g diPrPEH was still present, while PrPEH was no longer measurable. As shown in Figure 6-42, PrPEH was also detected in the brains of diPrPEH-treated animals at all timepoints tested, indicating that diPrPEH was depropargylated to PrPEH *in vivo*, although apparently to a very small extent.

6.3.7 Brain PEH levels

Comparison of the effects of PrPEH and diPrPEH with PEH on GABA-T, GABA and ALA levels suggested that the propargylated analogs are not effective prodrugs of PEH, since they do not produce the same neurochemical effects as PEH. However, evidence for the *in vivo* depropargylation of diPrPEH to PrPEH suggests that a small amount of PEH could potentially be formed from one or both analogs, but in such small amounts that no neurochemical effect would be observed. To determine whether a small, non-effectual amount PEH was being formed by the *in vivo* depropargylation of either analog, brains were collected from animals treated with modest doses of PrPEH (35 mg/kg) or diPrPEH (47 mg/kg) 3 hours following drug administration, and with high doses of PrPEH (77 mg/kg) or diPrPEH (94 mg/kg) 1 hour following drug administration. Three hours after drug administration, PEH was not detected in either PrPEH or diPrPEH-treated brains. One hour after administration of high drug doses, PEH was not

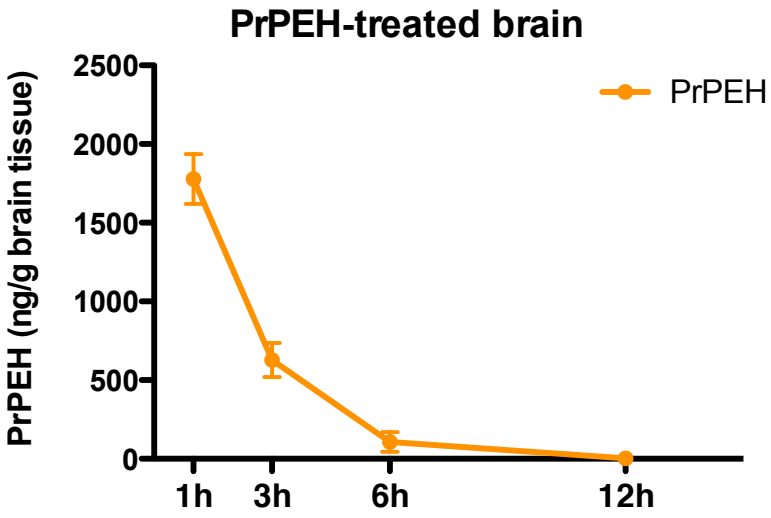

Figure 6-41. Brain levels of prodrugs in PrPEH-treated animals (38.5 mg/kg) at 1, 3, 6 and 12 hours following drug administration. n = 4-5 for each group. Results are expressed as mean ± SEM (ng/g brain tissue).

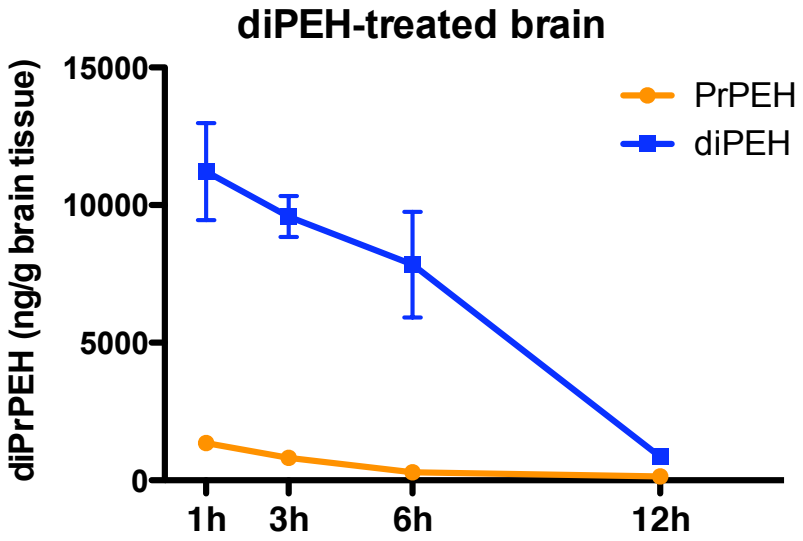

Figure 6-42. Brain levels of prodrugs in diPrPEH-treated animals (47 mg/kg) at 1, 3, 6 and 12 hours following drug administration. n = 4-5 for each group. Results are expressed as mean ± SEM (ng/g brain tissue).

detected in any of the diPrPEH-treated brains, although in the PrPEH-treated brains, a small but non-quantifiable PEH peak was detected in two of the three brains. These data indicate that while a miniscule degree of depropargylation of the analogs to PEH occurs *in vivo*, the significance of this depropargylation is negligible, and confirm the findings of the neurochemical studies that the PEH analogs did not behave as prodrugs. In a separate experiment, both PrPEH and diPrPEH were carried through the PEH assay procedure, and it was confirmed that neither drug was derivatized by PFBA nor did they produce an interfering peak with PEH.

6.4 DISCUSSION

PEH is a putative metabolite of PLZ and is thought to contribute to some of PLZ's neurochemical and therapeutic properties. On its own, PEH has been shown to be an inhibitor of GABA-T, significantly elevating brain GABA levels, while its effects on MAO activity are weak and transient (Paslawski *et al.* 2001). Its potential as a GABAergic agent warrants further investigation. In the present experiments, two putative prodrugs of PEH, PrPEH and diPrPEH, were investigated to determine their value as PEH prodrugs.

The neurochemical properties of PEH and those of the analogs were quite dissimilar. It is well-established that PEH is an inhibitor of GABA-T and causes marked, long-lasting increases in brain levels of GABA (Paslawski *et al.* 2001). This elevation in GABA by PLZ has been found by ourselves and others to occur at a relatively low inhibition of GABA-T (Popov and Matthies 1969; Todd and Baker 1995). While PrPEH and diPrPEH also inhibited GABA-T, the inhibition at the equivalent dose tested was to a significantly lesser degree than the inhibition observed following administration of PEH itself, and was apparently insufficient to significantly elevate brain GABA levels. The fact that these two analogs caused some inhibition of GABA-T activity *ex vivo* while being inactive *in vitro* (unpublished observation) could suggest that some metabolic conversion of the analogs to PEH may occur *in vivo*;

however this possibility is unlikely given that high doses of the analogs produced no or negligible amounts of PEH one hour after drug administration when levels of the parent drugs in the brain are very high.

PEH also elevated brain levels of ALA, while administration of PrPEH and diPrPEH did not alter ALA levels. Given that the breakdown of ALA is achieved by the action of ALA-transaminase (ALA-T), an enzyme structurally and functionally related to GABA-T, and that PLZ has been shown to inhibit ALA-T activity, it is likely that PEH inhibits ALA-T in a similar manner to the inhibition of GABA-T, causing increased ALA levels. PrPEH and diPrPEH, which were less potent than PEH at inhibiting GABA-T, would also be expected to be less potent than PEH in inhibiting ALA-T, resulting in the unaltered ALA levels observed here.

None of the drugs had any effect on brain levels of glutamate. However, whereas PEH did not significantly alter glycine levels, both diPrPEH and PrPEH caused marked increases in brain glycine levels. The significance of this finding is discussed further below. L-Serine is a precursor to glycine, and its brain levels were transiently reduced by PrPEH and diPrPEH, which may be an indication of its increased conversion to glycine in the presence of these drugs.

Previous experiments in our laboratories have shown that PEH does not greatly affect the activity of MAO-A or -B (Paslawski *et al.* 2001), and these findings were replicated here. Not surprisingly, there were few significant changes in brain levels of biogenic amines following PEH administration. PrPEH had modest inhibitory effects on MAO-A at two timepoints and diPrPEH had no significant effects at any timepoint tested; however unlike PEH, both diPrPEH and PrPEH had relatively strong inhibitory effects on MAO-B, particularly 6 and 12 hours after drug administration. This was unexpected, since neither diPrPEH nor PrPEH caused any inhibition of MAO-A or -B *in vitro* at a concentration of 10μM, which is much higher than the concentrations of PrPEH and diPrPEH found *in vivo* at 6 and 12 hours post-injection. It is possible that one or more as-of-yet-unknown metabolites are

formed from PrPEH and diPrPEH, resulting in the inhibition of MAO. The inhibition of MAO by the PEH analogs was dose-dependent, with higher doses inhibiting MAO to a greater degree than lower doses.

A greater degree of inhibition of MAO than seen with any of the drugs tested here is required in order to produce marked increases in brain levels of these neurotransmitter amines (Ling *et al.* 2001; Emilsson *et al.* 2002) than is apparently the case with GABA-T and GABA; this was reflected in the experiments here, as very few changes to brain levels of biogenic amines were observed. The decreases in DOPAC with PrPEH and diPrPEH are probably a reflection of inhibition of MAO-B with these two drugs. The increases in brain levels of 5-HT at 6 hours and DA and NA at 12 hours, even though small, were unexpected, and may reflect other effects of these drugs since some related phenylethylamine-type drugs affect brain levels and transport of amino acids that are precursors of 5-HT and the catecholamines (Wong 1990); in the study by Wong (1990), PLZ was shown to cause a small but significant increase in tryptophan, It is unlikely that PEH and its analogs (except perhaps at very high doses) would be effective agents in altering the brain levels of biogenic amines for any clinical purpose.

It is clear from these neurochemical results that PrPEH and diPrPEH are not efficient prodrugs of PEH, given their markedly different effects from PEH on the enzymes, amino acids and biogenic amines tested here. To further confirm that the analogs were not being metabolized to PEH to any neurochemically relevant degree, animals were treated with high doses of both PEH analogs and brains were collected 1 hour after drug injection. This timepoint was chosen because, as shown in Figures 6-41 and 6-42, the drug levels in the brain were highest at 1 hour post-injection, and due to the rapid rate of PEH clearance from the brain (Chapter 1), it was inferred that the highest amount of metabolite (if formed) would be present at this time. This is supported by the findings by Rao *et al* (1987b), who demonstrated that high PEA levels were found in brain 1 hour after administration of propargyl-PEA (a prodrug of PEA, which is similar in structure to PEH). PEH was not

quantifiable at 1 hour in brains treated with high doses of either analog, confirming the neurochemical studies indicating that the analogs were not depropargylated *in vivo*.

The finding that PrPEH and diPrPEH are not effective PEH prodrugs was somewhat surprising, since a number of studies have demonstrated the extensive *N*-depropargylation of drugs such as (-)-deprenyl, rasagiline and pargyline (Durden *et al.* 1975; Pirisino *et al.* 1978; Reynolds *et al.* 1978; Philips 1981; Weli and Lindeke 1985, 1986; Yoshida *et al.* 1986; Heinonen *et al.* 1989; Kalasz *et al.* 1990; Shin 1997; Bach *et al.* 2000; Am *et al.* 2004; Rittenbach *et al.* 2007), and the utility of *N*-propargyl forms of β-phenylethylamine as prodrugs (Rao *et al.* 1987a, 1987b). However, despite the fact that diPrPEH and PrPEH do not appear to be suitable PEH prodrugs, these drugs have interesting neurochemical properties in their own right. They are inhibitors of MAO-B *ex vivo*, which is interesting given that other MAO-B inhibitors such as l-deprenyl and rasagiline have been reported to be useful in treatment of neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD). Inhibition of MAO-B could increase brain levels of DA in patients suffering from PD, and could counteract the increased MAO activity and the increased MAO mRNA expression observed in AD (Emilsson *et al.* 2002). The neuroprotective abilities of the *N*-propargylated drugs mentioned above (l-deprenyl, rasagiline) are not completely understood, and are not thought to be solely related to their ability to inhibit MAO-B; they have in common with our analogs the presence of the *N*-propynyl moiety. Based on this evidence, diPrPEH and PrPEH may also be interesting candidates for novel neuroprotective agents. Researchers in the Neurochemical Research Unit have previously prepared *N*-propynyl analogs of PLZ and found them to be weaker inhibitors of MAO than PLZ (but stronger than the analogs of PEH described in the present study) and to be reasonably potent at preventing NA depletion caused by the neurotoxin DSP-4 (Ling *et al.* 2001).

The most unexpected finding in the present experiments was the exciting observation that PrPEH and diPrPEH markedly increased brain levels of glycine. Glycine acts as an inhibitory amino acid in the brain stem and spinal cord (Curtis *et al.* 1968), but is also a co-agonist at the glutamate NMDA receptor (Johnson and Ascher 1987; Olsen and Betz 2006) in other parts of the brain, and exerts excitatory effects in other brain areas such as the cortex and hippocampus (Cooper *et al.* 2003). Interestingly, NMDA receptor hypofunction is believed by many to contribute to the symptoms of schizophrenia (Coyle 2006). Preclinical and clinical studies have consistently demonstrated that NMDA antagonists produce a range of symptoms characteristic of the illness (Coyle 2006; Javitt 2008). While direct glycine agonists have been reported to be useful in treating negative and possibly cognitive symptoms of schizophrenia, they have been of limited clinical utility because of the high doses required and their relatively poor penetration of the blood-brain barrier (Javitt 2008). However, indirect increases in brain glycine *via* inhibition of glycine transporters (proteins that remove glycine from the synapse) have shown substantial promise (Javitt 2008). PrPEH and diPrPEH could also be useful as therapeutic agents in schizophrenia, since they offer another indirect mechanism by which brain glycine is elevated (and therefore NMDA receptor activity possibly facilitated). The primary route of glycine metabolism in animals involves the glycine cleavage system (GCS), a mitochondrial complex of four enzymes that is present in many vertebrate organs, including the brain. It is possible that PrPEH and diPrPEH inhibit GCS activity, resulting in the dramatic increases in glycine observed here; this hypothesis warrants further investigation.

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CHAPTER 7.
GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 GENERAL DISCUSSION

First and foremost, the data presented in this thesis provide definitive evidence that β-phenylethylidenehydrazine (PEH) is an active metabolite of the antidepressant/antipanic drug phenelzine (PLZ), formed *in vivo* by the actions of MAO. This was an exciting finding, since many years of research have until now yielded only indirect evidence for PLZ metabolism to PEH given that an analytical assay for PEH has not been available. A study published 40 years ago suggested that a metabolite of PLZ was likely contributing to its GABAergic effects (Popov and Matthies 1969), and an unrelated study published 3 years later suggested that PEH was a direct metabolite of PLZ (Tipton 1972). Today, because of the assay developed for PEH and described in this thesis, I have been able to confirm that PEH is the metabolite responsible for PLZ's GABAergic effects. Furthermore, my data provide strong support for the potential of PEH as a therapeutic agent in its own right.

The data presented here also advance our knowledge of the neurochemical actions of both PLZ and PEH that may contribute to their neuroprotective actions recently described (Tanay *et al.* 2002; Wood *et al.* 2006). The role of neuroprotective agents in degenerative conditions such as cerebral ischemia and Alzheimer's disease (AD) is a critically important area of research, and the data presented here are noteworthy in that they support a possible role for both a currently-prescribed drug (PLZ) and a novel neuroprotective agent (PEH) in the treatment of these, and other, neurodegenerative conditions. While the tolerability of PEH administration in humans remains to be determined, PLZ is a well-tolerated medication, and despite its potentially serious side effects involving interactions with certain foods, its neuroprotective actions certainly outweigh the inconvenience of the dietary restrictions associated with its use, and therefore support a role for this drug in inhibiting and/or reducing neurotoxic processes. PEH may potentially be of greater value (provided demonstration of its safety in

humans), given that it does not appreciably inhibit MAO activity (Paslawski *et al.* 2001) and therefore the dietary restrictions imposed upon patients taking PLZ would not be of concern. It is of interest that members of the Neurochemical Research Unit have observed no toxic effects of PEH after numerous studies in rats and gerbils.

We and others have hypothesized for many years that PEH formation *in vivo* mediates a number of PLZ's neurochemical effects, most notably its ability to inhibit GABA-transaminase (GABA-T) and to increase brain levels of GABA (Popov and Matthies 1969; Todd and Baker 1995). While the present results agree with this in part, *in vitro* studies from this thesis demonstrated that PLZ itself also most likely possesses some inhibitory potential on GABA-T. Indeed, MAO inhibition *in vitro* prior to PLZ application (presumably inhibiting PEH formation) did not completely abolish the inhibitory effects on GABA-T. These results suggest that the changes in GABAergic transmission resulting from chronic PLZ administration may need to be considered independently from those arising from acute drug administration. Since the exact mechanism in which PLZ (and PEH) inhibit GABA-T, thereby increasing brain GABA, are not known, and since it is not known for certain if the GABAergic effects of these drugs are due solely to the inhibition of GABA-T, more in-depth studies on the kinetics of these drugs on GABA-T inhibition and other components of the GABAergic system are warranted. It is important to understand this more completely, as it is believed that the GABAergic effects of these drugs play an integral role in their therapeutic and neuroprotective actions. Determining the complete mechanism that is used by these drugs to elevate GABA levels will not only clarify the mechanisms of action of the current drugs of interest, but will also provide a strong basis for drug development for GABAergic compounds in the future.

The results from the present series of experiments highlight a number of putative mechanisms by which PLZ and PEH may counteract degenerative processes thought to underlie toxicity in conditions such as cerebral ischemia and AD. A summary of these relationships is illustrated in Figure 7-1. In

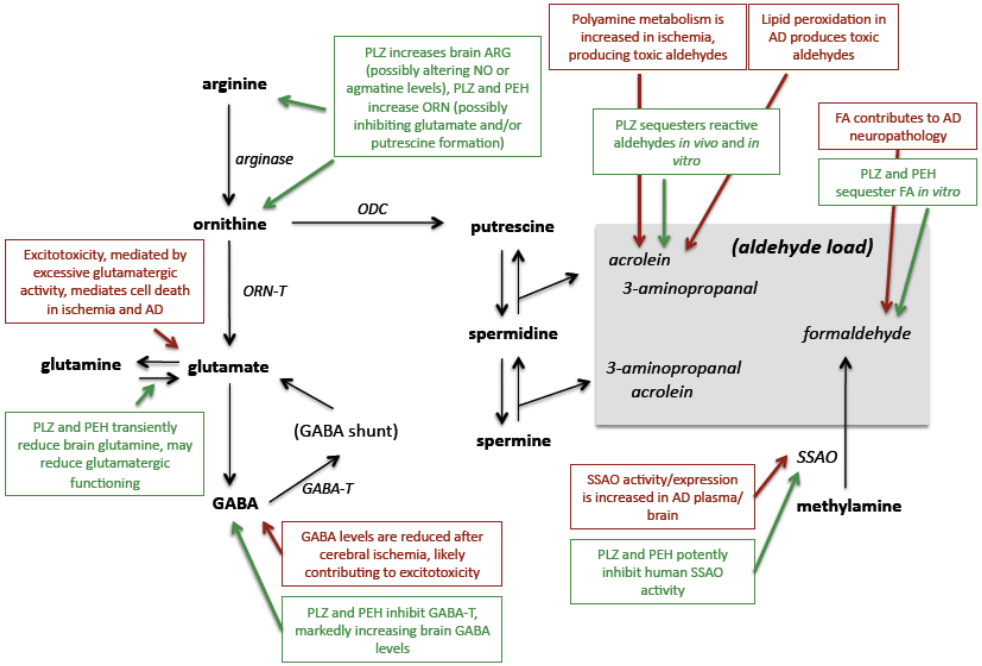

Figure 7-1. A summary of the putative neuroprotective mechanisms of action of PLZ and PEH

cerebral ischemia, neuronal loss has been attributed in large part to excitotoxicity due to the marked increase in brain glutamate that is unopposed by low brain GABA levels (Green *et al.* 2000), and to the excessive formation of toxic aldehydes produced from the increased metabolism of polyamines (Ivanova *et al.* 1998). PLZ and PEH markedly increase brain GABA and quite possibly decrease glutamatergic transmission, both by potentially reducing brain glutamate levels (although this effect requires further study), and by decreasing brain levels of glutamine and D-serine (which is also an important modulator of the glutamate NMDA receptor). While polyamine metabolism might be slowed by drug-induced inhibition of ODC (reflected here by a significant elevation in brain ORN), it was shown here and elsewhere that PLZ and PEH sequester reactive aldehydes, an effect that would prevent the aldehydes from exerting their toxic effects.

In AD, excitotoxic processes are also believed to play a role in the neurodegeneration characteristic of the illness, in addition to lipid peroxidation (i.e. oxidative stress) that may be caused in part by the overproduction of reactive aldehydes. The aldehyde formaldehyde (FA) has also been shown in numerous studies to be involved in the AD neuropathology (i.e. in the deposition of intracellular fibrillary tangles and extracellular neuritic plaques) (Chen *et al.* 2006), and FA levels in AD brains are probably elevated given that the expression and activity of SSAO, the enzyme catabolizing the deamination of methylamine (MA) to FA, is increased in AD (Ferrer *et al.* 2002; del Mar Hernandez *et al.* 2005). PLZ and PEH would not only counteract excitotoxic processes via their effects on GABAergic (and possibly glutamatergic) transmission, but both drugs also potently inhibit the activity of human SSAO, an effect that would be expected to decrease the formation of FA. MA metabolized by SSAO to FA would be sequestered by PLZ and/or PEH, given that both drugs are able to decrease free FA by forming a hydrazone product, thereby reducing the toxic effects mediated by FA in the AD brain. The finding that PLZ and PEH inhibited

human SSAO was particularly exciting, as this marks the first report to demonstrate that these drugs inhibit the human isoform of the enzyme.

Given the promising results suggesting that PEH should be considered as a therapeutic agent itself, we sought to develop propargyl prodrugs of PEH to maximize the neurochemical (and potentially the therapeutic) effects of the drug by producing more consistent, longer lasting levels of PEH in the brain (such effects have been observed previously with propargyl analogs of other phenylethylamines (Rao *et al.* 1987a, 1987b). Mono- and di-propargyl analogs of PEH (PrPEH and diPrPEH, respectively) were synthesized and characterized, and assay methods were developed for both of these novel drugs in brain, but surprisingly these compounds were not particularly effective prodrugs of PEH. Both drugs caused marked elevations in brain glycine levels, however, which is a timely and serendipitous finding in light of the recent interest in glycine and NMDA receptor hypofunction in conditions such as schizophrenia (Javitt 2008). Glycine is a potent co-agonist at the NMDA receptor, and thus PrPEH and diPrPEH should be further investigated in this context.

7.2 SUMMARY OF NOVEL FINDINGS REPORTED IN THIS THESIS

The data presented in Chapter 2 clearly demonstrate that PEH is a metabolite of PLZ that is formed *in vivo* by the action of MAO. This finding was made possible by the development of a novel procedure for the anlysis of PEH. While previous studies have demonstrated the effects of PLZ and PEH on whole brain amino acid levels, the effects of these drugs on brain L-serine, D-serine and arginine (ARG) levels have not, until now, been reported. Both PLZ and PEH increased brain levels of ARG (important in that ARG is a precursor to a number of important molecules involved in cell signaling, including agmatine and nitric oxide (NO)). PEH decreased brain levels of D-serine, which could contribute to neuroprotection given that D-serine is a potent co-agonist at the glycine binding site of the glutamate NMDA receptor.

Other possible mechanisms of neuroprotection were also reported in this thesis. In Chapter 4 it was shown that PLZ and PEH caused marked elevations in brain ORN, which could potentially lead to neuroprotective effects by either reducing glutamate formation or polyamine formation. Furthermore, in Chapter 5 it was demonstrated that PLZ and PEH sequestered FA *in vitro*, shown by the reduction of free FA and the formation of a hydrazone product. Both PLZ and PEH also inhibited the activity of SSAO, the enzyme responsible for the formation of the toxic aldehyde FA. Since PLZ and PEH both reduce the formation of FA and sequester the FA that is formed, it can be confidently hypothesized that both drugs would be neuroprotective against FA-mediated pathology. Importantly, PLZ and PEH potently inhibited the human isoform of SSAO, a finding that has not been shown previously and that is very relevant clinically given the documented differences in SSAO isoforms with respect to inhibitor specificity; studies using animal isoforms of the enzyme do not provide accurate information for clinical applications.

The elevation in brain levels of GABA by PLZ and PEH is well established, and it has been suggested that the facilitation of GABAergic activity may contribute to the neuroprotective effects of these drugs. The data presented in Chapter 3 demonstrated that while PEH formation likely contributes to the inhibition of GABA-T observed by PLZ, PLZ itself also possesses intrinsic inhibitory potential on GABA-T activity, since GABA-T activity was inhibited *in vitro* despite the fact that the formation of PEH was (most likely) inhibited. Since previous work has shown that inhibition of PEH formation in vivo completely abolishes the GABAergic effects of PLZ, the results in Chapter 3 demonstrate that the GABA-T inhibition kinetics are more complicated than perhaps initially believed.

Finally, since the data presented throughout this thesis indicate that PEH should indeed be considered as a novel therapeutic agent in and of itself, two PEH analogs (PrPEH and diPrPEH) were synthesized and tested for their abilities to act as PEH prodrugs. Contrary to our hypotheses, these drugs did not appear to be particularly effective PEH prodrugs. Interestingly, however,

both analogs (particularly diPrPEH) caused significant elevations in whole brain levels of glycine, a co-agonist at the glutamate NMDA receptor. This finding has great therapeutic potential in conditions such as schizophrenia, where hypofunction of the glutamatergic system is believed to be related to illness pathology.

7.3 FUTURE DIRECTIONS

The development of an analytical assay to measure brain and liver levels of PEH drastically improves our ability to explore the metabolism of PLZ and PEH and the role of PEH formation in the neurochemical and therapeutic effects of PLZ. It was recently suggested that PLZ is metabolized by MAO to produce PEH as a reaction intermediate, which in turn is rapidly metabolized to phenylacetic acid (PAA) (Binda *et al.* 2008). Indeed, preliminary studies in the Neurochemical Research Unit have now confirmed that both PLZ and PEH are metabolized to PAA (Banasch, MacKenzie, Rauw and Baker, unpublished). Furthermore, the trace amine β-phenylethylamine (PEA) is also a metabolite of PLZ (Baker *et al.* 1982), and with the development of the PEH assay described in this thesis, researchers in the Neurochemical Research Unit are now in the unique position to conduct a comprehensive metabolite study in which PEH, PEA, p-tyramine, PAA and p-hydroxy-PAA are studied simultaneously. Robinson and colleagues (1985), in their mass spectrometric investigation of metabolites of PLZ in human urine, reported PAA and p-hydroxy-PAA as important metabolites of PLZ, but as described by Kennedy *et al* (2009), those metabolites could arise from PEA and/or p-tyramine through various routes. We now also know that PEH could be a major source of PAA, but Robinson *et al* (1985) did not investigate PEH. Both PEA and p-tyramine are so-called trace amines that normally occur in low concentrations in the brain but have been implicated in the etiology and pharmacotherapy of a number of neurological and psychiatric disorders, including depression (Dewhurst 1968; Sabelli and Mosnaim 1974; Sandler *et*

al. 1979; Boulton 1985; Paterson *et al.* 1990; McManus *et al.* 1991; Tomlinson and Baker 2009). PEA and p-tyramine are known to interact intimately with the catecholamine neurotransmitters (Boulton 1985; Greenshaw *et al.* 1986; Greenshaw 1989), but have also been proposed to have receptors of their own, although this was a matter of controversy of many years (Vaccari 1986). However, the trace amines have been the subject of renewed interest in recent years with the discovery of a family of G-protein coupled receptors, some of which are selectively activated by these and other trace amines; these receptors have been named trace amine-associated receptors (Borowsky *et al.* 2001; Lindemann *et al.* 2005; Berry 2007). High doses of PEA have also been suggested to interact with the GABAergic system (Dourish and Cooper 1983), and the role of this and other metabolites in the neurochemical actions of PLZ should be studied. Further investigation into the metabolism of PLZ and PEH is also warranted to obtain a greater understanding of the pharmacology and pharmacokinetics of these drugs. It is surprising that despite being commercially available for approximately 50 years, so little is actually known about the metabolism of PLZ (Kennedy *et al.* 2009).

While it has been presumed until now that PEH formation mediates the GABAergic effects of PLZ, results from this thesis suggest that PLZ itself may possess inhibitory actions on GABA-T. This finding fits with previous findings demonstrating that in rodents chronically treated with PLZ (where MAO would be significantly, if not completely, inhibited and thus PEH formation nearly, if not completely, prevented), brain GABA levels remain significantly elevated (McManus *et al.* 1992; McKenna *et al.* 1994; Kennedy *et al.* 2009). Since the antidepressant and anxiolytic properties of PLZ are apparent only after chronic treatment, studies investigating PEH levels in rodents chronically treated with PLZ are essential to understand the individual roles of PLZ and its metabolite in the therapeutic benefits of PLZ. It is of great interest and importance to determine PEH levels in animals chronically treated with PLZ and with PEH itself.

The development of the analytical method for PEH is also exciting because the metabolism of PLZ can now potentially be assessed in patients taking PLZ. Although the assay for the determination of PEH levels in rat brain and liver was not extended to measure plasma PEH levels, presumably this could be achieved relatively easily given the simple nature of the procedure (although the presence in plasma samples of water may cause problems with stability of the final derivative and it may be necessary to do solid phase extractions of the plasma samples before conducting the assay). Determination of the ratio of PEH in brain to plasma in animals could potentially provide some insight to brain levels of PEH in humans after an acute PLZ dose and during the course of chronic PLZ treatment. Potential links between PEH plasma levels and symptom improvement and treatment resistance could be investigated in humans.

While the results of the experiments described here provide a broad range of mechanisms by which neuroprotection may be achieved with PLZ and PEH, further studies are warranted. The mechanism by which ORN levels in the brain are increased by PLZ and PEH an important to investigate for example, since at this time it is not known whether inhibition of ORN-T or of ODC (or of both) is the cause the significant increase in brain ORN observed. It is hypothesized that ORN-T is inhibited rather than ODC, and my preliminary studies suggested that ODC activity may have actually been slightly induced by PLZ administration, although these findings need to be confirmed. If inhibition of ORN-T is the cause of this effect, this may contribute to the possible decrease in glutamatergic transmission that has been reported following PLZ administration. Indeed, since the effects of PLZ and PEH on glutamate levels appear to be condtradictory and at least transitory in acute studies, long-term studies should now be conducted. Such studies and additional short-term investigations should now include analyses of glutamate in various brain regions since whole brain studies may be masking subtle changes in glutamate levels in discrete regions. As noted previously in this thesis, Sowa (2006) found that 4-fluoroPEH caused a

marked decrease in elevated brain levels of glutamate that were observed in gerbil frontal cortex one week after induction of global ischemia and reperfusion. The role of increased arginine (ARG) (a precursor of ORN) in the ORN increase should also be investigated, since the clinical relevance of ARG is also of great interest given its association with nitric oxide (NO) and the neuromodulator agmatine (Gilad *et al.* 1996; Wu and Morris 1998) as well.

The results presented here are extremely exciting with respect to their possible implications for neuroprotection in AD; given their effects upon the SSAO system, the putative neuroprotective roles of PLZ and PEH deserve further investigation. The findings that PLZ and PEH both inhibited human SSAO and sequestered FA are particularly exciting, and warrant further study of how these two neurochemical effects act together *in vivo*. At this time it is unknown, for example, if PLZ and PEH have a greater affinity for the quinone cofactor of SSAO or for FA. Time- and dose-studies are also warranted to determine the efficacy of both drugs in sequestering FA, the stability of the hydrazone produced, and the actual protective effects in the brain. Recent advances in animal models of AD could provide valuable tools for assessing the putative neuroprotective role of these drugs against the neuropathological changes observed in AD brains.

Finally, the effects of PrPEH and diPrPEH on brain glycine, although unexpected, could potentially lead to the development of therapeutic agents for a completely different use than was the focus of this thesis. The mechanism by which the PEH analogs cause such marked increases in brain glycine certainly merit investigation, given the recent interest in pharmacological agents increasing activity of the glycine system for the treatment of glutamatergic hypofunction in conditions such as schizophrenia. Further studies are also warranted to identify other putative analogs of PEH which may effectively act as prodrugs; ironically, PLZ could be considered to be a PEH prodrug since higher brain levels of PEH were measured after PLZ administration compared to an equivalent dose of PEH.

7.4 FINAL REMARKS

The results presented in this thesis have unequivocally identified PEH as a metabolite of PLZ and have provided further evidence to suggest that PEH likely mediates many of PLZ's therapeutic effects, and that it warrants further investigation as a GABAergic and neuroprotective agent in its own right. For the treatment of depression and anxiety-related disorders, chronic administration of PLZ is necessary, and the role of PEH formation in this therapy remains to be elucidated with chronic studies. Chronic PEH treatment would be expected to produce antipanic effects, since PEH shares all of PLZ's currently known neurochemical actions except for MAO inhibition; for this reason, the antidepressant effects of chronic PEH are less certain, but undoubtedly warrant investigation given the evidence for the antidepressant effects of GABA facilitation. Chronic administration of PLZ or PEH is also likely to be necessary to confer neuroprotection in AD, since the neurotoxicity observed in this and other progressive degenerative conditions is ongoing. Whether a lower dose of PLZ than that used for the treatment of depression and anxiety will be required remains to be determined, and may depend on the concentrations of PLZ required to inhibit SSAO in the brain and to sequester any excess FA produced. On the other hand, the neuroprotective effects of PLZ and PEH in ischemia would likely not require chronic dosing, but rather subchronic dosing until the excitotoxic processes and increased production of aldehydes from excessive polyamine metabolism abate. In most cases, either PLZ or PEH could probably be used without much difference in doses between the two drugs; while PLZ administration results in higher brain levels of PEH (at least after acute administration), PLZ also inhibits MAO, thereby altering concentrations of amine neurotransmitters and increasing the risk of hypertensive side effects. Investigation of the neurochemical actions of both PLZ and PEH are ongoing in the Neurochemical Research Unit.

7.5 REFERENCES

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