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The Antidepressant/Antipanic/Neuroprotective Drug Phenelzine: Neuropharmacological and Drug Metabolism Studies

by

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Abstract

Phenelzine (PLZ) is a monoamine oxidase (MAO)-inhibiting antidepressant with anxiolytic and neuroprotective properties. Metabolites of PLZ were investigated in rat brain. Levels of phenylacetic acid (PAA) and β-phenylethylamine (PEA) were found to be elevated after administration of PLZ and PEH (a major metabolite of PLZ) to rats, but the effect was greater with PLZ in both cases. Acute administration of PLZ or PEH increased brain levels of alanine (ALA) and GABA and decreased glutamine (GLN) levels. When MAO was inhibited prior to PLZ administration, levels of GABA and ALA were not increased, supporting the idea that the metabolite PEH formed by MAO is responsible for the increases. Chronic PLZ administration significantly reduced weight gain in rats, and brain levels of amino acids were quite different after 1 and 3 weeks of PLZ administration, perhaps as a result of its decreased metabolism to PEH with time.

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Abbreviations and Symbols:

3-AP	3-aminopropanal
5-HT	5-hydroxytrypamine/serotonin
ALA	L-alanine
ALA-T	alanine transaminase
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
ARG	L-arginine
ASP	L-aspartate
BZA	benzylamine
CNS	central nervous system
DA	dopamine
DCC	N,N'-dicyclohexylcarbodiimide
D-SER	D-serine
ECD	electron capture detector/detection
EOS	ethanolamine-O-sulfate
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyric acid transaminase
GAD	glutamic acid decarboxylase
GAT	γ-aminobutyric acid transporter
GLN	L-glutamine
GLU	L-glutamate
GLY	glycine
GC	gas chromatography
GC-ECD	gas chromatography with electron capture detection
$HClO_4$	perchloric acid
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography

IBLC	N-isobutryl-L-cysteine
i.p.	intraperitoneal
LH	lateral hypothalamus
L-SER	L-serine
MAO	monoamine oxidase
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
MAOIs	monoamine oxidase inhibitors
MeOH	methanol
MP H_2O	millipore water
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
Na_2HPO_4	disodium hydrogen phosphate
NaOH	sodium hydroxide
NA	noradrenaline
NH_3	ammonia
NH_4OH	ammonium hydroxide
NMDA	N-methyl-D-aspartate
OPA	o-pthalaldehyde
p-OH-PAA	p-hydroxyphenylacetic acid
PAA	phenylacetic acid
pCPAA	p-chlorophenylacetic acid
PEA	β-phenylethylamine
PEH	β-phenylethylidenehydrazine
PFBA	pentafluorobenzaldehyde
PFBS	pentafluorobenzenesulfonyl chloride
PFPhOH	pentafluorophenol
PLP	pyridoxal-5'-phosphate

PLZ	phenelzine
PrAO	primary amine oxidase
RIMA	reversible inhibitor of monoamine oxidase A
ROS	reactive oxygen species
SEM	standard error
SSAO	semicarbazide sensitive amine oxidase
SSRI	selective serotonin reuptake inhibitor
TAUR	L-taurine
TCP	tranylcypromine
UV	ultraviolet
Vigabatrin	γ-vinylGABA

1.0. INTRODUCTION:

1.1. Preamble

Phenelzine (PLZ) (Figure 1) is an irreversible monoamine oxidase (MAO) inhibitor used in the treatment of atypical depression and anxiety disorders. In addition to inhibiting MAO, PLZ inhibits the catabolic enzymes GABA-transaminase (GABA-T) and alanine transaminase (ALA-T), leading to marked increases in rat brain levels of the amino acids γ-aminobutyric acid (GABA) and alanine (ALA). There is less information available about its effects on rat brain levels of other amino acids that may be involved in the etiology of mood and anxiety disorders. It is important to note that some of the effects of PLZ administration may be mediated by active metabolites such as β-phenylethylidenehydrazine (PEH) (Figure 1) and 2-phenylethylamine (PEA) (Figure 1). This thesis presents the results of studies comparing the effects of PLZ and PEH (a major metabolite) on levels of PEA, phenylacetic acid (PAA) and several amino acids in rat brain. The effects of PLZ on the weights of rats after acute and chronic administration are also reported.

1.2. Monoamine Oxidase

The enzyme MAO is responsible for oxidative deamination of serotonin (5-hydroxytryptamine, 5-HT), noradrenaline (NA) and dopamine (DA) (Pisani et al. 2011). Aldehydes, hydrogen peroxide (H_2O_2) and ammonia (NH_3) are the products of such metabolism (Youdim et al. 2006). There are two isoforms of MAO, namely MAO-A and MAO-B (Johnson, 1968). The genes for both isoforms are located on the X-chromosome (Xp11.23) (Lan et al. 1989). The

isoforms differ in substrate specificity, sensitivity to inhibitors, and tissue localization. 5-HT, NA and adrenaline are preferential substrates of MAO-A and benzylamine (BZA) and β-phenylethylamine (PEA) are preferential substrates of MAO-B. (Pisani et al. 2011). DA degradation by MAO is mainly carried out by MAO-B in humans and other primates (Johnston 1968). There are selective inhibitors of each isoform. MAO-A is selectively inhibited by clorgyline and MAO-B is selectively inhibited by l-deprenyl (selegiline) and rasagiline (Stahl et al. 2008).

The MAO isoforms have specific tissue expressions. Many tissues, including the liver and the brain, express both MAO-A and MAO-B (Thorpe et al. 1987). The placenta expresses mostly MAO-A (Weyler and Salach 1985) and specific blood components including lymphocytes and platelets only express MAO-B (Bond and Cundall 1977; Donnelly and Murphy 1977). In the brain, expression of MAO isoforms displays regional variation. In rat brain, MAO-A mRNA is localized primarily in the locus ceruleus, ventral tegmental area, substantia nigra, hippocampus, thalamus, hypothalamus and cerebral cortex (Jahng et al. 1997). Highest levels of MAO-B mRNA are seen in the area postrema, subfornical organ and dorsal raphe (Jahng et al. 1997).

Expression of brain MAO has been reported to change with aging and with neurodegenerative disease. Levels of MAO-A in brain have been reported to be unchanged (Gottfries et al. 1975; Fowler et al. 1980; Saura et al., 1997; Mahy et al., 2000; Volchegorskii et al. 2001) or increased (Shih 1979) with age. There is an age-related increase in brain MAO-B expression and activity (Mahy et al.,

2000; Saura et al., 1997). In Alzheimer's and Parkinson's disease MAO-B activity has been reported to be increased to an even greater extent (Benedetti and Dostert, 1989; Jossan et al. 1991; Sherif et al. 1992; Saura et al. 1994).

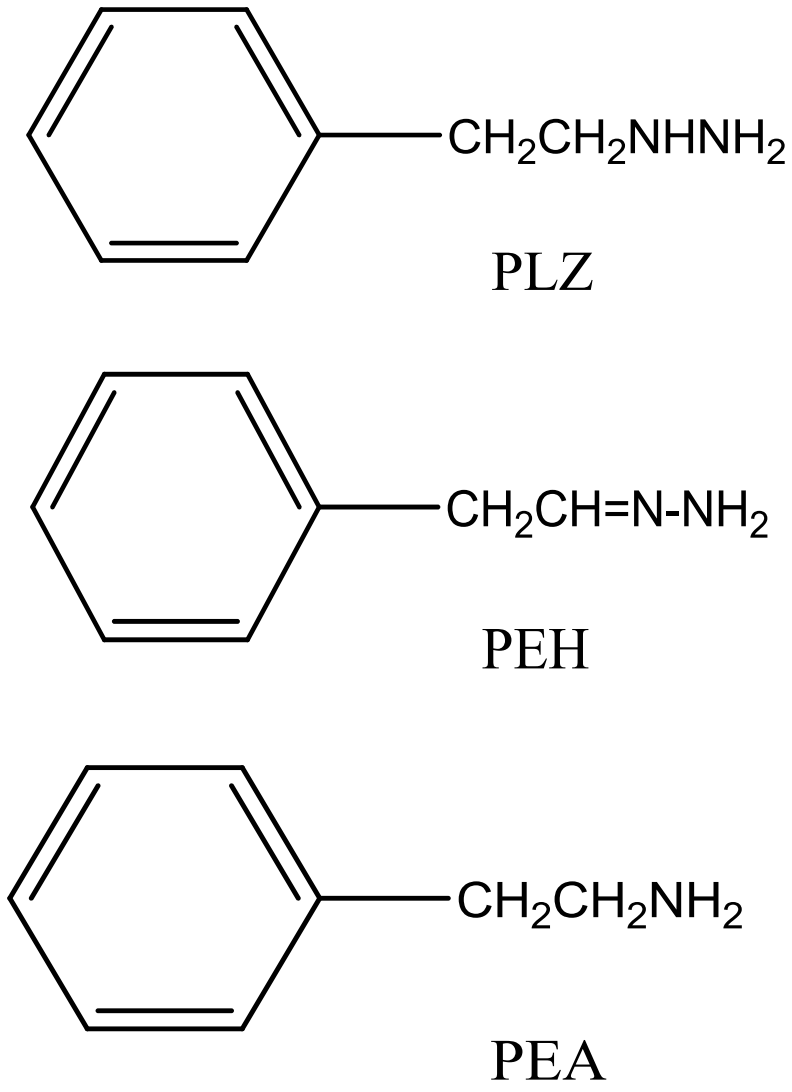


Figure 1. Structures of phenelzine (PLZ), β-phenylethylidenehydrazine (PEH) and β-phenylethylamine (PEA)

1.3. Monoamine Oxidase Inhibitors

In the 1950s a drug used to treat tuberculosis, iproniazid, was clinically observed to have mood enhancing qualities in patients (Fox and Gibas 1953). The antidepressant actions of iproniazid were attributed to its MAO inhibition and the resultant elevation of levels of monoamines in the CNS (Zeller and Barsky, 1952). Iproniazid was of limited value as an antidepressant as it has serious adverse affects such as liver toxicity (Youdim and Bakhle, 2006). Additional MAO inhibitors with more favorable side effect profiles such as phenelzine and tranylcypromine were developed soon after iproniazid for the treatment of depression.

MAO inhibitor (MAOI) profiles are classified based on selectivity and reversibility of inhibition. Selective MAOIs selectively inhibit one of the two isoforms (although many inhibit both isoforms at high doses). Non-selective MAOIs inhibit both isoforms relatively equally. Irreversible MAOIs bind irreversibly to the MAO enzyme or its cofactors, requiring new enzyme to be synthesized for a restoration in function. Reversible MAO inhibitors temporarily bind to MAO, and substrate can competitively displace a reversible MAO inhibitor (Krishnan 2007).

The MAOIs have been used in psychiatry as antidepressants and antianxiety agents and in the treatment of Parkinson's disease (Sowa et al. 2004). The pharmacological effect of a MAOI is primarily related to which MAO isoenzyme is inhibited. The antidepressant action of MAOIs is mainly due to the

inhibition of MAO-A, as this isoform is responsible for degrading 5-HT and NA. Inhibition of MAO-A leads to an increase in functional availability of NA and 5-HT at the synapse. Selective MAO-B inhibitors are not generally effective antidepressants, although l-deprenyl has been reported in recent years to be effective for treating depression if administered transdermally (Lee and Chen 2007). MAO-B inhibitors such as l-deprenyl and rasagiline are used in the treatment of Parkinson's disease to increase concentrations of dopamine in the substantia nigra. Interestingly, these drugs have also been reported to be neuroprotective or neurorescue drugs in a wide variety of toxicity models in vivo and in vitro (Chetsawang et al. 2008, Ebadi et al. 2002, Finnegan et al. 1990, Tatton et al. 2003, Abu-Raya et al. 2002, Buys et al. 1995, Knollema et al. 1995, Abu-Raya et al. 1999, Maruyama et al. 2002, Maruyama et al. 2001, Ou et al. 2009). Reports of neuroprotection/neurorescue with these drugs has increased interest in MAOIs in general and stimulated research on neuroprotective/neurorescue properties of a wide variety of psychiatric drugs (Li and Xu, 2007; Jantzie et al., 2007).

Although MAOIs are effective for treating a number of psychiatric disorders, they have several adverse side effects, which often preclude their use as first line agents. First generation (nonselective and irreversible) MAO inhibitors (eg. PLZ, tranylcypromine, isocarboxazid) may produce side effects such as orthostatic hypotension and weight gain (Yamada and Yasuhara 2004). In contrast, a side effect known as "the cheese effect" can result when irreversible MAO-A inhibitors such as PLZ and tranylcypromine are combined with foods

rich in the sympathomimetic amine tyramine such as aged cheese or red wine. Tyramine, normally metabolized by MAO-A in the gut enters the systemic circulation and causes the release of NA (which is normally metabolized by MAO-A), resulting in hypertension and possibly a hypertensive crisis leading to cerebral hemorrhage (Bortolato et al. 2008). Thus foods containing high amounts of tyramine and related sympathomimetic amines must be avoided by patients taking these drugs. Reversible inhibitors of MAO-A (RIMAs) such as moclobemide usually do not lead to a hypertensive crisis since the enzyme can still partially metabolize tyramine in the gut (Krishnan, 2007). Another important adverse effect of MAOIs, serotonin syndrome, can occur when an MAOI is combined with other drugs that enhance serotonergic neurotransmission in the CNS. The syndrome produces restlessness, confusion, tremors, hyperpyrexia and diarrhea and can result in a coma (Nierenberg and Semprebon 1993). Drugs that may interact with MAOIs to produce serotonin syndrome include the selective-serotonin reuptake inhibitors (SSRIs), tricylic antidepressants, meperidine and dextromethorphan (Nierenberg and Semprebon 1993).

1.4. Phenelzine

Although it is not usually used as a first line antidepressant agent, PLZ is effective in the pharmacotherapy of atypical depression (Quitkin et al. 1988, Quitkin et al. 1990), social anxiety disorder (Heimberg et al. 1998) and panic disorder (Ballenger, 1986). In animal models, PLZ has been observed to significantly reduce neuronal loss in transient forebrain ischemia (Wood et al. 2006) and to reverse some of the neurochemical (NA, 5-HT, GABA) and

behavioral deficits in an animal model of multiple sclerosis (Musgrave et al., 2011 a,b).

Due to its irreversible inhibition of MAO, PLZ raises brain levels of 5-HT, DA, and NA (McKim et al. 1983, Baker et al. 1984, Blier et al. 1986, McKenna et al. 1991) and the trace amines β-phenylethylamine (PEA), tryptamine and tyramine (Philips and Boulton 1979). PLZ irreversibly inhibits both MAO isoenzymes. The proposed mechanism by which PLZ inhibits MAO involves oxidation by MAO to a reactive diazine metabolite that binds to the 4a carbon of the FAD cofactor (Holt et al, 2004).

PLZ also inhibits the catabolic enzymes GABA-transaminase (GABA-T) and alanine transaminase (ALA-T), leading to a marked increase in brain levels of the amino acids γ-aminobutyric acid (GABA) (Popov and Matthies 1969, Baker et al. 1991, McManus et al. 1992) and ALA (Tanay et al. 2001). The antianxiety effects of PLZ have been suggested to be due to its elevation of brain GABA (Paslawski et al. 1996). The proposed mechanism in which PLZ inhibits GABA-T and ALA-T may be by binding to the pyridoxal L-phosphate (PLP) cofactor required by both enzymes as PLZ has been shown to inhibit a number of PLP-dependent enzymes (Dyck and Dewar 1986; Yu and Boulton 1992; Holt et al. 2004).

In rats, PLZ has been reported to increase brain GABA levels to be 3-4 times control values (Popov and Matthies, 1969; Baker et al. 1991). Pre-administration of tranylcypromine (TCP) to inhibit MAO was observed to prevent the GABA-elevating effects of PLZ, suggesting that an active metabolite of PLZ may be responsible for its GABA-elevating properties (Popov and Matthies 1969; Todd

and Baker, 1995). Acute and chronic administration of PLZ did not lead to changes in the expression of mRNA encoding for GABA-T or for GAD65 and GAD67, the rate limiting enzyme in GABA synthesis (Lai et al. 1998). Chronic administration of PLZ has been reported to lead to an increase in expression of mRNA for the GABA transporter-1 (GAT-1), which takes up GABA from the synaptic cleft (Lai et al. 1998) and may protect it from catabolism by GABA-T.

Administration of PLZ has been shown to significantly reduce glutamatergic neurotransmission as it reduces potassium induced outflow of glutamate from the prefrontal cortex (Michael-Titus et al. 2000), and significantly reduces the glutamate-glutamine cycling rate (Yang and Shen 2005). However it is important to note that PLZ was also observed to have no significant effect on whole brain glutamate levels (Parent et al. 2000). More studies on PLZ's effect on glutamatergic transmission are required to determine why these inconsistencies are observed. Recently, the reactive aldehyde formaldehyde has been shown to decrease glutamate uptake in astrocytes, and PLZ has been shown to reverse this decrease in glutamate uptake (Song et al. 2010).

PLZ also has other actions which may contribute to its neuroprotective effects. It is an inhibitor of primary amine oxidase (PrAO), formerly called semicarbazide-sensitive amine oxidase (SSAO) (Holt et al., 2004) and also sequesters reactive aldehydes (Wood et al., 2006). PrAO is responsible for oxidation of amines like methylamine and aminoacetone to potentially toxic reactive aldehydes (formaldehyde and methylglyoxal respectively). This amine oxidase has been reported to be overexpressed in Alzheimers' disease and a

variety of cardiovascular disorders (Yu et al., 2003; Chen et al., 2006; Jiang et al., 2008). Other reactive aldehydes that have been proposed to be produced in excessive amounts in various neurodegenerative disorders include 3-aminopropanal (3-AP), acrolein, 4-hydroxynonenol and malondialdehyde. Abnormally high levels of these aldehydes and their adducts have been reported in a variety of neurodegenerative disorders, including Alzheimer`s disease (Kuhla et al., 2005; Lovell et al., 2001; Gustaw-Rothenberg et al., 2010; Sinem at al., 2010; Greilberger et al., 2008; Martin-Aragon et al., 2009; Casado et al., 2008; Markesbery and Lovell, 1998), Parkinson`s disease (Serra et al., 2009; Ilic et al., 1999; Baillet et al., 2010; Selley, 1998; Chen et al., 2009), multiple sclerosis (Leung et al., 2011) and amyotrophic lateral sclerosis (ALS) (Baillet et al., 2010; Bonnefont-Rousselot et al., 2000; Oteiza et al., 1997; Ferrante et al., 1997; Smith et al., 1998; Simpson et al., 2004; Pedersen et al., 1998; Pedersen et al., 1999). Being a hydrazine, PLZ forms inactive hydrazones with aldehydes (Wood et al., 2006). A similar sequestering action has also been shown with PEH, a proposed metabolite of PLZ (Clineschmidt and Horita, 1969a; Tipton and Spires, 1972; Binda et al., 2008; Mackenzie, 2009). Thus PLZ has a dual action, ie. inhibition of PrAO and direct sequestration that can counteract the deleterious effects of reactive aldehydes.

1.5. Metabolism of Phenelzine

For a long time it was assumed that the main route of catabolism of PLZ is acetylation in the liver. This assumption was based on evidence that structurally related compounds such as isoniazid, hydrazine and many sulfa drugs are

metabolized by acetylation in the liver (Robinson et al. 1985). Early studies on PLZ metabolism suggested a link between the acetylator phenotype and PLZ's therapeutic effect, although subsequent studies have failed to make an association between phenelzine treatment efficacy and acetylator status (Robinson et al. 1985). Additional studies in the 1980s concluded that N-acetylphenelzine is only a minor metabolite (Robinson, 1985; Mozayani et al., 1988). However, evidence suggests that oxidation, N-methylation, and ring hydroxylation may be other steps in the metabolism of PLZ (Clineschmidt and Horita, 1969 a,b; Tipton and Spires, 1972; Baker et al. 1999).

In addition to being an inhibitor of MAO, PLZ is a substrate of MAO (Clineschmidt and Horita 1969a, 1969b). Steady-state plasma levels of PLZ increase gradually over 6-8 weeks of treatment as a greater percentage of MAO is inhibited (Robinson at al., 1978, 1980 ; Mallinger and Smith 1991). Inhibition of MAO prior to PLZ administration in rats negates its GABA-elevating properties in brain (Popov and Matthies 1969, Todd and Baker 1995), suggesting that an active metabolite of PLZ is responsible for this effect. Phenylethylidenehydrazine (PEH) (Figure 1) was suggested to be that metabolite (Tipton and Spires 1971; Patek and Hellerman 1974) and metabolism of PLZ to PEH has now been demonstrated in vitro and ex vivo (Mackenzie, 2009; Tipton and Spires, 1972; Matveychuk, Baker and Holt, unpublished). When administered to rodents, PEH leads to inhibition of GABA-T and elevations in brain GABA levels (Paslawski et al. 2001). PEH increases extracellular GABA in the striatum when administered to rats (Parent et. al, 2002), increases rat brain levels of ornithine (MacKenzie et

al., 2008) and has neuroprotective actions in a gerbil model of global ischemia (Todd et al., 1999; MacKenzie et al. 2011). Because of its ability to elevate brain GABA levels and to produce neuroprotective effects, PEH may be a useful drug in the treatment of a variety of psychiatric and neurological disorders, and such potential activity is currently being investigated by other researchers. Unlike PLZ, PEH only weakly inhibits MAO (Paslawski et al. 2001; Mackenzie et al., 2008), suggesting that PEH would lack the potential side effects of PLZ resulting from MAO inhibition.

Another possible active metabolite of PLZ, the trace amine β-phenylethylamine (PEA) (Figure 1), may also have important implications. PEA is called a trace amine because at physiological levels there is a very low concentration of PEA in the CNS relative to the catecholamines and 5-HT (Baker et al. 1982; Boulton, 1984). However, the turnover rate (synthesis and catabolism) of PEA is very rapid and thus the amount of PEA being processed by the brain is much larger than would be indicated by its absolute brain levels (Boulton, 1984). It can also cross the blood-brain barrier readily, and a deficiency of brain PEA has been implicated in depression (Dewhurst, 1968; Sabelli and Mosnaim, 1974; Boulton et al., 1984; Tomlinson and Baker, 2009). The discovery and cloning of a unique family of G protein coupled receptors, some of which are selectively activated by trace amines (Borowsky et al., 2001; Bunzow et al., 2001) has caused a flurry of research interest in PEA and other trace amines (Berry, 2007; Narang et al., 2011). Dyck et al. (1985), in studies with deuterated PLZ demonstrated that part of PLZ is converted to PEA in rats (Dyck et al. 1985). After acute

administration (1 and 2 days) of PLZ to rats, whole brain PEA levels are not significantly different from control values, but after chronic administration (19 days), levels of PEA are markedly increased (Baker et al. 1982). Since it has been proposed that a functional deficiency of PEA may be involved in the etiology of certain types of depression, elevation of PEA after chronic PLZ administration may contribute to the observed antidepressant effect. A major metabolite of PLZ identified in urine samples of human subjects taking PLZ is phenylacetic acid (PAA) (Robinson et al., 1985), the major metabolite of PEA. Studies with radiolabelled PLZ incubated with rat liver homogenates and in urine of rats treated with radiolabelled PLZ also reported PAA as a major metabolite of PLZ (Clineschmidt and Horita, 1969a,b), although it was suggested by Clineschmidt and Horita (1969a) that the PAA might be formed from an intermediate metabolite such as PEH.

1.6. Phenelzine and Weight Gain

There have been reports in the literature that γ-vinylGABA (vigabatrin), a GABA-T inhibitor, decreases weight gain in Sprague-Dawley rats and in Zucker fatty rats (Huot and Palfreyman, 1982; Morgan and Dewey, 1998; De Marco et al. 2008). Since PLZ is an even stronger inhibitor of GABA-T than vigabatrin (Todd and Baker, 2008) and is a more potent elevator of GABA levels (Yang and Shen, 2005), it was of interest to determine its effects on weight gain in rats.

1.7. Chromatography

Chromatography is a technique used to separate mixtures and to identify and quantify substances of interest. Gas chromatography (GC) with electron capture detection (ECD) and high-performance liquid chromatography (HPLC) with fluorescence detection were used in this thesis for analysis of PLZ metabolites and amino acids, respectively.

1.7.1 Gas chromatography (GC)

The technique of GC involves vaporization of a sample and separation of its components in a specialized chromatography column, followed by identification and quantification of the individual components. Compounds analyzed in GC must be volatile. However, heat stability is required so that the compound of interest is not altered by high oven temperatures. Chemical derivatization of a compound is often employed to produce a derivative with the desired properties of greater volatility, heat stability, and sensitivity to detection (Sparkman et al., 2011).

There are two phases used in GC, a mobile and a stationary phase. The mobile phase consists of a inert carrier gas such as helium, nitrogen or hydrogen that is used to carry the sample from the injector site, through the column and to the detector (Sparkman et al. 2011). The stationary phase is a nonvolatile liquid that lines the analytical column. Individual components of the analyte are separated based on differential affinities for the stationary phase (Skoog et al., 1996a). Compounds that have a high affinity for the stationary phase remain in

the column longer and have a greater retention time than compounds that have a low affinity for the stationary phase. Compounds that elute close together in time can be separated by the use of a specific oven temperature program that has a specific rate or pattern of temperature increase. Variations in temperature can alter the retention times of compounds relative to one another.

Electron capture detection (ECD) is a highly sensitive method of detection that detects compounds containing electron-absorbing components. A radioactive isotope (such as ^{63}Ni) is used to create a source of electrons by continuous beta decay. The beta particles act on the makeup gas (argon-methane in our case) at the detector to produce electrons. The electrons generated migrate to the anode and create a baseline level of current. Absorption of electrons by gaseous analytes disrupts the baseline level of current, and this is recorded as a peak (Dybowski and Kaiser 2002). Although the ECD is very sensitive, often compounds of interest do not contain electrophilic groups and must be derivatized to add such groups; this was the case in the analyses reported in this thesis. GC-ECD was used in this thesis to measure brain and liver levels of PEH, PEA, and PAA following assay-specific methods of derivatization.

1.7.2. High performance liquid chromatography (HPLC)

HPLC has an advantage over GC in that it does not require a sample to be volatile or heat stable and can be run at room temperature. HPLC involves separation of a mixture of compounds using a liquid mobile phase on a solid analytical column (stationary phase) (Odontiadis and Rauw, 2007). A sample is

introduced into the liquid mobile phase and separation of the compound is achieved through differential interactions of the individual components with the solid stationary phase of the analytical column and the liquid mobile phase (Skoog et al. 1996b). Gradient elution refers to the programmed use of two or more mobile phases in a time-dependent manner in order to achieve adequate compound separation and a desired retention time. Gradient elution was used in this thesis.

In the studies reported in this thesis, a fluorescence detector was used in combination with HPLC to measure levels of amino acids in brain. In this type of detection, the compound is excited with electromagnetic energy from the ultraviolet (UV) spectrum and emits energy at a longer wavelength (largely in the visible spectrum) (Baker et al., 1985). A fluorescence detector detects the amount of energy emitted by the derivatized compound. Derivatization of compounds of interest with fluorescent reagents is often done to introduce fluorescent groups, and such derivatization of amino acids was utilized in the studies reported here.

1.8. Objectives and Hypotheses

The objectives of the studies reported in this thesis were to study the formation of PEA and PAA from PLZ and PEH in rat brain. The effects of PLZ and PEH on amino acid levels in rat brain were also compared directly at a number of time intervals. The effects of chronic administration of PLZ on brain amino acid levels and weight gain in rats were also investigated.

The following hypotheses were put forward:

-Both PLZ and PEH will be metabolized to PEA, but the effect will be much greater with PLZ

-Both PLZ and PEH will be metabolized to PAA, but the effect will be greater with PLZ.

-PLZ and PEH will have similar effects on rat brain levels of amino acids, but the effects of PLZ, but not PEH, will be blocked by inhibition of MAO prior to administration of PLZ.

-After chronic administration of PLZ (1 week and 3 weeks), its effects on alanine and GABA will diminish, presumably because of increased inhibition of MAO, the enzyme that converts it to PEH.

-PLZ will decrease weight gain in rats.

2.0. METHODS

2.1. General Methods

2.1.1 Animals

All animal procedures employed were approved by the University of Alberta Biosciences Animal Policy and Welfare Committee and were performed according to the guidelines set forth by the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 200-300g were housed in pairs in polycarbonate cages with free access to food (Purina Rat Chow) and water. Temperature was controlled at 19-21°C and a 12h light-dark cycle was maintained.

2.1.2 Drug Administration

All drugs were administered by intraperitoneal (i.p.) injection. PEH was synthesized at the University of Alberta (Dr. E. E. Knaus, Faculty of Pharmacy and Pharmaceutical Sciences for synthetic details). For the acute administration studies, rats were injected with PLZ sulfate (30mg/kg in distilled water) or PEH (30mg/kg in pure corn oil, Mazola brand) or their respective vehicle solutions (saline or corn oil) and were euthanized by decapitation 1hr, 3 hr or 6 hr after drug administration. For the chronic PLZ administration study, rats were injected daily in the morning with PLZ sulfate (15mg/kg i.p.) for 1 week or 3 weeks, and the rats were euthanized 24 hr after the last treatment of PLZ. In the chronic studies a dose of 15mg/kg PLZ was used since preliminary studies by members of the Neurochemical Research Unit had suggested that the 30mg/kg dose might cause

excessive weight loss. In addition, previous studies in the Neurochemical Research Unit had also shown that both doses of PLZ produced similar effects on amino acid levels after acute administration. For acute studies in which TCP was used to pre-inhibit MAO, TCP or vehicle (saline) was injected 1hr before PLZ or PEH treatment, and animals were euthanized by decapitation at 0.5 hr or 1 hr following PLZ or PEH administration. After decapitation, livers and brains were quickly removed and flash-frozen in 2-methlybutane on dry ice, then stored in containers on dry ice. The containers were then stored at -80°C until analysis.

2.1.3 Ex vivo determination of PEH in liver

Partially thawed liver tissue was homogenized in 8 volumes of ice-cold methanol (MeOH). A portion (1ml) of this homogenate was used in the assay. For the standard curves, standard dilutions of PEH in MeOH were added to 1ml aliquots of naïve homogenate. MeOH (20µl) was also added to each PEH-treated sample in order to keep the amount of MeOH constant between the standards and the samples. All homogenates were vortexed, then centrifuged at 13000g at 4°C for 10 min to precipitate protein. The supernatant (500µl) for the standards, blank, and samples was pipetted into a Savant size (10x100mm) drying tubes. Pentafluorobenzaldehyde (PFBA) (10µl) was added to each tube. The tubes were capped, vortexed and left to stand for 30 min in the dark. Solutions in the tubes were then taken to dryness in a Savant evaporator at ambient temperature. The residue was then reconstituted in 200µl of cylcohexane. The solution was then transferred to microfuge tubes and centrifuged at 13000g for 5 min. The

supernatant was transferred to GC inserts and 2ul were injected into a GC-ECD. The structure of the final derivative is shown in Figure 2.

An Agilent 6890 Series GC system was used with a HP-5MS column (length = 30m, internal diameter = 0.250mm, film thickness = 0.25 µm) and a micro-electron capture detector (ECD; Agilent). The carrier gas was helium, and argon-methane (95% argon: 5% methane) gas was used as the makeup gas at the ECD. The injector temperature was 250°C. At initial conditions, the oven temperature was set at 80°C, and increased at a rate of 15°C/min until a final temperature of 290°C was reached. The oven was held at the final temperature of 290°C for 10 minutes before returning to initial conditions.

2.1.4 Ex vivo determination of PEA

Partially thawed tissues were homogenized in 5 volumes of 0.1N $HClO_4$ for brain tissue and in 0.4N $HClO_4$ for liver tissue. Homogenized samples were centrifuged at 12000 xg for 5 min at 4°C. To large tubes on ice 2ml of 0.1N $HClO_4$ (brain) or 0.4N $HClO_4$ (liver) were added for the standard curve and blanks. Internal standard benzylamine (BZA) (5µl= 50ng) was added to all tubes including the blank. Supernatants (0.1-2ml) of the brain and liver samples were added to tubes for the unknown samples. Appropriate dilutions of standard concentrations of PEA (diluted in Millipore-filtered water [MP H_2O]) were then added to the standard tubes. All tubes were then vortexed and the solutions basified with solid $NaHCO_3$ in excess, and then vortexed again thoroughly. The derivatizing reagent solution containing pentafluorobenzenesulfonyl chloride

(PFBS; 9ml of ethyl acetate: 1ml acetonitrile: 10µl of PFBS) was mixed and 4ml was added to each tube. Afterwards, the tubes were shaken for 5 min, and centrifuged for 5 min at 2000xg. Most of the top layer (organic) was removed into drying tubes (with care not to include any of the aqueous layer) and then left to dry in a Savant evaporator at ambient temperature. The residue was reconstituted in 100µl of toluene and the solution was transferred to microfuge tubes and centrifuged at 12000 xg for 5 min. The supernatant was transferred to GC inserts and 2µl were injected into a GC-ECD.

An Agilent 6890 Series GC system was used with a HP-5MS column (length = 30m, internal diameter = 0.250mm, film thickness = 0.25 µm) and a micro-electron capture detector (ECD; Agilent). The carrier gas was helium, and argon-methane (95% argon: 5% methane) gas was used as the makeup gas at the ECD. The injector temperature was 230°C. At initial conditions, the oven temperature was set at 100°C, and increased at a rate of 25°C/min until a final temperature of 295°C was reached. The oven was held at the final temperature of 295°C for 5 minutes before returning to initial conditions. The structure of derivatized PEA is shown in Figure 3.

2.1.5 Ex vivo determination of PAA

Partially thawed brains were homogenized in 5 volumes of 0.1N $HClO_4$. Homogenates (1.5ml) were then centrifuged at 12000 xg at 0-4°C for 5 min. To tubes (15x100 mm) on ice, 1ml of 0.1N $HClO_4$ (brain) was added for the standards and the blank, and 1ml of supernatant was added for the brain samples.

The internal standard p-chlorophenylacetic acid (pCPAA) (5µl= 50ng) was added to all the tubes. Appropriate standard dilutions of PAA (in MP H_2O) were then added to the standard tubes. All tubes were then vortexed. Sodium phosphate buffer (21.48 g NaH_2PO_4, 3.16g Na_2HPO_4, 100ml of MP H_2O) (1ml) of pH 5 to 6 (checked by a pH indicator strip) was added to each tube, and the tubes were vortexed. N,N'-Dicyclohexylcarbodiimide (DCC) solution (900µl toluene, 100µl acetonitrile, 5µl DCC) (200µl) was then added to each tube. Following the addition of the DCC solution, 200µl of the derivatizing agent pentafluorophenol (PFPhOH) (900µl toluene, 100µl acetonitrile, 5µl PFPhOH) was added. The tubes were then shaken for 20 min and centrifuged at 2000xg for 5 min. The top layer (organic) was then transferred to 2ml microfuge tubes, and 400µl of 0.5N NH_4OH was then added to each microfuge tube, and the tubes were vortexed, then centrifuged at 12000xg for 4 min. Part of the top layer (organic) was transferred to GC vials and 2µl was injected on a GC-ECD.

An Agilent 6890 Series GC system was used with a HP-5MS column (length = 30m, internal diameter = 0.250mm, film thickness = 0.25 µm) and a micro-electron capture detector (ECD; Agilent). The carrier gas was helium, and argon-methane (95% argon: 5% methane) gas was used as the makeup gas at the ECD. The injector temperature was 250°C. At initial conditions, the oven temperature was set at 100°C, and increased at a rate of 15°C/min until a final temperature of 295°C was reached. The oven was held at the final temperature of 295°C for 15 minutes before returning to initial conditions. The structure of derivatized PAA is shown in Figure 4.

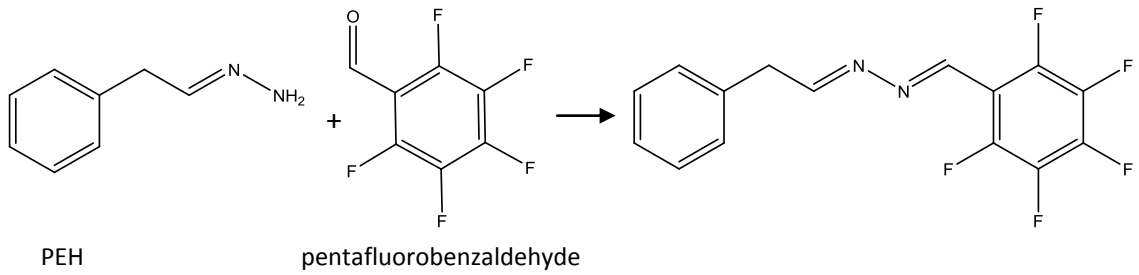


Figure 2. Derivatization of PEH with pentafluorobenzaldehyde.

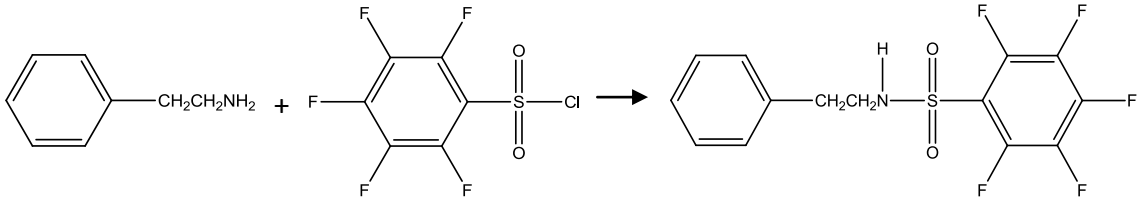


Figure 3. Derivatization of PEA with pentafluorobenzenesulfonyl chloride

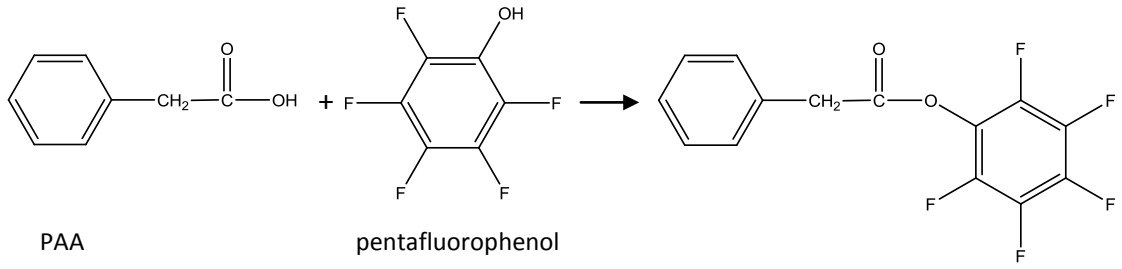


Figure 4. Derivatization of PAA with pentafluorophenol produces a fluorinated ester.

2.1.6 Ex vivo determination of whole brain amino acid levels

Whole brain levels of the amino acids L-aspartate, L-glutamate, L-serine, D-serine, L-glutamine, glycine, L-arginine, L-taurine, ALA, and GABA were analyzed by HPLC using a slight modification of the procedure developed by Grant et al (2006) which uses o-pthalaldehyde (OPA) and N-isobutryl-L-cysteine (IBLC) as derivatizing reagents. Partially thawed brain tissues were homogenized in 5 volumes of ice-cold MP H_2O. Homogenate (100µl) was added to 400µl of ice-cold MeOH in microfuge tubes, and the solution was vortexed and left to stand for 10 min. The microfuge tubes were then centrifuged at 12000xg for 5 min at 4°C. A portion of the supernatant (100µl) was removed and added to 100µl of MP H_2O and the resultant solution was vortexed and transferred to HPLC vials. Supernatant (5µl) was reacted with a 5µl mixture of OPA/IBLC in the injection loop of a Waters Alliance 2690XE system for 5min before injection into the analytical column. Preparation of the OPA/IBLC mixture involved dissolving 2mg of OPA and 3mg of IBLC in 150µl of methanol, followed by the addition of 1350µl of 0.1M sodium borate buffer, and the resulting solution was vortexed. All samples were stored at 4°C before derivatization.

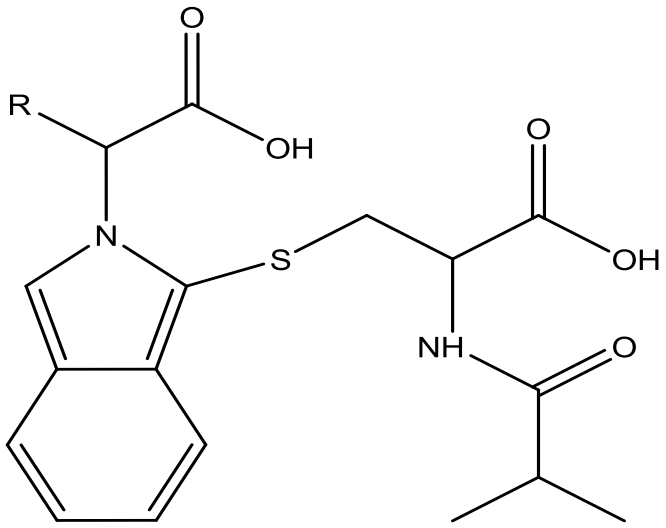
Derivatized amino acids were quantified by HPLC in combination with fluorescence detection. Compounds were separated using a Waters Alliance 2695 system using a Symmetry C_{18} 5µm (4.6 x 150mm) column connected to a Symmetry C_{18} guard column. The temperature of the system was maintained at 30°C. Two mobile phases were used and were filtered under a vacuum using 0.2µm filters. Mobile phase A consisted of 85% 0.04M Na phosphate buffer and

15% methanol, with pH adjusted to 6.2 using a phosphoric acid solution. Mobile phase B consisted of 53.4% 0.04M Na phosphate buffer, 44.2% methanol and 2.4% tetrahydrofuran, with pH adjusted to 6.2 with NaOH solution.

The relative amounts of mobile phases used was time programmed. At starting conditions the mobile phase consisted of 85% mobile phase A and 15% mobile phase B. Over a time period of 35min the composition of mobile phases was shifted to 100% mobile phase B, which was maintained for 10 minutes before returning to initial conditions. The flow rate was 0.5ml/min. A Waters 474 fluorescence detector was used to measure the energy emitted by the amino acid derivatives. The detector was set to an excitation wavelength of 344nm and an emission wavelength of 443 nm. The Empower Pro software package (Waters) was used to collect data. A standard curve was obtained by running standard concentrations of amino acids of interest with each assay run. The general structure of the derivatized amino acids is shown in Figure 5.

2.1.7. Statistical Analysis

The data were analyzed by either one-way or two-way ANOVA followed by independent t-tests for single pair comparisons or by the Newman-Keuls test for comparing multiple means. A probability value (p-value) of <0.05 indicated statistical significance.



fluorescent isoindole derivative

Figure 5. General structure of the derivatized amino acids using the method described here.

3.0. RESULTS

3.1. PEH levels in liver of PEH- and PLZ-treated animals following acute i.p. administration

A typical standard curve for the PEH assay is shown in Figure 6.

Results from acute administration with PEH indicate that liver levels of PEH steadily decline with time after acute PEH administration. One hour following a single i.p. injection of PEH (30mg/kg), levels of PEH in liver were 9198 ± 1218 ng/g (mean ± SE). At 6hrs following i.p. injection of PEH (30mg/kg), levels of PEH were (1775 ± 336 ng/g). Acute PLZ treatment produces marked increases levels of PEH in liver, indicating that PLZ is metabolized to PEH in vivo. Liver levels of PEH in PLZ-treated animals were observed to steadily decrease with time. One hour following a single i.p. injection of PLZ (30mg/kg), liver levels of PEH were 12131 ± 887 ng/g. At 6hrs following i.p. injection of PLZ (30mg/kg), liver levels of PEH were reduced to 1242 ± 180 ng/g. Data for acute administration of PEH and PLZ are shown in Figure 7. Brain levels of PEH were not measured in these studies since the brain tissue was required for analyses of PAA and PEA. MacKenzie (2009) had already shown similar levels to the ones reported here for brain levels of PEH, and liver levels were measured here to demonstrate that the PLZ and PEH had been administered effectively.

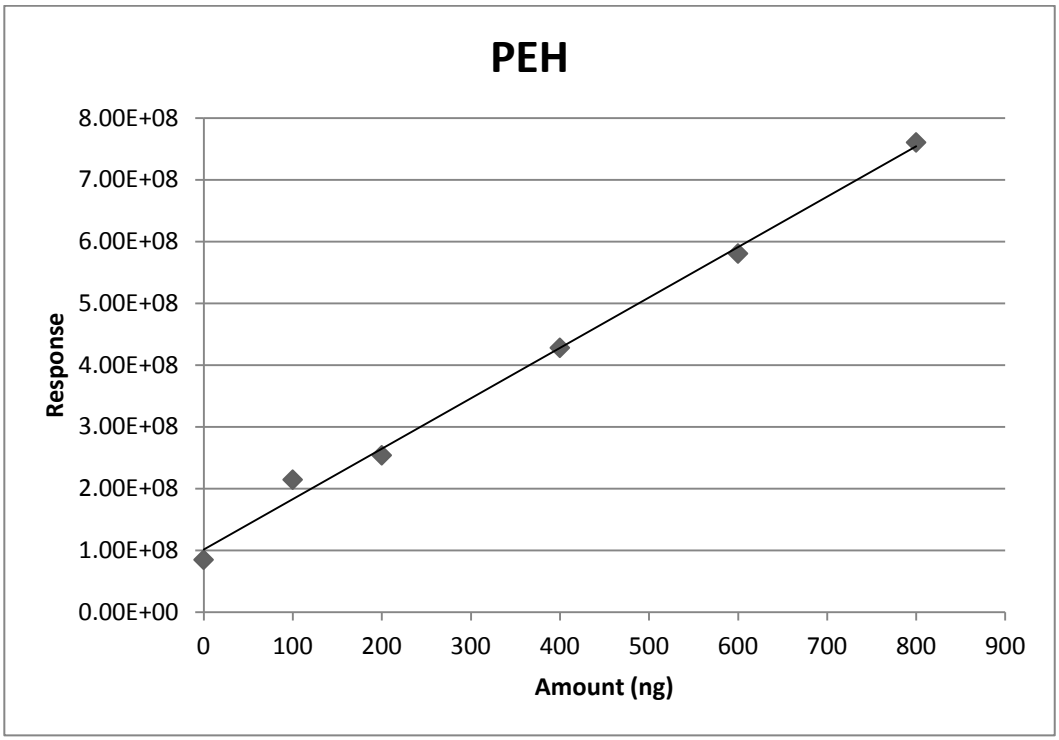


Figure 6. Typical standard curve for the PEH assay.

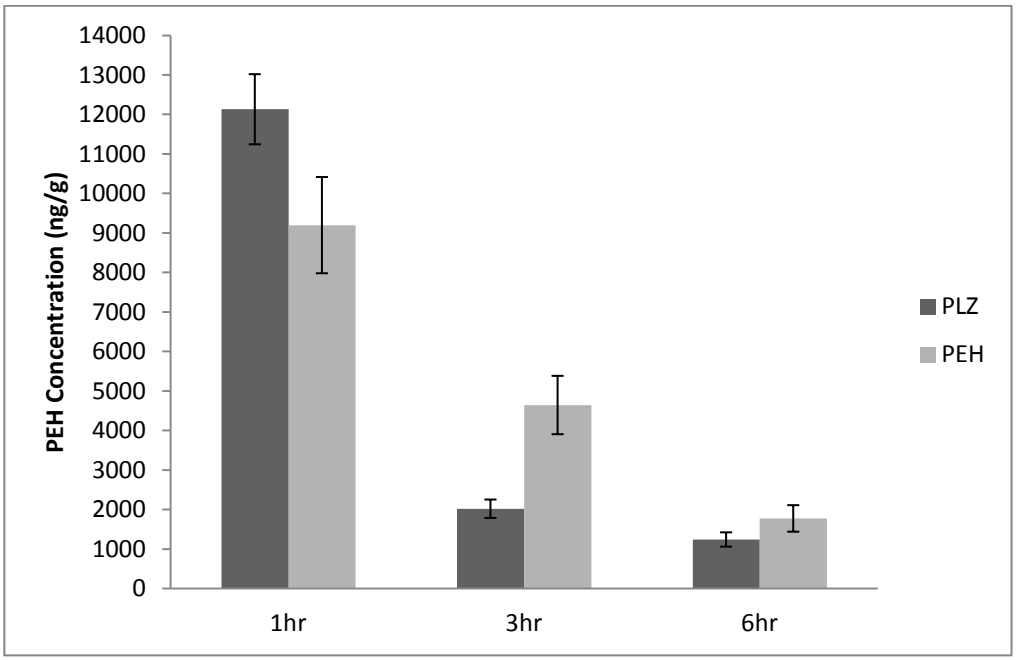


Figure 7. PEH levels in liver after administration of PLZ or PEH (30mg/kg).

Results are means ± SEM (N = 5)

3.2. PEA levels in brain and liver of PEH- and PLZ-treated animals following acute i.p. administration

A typical standard curve for the PEA assay is shown in Figure 8.

Brain levels of PEA were slightly increased 1hr after administration of either 30mg/kg of PLZ or 30mg/kg of PEH (5.7 ± 0.7 ng/g and 2.6 ± 0.3 ng/g respectively) compared to control values of PEA (0.8 ± 0.3 ng/g). This indicates that some of PLZ and PEH is metabolized to PEA in vivo in brain. The levels decreased to control values with both drugs at 3h and 6h.

Liver levels of PEA were also increased following i.p. administration of PLZ. One hour after i.p. injection of PLZ (30mg/kg), PEA levels in liver were increased to 42.3 ± 8.8 ng/g (control values of PEA were 12.4 ± 10.9 ng/g). At 6hrs following injection of PLZ, liver levels of PEA were reduced to 13.9 ± 0.5 ng/g compared to control values of 3.8 ± 0.5 ng/g. Liver levels of PEA for PEH-treated animals (30mg/kg) were not significantly different from control values at the time periods of 1hr, 3hr and 6hr after injection. Thus it appears that PEA is formed to a small extent from PLZ in liver and apparently not at all from PEH in this organ. The liver is the major metabolizing organ in the body and contains high activity of MAO: PEA is an excellent substrate for MAO and is probably metabolized very quickly after being formed; thus it was important to also conduct these studies in the presence of previous inhibition of MAO.

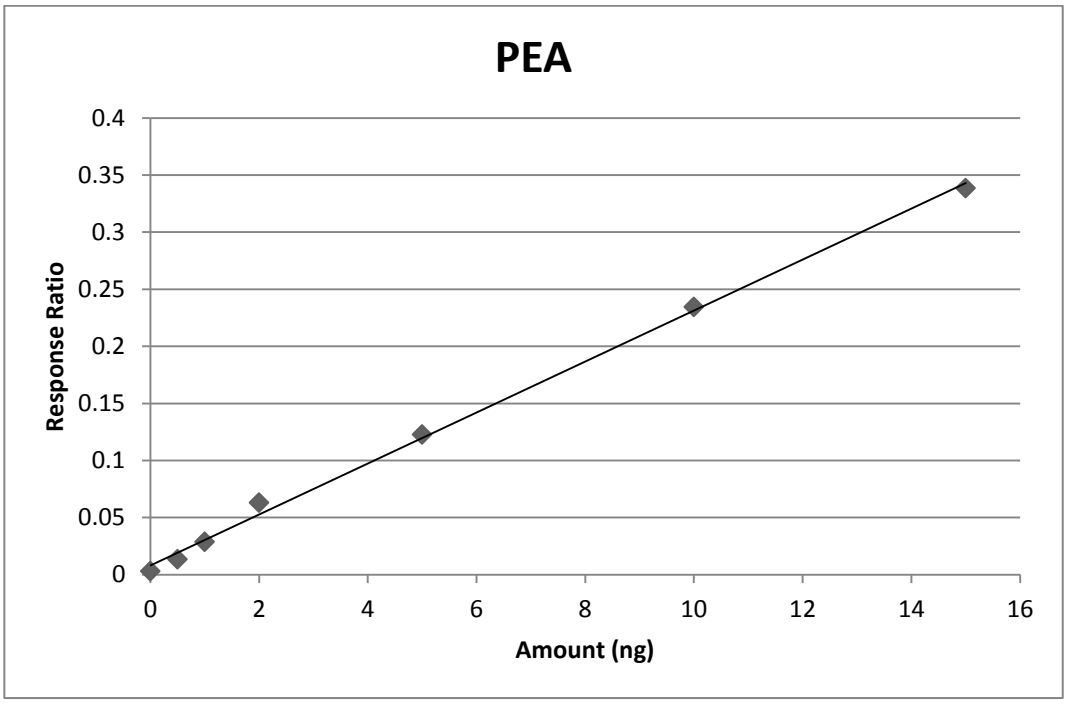


Figure 8. Typical standard curve for the PEA assay.

3.3. PAA levels in rat brain after administration of PLZ and PEH

A typical standard curve for the PAA assay is shown in Figure 9.

Marked increases in brain levels of PAA were observed 1h after treatment with each drug, but the effect was greater with PLZ administration (Figure 10). PAA is cleared rapidly from the brain and was not different from control levels at 3 or 6h after drug administration.

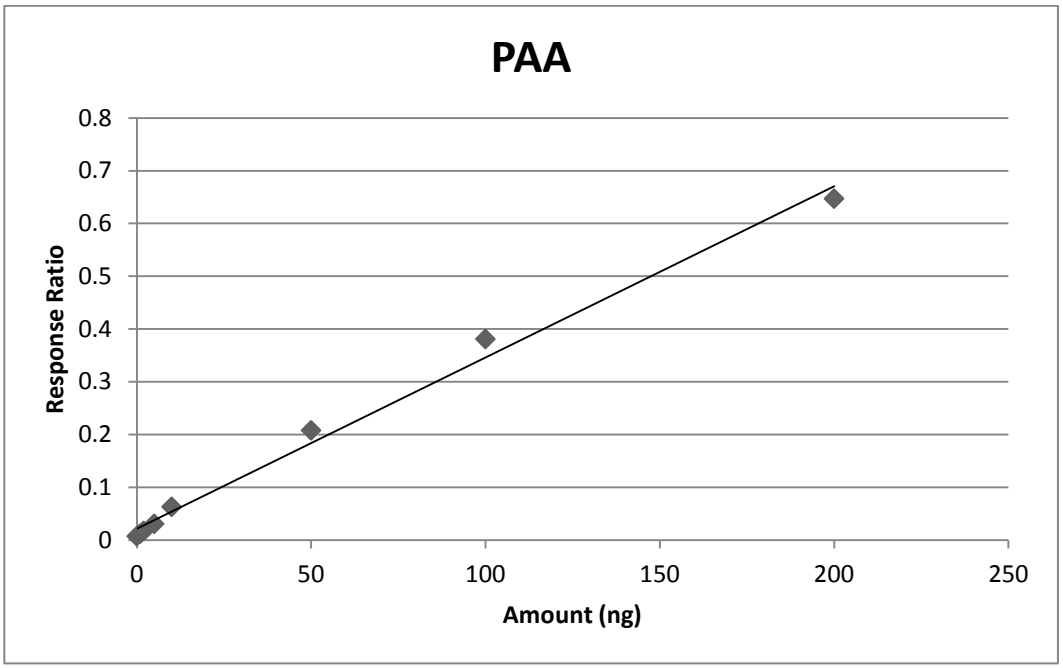


Figure 9. Typical standard curve for the PAA assay.

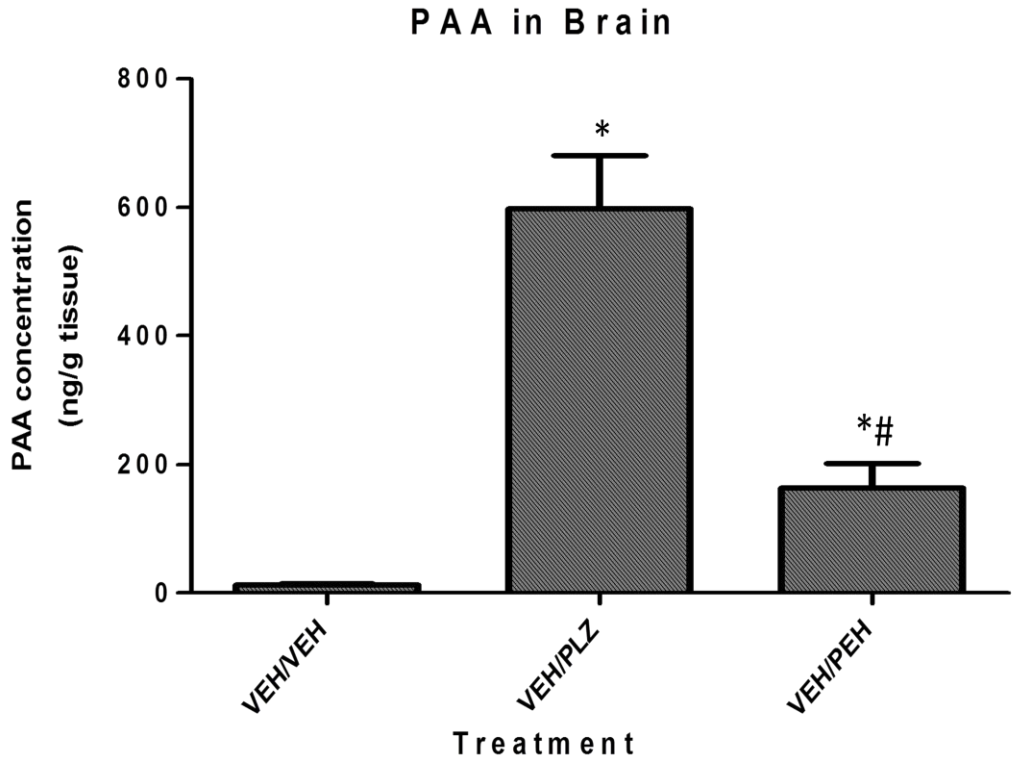


Figure 10. Whole brain levels of PAA (mean ± SEM) in PEH or PLZ treated animals. N=3-5 for each group and the rats were euthanized 1 hour after drug administration. (Done in collaboration with H. Banasch). * = significantly different from VEH/VEH; #= significantly different from VEH/PLZ.

3.4. Effect of prior MAO inhibition on levels of PEA in brain and liver of rats treated with PLZ and PEH

Prior inhibition of MAO by tranylcypromine (TCP) led to massive increases in brain levels of PEA for PLZ-treated animals and led to much smaller increases in brain PEA levels for PEH-treated animals which appear to be due primarily to the inhibition of MAO by TCP. TCP (1mg/kg) was administered by i.p. injection 1hr before i.p. injection of either PLZ or PEH treatment (30mg/kg) and animals were euthanized at 0.5hr and 1hr following PLZ or PEH administration. Brain levels of PEA in PLZ-treated animals (30mg/kg) pre-inhibited by TCP (1mg/kg) were massively increased to 470.6 ± 113.1 ng/g at 0.5hr and 712 ± 115.4 ng/g at 1hr compared to control VEH/PLZ levels of 3.5 ± 0.7 ng/g (0.5 hr) and 5.7 ± 1.7 ng/g (1hr). This demonstrates that under circumstances of acute administration of PLZ, large amounts of PLZ are converted to PEA, which is rapidly catabolized by MAO since pre-inhibition of MAO with TCP leads to massive increases of PEA in rat brain (much greater than produced by inhibition of MAO by TCP alone). Brain levels of PEA in PEH-treated animals (30mg/kg) pre-inhibited by TCP (1mg/kg) were slightly increased to 18.1 ± 6.0 ng/g at 1hr compared to VEH/PEH levels of 2.6 ± 0.3 and VEH/VEH levels of 0.8 ± 0.3 (there were no TCP/PEH treated animals at 0.5hr). However, brain levels of PEA in this case were not significantly higher than those produced by TCP alone, indicating that the increase in PEA levels in the TCP/PEH animals was due primarily to the MAO-inhibiting effect of TCP and

that conversion of PEH to PEA was minimal. The brain results are shown in Figures 11 and 12.

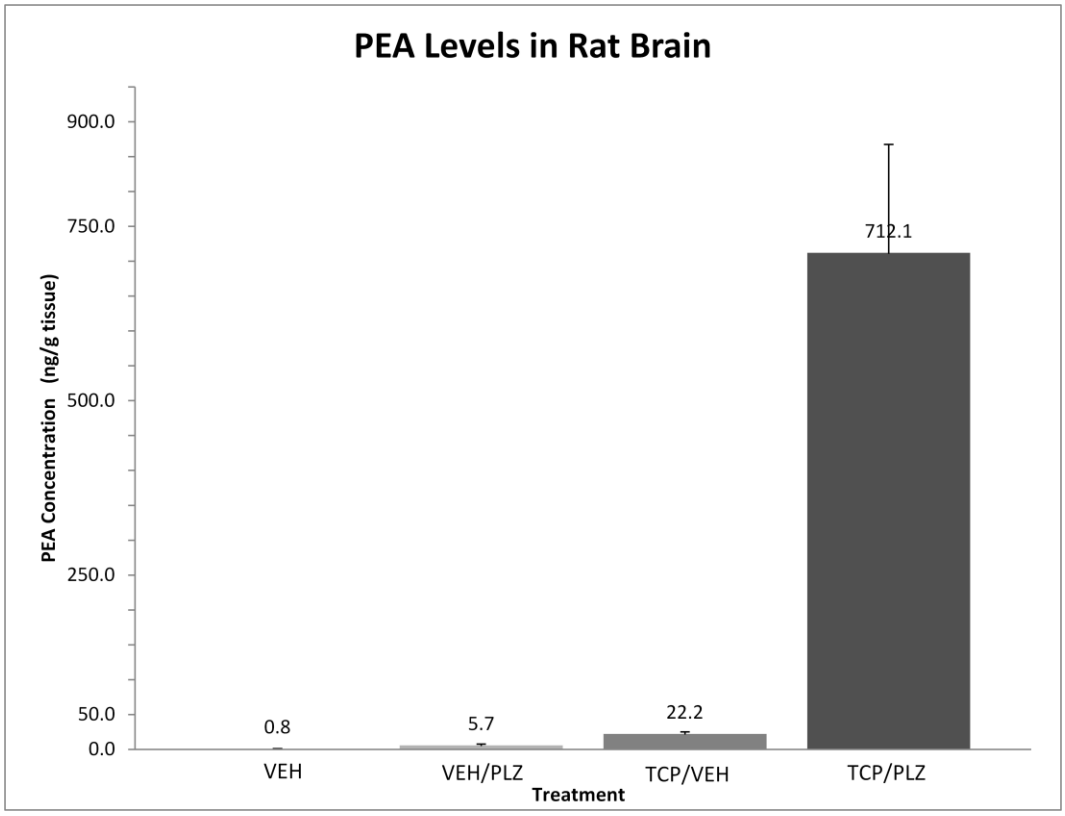


Figure 11. PEA levels in rat brain following injection of TCP (1 hour) and PLZ (1 hour). Results are means ± SEM (N=6). VEH/PLZ, TCP/VEH and TCP/PLZ values are all significantly different from control (VEH/VEH) values.

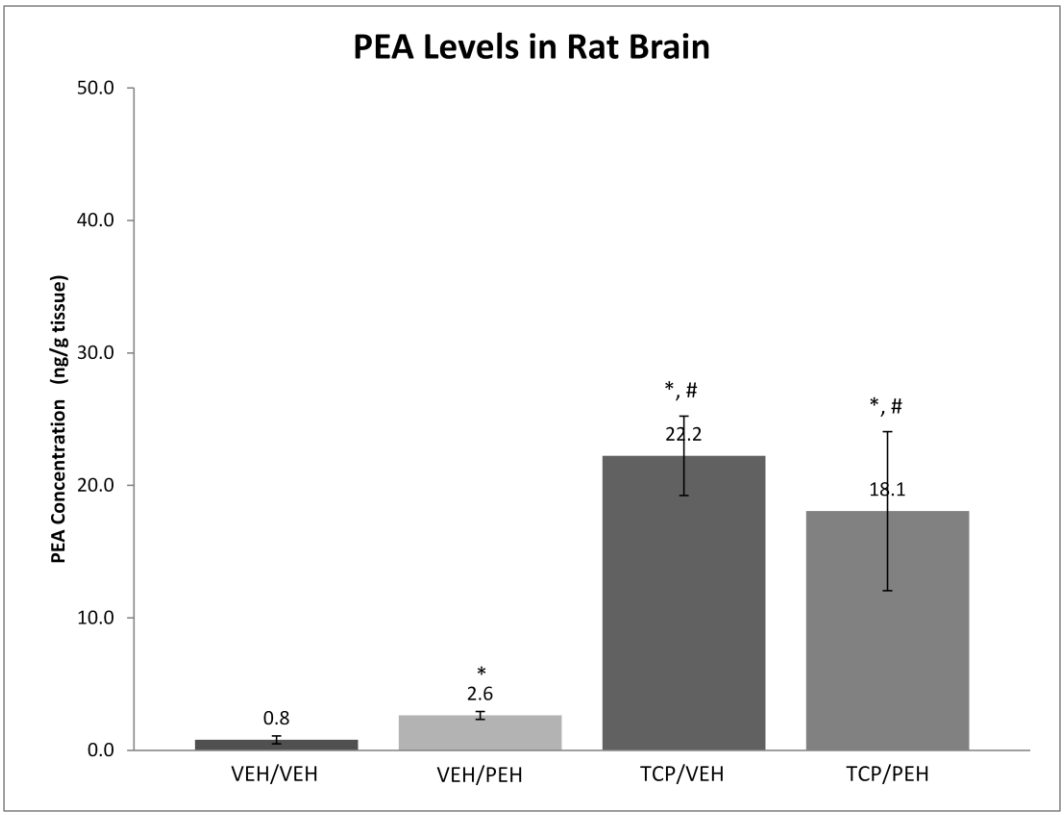


Figure 12. PEA levels in rat brain following injection of TCP (1 hour) and PEH (1 hour). Results are means ± SEM (N=6). *= significantly different from VEH/VEH values; #= significantly different from VEH/PEH values.

3.5. Amino Acids in Rat Brain after Acute Administration of PLZ and PEH

The assay used provides good separation of amino acids of interest (Figure 13). Typical standard curves for each of the amino acids are shown in Figures 14 to 23.

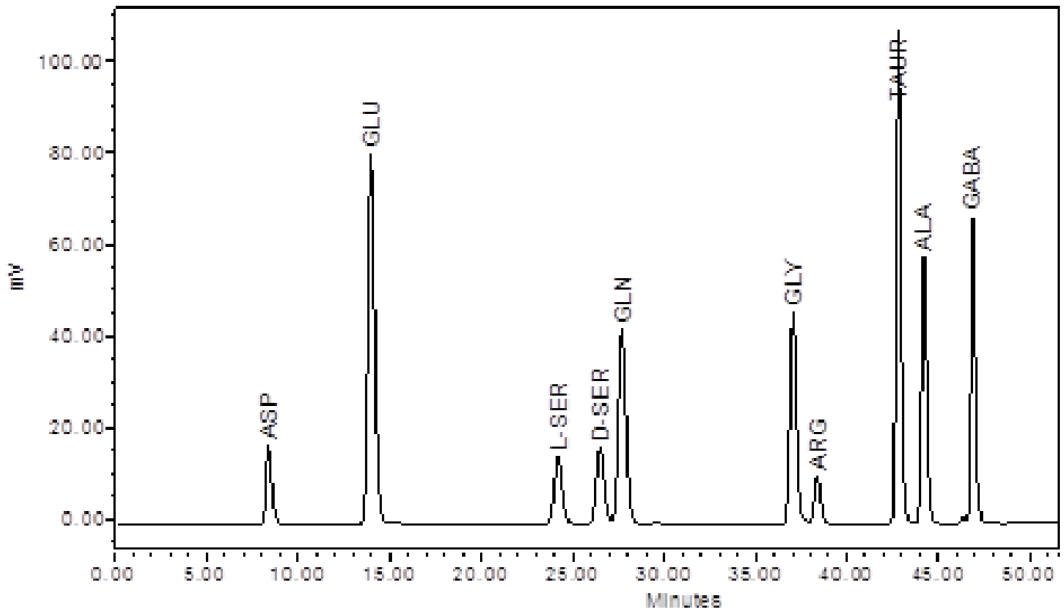


Figure 13. Example of an HPLC amino acid trace from the assay used for analysis of amino acid levels. ASP = L-aspartate; GLU =L-glutamate; L-SER = L-serine; D-SER = D-serine; GLN = L-glutamine; GLY = glycine; ARG = L-arginine; TAUR = L-taurine; ALA =L-alanine; GABA = γ-aminobutyric acid. Hereafter in the thesis, D- and L- are only used with D- and L-serine.

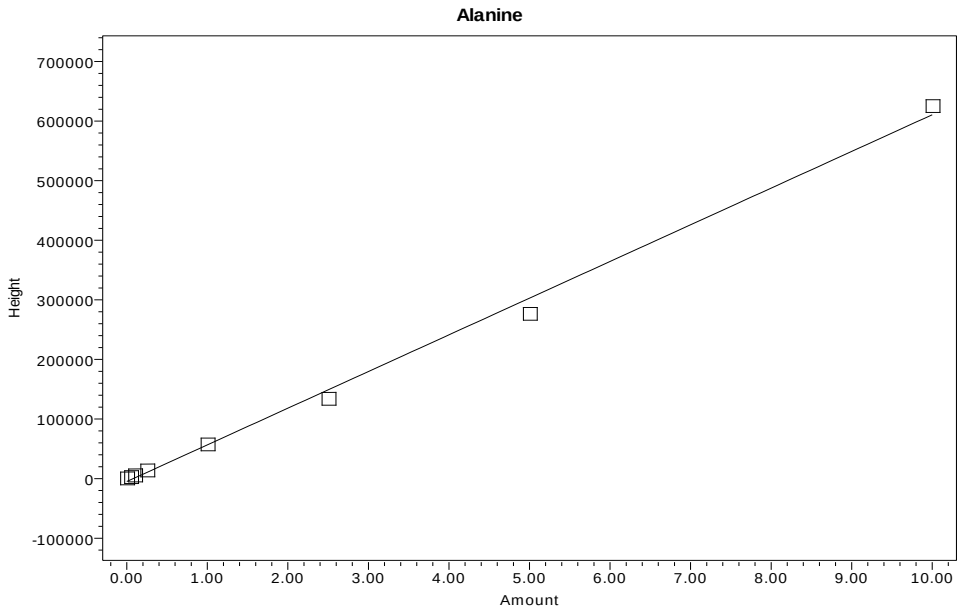


Figure 14. Typical standard curve for alanine.

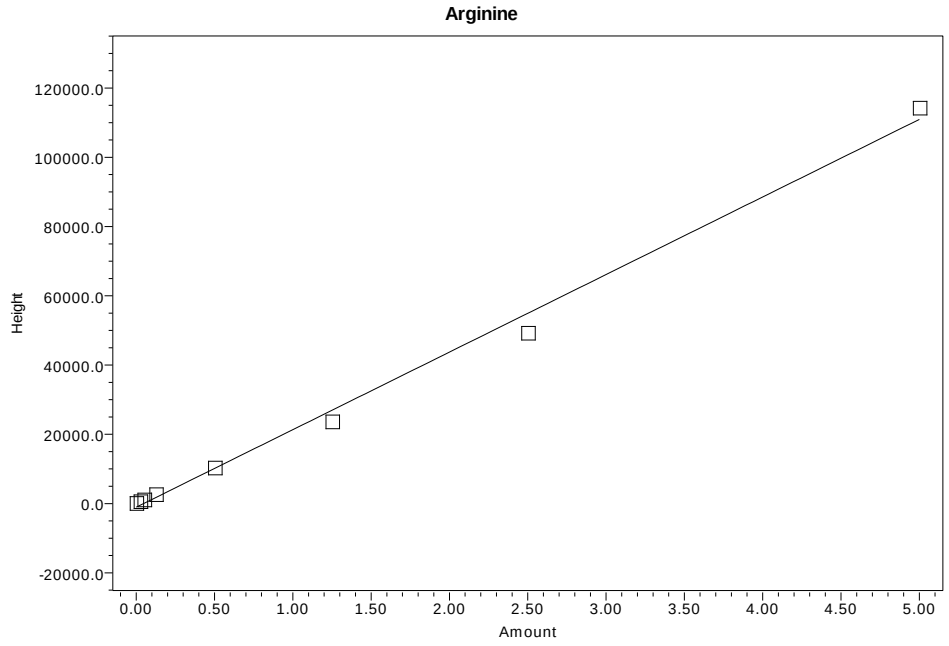


Figure 15. Typical standard curve for arginine.

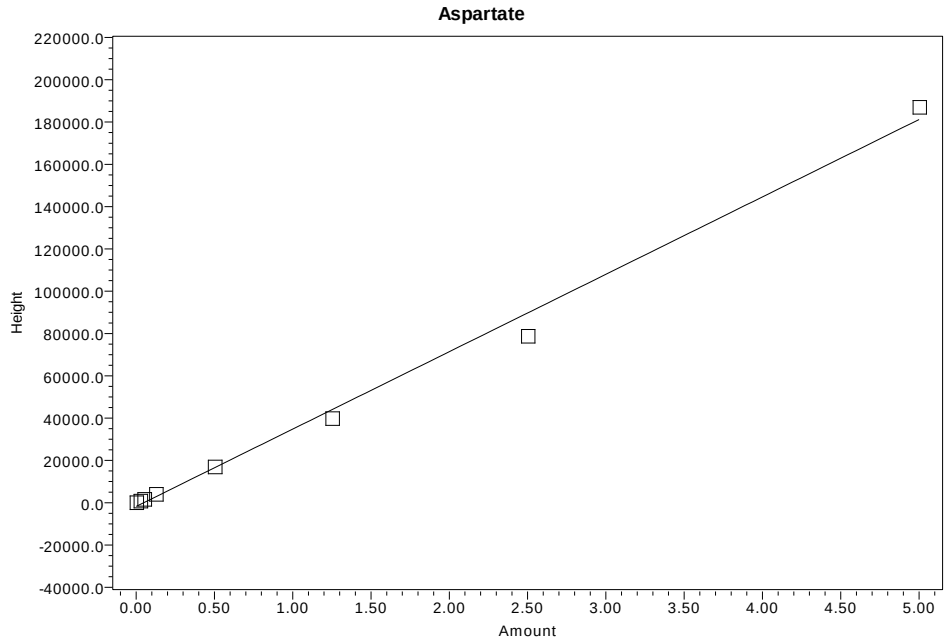


Figure 16. Typical standard curve for aspartate.

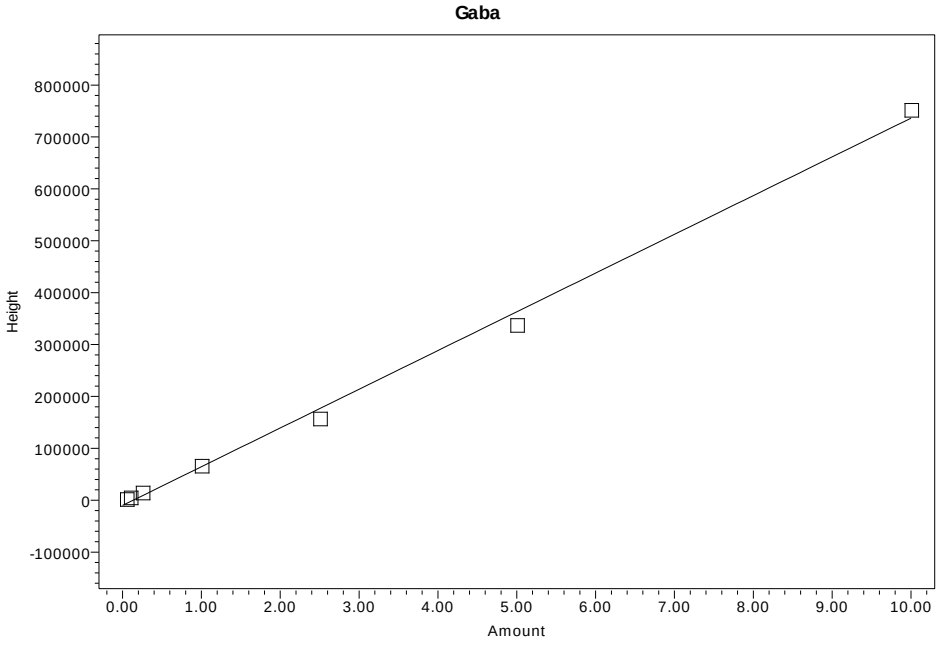


Figure 17. Typical standard curve for GABA.

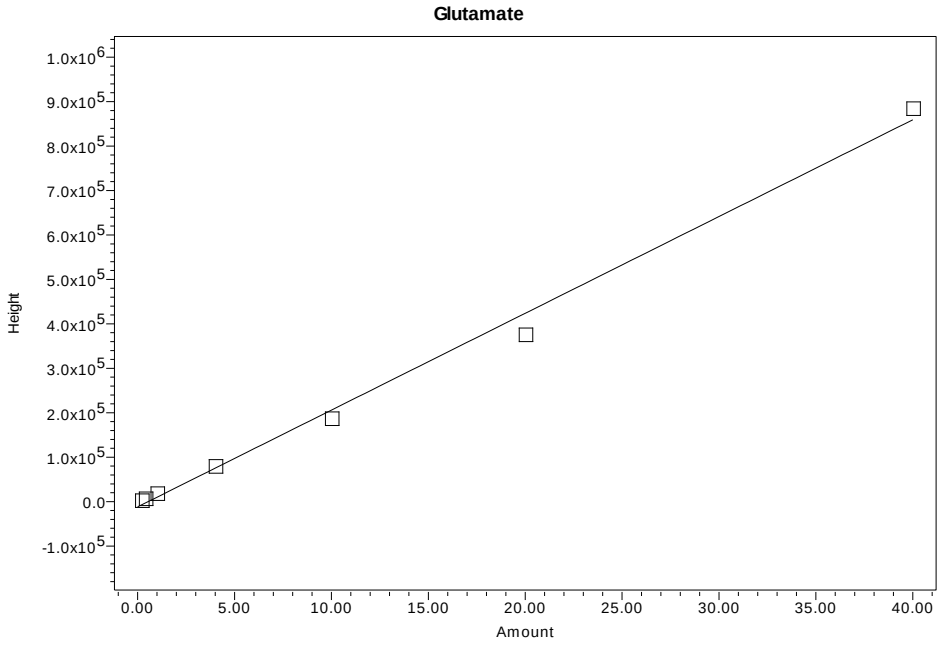


Figure 18. Typical standard curve for glutamate.

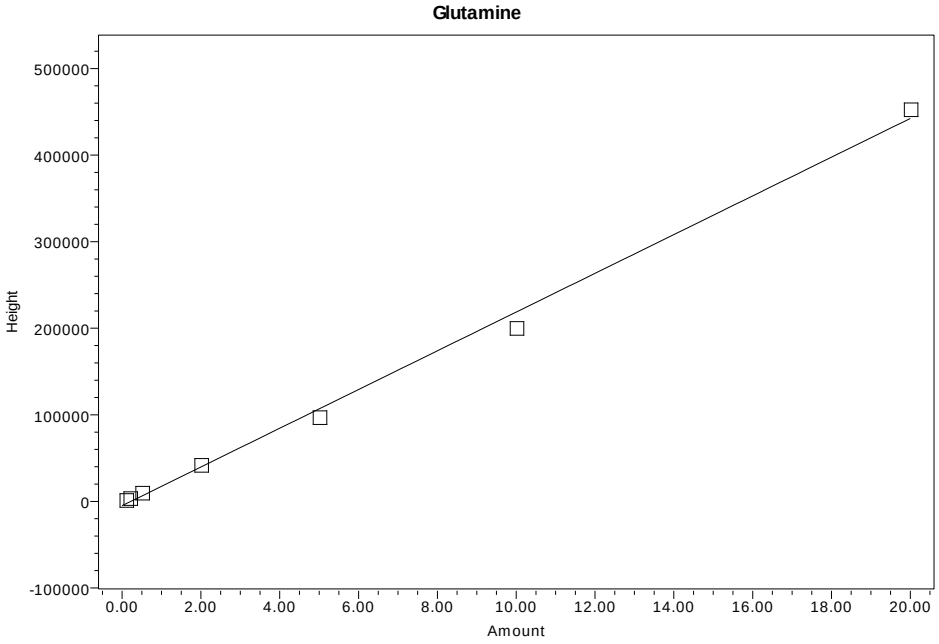


Figure 19. Typical standard curve for glutamine.

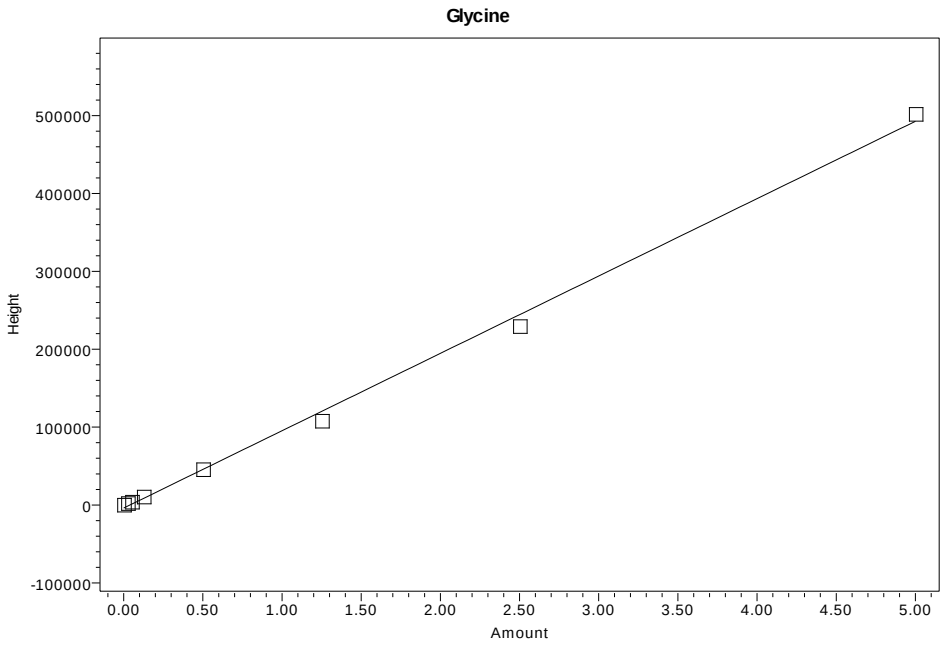


Figure 20. Typical standard curve for glycine.

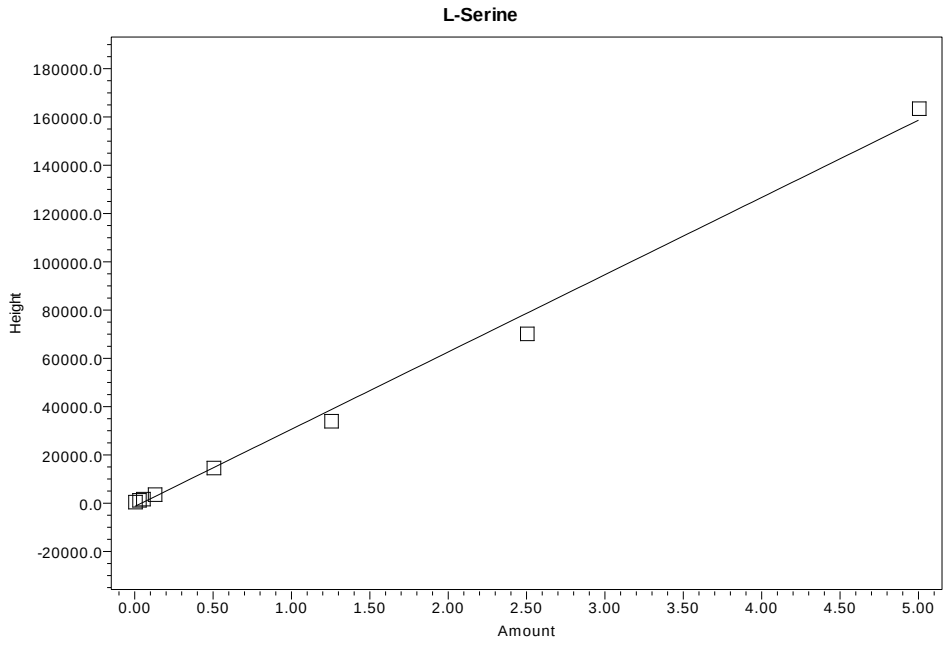


Figure 21. Typical standard curve for L-serine.

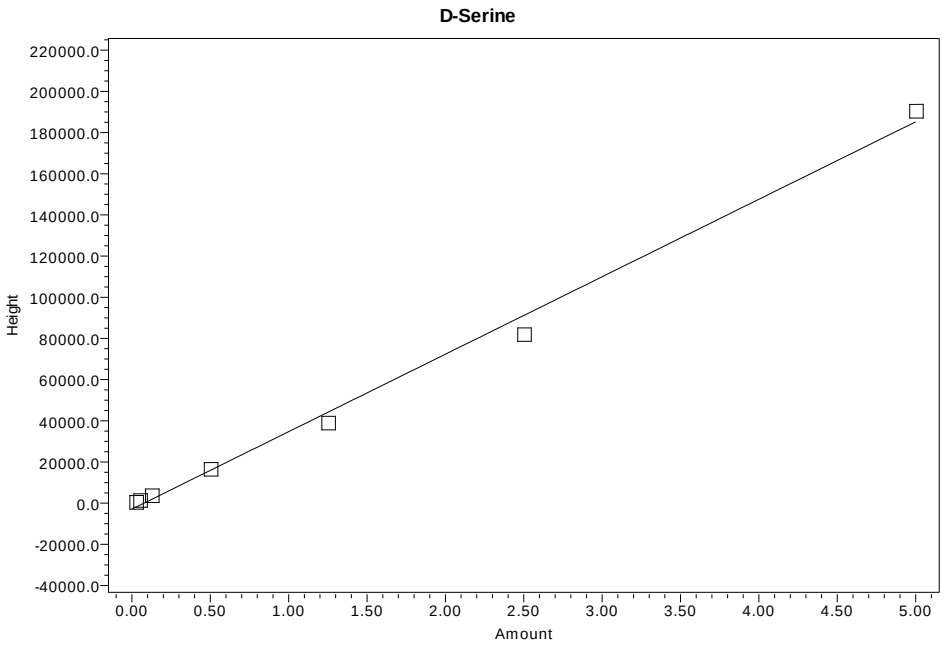


Figure 22. Typical standard curve for D-serine.

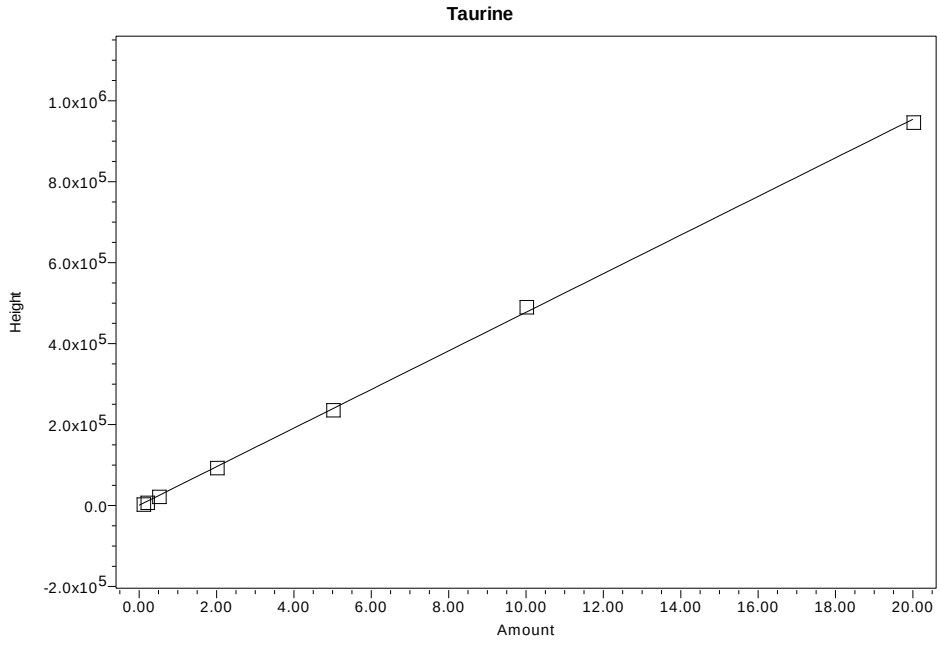


Figure 23. Typical standard curve for taurine.

Amino acids in rat brain were measured after acute administration of PLZ (30mg/kg) or PEH (30mg/kg) at time intervals of 1hr, 3hrs and 6hrs after treatment administration. Whole brain levels of the amino acids glutamine (GLN) and glycine (GLY) were decreased at 3hrs and 6hrs following administration of PLZ (30mg/kg), and levels of ALA and GABA progressively increased with time following administration of PLZ (30mg/kg). L-Serine levels were decreased at 3 and 6hrs. Results are shown in Figures 24, 26 and 28.

Whole brain levels of amino acids following administration of PEH (30mg/kg) at 1hr, 3hr and 6hr are shown in figures 25, 27 and 29. Levels of GLN and L-serine were decreased at 3hr and 6hr. Levels of GLY were not significantly changed after administration of PEH. Whole brain levels of ALA and GABA were increased at 1hr, 3hrs and 6hrs. The largest increase of ALA and GABA was observed at 6hr.

3.6. Effect of Prior MAO Inhibition on Amino Acid Levels in Rat Brain

When MAO was inhibited by TCP prior to administration of PLZ, levels of ALA and GABA were not increased above control levels, GLY levels returned to control levels and GLN levels remained reduced (Figures 30 and 31). When MAO was inhibited by TCP prior to administration of PEH, levels of ALA and GABA remained increased (Figures 32 and 33).

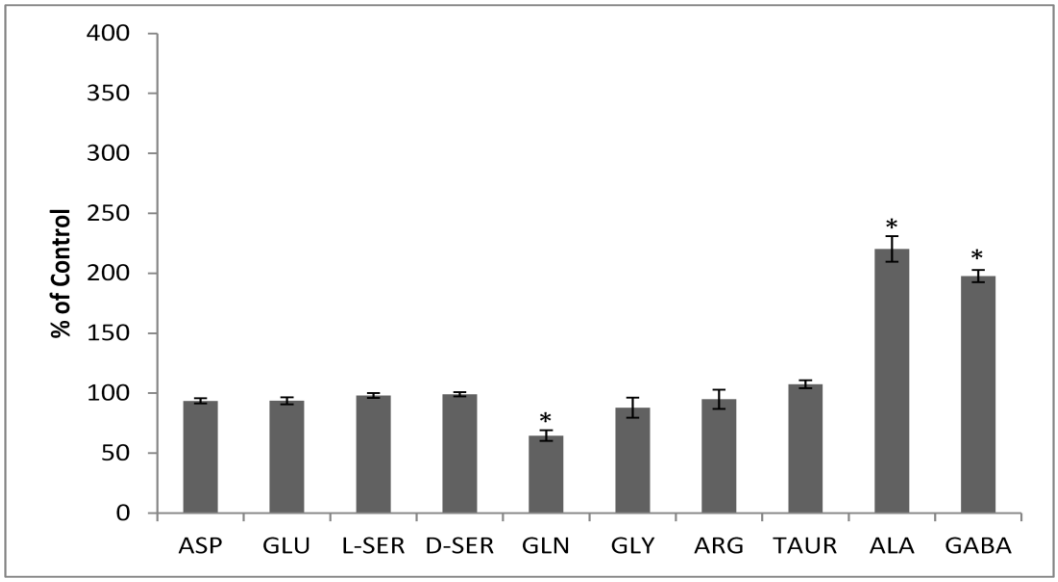


Figure 24. Amino acids in brain (means ± SEM, N=3) 1hr after administration of 30mg/kg PLZ

. *= significantly different from control values.

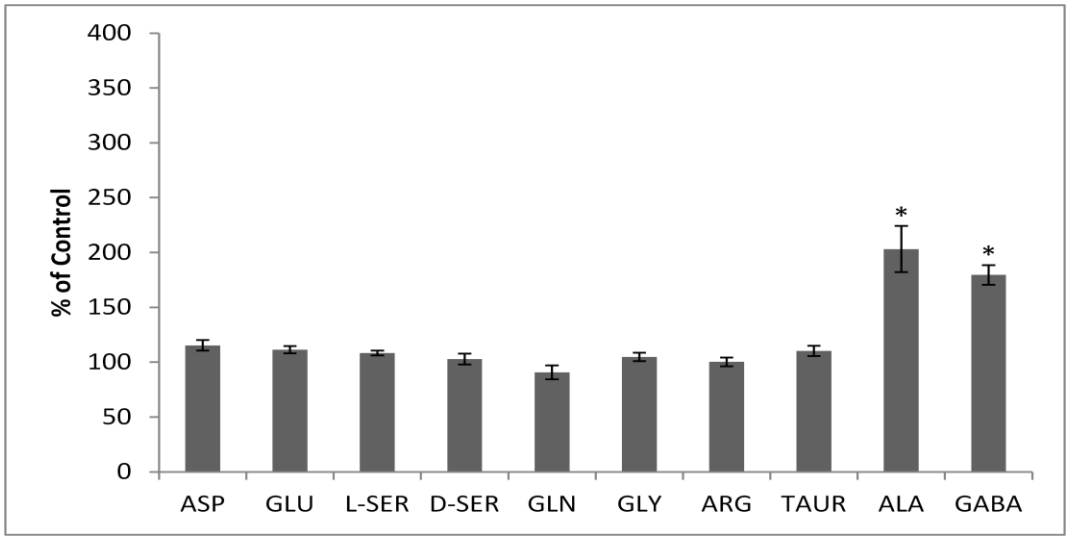


Figure 25. Amino acids in brain (means ± SEM, N=4) 1hr after administration of 30mg/kg PEH.

*= significantly different from control values.

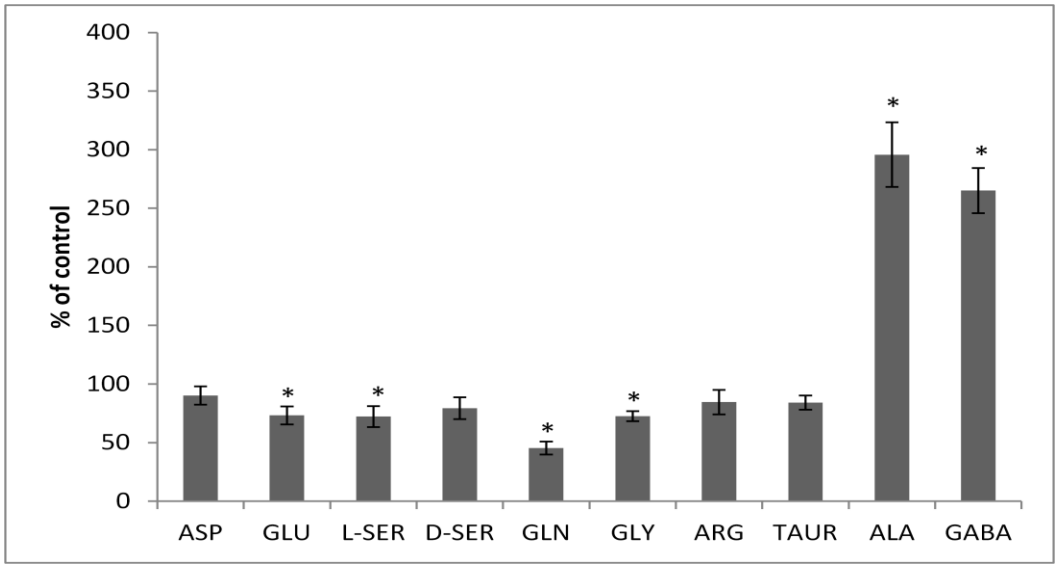


Figure 26. Amino acids in brain (means ± SEM, N=6) 3hr after administration of 30mg/kg PLZ.

*= significantly different from control values.

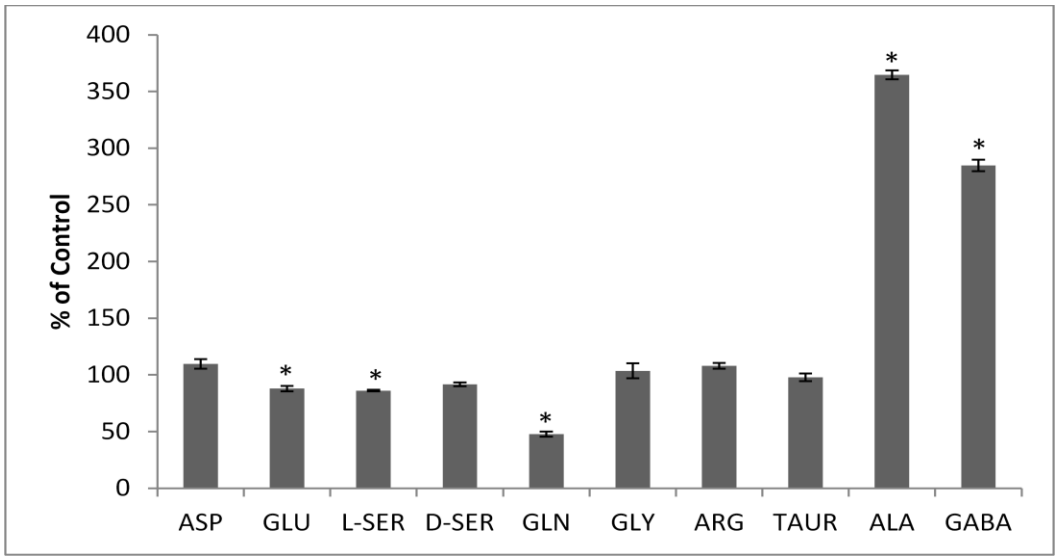


Figure 27. Amino acids in brain (means ± SEM, N=4) 3hr after administration of 30mg/kg PEH.

*= significantly different from control values.

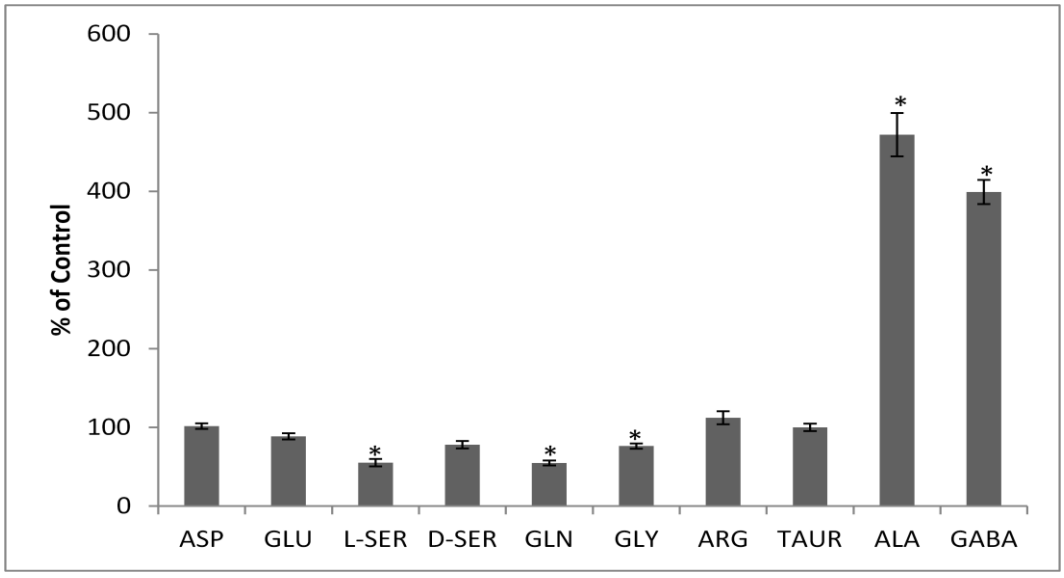


Figure 28. Amino acids in brain (means ± SEM, N=5) 6hr after administration of 30mg/kg PLZ.

*= significantly different from control values.

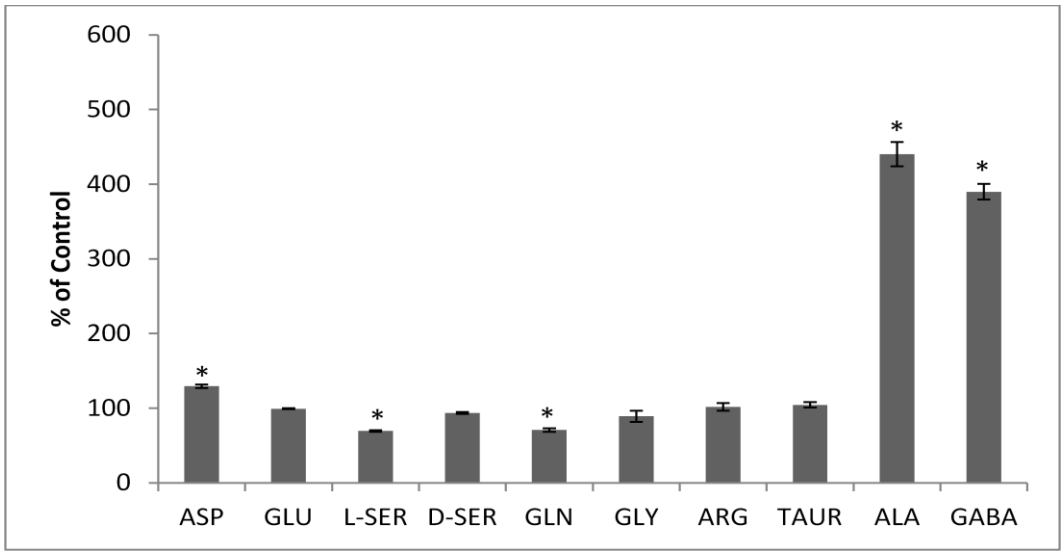


Figure 29. Amino acids in brain (means ± SEM, N=4) 6hr after administration of 30mg/kg PEH.

*= significantly different from control values.

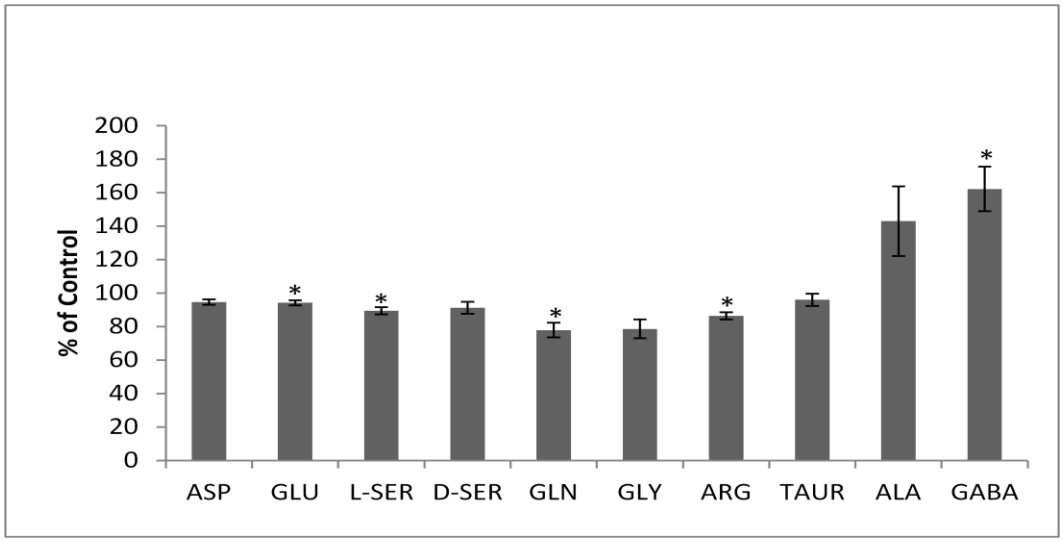


Figure 30. Amino acids in brain 1hr after administration of VEH/PLZ (30mg/kg). Results are means ± SEM (N=5).

*= significantly different from control values.

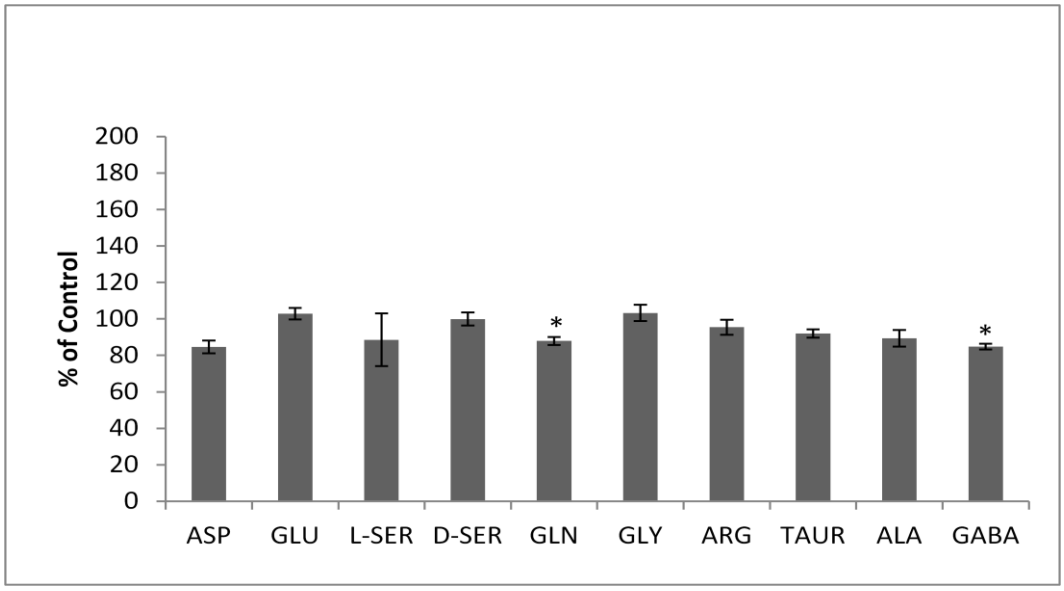


Figure 31. Amino acids (means ± SEM, N=5) in brain 1hr after administration of TCP/PLZ (30mg/kg).

*= significantly different from control values.

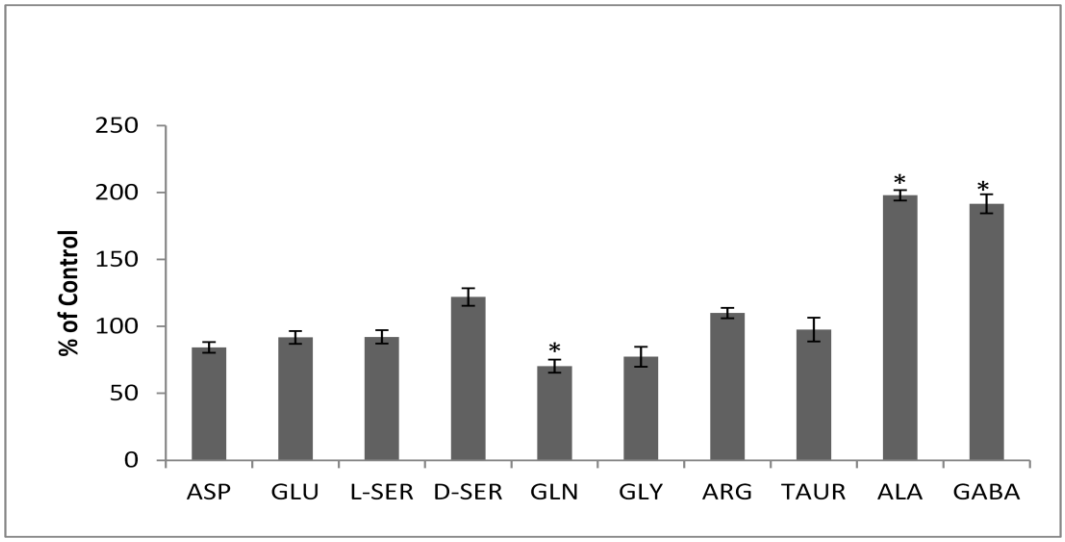


Figure 32. Amino acids in brain (means ± SEM, N=5) 1hr after administration of VEH/PEH (30mg/kg).

*= significantly different from control values.

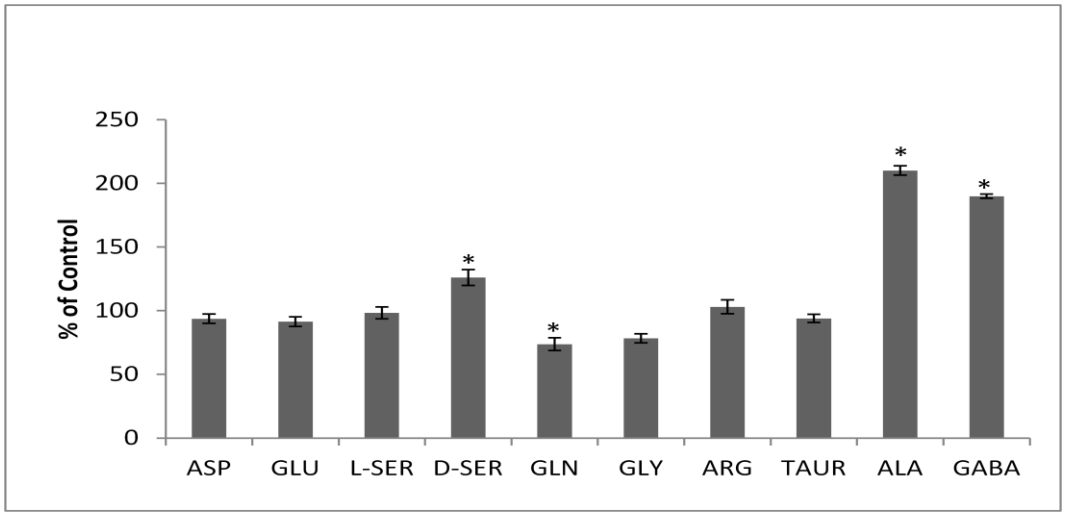


Figure 33. Amino acids (means ± SEM, N=5) in brain 1hr after administration of TCP/PEH (30mg/kg). TCP has no effect on PEH-induced changes in amino acid levels.

*= significantly different from control values.

3.7. Effects of chronic PLZ on weight gain and brain amino acid levels in rats.

PLZ (15 mg/kg ip) produced a reduction in weight gain compared to vehicle-treated rats that was significant by day 3. The reduction in weight gain was persistent for the remainder of the 3 week period studied. Results are shown in Figures 34 and 35.

Amino acid levels (work done in collaboration with I. Armstrong) were also measured in the brains of these rats 24 h after administration of the last (7 day or 21 day) dose of PLZ. At 1 week the levels of the amino acids were quite different from those at 3 weeks, with levels of aspartate, L-serine, D-serine, GLN, GLY and arginine decreased. GABA levels had actually decreased to below control levels (Figure 36). At 3 weeks, GLN levels were still reduced compared to control values, and ALA and GABA levels were much reduced compared to the peak values in the acute PLZ-treated rats (Figure 37).

PAA levels were also measured in the brains treated for 1 week and 3 weeks with PLZ and were not significantly different from values in the vehicle-treated controls. Values were 6.4 ± 0.6 (vehicles) and 9.7 ± 2.9 (PLZ) at 1 week and 7.1 ± 1.5 (vehicles) and 3.1 ± 0.6 (PLZ) at 3 weeks. In these chronic studies there was a peak that showed up and interfered with the PEH analysis; we were unable to separate that peak from PEH and thus PEH levels were not recorded. There was not sufficient tissue left to measure PEA levels after all the other measurements mentioned above were done.

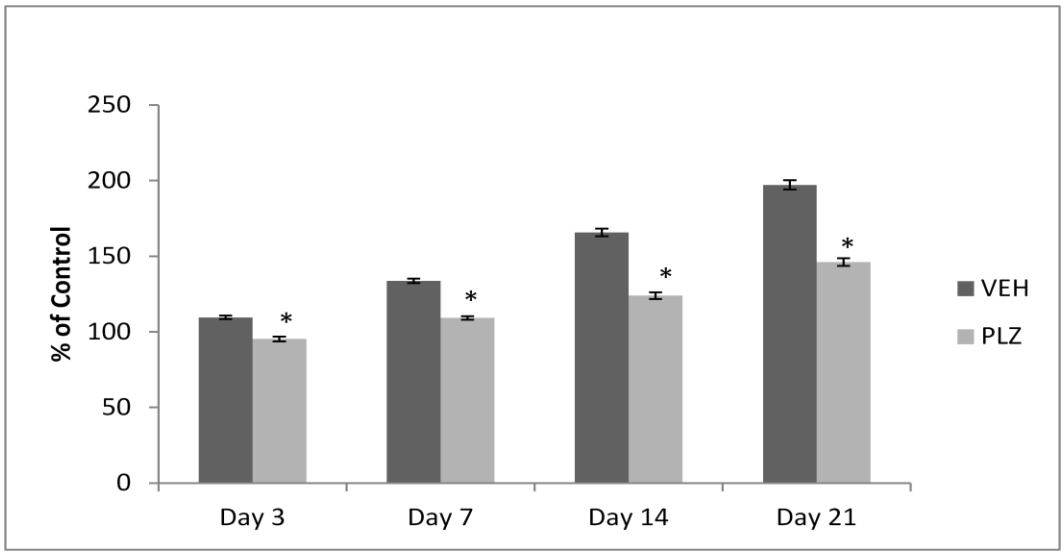


Figure 34. Weights of rats (as % of vehicle controls, means ± SEM, N=6) over 3wks of treatment with PLZ (15mg/kg ip.).

*= significantly different from control values.

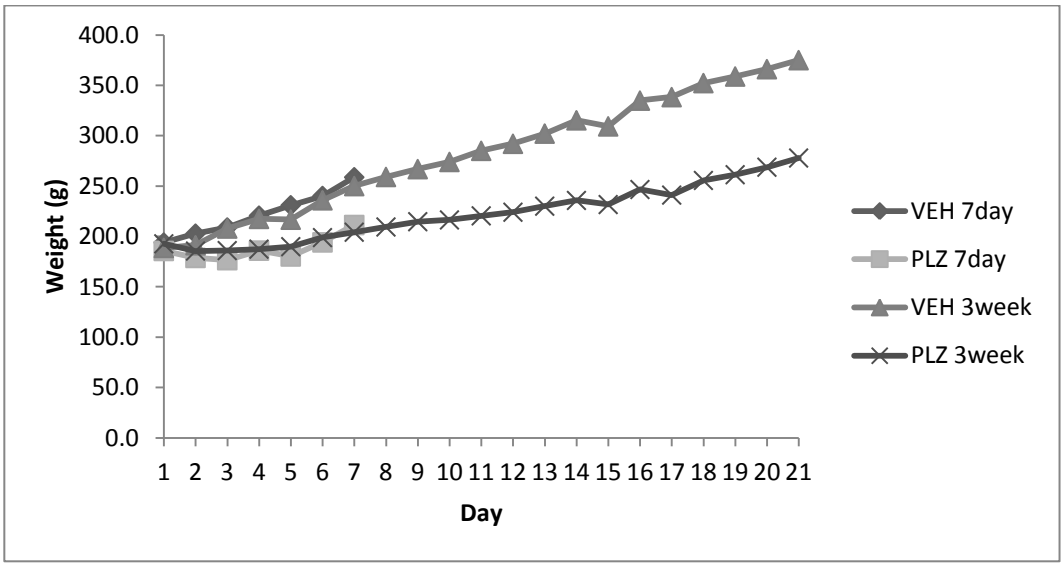


Figure 35. Daily weights of rats over 3wks of daily treatment with PLZ (15 mg/kg ip.).

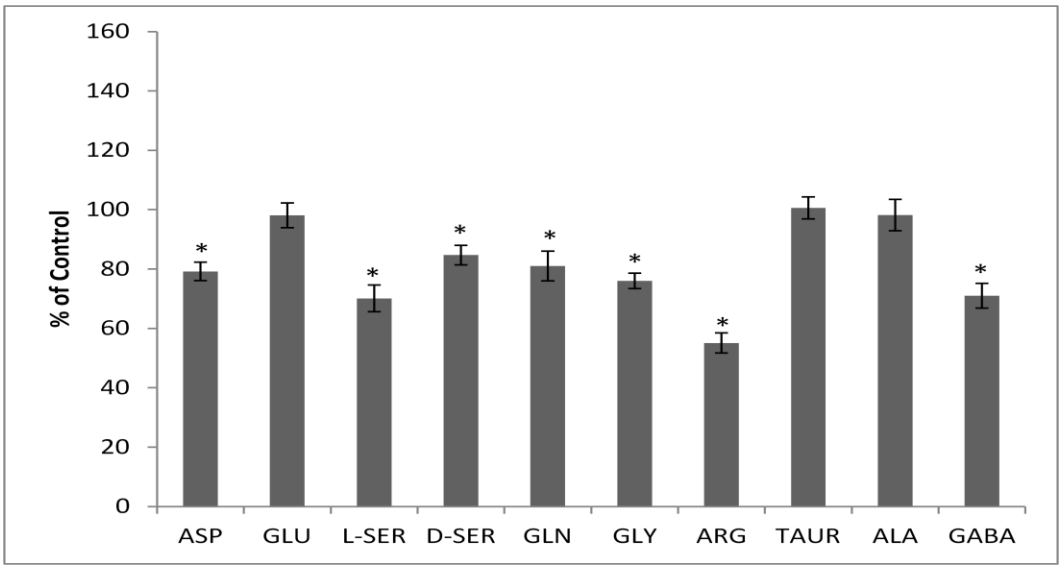


Figure 36. Rat brain levels of amino acids 1 week after daily administration of PLZ (15mg/kg ip.).

*= significantly different from control values.

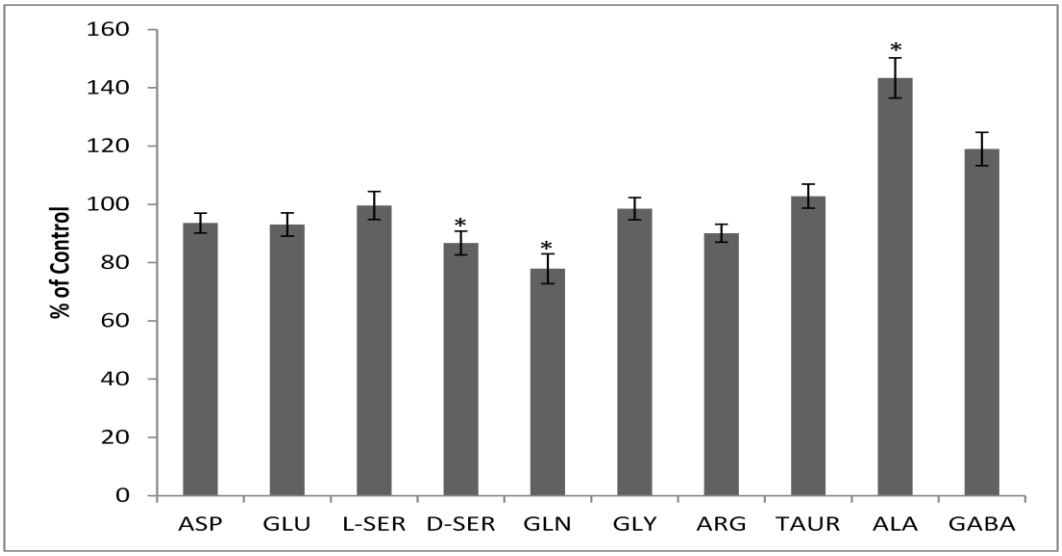


Figure 37. Rat brain levels of amino acids 3weeks after daily administration of PLZ (15mg/kg ip.).

*= significantly different from control values.

4.0. DISCUSSION

The findings in rat liver after acute administration of PLZ (30mg/kg) show extensive conversion of PLZ to PEH (Fig. 7). These results are similar to those found in brain by MacKenzie (2009) and highlight the marked metabolism of PLZ into the metabolite PEH. Recent mass spectrometric analysis (Matveychuk, Holt and Baker, unpublished) has shown that in the assay procedure described here the final derivative is actually converted to a hydrazine derivative that is proportional to the amount of PEH present, ie. it is an indirect assay. Using another assay, those researchers have also shown that PEH is formed from PLZ when PLZ is incubated with human MAO-B.

The studies on formation of PEA from PLZ (Fig. 11) in the absence and the presence of TCP indicate that PEA is also an important metabolite of PLZ, but that it is rapidly metabolized by MAO as it is formed. A similar formation of amphetamine from the hydrazine analogue of amphetamine (pheniprazine) has been reported (Cristofoli et al., 1982), but the enzyme responsible was not identified. There also appears to be some PEA formed from PEH, but to a much lesser extent than from PLZ (Fig. 12). PEA may contribute to the antidepressant effects of PLZ since it has been proposed to have antidepressant-like effects in lab animals (Dewhurst, 1968) and to be excreted in lower amounts in depressed patients than in controls (Sabelli and Mosnaim, 1974; Boulton and Milward, 1971). MAO-B knockout mice have a profound increase in central levels of PEA; these mice display behavioral characteristics similar to those induced by clinically effective antidepressants (Berry, 2007). In a clinical study, the MAO-B inhibitor

(-)deprenyl was combined with phenylalanine (a precursor of PEA) and was reported to reduce depressive symptoms in 70% of patients (Sabelli et al. 1996). Baker et al. (1982), in a study in rats, found an increase to 3 times brain control PEA levels after 8 days of administration of PLZ, but a massive increase (30 fold) after 19 days of PLZ administration, suggesting that after chronic administration of PLZ, PEA levels in brain may be high enough to contribute to the antidepressant effect of PLZ.

It is interesting that PAA is formed from both PLZ and PEH. The possible metabolic routes of PLZ are summarized in Figure 38. PAA is the major metabolite of PEA under normal circumstances. Clineschmidt and Horita (1969a,b) reported PAA as a major metabolite of PLZ in vitro in rat liver homogenates and in urine samples collected from rats treated with radiolabelled PLZ, but suggested that it may have come from an intermediate metabolite such as PEH. Robinson et al. (1985) reported that PAA was a major metabolite in urine of patients treated with a single dose of PLZ but did not measure PEH levels. Robinson et al. also found large amounts of p-hydroxyPAA but did not speculate on the origin of that potential metabolite. However, Kennedy et al. (2009) have speculated that it could arise from hydroxylation of PAA, from hydroxylation of PEA to p-tyramine followed by metabolism of p-tyramine to p-hydroxyPAA or by hydroxylation of PEH and then conversion to p-hydroxyPAA; none of these steps has been investigated. I did not find significant increases in brain levels of PAA in the brains of rats treated with PLZ for either 1 or 3 weeks, but this may be

because PAA is rapidly conjugated and cleared from the brain by a transport system after being formed (Dyck et al., 1993).

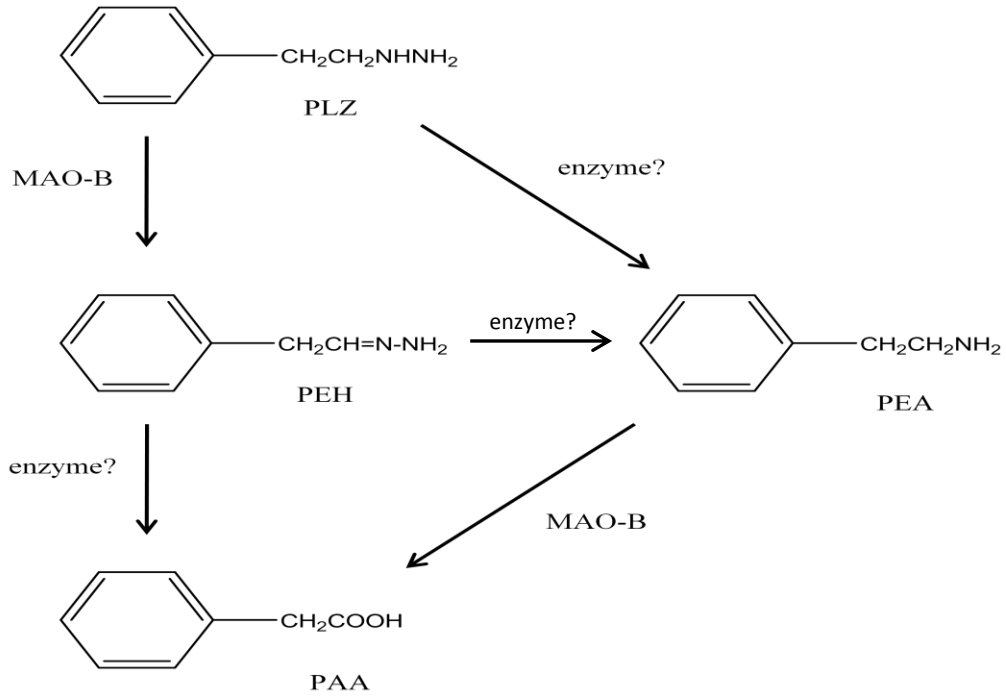


Figure 38. Proposed metabolism of phenelzine (PLZ). PEH = phenylethylidenehydrazine; PEA = 2-phenylethylamine; PAA = phenylacetic acid.

In the acute administration studies, both PLZ and PEH produced changes in brain levels of several amino acids, particularly GABA, ALA, and GLN. The importance of the observed changes of these amino acids is that they may be related to changes in brain functioning. Alanine is a weak coagonist at the NMDA glutamate receptor (McBain et al. 1989). Glutamine is interconvertable with glutamate metabolically (Nestler et al., 2001). Glycine acts as a neurotransmitter and mediates inhibition in spinal cord motor neurons, but in higher brain regions acts as a coagonist at excitatory NMDA receptors (Mogaddam and Javitt, 2012). GABA is the major inhibitory neurotransmitter in the CNS (Olsen and DeLorey, 1999). D-Serine is a coagonist at the NMDA glutamate receptor and is actually more potent than GLY in this regard (Labrie et al., 2010); it is formed metabolically from L-serine via the enzyme serine racemase (Labrie et al., 2009).

The increases in brain levels of ALA and GABA are due, at least in part, to inhibition of alanine transaminase and GABA-T, respectively, as discussed earlier in this thesis. The decrease in levels of GLN may be indicative of effects of PLZ or PEH on both glia and neurons. Glutamate from the synapse is taken up by glia and converted to GLN by glutamine synthetase. The GLN can then pass to the neurons where it is converted to GLU by glutaminase (Nestler et al., 2001). It is possible that PLZ and PEH can alter the activity of one or both of these enzymes and/or reduce uptake of glutamate into glia, reducing the amount available in glia to be converted to GLN.

PLZ causes a decrease in brain levels of GLY, a coagonist at the NMDA glutamate receptor. There is a great deal of evidence available now suggesting

hyperactivity of the NMDA receptor in depression (Gur and Arnold, 2009), and the NMDA receptor antagonist ketamine has been shown to produce very rapid antidepressant effects after intravenous administration (Zarate et al., 2006; Machado-Vieira et al., 2008; Sanacora et al., 2008). However nobody, to my knowledge, has looked at the effects of ketamine on levels of GLY, a coagonist required in order for the NMDA receptor to function. PLZ produced a decrease in GLY levels at all time intervals studied except 1hr and 3 weeks.

The acute study on levels of amino acids in brain after administration of PLZ and PEH in the presence and absence of TCP pretreatment (Figures 30-33) indicate that changes in levels of GABA and ALA, but not GLN, observed in brains of rats treated with PLZ are actually related to its conversion to PEH, since TCP blocks formation of PEH from PLZ. It would appear that both PLZ and PEH contribute to the reduction of GLN levels. As expected, prior treatment with TCP had no effect on the amino acid level changes produced by PEH, providing further support that PEH, unlike PLZ, is not a substrate of MAO. It is interesting that chronic administration of PLZ for 1 week results in a reduction of brain levels of several amino acids, including, surprisingly, GABA. The reason for the "overshoot" depletion of GABA levels is not clear at this time. Recent studies in the Neurochemical Research Unit (Matveychuk and Baker, unpublished) on 1 week administration of PEH (30mg/kg) showed that PEH had similar effects to PLZ in depleting D-serine, GLY and GLN levels, but increased levels of GABA and alanine above control levels and above levels observed with PLZ. These findings suggest that conversion of PLZ to PEH has declined by 1 week of PLZ

administration. By 3 weeks of PLZ administration, levels of all the amino acids except D-serine and GLN have returned to control values, presumably as a result of lack of PEH formation (inhibition of MAO-A and -B was at 95% by 3 weeks). ALA levels were still slightly above control values but reduced from the levels observed in the acute studies.

The effects of PLZ on reducing weight gain in normal rats has not, to my knowledge, been reported previously. However, PLZ has also been shown to lower body weight in obese Zucker rats (Carpene et al. 2007). The reduction in weight gain observed with PLZ in normal rats is similar to the findings of De Marco et al. (2008) with vigabatrin, another GABA-T inhibitor. It is possible that PLZ could be a more useful drug than vigabatrin at preventing weight gain since it is 50-100x more potent as an inhibitor of GABA-T (Todd and Baker, 2008). Also, vigabatrin produces a high incidence of visual field deficits due to retinopathy from the drug (Fecarotta and Sergott, 2012). Elevation in brain GABA may be a common mechanism responsible for the observed anorectic effects of PLZ and vigabatrin. Ethanolamine-O-sulfate (EOS), an irreversible inhibitor of GABA-T was reported to result in a dose-dependent decrease in food consumption and body weights in rats (Cooper et al. 1980). Muscimol, a $GABA_A$ receptor agonist, was observed to decrease consumption of sweetened milk in rats (Cooper et al. 1980). These findings suggest that brain GABA levels are important for satiety and decreased food consumption. Future studies on weight loss with PLZ or PEH should measure the amount of food consumed to see if there is a reduction in food intake. The area of the brain involved in the GABA-

mediated reduction in food consumption and satiety may be the lateral hypothalamus (LH). In one study, the relative levels of glutamate and GABA in the LH were seen to influence food consumption and satiety, with increased glutamate resulting in increased food consumption and increased GABA resulting in decreased food consumption (Rada et al. 2003). However, only whole brain levels of GABA were looked at in my studies, and measuring regional levels of GABA is warranted in the future. PLZ's weight reducing effect may also be due to peripheral effects such as decreased lipogenesis. PLZ appears to act directly on adipocytes to limit lipogenesis, an effect only partially due to SSAO/PrAO inhibition (Carpene et al. 2012). Future studies should also look at the relative contributions of CNS and peripheral effects of PLZ with regard to effects on weight gain.

It will now be of interest to repeat these weight studies with PEH since it would be expected that PEH would continue to elevate brain GABA levels for a longer period of time than PLZ.

4.1. Possible Future Directions

Based on the findings of this project, the following should be studied in the future:

- Effects of chronic administration (3 weeks) of PEH on whole brain levels of amino acids. Regional brain analysis of amino acids should be conducted after acute and chronic administration of PLZ and PEH.
- Effects of chronic administration (at least 3 weeks) of PEH on weight and food intake in rats.
- The studies on PLZ and weight gain should be repeated to include studies on food intake.
- The effects of PEH as an antidepressant, anxiolytic, and anticonvulsant because of its GABAergic actions.
- Based on the repeated effectiveness of PLZ on relieving some of the symptoms in the EAE mouse model of MS (Musgrave et al. 2011a), such studies should also be extended to PEH to determine if effects of PLZ on biogenic amines or GABA are more important in its positive effects in this animal model.

5.0. SUMMARY

A series of experiments with administration of PLZ and PEH in the presence and absence of prior inhibition of MAO by TCP indicated that PEA and PAA are present as metabolites of PLZ and PEH in rat brain, although formed in much larger quantities from PLZ. Direct comparisons between PLZ and PEH at various time intervals after injection showed that both had similar effects on brain levels of several important amino acids. Studies of brain levels of these amino acids in PLZ- and PEH-treated rats after prior inhibition of MAO by TCP provided further evidence that PLZ is metabolized to PEH by MAO. Another chronic experiment with PLZ (up to 3 weeks of PLZ administration) showed that PLZ decreased weight gain in normal rats; parallel studies on amino acid brain levels at 1 week and 3 weeks showed a marked difference of PLZ on amino acids levels between 1 and 3 weeks, providing further evidence suggesting that less PLZ is converted to PEH by MAO over time.

6. 0. REFERENCES

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