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UNIVERSITY OF ALBERTA

The Antipanic Drug Phenelzine and Its Effects on GABA and Related Amino Acids

BY

Teresa M. Paslawski

A thesis
submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: THE ANTIPANIC DRUG PHENELZINE AND ITS EFFECTS ON GABA AND RELATED AMINO ACIDS hereby submitted by TERESA M. PASLAWSKI in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Panic disorder (PD) affects approximately 3% of the population. Phenelzine (PLZ) is an antidepressant which also possesses anxiolytic properties, and is particularly useful as an antipanic agent. In this thesis, the mechanism of action of PLZ is explored in relation to the GABAergic system, which has been suggested to play a significant role in PD.

Investigations in rat hypothalamus and whole brain included acute time- and dose-response studies and chronic studies. In addition to elevating GABA and inhibiting MAO-A and -B in brain tissue, PLZ also decreased glutamate and glutamine levels in a dose-dependent manner. The effects of PLZ on glutamine, glutamate and GABA were blocked by prior treatment with another MAO inhibitor, suggesting that the effects of PLZ on these aspects of the GABA shunt are produced by a metabolite of PLZ formed by the action of MAO on the parent drug. $GABA_A$ receptor activity was also measured following chronic PLZ administration. PLZ did not significantly alter $^{36}Cl^-$ uptake in synaptoneurosomes, a measure believed to reflect $GABA_A$ receptor activity.

The elevated plus-maze was employed to compare the behavioral effects of PLZ and its acetylated metabolite, N^2AcPLZ. Like PLZ, N^2AcPLZ elevates brain levels of the biogenic amines and inhibits MAO-A and -B, but unlike PLZ, has no GABA-elevating properties. PLZ had an anxiolytic effect

in the elevated plus-maze whereas N^2AcPLZ did not. These results provide further support for the role of GABA in anxiety disorders.

Clinical analyses were conducted using blood samples taken from PD patients undergoing treatment with PLZ over an 8-week period. Plasma samples showed significant differences in GABA but not in alanine levels during treatment, or between treatment and control groups.

Studies on a proposed metabolite of PLZ, phenylethylidene hydrazine (PEH), revealed that, like PLZ, PEH elevated brain GABA and alanine levels and inhibited GABA-T and ALA-T. PEH differs from PLZ in that it does not significantly inhibit MAO-A and MAO-B, suggesting that PEH may have the anxiolytic properties of PLZ but lack some of the side effects and interactions of PLZ. Attempts were made to develop an assay for the detection of PEH in tissues.

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5-HT	serotonin, 5-hydroxytryptamine
ACh	acetylcholine
ALA	alanine
ALA-T	alanine-transaminase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BZD	benzodiazepine
CCK	cholecystokinin
CMI	clomipramine
CNS	central nervous system
CTRF	corticotropin-releasing factor
d	distilled
dd	double-distilled
DA	dopamine
DMI	desipramine
DSM	Diagnostic and Statistical Manual of the American Psychiatric Association
ECD	electron-capture detector
FID	flame ionization detector
g	grams
GABA	γ-aminobutyric acid

GABA-T	GABA-α-oxoglutarate transaminase, GABA-transaminase
GAD	glutamic acid decarboxylase
GC	gas chromatography
GTP	guanosine triphosphate
h	hour(s)
HPLC	high performance liquid chromatography
i.v.	intravenously
ICD	International Classification of Diseases
IMI	imipramine
m	meter(s)
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MDD	Major Depressive Disorder
µg	microgram(s)
min	minute(s)
mRNA	messenger ribonucleic acid, messenger RNA
N^2AcPLZ	2-acetyl-1-(2-phenylethyl)hydrazine, N^2-acetyl-PLZ
NA	noradrenaline
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
ng	nanogram(s)
NPD	nitrogen-phosphorus detector

OCD	Obsessive Compulsive Disorder
ODS	octadecylsilane
OPA	o-Phthalaldehyde
PAA	phenylacetic acid
PD	panic disorder
PEA	2-phenylethylamine
PEH	phenylethylidene hydrazine
PFBC	pentafluorobenzoyl chloride
PFBSC	pentafluorobenzenesulfonyl chloride
PFPA	pentafluoropropionic anhydride
PLP	pyridoxal-5-phosphate
PLZ	phenelzine
PTSD	post-traumatic stress disorder
SCOT	support-coated open tubular
SEM	standard error of the mean
SSADH	succinic semialdehyde dehydrogenase
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TCD	thermal conductivity detector
UV	ultraviolet
veh	vehicle
WCOT	wall-coated open tubular

1 Introduction

1.1 General Introduction - Anxiety Disorders

Anxiety is one of the most frequently reported symptoms in mental illnesses, and anxiety disorders are among the most common of psychiatric diagnoses. In the United States, anxiety disorders are the most prevalent psychiatric illnesses (Lydiard et al., 1996), and some believe that they are still underdiagnosed (Valente, 1996). Included in the category of Anxiety Disorders are: generalized anxiety disorder, panic disorder (PD) with and without agoraphobia, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD) and the phobias (e.g. social phobia, simple phobias). The DSM-IV (Diagnostic and Statistical Manual of the American Psychiatric Association, 4th edition, 1994) estimates that up to 3% of the general population suffers from PD, while generalized anxiety disorder is estimated to afflict 3 to 5% of the population.

1.2 Differentiating Panic Disorder and Generalized Anxiety Disorder

The separation of the clinical diagnostic category of anxiety neurosis into PD and generalized anxiety disorder can be traced back to the late 1950s and early 1960s when researchers noted that the anxiety syndromes appeared to be differentially responsive to drugs (Torgersen, 1986). West and Dally described, in 1959, an atypical depression with concomitant

anxiety and phobias that was responsive to monoamine oxidase inhibitors (MAOIs) (Brandon, 1993). That same year, Martin Roth defined the Phobic Anxiety Depersonalization or Calamity Syndrome which recognized panic as a core symptom. Klein, in 1964, delineated two distinct drug-responsive anxiety syndromes and showed that imipramine (IMI) could block the occurrence of panic attacks. Prior to this, most clinicians considered panic attacks to be an acute element in the symptomology of generalized anxiety disorder, described by various names including "effort syndrome" and "neurocirculatory asthenia" (Hollister, 1986; Noyes et al., 1987). In the DSM-III (Diagnostic and Statistical Manual of Mental Disorders - III, 3rd ed., 1980), PD was recognized as a distinct diagnosis. Anxiety neurosis was divided into a chronic, free-floating type of anxiety, generalized anxiety disorder, and a disorder which was more acute in nature, PD (Kendler et al., 1995). The DSM-III defined PD by 3 or more panic attacks over a 3 week period while generalized anxiety disorder was characterized by less than 3 panic attacks accompanying 1 or more months of persistent anxiety, including such symptoms as motor tension, autonomic hyperactivity, apprehension and hypervigilance (Breier et al., 1985; Torgersen, 1986). In the DSM-III, generalized anxiety disorder was considered a residual category and could not be diagnosed concurrently with PD (Anderson et al., 1984). The revised edition of the DSM-III (DSM-III-R, Diagnostic and Statistical Manual of

Mental Disorders, revised, 1987) changed the criteria slightly, increasing the duration for generalized anxiety disorder from 1 month to 6 months and eliminating the hierarchy such that generalized anxiety disorder could be diagnosed in the presence of PD (Di Nardo et al., 1993). DSM-III-R also introduced the concept of limited symptom attacks in PD (Goisman et al., 1995). A panic attack in DSM-III-R is characterized by 4 or more panic symptoms, including palpitations, unsteadiness, sweating, and feelings of unreality. A limited symptom attack is defined by less than 4 of these symptoms. The DSM-III-R also established the primacy of PD over agoraphobia (Brandon, 1993).

The essential features of PD as defined by the most current edition of the DSM, DSM-IV, are as follows: recurrent, initially unexpected panic attacks followed by 1 or more months of persistent anxiety focusing on the possibility of an attack and worry about the potential implications or consequences of the attack. At least 2 attacks are required for the diagnosis of PD. Associated features include: constant or intermittent unfocussed feelings of anxiety, apprehension about outcomes of routine events, demoralization, and agoraphobia. PD is commonly associated with agoraphobia (Noyes et al., 1992; Hollister, 1986). Goisman and colleagues (1995) have proposed that agoraphobia is a more severe variant of PD rather than a separate entity. PD without agoraphobia is diagnosed twice as

often in women as in men, and panic with agoraphobia is diagnosed 3 to 1, female to male (DSM-IV).

The DSM-IV defines the essential features of generalized anxiety disorder as excessive anxiety and worry (apprehensive expectation) occurring more days than not for a minimum of 6 months. The focus of this worry cannot be confined to any feature of the other Axis I anxiety disorders (i.e. OCD, PD, Phobias, PTSD) but is about a number of events or activities and the intensity, duration or frequency of the worry is disproportionate to the impact of the feared event (DSM-IV). Just over one half of those diagnosed with generalized anxiety disorder are women (55-60%) (DSM-IV).

Some researchers have suggested that generalized anxiety disorder may, in some instances, represent a precursor or substrate for a more pervasive disorder such as PD or Major Depressive Disorder (MDD) (Noyes et al., 1992; Massion et al., 1993; Nisita et al., 1990; Barlow et al., 1986; Breier et al., 1985). Alternatively, Pollack and Smoller (1995) suggest that panic and other anxiety disorders in adulthood may represent the manifestation of an underlying constitutional vulnerability or diathesis for anxiety that is familial and probably genetic, and variably expressed i.e. as generalized anxiety disorder or PD, over the life-cycle. Generalized anxiety disorder and PD cannot be differentiated by mean age of onset, both having a mean age of onset in the 20s (Noyes et al., 1992; Nisita et al., 1990).

However, it is generally considered that generalized anxiety disorder has a more variable, often earlier, and gradual onset than does PD (Noyes et al., 1992; Barlow et al., 1986; Hollister, 1986; Anderson et al., 1984; Nisita et al., 1990; Schweizer, 1995).

Differences do appear to exist between clinical populations in terms of symptomology. More generalized anxiety disorder patients report experiencing symptoms belonging to the category of vigilance and scanning, including trouble getting to sleep, trouble concentrating, irritability, impatience, and feelings of restlessness or inability to relax, than do PD patients. PD patients report more cognitive symptoms, including fear of dying or going crazy, feelings of impending doom, depersonalization and derealization, than do generalized anxiety disorder subjects. Overall, PD subjects report a greater number of symptoms over the course of their illness than do subjects with generalized anxiety disorder, and more of the symptoms are autonomic in PD than in generalized anxiety disorder (Noyes et al., 1992; Nisita et al., 1990; Anderson et al., 1984; Clark et al., 1994). The PD profile shows more symptoms indicative of autonomic hyperactivity (the kinds of symptoms that typically accompany a panic attack) and the generalized anxiety disorder profile shows more symptoms indicative of CNS hyperarousal (Noyes et al., 1992; Hollister, 1986).

1.2.1 Differentiating Anxiety Disorders and Depression

There is considerable overlap in the symptomology of anxiety disorders and depressive states. The comorbidity rates of MDD and generalized anxiety disorder or PD are often cited in the literature, although there is considerable disagreement as to whether MDD-generalized anxiety disorder or MDD-PD is more common (Noyes et al., 1992; Massion et al., 1993; Hollister, 1986; Anderson et al., 1984; Nisita et al., 1990; Schweizer, 1995; Westenberg, 1996). Breier and colleagues (1985) found a very strong relationship between PD and MDD, reporting that as many as 75% of persons who experience panic attacks go on to develop a depressive syndrome (Breier et al., 1985). They theorize that PD is a separate disorder from MDD while symptoms of generalized anxiety disorder contribute to the overlap between the two disorders. Nisita and colleagues (1990) suggest that generalized anxiety disorder and MDD are distinct disorders that can occur concurrently. Alternatively, Sheehan and associates (1980) suggest that antidepressants are actually treating a polysymptomatic anxiety disorder that is independent of biological depression. The suggestion of a separate and distinct disorder is reflected in the inclusion of a "mixed anxiety-depression" category in the International Classification of Diseases - 10 (ICD-10), because of the observation that many patients suffer from

symptoms of both depression and anxiety but do not meet the established criteria for either an anxiety or a mood disorder (Zinbarg et al., 1994).

1.3 Treatment of Generalized Anxiety Disorder and Panic Disorder

Just as there is overlap in the symptoms of anxiety and depression, there is also overlap in the pharmacological methods used in the treatment of anxiety disorders and depression. Many different classes of drugs, most with some antidepressant activity, have been employed in the treatment of anxiety disorders; these include the benzodiazepines (BZDs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and MAOIs (Westenberg, 1996). Tricyclic antidepressants, including desipramine (DMI), IMI, and clomipramine (CMI), have been shown to be effective antipanic agents (Lydiard et al., 1996; Sheehan et al., 1980; Suranyi-Cadotte et al., 1990; Zitrin et al., 1983); IMI also has been found to be effective in the treatment of generalized anxiety disorder (Sheehan et al., 1980; Suranyi-Cadotte et al., 1990; Zitrin et al., 1983). The BZDs alprazolam and diazepam, long considered standard treatment for generalized anxiety disorder (Noyes et al., 1987), have been found to be effective antipanic agents (Ballenger, 1993). Clonazepam, another BZD, is also used in the treatment of generalized anxiety disorder and PD. The SSRIs, including fluoxetine, paroxetine, fluvoxamine, sertraline, and citalopram appear to attenuate panic attacks and control the secondary symptoms of PD

(Denboer and Westenberg, 1995; Lydiard et al., 1996).

As Burrows and colleagues (1993) discuss, the choice of pharmacological treatment for PD is not obvious. The side effects of each drug, as well as the specific profile and tolerance levels of the patient, play major roles in the drug treatment chosen (Burrows et al., 1993). TCAs are popular treatment choices, but there are a number of undesirable side effects, including an initial jitteriness, a 4-6 week lag in therapeutic efficacy, weight gain, anticholinergic effects (including dry mouth, blurred vision, constipation) and cardiovascular effects, as well as neurotoxicity in overdose (Westenberg, 1996; Lydiard et al., 1996). BZDs have been shown to be effective in the long-term treatment of PD but have a risk of dependence and withdrawal syndromes (Westenberg, 1996). Also, following discontinuation, the emergence of depressive symptoms is frequently reported (Lydiard et al., 1996). SSRIs frequently cause sleep disturbance and gastrointestinal distress (diarrhea, nausea) but do not appear to cause weight gain or have anticholinergic or cardiovascular side effects (Lydiard et al., 1996; Westenberg, 1996). MAOIs are at least as effective as TCAs in the treatment of PD, and have been shown to be effective in the long-term treatment of PD (Burrows et al., 1993) but have significant side effects. MAOIs are associated with weight gain, orthostatic dizziness and hyposomnia. Treatment with the irreversible MAOI phenelzine (PLZ) increases the risk of

hypertensive crisis if foods containing high concentrations of sympathomimetic amines (e.g. tyramine) are ingested (Lydiard et al., 1996; Westenberg, 1996; Miller Federici and Tommasini, 1992). Like TCAs, MAOIs show a high rate of relapse following discontinuation of drug treatment (Burrows et al., 1993).

Relapse is not uncommon in anxiety disorder populations. Studies suggest that although many patients improve with pharmacological treatment, they rarely become symptom-free (Pollack and Smoller, 1995). This supports the notion that patients have an inherent predilection to anxiety and perhaps a biochemical predisposition.

1.3.1 Phenelzine

Phenelzine (PLZ) or 2-phenylethylhydrazine (see Figure 2), is marketed under the trade name of Nardil by Parke-Davis. Originally it was marketed as an antidepressant, but through clinical use was also found to have therapeutic efficacy in the treatment of anxiety disorders, particularly PD. It is a nonspecific, irreversible MAOI (Popov and Mathies, 1969; Baker et al., 1991; McKenna et al., 1991; McManus et al., 1992) that is also a substrate for MAO (Clineschmidt and Horita, 1969a and b). It causes marked increases in brain levels of the biogenic amines dopamine (DA), noradrenaline (NA), serotonin (5-hydroxytryptamine; 5-HT), 2-phenylethylamine (PEA), p-tyramine, and tryptamine as well as increasing

brain levels of the amino acids γ–aminobutyric acid (GABA) and alanine (ALA) (Popov and Mathies, 1969; Philips and Boulton, 1979; Baker et al., 1988; Baker et al., 1991; McKenna et al., 1991; McManus et al., 1992; Wong et al., 1990b).

PLZ is typically administered orally with an initial dose of 15 mg 3 times/ day, increasing to 60-90 mg/daily, although maintenance doses can be as low as 15 mg/day or 15 mg every two days (Compendium of Pharmaceuticals and Specialties, 30th ed., 1995). It is considered safe for long-term use. PLZ is readily absorbed after oral administration in humans, with a maximum inhibition of MAO in brain after 5 to 10 days of treatment. A noticeable clinical improvement is usually seen after two weeks of treatment, but a pronounced therapeutic effect typically requires 4 weeks. The most commonly reported side effects with PLZ are drowsiness, dizziness and fainting, but one of the principal reasons why PLZ is used with caution is the risk of hypertensive crisis. Hypertensive crisis, or the 'cheese effect" is due to potentiation of sympatho-mimetic substances by inhibition of MAO (Merck Manual, 16th ed., 1992). Hypertensive crisis is characterized by an occipital headache, nausea and vomiting, neck soreness, sweating, and tachycardia or bradycardia (Compendium of Pharmaceuticals and Specialties, 30th ed., 1995). Hypertensive crisis is avoided with a strict diet, avoiding any foods high in tyramine or related amines as well as many over-the-counter drugs,

including preparations containing sympathomimetic amines (Compendium of Pharmaceuticals and Specialties, 30th ed., 1995; Merck Manual, 1992). Clinicians are reportedly reluctant to prescribe PLZ due to concern about the restrictions and apparent dangers of the drug. It should be noted, however, that patients with PD are often more sensitive to specific side effects such as the jitteriness associated with TCA administration, and while MAOIs may also cause this side effect, they do so to a lesser extent, making them a reasonable alternative (Johnson et al., 1987).

PLZ is currently prescribed for PD in cases where patients are unresponsive to other medications or where patients have a medical condition that precludes use of other classes of drugs. While the MAOIs have been found to be effective tools in the treatment of PD, their current frequency of use does not adequately reflect their efficacy (Johnson et al., 1994; Kennedy and Glue, 1994). Many studies document PLZ's effectiveness in treating PD (see Johnson et al.,1994). For example, PLZ was found to be superior to TCAs and placebo in the treatment of atypical depression, specifically in the treatment of depression with a history of spontaneous panic attacks (Liebowitz, et al., 1988; Kayser et al., 1988). Atypical depression, and especially major depression with PD, is quite common and is more difficult to treat than major depression alone. PLZ appears to be more efficacious than TCAs in the treatment of this form of

atypical depression (Dassylva and Fontaine, 1995; Johnson et al., 1994). In keeping with this observation, Kayser and colleagues (1988) reported that PLZ was more effective than amitriptyline in the treatment of MDD with panic attacks. Klein and Metz (1990) report a case study where clomipramine (CMI) was used to treat a case of PD with agoraphobia. The patient's panic symptoms subsided with CMI but the agoraphobic symptoms remained. The addition of PLZ to the treatment regime resulted in the cessation of the agoraphobic symptoms. PLZ was also found to be effective in the long-term treatment of panic attacks, with successful treatment of 97% of patients who completed a 6 month study (Burrows et al., 1993).

Despite its widespread use, the metabolism of PLZ is not well understood in humans. At present, it appears that the main metabolic routes in humans are formation of phenylacetic acid and *p*-hydroxyphenylacetic acid, accounting for approximately 80% of PLZ metabolism (Clineschmidt and Horita, 1969b; Robinson et al., 1985; McKenna et al., 1990) (See Figure 1 for metabolism of PLZ). Other metabolites of PLZ include 2-phenylethylamine (PEA), *p*-hydroxy-PLZ (*p*-OH-PLZ) and *p*-tyramine (*p*-TA) (Baker et al., 1982b; Dyck et al., 1985; McKenna et al., 1990). Hydrazine has also been suggested to be a metabolic end-product (Perry et al., 1981). Acetylation was thought for many years to be a major route of metabolism of PLZ, however studies have indicated that, at most, acetylation is only a

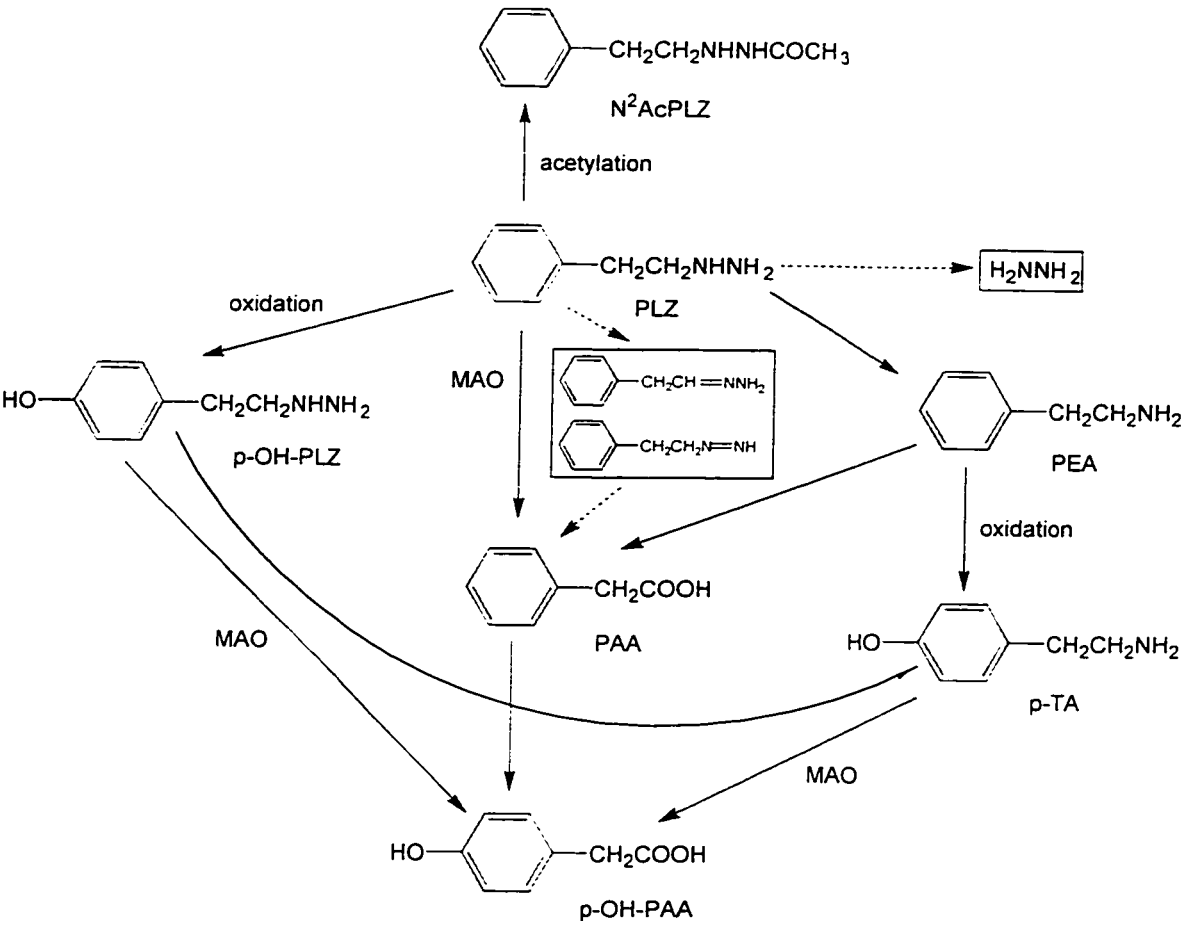


Figure 1: Metabolism of PLZ, adapted from K. McKenna's Ph.D. thesis (1995). Dotted lines and boxes indicate hypothesized metabolites.

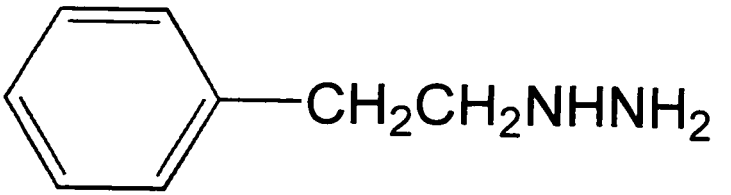
minor metabolic route (Robinson et al., 1985; Mozayani et al., 1988). Two compounds, phenylethylidene hydrazine ($PhCH_2CH=N-NH_2$) (Tipton, 1972) and phenylethyldiazene ($PhCH_2CH_2N=NH$) (Patek and Hellerman, 1974; Kenney et al., 1979), have been proposed to be intermediates in PLZ metabolism, but their formation has not yet been confirmed.

The exact mechanisms by which PLZ exerts its effects on the GABA system are not yet known. Initially, it was assumed that PLZ elevated GABA and ALA by inhibiting GABA-T and alanine-transaminase (ALA-T) respectively; however, studies have shown that PLZ produces a three-to-four fold increase in GABA and ALA levels while only inhibiting the transaminases by less than 50% (Popov and Mathies, 1969; Baker et al., 1991; McManus et al., 1992; McKenna, 1995), suggesting that some alternate or additional mechanism must be operating. Studies that involved the administration of other MAOIs prior to administration of PLZ suggested that a metabolite of PLZ, produced by the action of MAO on PLZ, may be contributing to the elevation of GABA levels, as the prior administration of another MAOI (e.g. tranylcypromine, TCP) blocked the effects of PLZ on GABA (Popov and Mathies, 1969; Todd and Baker, 1995). The effects of PLZ on ALA levels in brain are also blocked by pretreatment with another MAOI.

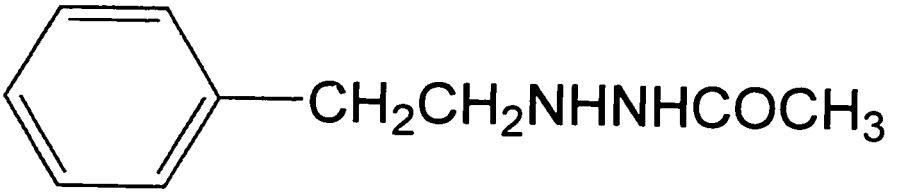
PAA and its hydroxylated metabolite, *p*-hydroxy-PAA, identified metabolites of PLZ (Clineschmidt and Horita ,1969b; Robinson et al., 1985),

have been found to have no GABA-elevating properties (Baker, Leung and McKenna, unpublished). The ring-hydroxylated metabolite, *p*-hydroxy-PLZ exhibits some GABA-T inhibition *in vitro* (Baker, McKenna and Coutts, unpublished), but prior inhibition of MAO would not likely interfere with its formation from PLZ. PEA has also been examined and found to exhibit no GABA-T inhibiting properties (Baker, personal communication). Acetylation to N^2-acetylphenelzine (N^2AcPLZ) is only a minor pathway for PLZ (Robinson et al., 1985; Mozayani et al., 1988), and N^2AcPLZ does not cause an elevation of GABA (McKenna et al., 1994; Paslawski et al., 1996).

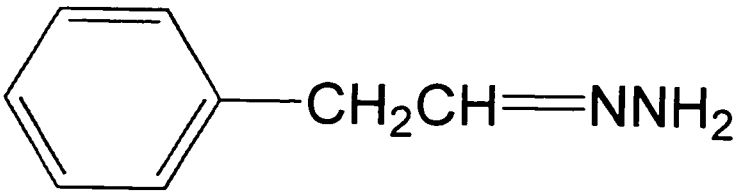
PLZ and N^2AcPLZ differ by substitution on the hydrazine moiety (see Figure 2). It has been suggested that the free hydrazine component in PLZ is essential to producing the elevated GABA levels observed (McKenna et al., 1994). Interestingly, hydrazine itself has been shown to elevate GABA and inhibit GABA-T (Perry et al., 1981). The structure of phenylethylidene hydrazine (PEH) (see Figure 2), one of the putative intermediate metabolites suggested by Tipton (1972), maintains the hydrazine portion present in the parent molecule and therefore it is possible that PEH, like PLZ, elevates brain GABA.



PLZ



N^2AcPLZ



PEH

Figure 2: Structures of PLZ, N^2AcPLZ and PEH (phenylethylidene hydrazine).

1.4 GABA (γ-aminobutyric acid)

1.4.1 GABA and Panic Disorder

Several neurotransmitter systems have been implicated in the neurobiology of anxiety and its treatment. These systems include the noradrenergic system, the mesocortical dopaminergic system, the central serotonin (5-hydroxytryptamine, 5-HT) system, adenosine, neuropeptides including cholecystokinin (CCK) and corticotropin-releasing factor (CTRF), and the GABA (γ-aminobutyric acid) system (Enna and Mohler, 1987; Iversen, 1984; Suranyi-Cadotte et al., 1990; Coupland et al., 1992; Lydiard et al., 1996).

GABA has been implicated in the etiology and pathophysiology of a number of disorders, including Huntington's disease and Parkinson's disease, schizophrenia, epilepsy, senile dementia and tardive dyskinesias (Cooper, Bloom and Roth, 1991; DeLorey and Olsen, 1994). Many of the drugs used in psychiatry, including the BZDs and barbiturates, have an effect on the GABAergic system (Schwartz et al., 1986). GABA has also been suggested to play a role in PD (Johnson et al., 1994; Petty et al., 1993; Bourin et al., 1998), in part due to increasing evidence that GABA is implicated in the actions of many of the drugs used in the treatment of PD (Breslow et al., 1989).

The ability to augment GABA transmission is a consistent finding with many of the drugs used to treat PD (Breslow et al., 1989; Lloyd et al., 1985, 1989; Suranyi-Cadotte et al., 1990). For example, IMI has been reported to cause release of GABA in studies with push-pull cannulae in rat thalamus (Korf and Venema, 1983). Alprazolam is a BZD reported to have both antidepressant and antipanic efficacy (Breslow et al., 1989), and, being a BZD, acts through facilitation of GABAergic transmission. Brofaromine, a selective, reversible inhibitor of MAO-A with antipanic efficacy (Bakish et al., 1993; Johnson et al., 1994), has been shown to have relatively strong GABA uptake inhibiting properties (Urichuk, personal communication) and PLZ, as mentioned previously, is a nonselective irreversible MAOI that also increases brain levels of several inhibitory amino acids, including ALA, β-alanine and GABA (Wong et al., 1990b; Baker et al., 1991; Erecinska et al., 1994).

A number of studies have documented interactions between GABA and the monoamines (Bartholini, 1984; Sheehan et al., 1980) as well as other neuroactive molecules (Bradwejn, 1993), suggesting that a GABAergic hypothesis of PD could coexist with other theories regarding the etiology of PD. Akasu (1988) showed that 5-HT increases the sensitivity of the $GABA_A$ receptor to GABA, while Bosler (1989) revealed a serotonin/GABA axonal interface, representing a "privileged" site of interaction between the two neurotransmitters. In keeping with this observation, $GABA_A$ and $GABA_B$

receptors have been reported to differentially regulate 5-HT release in discrete brain regions (Tao et al., 1997). Interestingly, Morales and associates (1996) found that the 5-HT_3 receptor is expressed in a subpopulation of GABAergic neurons in the rat neocortex and hippocampus. Lloyd et al. (1989) reported a NA-GABA interaction, in that $GABA_A$ receptor stimulation enhanced NA release in the ventral tegmental NA pathway. As well, GABA is co-localized in some brain regions with CCK (Kosaka et al., 1985), a peptide which has been demonstrated to produce panic symptoms in humans (review: Bradwejn, 1993).

1.4.2 GABA Distribution in the CNS

GABA is the major inhibitory neurotransmitter in the mammalian CNS. It is estimated to be implicated in the activity of 30-50% of all CNS neurons (Breslow et al., 1989; Petty et al., 1993). It is also fairly specific to the CNS and retina, with only small amounts of GABA found in peripheral tissues such as the sciatic and splenic nerves, liver, heart and adrenal gland (DeLorey and Olsen, 1994; Petty et al., 1993). GABA has been shown to coexist with other neurotransmitters, e. g. somatostatin in cortical and hippocampal neurons and CCK in cortical neurons, and to interact with other neurotransmitters in the CNS (Cooper et al., 1991). For example, in certain areas of the brain, dopaminergic neurons have an inhibitory effect on GABAergic neurons (in the striatonigral GABA projection) (Perry, 1982).

Another example of GABA's interactions with other neurotransmitters is the activation of presynaptic GABA receptors resulting in a decrease in release of Substance P (a peptide neurotransmitter believed to be important in the pain fiber system) (Petty et al., 1993). GABA or GABA agonists inhibit *in vitro* and *in vivo* release of other transmitters such as dopamine, 5-HT and acetylcholine (Okakura-Mochizuki et al., 1996). Muscimol, a naturally occurring GABA-mimetic, causes a decrease in the turnover rate of acetylcholine (ACh) in rat midbrain and cortex (DeLorey and Olsen, 1994).

In many brain regions, GABA is present in millimolar concentrations, about 1000 times higher than the concentrations of the classical monoamine transmitters in the same regions (Cooper et al., 1991). In humans the highest concentrations of GABA are found in the substantia nigra (the main area implicated in Parkinson's and the principal area of the brain projecting dopaminergic neurons to the corpus striatum) and the globus pallidus (Perry, 1982). In rats the highest levels of GABA are found in the substantia nigra, the diencephalon and the corpora quadrigemina (Perry, 1982). There is no evidence for lateral assymmetry of GABA in the human brain except for a slightly higher concentration of GABA in the right nigral striatal region compared to the left (Rossor et al., 1980; Perry, 1982).

1.4.3 GABA Metabolism

GABA metabolism is highly regulated and dependent on the availability of precursors (Westergaard et al., 1995).It is closely associated with the oxidative metabolism of carbohydrates (also proteins and fats) in the CNS through the Kreb's citric acid cycle. GABA is formed by a metabolic pathway that basically bypasses the normal oxidative metabolism of carbohydrates and involves the enzymes α-ketoglutarate dehydrogenase and succinyl thiokinase. This bypass is referred to as the GABA shunt (Figure 3), a loop with the two-fold purpose of producing and conserving the supply of GABA. It is less efficient energy-wise than direct oxidation through the Kreb's cycle (3 ATP equivalents produced vs. 3 ATP and 1 GTP for the Kreb's cycle), yet it is estimated that 10 to 50% of total brain metabolism is through the GABA shunt (Petty et al., 1993; DeLorey and Olsen, 1994; Sieghart, 1995).

GABA synthesis is controlled by enzyme activity. Glutamic acid decarboxyiase (GAD) catalyzes the decarboxylation of glutamate to form GABA. Endogenous levels of GABA increase rapidly postmortem if tissues are not frozen immediately. This postmortem increase may be due to transient activation of GAD. Pyridoxal-5-phosphate (PLP) acts as a positive coenzyme in the GAD reaction. PLP is the main coenzymatic form of pyridoxine, a vitamin B_6 vitamer. Of the over 100 known PLP-dependent

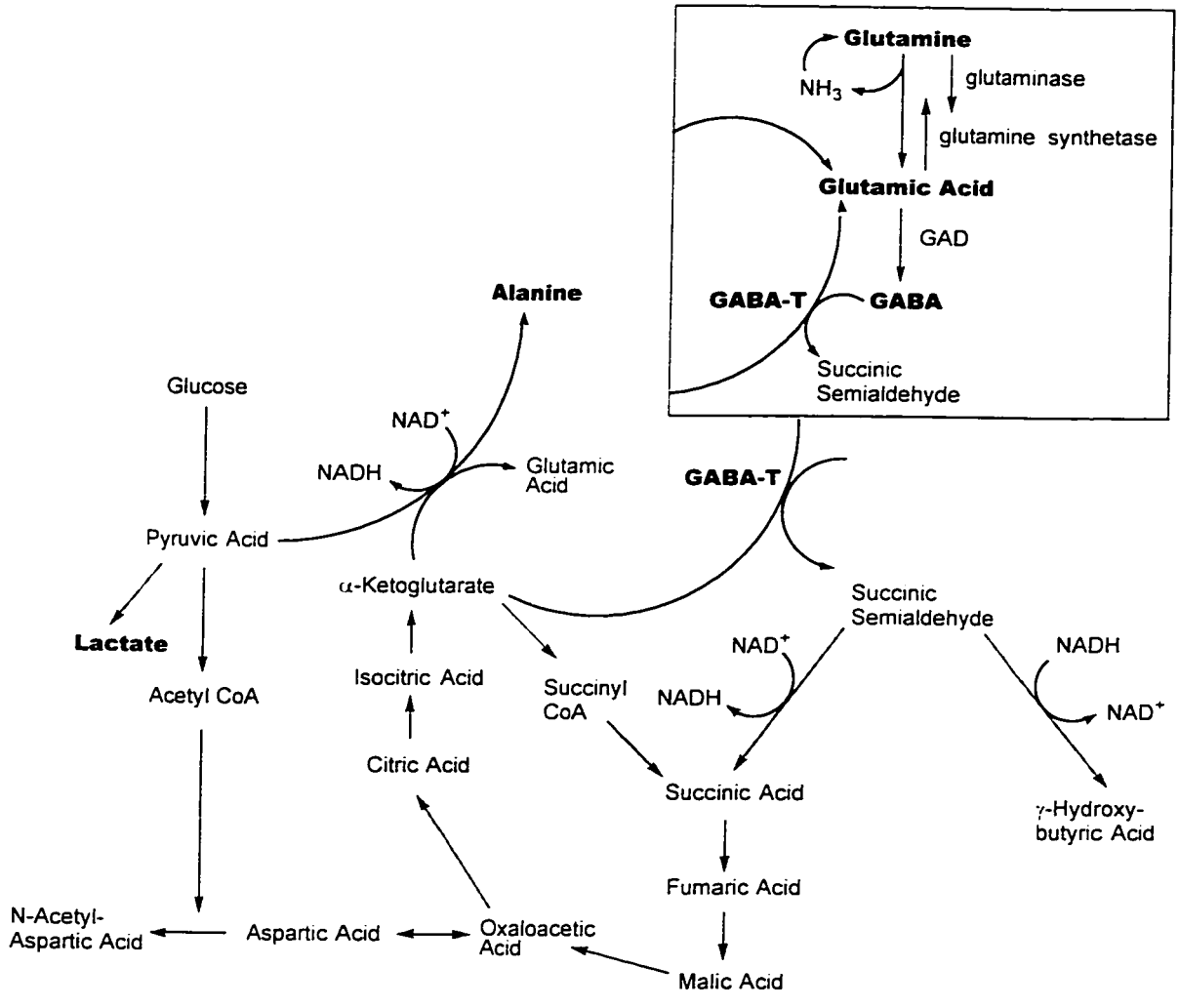


Figure 3: Diagram of the Citric Acid Cycle and GABA shunt (in box) (adapted from Cooper, Bloom and Roth, 1991, p. 136).

reactions, most are involved in amino acid metabolism (Dakshinamurti et al., 1992; Petty et al., 1993; DeLorey and Olsen, 1994).

Neither GABA nor its precursor glutamate crosses the blood-brain barrier very easily. GAD is the main enzyme involved in the synthesis of GABA. Levels of GABA and GAD activity in the various brain regions have a high correlation. In fact, GAD appears to be unique to the CNS and retina in mammals, and is apparently exclusively expressed in cells that use GABA as a neurotransmitter. GABA-α-oxoglutarate transaminase (GABA-transaminase; GABA-T) is the main enzyme involved in the degradation of GABA, but GABA levels and GABA-T activity in brain tissue have no consistent relationship. For example, the substantia nigra and globus pallidus have the highest levels of GABA and relatively low levels of GABA-T, while the dentate nucleus and inferior colliculus have high levels of both GABA and GABA-T (DeLorey and Olsen, 1994).

Like GAD, GABA-T also requires PLP as a coenzyme. PLP is more tightly bound to GABA-T than it is to GAD. Hydrazines and other carbonyl-trapping agents react with PLP's aldehyde group, which decreases the availability of PLP to act as a coenzyme. GAD is often preferentially inhibited over GABA-T by such compounds because of its lower affinity for PLP. Agents affecting this coenzyme will often affect GABA levels by interfering with its synthesis and degradation (Petty et al., 1993; DeLorey and Olsen,

1994). Epileptiform seizures can be caused by an inactivation or lack of PLP (Wasterlain et al., 1993).

GAD is primarily associated with the nerve terminal while GABA-T is associated with mitochondria (the energy source of the cell). However, studies have revealed that there is very little GABA-T activity in nerve-ending mitochondria. This finding suggests that GABA is broken down at some extraneuronal site. At a synapse, GABA's action is terminated by uptake both into presynaptic nerve terminals and surrounding glial cells. The membrane transport systems that mediate reuptake of GABA are temperature- and ion-dependent processes. The reuptake system can transport GABA against a concentration gradient; under normal physiological conditions the internal to external ratio of GABA is 200:1. The driving force for reuptake is supplied by movement of Na^+ down its concentration gradient, so the GABA transport system has an absolute requirement for Na+ ions and an additional dependence on Cl^- ions. GABA that is taken up into the nerve terminal may be reutilized and GABA taken into glial cells is metabolized by GABA-T (Petty et al., 1993; DeLorey and Olsen, 1994).

GABA cannot be resynthesized in glial cells because these cells do not have GAD. Instead the action of GABA-T forms succinic semialdehyde and glutamate. Glutamate is converted into glutamine via glutamine synthetase, which is only found in glial cells, and is transferred back to the

neuron where glutaminase can convert it to glutamate (Petty et al., 1993; DeLorey and Olsen, 1994). Many of the drugs that elevate brain GABA content act as enzyme inhibitors of GAD, but their effect is weaker on the synthesizing enzyme than on the degrading enzyme, GABA-T, and their net effect is to produce an increase in brain GABA content (Perry, 1982).

α-Ketoglutarate (α-oxoglutaric acid), formed from glucose metabolism in the Kreb's cycle, is transaminated with GABA by GABA-T to give succinic semialdehyde (SSA; an aldehyde intermediate) and to regenerate glutamate. SSA has not been detected as an endogenous metabolite of GABA *in vivo* because succinic semialdehyde dehydrogenase (SSADH) functions very effectively at low substrate concentrations, oxidizing SSA and bringing it back into the Kreb's cycle. The regional distribution of SSADH parallels the distribution of GABA-T, but the former enzyme is about 1.5 times as active as the latter (DeLorey and Olsen, 1994; Medina-Kauwe et al., 1994).

1.4.4 The $GABA_A$ Receptor

At present GABA receptors are classified into three major types, $GABA_A$, $GABA_B$ and $GABA_C$ receptors (Mody et al., 1994). $GABA_A$ receptors were initially identified as those GABA receptors that are blocked by bicuculline but are insensitive to baclofen, while $GABA_B$ receptors are activated by baclofen but are insensitive to bicuculline (Dunn et al., 1994). These two receptor subtypes also differ in structure and function. $GABA_A$

receptors are part of a family of ligand-gated ion channels believed to be hetero-oligomers consisting of five subunits making up a chloride ion channel and having both pre- and post-synaptic positions on neurons (Dunn et al., 1994). $GABA_B$ receptors are linked to a second-messenger system and also have both pre- and post-synaptic positions on neurons. The regional distribution of the two receptor subtypes differs considerably in the central nervous system (Johnston, 1994). A third class, $GABA_C$ receptors, are insensitive to both baclofen and bicuculline but are selectively activated by cis-4-aminocrotonic acid, and are linked to a chloride ion channel. This class has been localized predominantly to vertebral retinal neurons (Bormann and Feigenspan, 1995). A fourth GABA receptor, a G-protein coupled baclofen-resistant receptor, may also exist (Djamgoz, 1995).

The $GABA_A$ receptor is a multimeric membrane-spanning ligand-gated ion channel that is permeable to inorganic ions, primarily chloride (Rabow et al., 1995; Behringer et al., 1996; Cooper et al., 1991). It mediates fast inhibitory transmission in the CNS and is modulated by many endogenous and therapeutically important agents (Rabow et al., 1995; Behringer et al., 1996). The receptor has a variety of ligand binding sites, including the GABA site (which also binds muscimol), and other sites for several classes of drugs, including BZDs, barbiturates, picrotoxin, and probably steroids and ethanol (Johnston, 1994). Multiple distinct subunits and multiple subtypes of

each subunit, e.g. α_{1-6}, β_{1-3}, γ_{1-3}, δ subunits, have been identified by molecular cloning. Thus there are isoforms of the receptor, each with five subunits, which show ligand binding heterogeneity (Macdonald and Olsen, 1994; Bureau et al., 1995; Khan et al., 1996; Johnston, 1994). The recognition site for GABA analogs on $GABA_A$ receptors is jointly affected by all subunit classes present in a receptor (Luddens and Korpi, 1995).

The $GABA_A$ receptor complex has been implicated in the pathology of anxiety disorders in that inverse agonists of this receptor, i.e. some β-carbolines, have been shown to induce anxiety in human subjects (Enna and Mohler, 1987; Sieghart, 1995). As well, the BZD anxiolytics (e.g. alprazolam, clonazepam and diazepam) all increase the affinity for GABA at the $GABA_A$ receptor (review, Dunn et al. 1994). The $GABA_A$ receptor is currently believed to be the main mechanism for inhibition in the CNS of vertebrates (Dunn et al., 1994).

1.5 Animal Models of Anxiety Disorders

A number of animal models for anxiety have been developed, with an estimated 20 to 30 models currently in use. Most of the models use fear as an analogy to human anxiety (Green, 1991). Many of these tests involve the use of aversive stimuli, i.e. electric shock, or the implementation of food or water deprivation, the latter of which may conceivably alter the animal's response to a given drug (Pellow et al., 1985). Some tests, however,

including the elevated plus-maze, are based on the animal's reaction to negative stimuli (Treit et al., 1993). While the value of these tests may not be quantifiable in terms of face validity, their predictive validity makes them valuable tools in furthering our understanding of anxiolytic drugs (Greenshaw et al., 1988).

The most common means of validating an animal model of anxiety is pharmacologically -- drugs known to be clinically effective against anxiety are expected to show a specific profile of action in the various models (Pellow et al., 1985; File, 1987). Most animal models of anxiety can detect BZDs to varying degrees, but they have yielded unclear findings with regard to the anxiolytic effect of TCAs and MAOIs. In particular, antipanic agents are difficult to detect by animal models of anxiety (Commissaris and Fontana, 1991; Laino et al., 1993).

The elevated plus maze was developed from the work of Montgomery in 1955. There is now an extensive literature validating the plus maze as an animal model of anxiety in rats and in mice (Pellow, et al., 1985; Pellow and File, 1986; Lister, 1990). It is based on the relationship between fear induced by novel stimulation and exploratory behavior (Treit and Menard, 1998). The test involves placing the animal in the center of the apparatus and observing its behavior under quiet, dimly lit conditions (see Figure 4). An observer sits in a position where the entrance to all four arms is visible, often done with

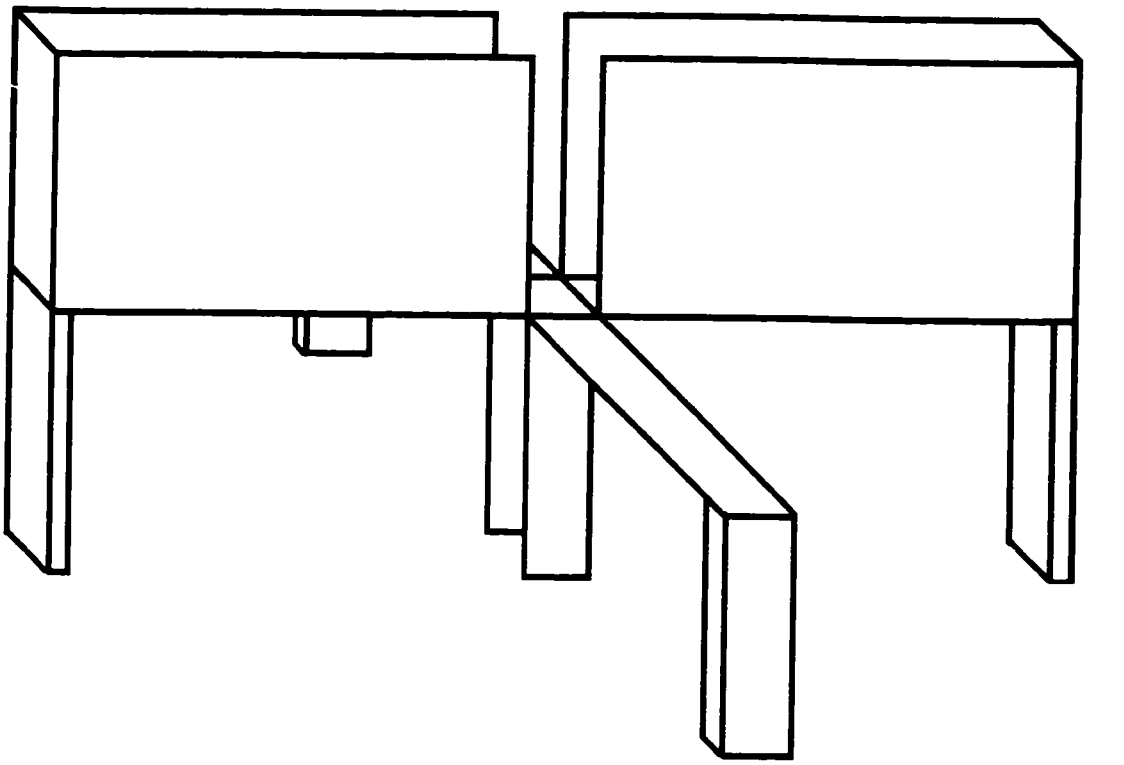


Figure 4: The elevated plus-maze.

the aid of an angled mirror above the maze. Typically the observer records four things: open arm time, closed arm time, open arm entries and closed arm entries. Two indices of anxiety are used: the proportion of entries made onto the open arms, often expressed as a percentage of total number of arm entries, and the time spent on the open arms of the maze expressed as a percentage of total time spent on open and closed arms. The plus maze has some face validity in that the reluctance of animals to explore the open arms probably results from a condition of rodents' aversion to open spaces and the elevation of the maze. Animals confined to the open arms of the maze have been shown to exhibit fear reactions, i.e. defecation (Treit et al., 1993). If a treatment increases an animal's preference for the open arms without altering total number of arm entries, this is taken to reflect an anxiolytic action. Similarly, if a treatment decreases an animal's preference for the open arms, again without altering total number of arm entries, then it is taken to reflect an anxiogenic effect. Total number of arm entries is used as a measure of general activity. A change in total number of arm entries suggests a locomotor effect or a sedation effect.

The elevated plus-maze has been shown to detect both anxiolytic, e.g. phenobarbital and diazepam, and anxiogenic, e.g. caffeine and yohimbine, compounds (Treit and Menard, 1998). Most BZD anxiolytics are detectable in the elevated plus-maze (Commissaris and Fontana, 1991).

BZDs enhance the affinity of the $GABA_A$ receptor for GABA and it is this effect that is believed to cause anxiolysis. Vigabatrin, a GABA-mimetic anticonvulsant, shows an anxiolytic effect in the plus-maze (Commissaris and Fontana, 1991; Sayin et al., 1992). One would expect that PLZ, because of its significant effect on GABA, would also be reliably detected on the plus-maze, but a review of the literature shows that most animal models of anxiolysis fail to reliably detect PLZ as an anxiolytic (Commissaris and Fontana, 1991; Johnston and File, 1988). Because other drugs that appear to rely on their effect on the GABAergic system to produce an anxiolytic or anxiogenic action are detectable on the elevated plus-maze, and because the effect of peripherally-administered PLZ on whole brain levels of GABA is time- and dose-dependent, it is possible that the lack of effect seen with PLZ on the plus maze may be due to inadequate time and dosing parameters.

1.6 General Analytical Techniques Relevant to this Thesis

1.6.1 Gas Chromatography

Chromatography refers to a group of processes that separate solutes in a solution by differential distribution between two phases, the mobile phase and the fixed or stationary phase (Tabor, 1989). The sample is carried by a mobile phase through a specially-treated column in which the components of the sample migrate at different speeds based on their

physical interaction with the stationary or fixed phase (Burtis et al., 1987; Baker et al., 1982a). Chromatographic techniques can be classified by the physical state of the mobile phase. Gas chromatography (GC) is a method whereby compounds are distributed or partitioned between a stationary and a mobile phase, with the mobile phase being an inert carrier gas, such as helium, nitrogen, hydrogen or argon (Burtis et al., 1987; Baker et al., 1982a).

The mobile phase or carrier gas takes the volatilized solute molecules through a chromatographic column where they partition between the two phases. The separated constituents of the sample are then carried by the carrier gas, in order of elution, to a detector (Tabor, 1989). The detector generates an electrical signal proportional to the amount of eluent present in the mobile phase stream, and this signal is amplified and recorded (Burtis et al., 1987; Baker et al., 1982a). The fundamental components of a GC system are: 1. an inlet or injector port where the sample is introduced to the system, vaporized and mixed with the carrier gas; 2. a variable-temperature oven housing the chromato-graphic column; 3. a detector that detects the components as they elute from the column; and 4. a data recorder that records and integrates the signals identified by the detector, recording the signals as a series of peaks versus time (Burtis et al., 1987; Coutts and Baker, 1982).

Both split and splitless injection systems are available for GC. In a

split system the carrier gas is divided and only a part of the injected sample is carried through the GC. The split injection system is employed in the analysis of concentrated samples. Conversely, the splitless system is used in the analysis of very dilute samples or wide-boiling range samples (Coutts et al., 1985), and this is the type of system that was employed in this thesis. In a splitless system the sample is introduced into the injection port, which is typically set at 25-50 °C higher than the boiling point of the highest boiling component, and vaporized in a glass-lined tube that extends to the GC column (Coutts et al., 1985).

The column oven maintains a constant and uniform temperature of the column, critical to maintaining reproducible retention times, the time interval between injection and apex of the recorded peak. Oven temperature can be kept constant (isothermal programming) or a temperature program can be employed. Temperature programming, stepwise and gradual changes in column temperature, allows for the analysis of components with a wider range of boiling points (Burtis et al., 1987) and generally results in sharper peaks and a shorter analysis time (Burtis et al., 1987). GC columns can be composed of glass, various metals or Teflon (Coutts et al., 1985; Burtis et al., 1987). Glass columns are used most often in the analysis of biological samples because of their inertness (Coutts et al., 1985). Experiments described in this thesis employed fused silica capillary columns.

Capillary columns are usually 10 - 75 m long and 0.25-0.5 mm in diameter and coated in a thin layer of stationary phase. In wall-coated open tubular (WCOT) capillary columns the liquid phase is deposited directly onto the inner surface of the glass column. Support-coated open tubular (SCOT) columns have a layer of solid support material between the glass and the liquid phase (Baker et al., 1982a; Coutts et al., 1985; Burtis et al., 1987). The experiments described in this thesis utilized WCOT columns, which are well-suited to lower analyte concentrations (Coutts et al., 1985).

Effluent from the column enters a detector which senses the sample components and generates an electrical signal. Many types of detectors have been developed for GC, including the thermal conductivity detector (TCD), the flame ionization detector (FID), the electron-capture detector (ECD), and the nitrogen-phosphorus detector (NPD). The experiments described here employed the ECD and the NPD. The ECD is a selective detector, sensitive to as little as 1 picogram of an organic compound containing an electrophoric substituent. The ECD contains two electrodes and a radioactive source, such as ^{63}Ni or ^{3}H, attached to the cathode, which emits high-energy β-particles. The β-particles collide with the carrier gas, producing low-energy secondary electrons and creating a small standing current. Some of these secondary electrons are absorbed by the sample components as they pass through the detector, causing changes in the

standing current. These changes are inverted and amplified, and recorded as peaks on the recorder. The NPD is sensitive to most compounds that possess nitrogen- or phosphorus-containing functions, and is able to detect low picogram quantities. As the effluent from the column enters the NPD it is mixed with hydrogen and enters a heated chamber containing an alkali source which forms a low-temperature plasma. A small current is produced, proportional to the amount of compound present in the carrier gas. This current is amplified and recorded (see Baker and Coutts, 1982; Coutts et al., 1985; Burtis et al., 1987).

Prior to analysis by GC, it is often necessary to derivatize the compounds of interest. Derivatization is done for several reasons, including: 1. to increase volatility; 2. to increase stability; 3. to reduce polarity since polar substances generally chromatograph poorly; 4. to improve the efficiency of extraction of a compound from aqueous solutions; and 5. to introduce a functional group that is sensitive to selective detectors, i.e. ECD or NPD. Derivatization for GC generally involves acylation, alkylation, silylation or condensation to replace an active hydrogen in a polar group, i.e. -NH, -OH, -SH (see Coutts et al., 1985; Coutts and Baker, 1982; Baker et al., 1982a).

1.6.2 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) differs from GC on a basic level in that the sample and mobile phase are in liquid form as the mixture flows over the stationary phase (Burtis et al., 1987; Warsh, et al., 1982). The basic components of an HPLC system are: 1. a solvent reservoir, which contains the mobile phase; 2. a pump, to drive the mobile phase from the reservoir through the system; 3. an injector, to introduce the sample into the mobile phase; 4. a chromatographic column; 5. a detector; and 6. a recorder (Burtis et al., 1987; Bowers, 1989).

The most common pump used in HPLC is a reciprocating pump. An asymmetric cam drives pistons in and out of two pumping chambers. On the fill stroke the piston is withdrawn from the chamber and a proportional volume of liquid is drawn from the solvent reservoir, through the inlet check valve, into the pumping chamber. The valve is then closed and the piston driven into the chamber, forcing the liquid into the column (Burtis et al., 1987). To avoid pump noise due to the pulsating mobile phase, a multihead reciprocating pump with two or more pistons operating out of phase, can be employed. The experiments described in this thesis utilized a multihead reciprocating pump system. The two methods of mobile phase delivery used in HPLC are isocratic mode, where composition of the mobile phase remains constant throughout the run, and gradient mode, where the mobile phase

composition changes in a stepwise or gradient manner (Stein, 1982; Burtis et al., 1987). The isocratic mode was used in the analyses described in this thesis. The sample is most commonly introduced into the mobile phase by a loop injector. A sample is injected into the external loop of the injector at atmospheric pressure, then the loop is rotated and the sample washed into the stream of mobile phase (Burtis et al., 1987).

The HPLC column is a stainless steel tube containing the stationary phase and packing. HPLC packing can be made of a number of materials, the most common being silica. Chromatographic packings are manufactured in three main types: microparticulate, porous spherical or irregular beads of 3-10 μm diameter; macroparticulate, 40+ μm diameter porous beads; and pellicular, with a thin porous layer of stationary phase coated on solid glass beads of varying diameters (Burtis et al., 1987; Stein, 1982). A guard column can be attached to the system ahead of the analytical column to filter out undesirable compounds. Bonded reversed-phase HPLC is the most widely used type of HPLC (Burtis et al., 1987), and was the type used in the experiments described in this thesis. 'Bonded' refers to the molecules of the stationary phase that have been chemically attached to the surfaces of the silica beads. Reverse-phased HPLC requires a nonpolar stationary phase [octadecylsilane (ODS) is commonly used], and a polar mobile phase, i.e. acetonitrile, water, or tetrahydrofuran (Burtis et al., 1987; Warsh et al., 1982;

Tabor, 1989). Reversed-phase HPLC is generally considered the most efficient for non-ionic compounds (Tabor, 1989).

The most common HPLC detectors are fluorescence, electrochemical (EC) and ultraviolet (UV) detectors. Fluorescence detectors are selective and sensitive to compounds that fluoresce within a specified wavelength range. A pre- or post-column reaction can tag a compound with a fluorescing label, such as an o-phthalaldehyde, dansyl or fluorescamine tag (Burtis et al., 1987; Anderson, 1985; Warsh et al., 1982). Flourescence detection was one of the methods utilized in the experiments described in this thesis. EC-HPLC requires that a compound be electrophoric. The sample is oxidized or reduced at an electrode surface using a constant potential. The change in current, either generated or consumed, is proportional to the concentration of the analyte (Burtis et al., 1987; Anderson, 1985). UV detectors measure the wavelength and magnitude of absorption of radiant energy by the compounds. Most pharmacologically active organic compounds absorb energy in the UV range (Burtis et al., 1987).

1.6.3 Spectrophotometry

Photometry refers to the measurement of radiant energy that is absorbed, reflected, emitted or transmitted by a substance. Most techniques used in the measurement of this energy employ some means of restricting the wavelength range that is measured; spectrophotometry uses prisms or

gratings to restrict the wavelength range (Caraway. 1987). Certain organic molecules have been shown to absorb radiant energy, which in turn raises the bond-energy of various interatomic bonds. This absorbed energy is dispersed in several ways, including as light energy. The number of photons of light emitted is proportional to the number of molecules involved (see Baker et al., 1985). A typical spectro-photometer consists of a light source, a monochromator, sample and reference absorption cells or cuvettes, a detector or photocell, and a recorder (see Caraway, 1987). The light source for measurements in the visible spectrum is usually provided by a tungsten lamp, and hydrogen or deuterium lamps are used for measurements in the UV spectrum. A monochromator isolates radiant energy of a desired wavelength. Cuvettes can be made from a variety of substances including glass, silica or plastic. For the experiments described in this thesis, square quartz cuvettes with a 1 cm light path were used. The most common detectors are barrier layer cells or photovoltaic cells and photomultiplier tubes. A photovoltaic cell was used in the experiments described in this thesis. The cell consists of a thin, semitransparent layer of silver on a layer of semiconductive selenium and mounted on an iron support. When light passes through the semitransparent silver onto the selenium surface, electrons are released in proportion to the intensity of light and collect on the silver layer, creating a negative charge. The iron base acts as a positive

pole. When connected to a recorder, a current flows with varying intensity dependent on the intensity of the light (review: Caraway. 1987).

Spectrophotometers can be equipped to allow for analysis of radiant energy over a range of wavelengths or over time. Change of absorbance over time is frequently used in the analysis of enzyme activity (Caraway, 1987), and was employed in the experiments conducted for this thesis.

Quantitative analysis of the results of spectrophotometry are based on Beer's law:

$$A = abc$$

where A= absorbance

a= a proportionality constant defined as absorptivity

b= the light path in cm

c= concentration of the absorbing compound

Absorbance values (A) have no units. When b= 1 cm and c is expressed in moles per litre, ϵ (molar absorptivity) can be substituted for a. ϵ is a constant for a given compound at a given wavelength under specified conditions of pH, temperature and solvent. It is then possible to solve for c: $c = A / (\epsilon \times b)$.

1.7 Introduction Summary

Anxiety disorders are among the most predominant psychiatric disorders in our society. Within the class of anxiety disorders, generalized

anxiety disorder and PD present an interesting combination of similarities and differences with regard to symptoms, suspected etiology, and treatment. Anxiety and depression also have a number of commonalities. Phenelzine (PLZ) is a drug used both in the treatment of anxiety disorders and depression and has a marked effect on the GABAergic system, which has been suggested to play a significant role in anxiety disorders, including PD. Although established animal models of PD have not yet been developed, there is potential for the use of animal models of anxiety to further our understanding of antipanic agents. It is also probable that a metabolite of PLZ contributes to the actions of the parent compound. The principles of a number of techniques that were used in this thesis to analyze the actions of PLZ on GABA and related systems have been described.

1.8 Objectives of this Study

This thesis focuses on GABAergic mechanisms of PLZ in an effort to gain further insight into the mechanisms of action of PLZ. The studies conducted included:

1. Analyses of the action of PLZ on GABA. These studies included acute time course investigations in hypothalamus and whole brain, acute dose-response and chronic analyses. These experiments measured the effect of PLZ on amino acid levels, including the precursors to GABA, glutamine

and glutamate, as well as on MAO and transaminase activity. In addition, $GABA_A$ receptor activity was measured in a chronic study.

2. Comparing PLZ and N^2AcPLZ - amino acid levels, MAO inhibition and transaminase activity were compared between PLZ and its acetylated metabolite acutely.
3. Behavioral analysis of PLZ - an acute study compared the effects of PLZ and N^2AcPLZ in an animal model of anxiety.
4. Clinical Studies - Blood samples across time were taken from patients with PD being treated with PLZ. These samples were analyzed for levels of GABA and ALA. This study necessitated extensive modification of a currently available assay in order to quantitate GABA in plasma samples.
5. Phenylethylidene hydrazine - this putative metabolite of PLZ was compared to PLZ with regard to its effects on MAO, amino acid levels, and transaminase activity.
6. Attempted development of an assay for the detection of PEH in tissues.

2 Materials and Methods

2.1 Chemicals

Chemicals	Suppliers
N^2-Acetylphenelzine (N^2-AcPLZ)	synthesized at Univ. of Alberta (Dr. R.T. Coutts)
2-Aminoethylisothiouronium Bromide (AET)	Sigma
Acetonitrile	BDH
(L)-Alanine	Sigma
γ-Aminobutyric Acid (GABA)	Aldrich
Ascorbic Acid	Sigma
Asparagine	Sigma
Aspartate	Sigma
Benzylamine	Sigma
Chloroform	BDH
Dicyclohexyl Carbodiimide (DCC)	Sigma
Di(2-ethylphenyl)phosphate (DEHPA)	Sigma
Dimethyl Sulfoxide (DMSO)	BDH
(D,L)-Dithiothreitol (DTT)	Sigma
Fluoraldehyde Reagent Solution	Pierce

Ethyl acetate	BDH
(L) – Glutamate	Sigma
(L) – Glutamine	Sigma
Glutathione (GSH)	Sigma
Glycerol (Glycerin)	Fisher
Glycine	Sigma
Homoserine	Sigma
Hydrazine	Matheson, Coleman & Bell (MCB)
Hydrochloric acid (HCl)	BDH
5-[2-^{14}C]-Hydroxytryptamine Binoxalate (^{14}C-5-HT)	Dupont
5-Hydroxy[G-^{3}H]tryptamine Creatinine Sulphate (^{3}H-5-HT)	Dupont
Isobutylchloroformate	Aldrich
(L) – Isoleucine	Sigma
α-Ketoglutarate	Sigma
Lactic Dehydrogenase (LDH)	Sigma
(L-)Leucine	Sigma
Methanol (MeOH; HPLC grade)	BDH

β-Nicotinamide Adenine Dinucleotide (NAD)	Sigma
β-Nicotinamide Adenine Dinucleotide-reduced form (NADH)	Sigma
(D, L-)Norleucine	Aldrich
Pentafluorophenol (PFPh-OH)	Aldrich
Perchloric acid ($HClO_4$), 60%	Fisher
Phenelzine (PLZ)	Sigma
Phenylethylidene hydrazine (PEH)	Synthesized at Univ. of Alberta (Dr. E. Knaus)
2-Phenyl[1-^{14}C]ethylamine hydrochloride (^{14}C-PEA)	Dupont New Products
Phosphoric Acid- 85%	Fisher
Potassium Carbonate (K_2CO_3)	Fisher
Potassium Chloride (KCl)	Fisher
Potassium Hydroxide (KOH)	Fisher
Potassium Phosphate dibasic (K_2HPO_4)	J.T. Baker
Potassium Phosphate monobasic (KH_2PO_4)	Fisher
Propylene Glycol	Fisher
Pyridoxal-5-Phosphate (PLP)	Sigma

Ready Safe® liquid scintillation cocktail	Beckman
(D, L)-Serine	BDH
Sodium Acetate (NaAc)	Fisher
Sodium Hydroxide (NaOH)	Fisher
Sodium Phosphate dibasic - anhydrous (Na_2HPO4)	Fisher
Sodium Phosphate monobasic - anhydrous (NaH_2PO_4)	Fisher
Tetrahydrofuran (THF)	Fisher
Toluene	BDH
Tranylcypromine (TCP)	Sigma
Tri-n-octylamine (TOA)	Fisher
Tris [(hydroxymethyl)methylamine]	Fisher
Triton X-100 (13%)	Terochem
(L-)Valine	Sigma

Table 1: List of chemicals used.

2.1.1 Drugs Administered in Studies

All drugs, with the exception of PEH, were dissolved in double-distilled water and doses were expressed as their free bases. N^2-AcPLZ was synthesized from phenyl-acetaldehyde and acetylhydrazine, according to a method described previously (Danielson et al., 1984; Coutts et al., 1990).

The structure and purity (>99%) of N^2-AcPLZ were confirmed by melting point, gas chromatography, and infrared, nuclear magnetic resonance and mass spectrometry (Coutts et al., 1990). Phenylethylidene hydrazine (PEH) was synthesized at the University of Alberta in the Faculty of Pharmacy and Pharmaceutical Sciences, under the supervision of Dr. E. Knaus and dissolved in a solution of DMSO: propylene glycol (1:1). Synthesis is described in Section 2.15 of this thesis.

2.2 Instrumentation

2.2.1 Gas Chromatography

For amino acid level determinations, either of two gas chromatographs was used: a Hewlett Packard (HP) 5890 GC equipped with a fused silica column, an ECD with a radioactive source of 15 mCI Nickel-63, an HP 7673A automatic sampler and an HP 3392A integrator; or a HP 5880A series GC and an ECD with a radioactive source of 15 mCI Nickel-63 and HP5880A integrator. The carrier gas, helium, was set at a flow rate of 2 ml/min. Argon-methane (95%- 5%), flow rate 35 ml/min, was the make-up gas used in the detector. The injection port temperature was 200°C and the detector temperature was 325°C.

2.2.2 High Pressure Liquid Chromatography

Chromatographic separations were performed using three systems.

The first system involved the use of a WISP 710B automatic injection system (Waters; Milford, MA, USA) and a flow of 1 ml/min (Waters model 510 pump). The compounds of interest were separated on a 5 ODS 2 column (5 μm, 250x4.6mm; Phenomenex, Torrence, CA, USA). Peak height was integrated using a HP 3392A integrator. The second system was a Gilson Sampling Injector (Model 231 XL). Both systems were linked to a Waters Fluorescence Detector (Model 420-AC).

GABA determination in plasma was accomplished using a Waters Alliance HPLC system consisting of a 2690XE separations module with an in-line degasser, integral sample cooler set at 4°C, and a column heater held at 30°C. The column was a Waters Spherisorb ODS 2 (5 μm, 250x4.6mm; Phenomenex, Torrence, CA, USA) with a Waters μBondapak C_{18} guard column. The detector was a Shimadzu RF-10A fluorescence detector (Mandel Scientific) with an excitation wavelength of 260nm and an emission wavelength of 455 nm. Flow rate was set at 0.5 ml/min and temperature was held constant at 30°C. Data acquisition and sample management was done using a Digital 5100 Venturis computer (Waters Corp.) and Millenium Multisystem Software (Waters Corp.).

2.2.3 Liquid Scintillation Spectrophotometry

A Beckman LS 7500 liquid scintillation spectrometer coupled to a Datamex 43 printer was used for counting radioactivity in MAO and GABA-transaminase assays.

2.2.4 UV Spectrophotometry

A Hitachi Double Beam Spectrophotometer (Model U-2000) was used for the analysis of ALA-T. The spectrophotometer was attached to a water bath to allow cuvettes to be incubated at 37 °C.

2.3 Apparatus

2.3.1 Elevated Plus Maze

The elevated plus-maze apparatus is a standard wooden, plus-shaped apparatus elevated 50 cm above the floor on a wooden stand, with two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms with open roofs (see Pellow et al., 1985 and Figure 4 in the introduction of this thesis).

2.3.2 Glassware

All glassware was rinsed with tap water and washed out with biodegradable Sparkleen (Fisher Scientific, Fairlawn, NJ, USA) solution. Further washing was accomplished with a dishwasher (Miele Electronic

6715). For test tubes, an additional cleaning step was added: test tubes were sonicated (Ultra-sonic cleaner. Mettler Electronics) in a solution of Decon 75 concentrate (BDH Chemicals) before the dishwasher wash. All glassware was then air-dried at a temperature of 250°C in a mechanical convection oven (Model 28, Precision Scientific Group, Baxter Corp., Edmonton, AB, Canada).

2.3.3 Homogenizer

A combination of a TRI-R S63 C variable speed laboratory motor (Tri-R Instruments, Rockville, NY, USA) with a Teflon pestle and a glass grinding tube was used for homogenizing samples.

2.3.4 Shaker-Mixers

Three types of vortex-shakers were used: a Thermolyne Maxi Mix 1 vortex mixer (Sybron Corp) for individual tubes and for larger volume vortexing; a benchtop Thermolyne Type 1000 (Sybron); and a bench top Thermix Model 210T mixer (Fisher Scientific, Fairlawn, NJ, USA).

2.3.5 Pipetters

The pipetters were of two types: variable volume (20, 200, 1000 μl; Gilson, France) and repeated volume (1.25, 5.0, 12.5, 50.0 ml capacity; Eppendorf, USA).

2.3.6 pH Meter

The pH values of the buffer solutions were determined using an Accumet Model 915 pH meter (Fisher Scientific, Fairlawn, NJ, USA) standardized with a certified buffer solution (pH 7.00 ± 0.01, 23°C, Fisher Scientific).

2.3.7 Centrifuges

Small volume, high speed centrifugations were performed using an MSE Micro-Centaur benchtop centrifuge (Baxter Corp., Edmonton, AB, Canada). Larger volume centrifugations requiring refrigeration were carried out in a Damon-1EC-B-20 refrigerated high-speed centrifuge or a Beckman L755 vacuum refrigerated ultracentrifuge. Large volume centrifugations not requiring refrigeration were carried out using a Du Pont Instruments Sorvall GLC-2B General Laboratories centrifuge.

2.3.8 Weighing Balances

A Metler AE 160 electronic balance (0.1 mg sensitivity, Mettler Instrument Co., Highstown, NJ, USA) was used for weighing chemicals and biological samples. Animals were weighed on a 700 series triple beam balance with a 2610 g capacity (Ohaus, Florham Park, NJ, USA).

2.4 Animals

2.4.1 Strain

All animal experiments conducted for this thesis utilized drug-naive, male albino Sprague-Dawley rats weighing 200-350 g, purchased from BioScience Animal Services (Ellerslie, AB).

2.4.2 Housing

The animals were individually or group housed (2 per cage) under a 12 h light/dark cycle at a temperature of 20 ± 1°C with free access to food and water. The animal feed (Lab-Blox feed, Wayne Feed Division, Continental Grain Co., Chicago, USA) composition was 4.0% crude fat (minimum), 4.5% crude fiber (maximum) and 24% crude protein (minimum).

2.4.3 Drug Administration

In all of the studies described in this thesis, animals were randomly allocated to drug or vehicle treatment conditions. Acute drug administration was by i.p. (intraperitoneal) injection with a tuberculin 1 ml syringe equipped with a 27 G $^1/_2$" needle (Becton Dickinson, Closter, NJ, USA). Drugs were dissolved in saline or double distilled water unless otherwise stated, and all control animals were injected with the corresponding vehicle.

Chronic administration studies employed osmotic minipumps for drug delivery. Each animal was deeply anesthetized with metaphane, and an

osmotic minipump (Alzet 2ML4, Alzo Corp., Palo Alto, CA) was implanted s.c. (subcutaneously) in the dorsal thoracic region. Prior to surgery, each pump was filled with a drug solution or with the distilled water vehicle, according to each animals' group allocation, to provide constant infusion over the course of the study. A computer program for weight-adjusted filling concentrations yielded the concentration of drug for each pump to ensure delivery of the required daily dose (Greenshaw, 1986). The incisions were sutured and, after recovery from the anesthesia, the animals were placed in normal housing conditions.

2.4.4 Animal Sample Collection and Storage

Following the experimental manipulations, the animals were killed by rapid decapitation and their brains removed immediately to isopentane over solid carbon dioxide. GABA content has been shown to rise appreciably in rat brain after death unless the brain is frozen immediately or fixed by some other method (Perry, 1982). Tissues were stored at -80°C until the time of neurochemical analysis.

2.5 Clinical Samples Collection and Storage

Patients were recruited by Dr. K. F. McKenna to participate in a clinical study of PLZ. Patients were included who were between 18-60 years of age with a DSM-III-R diagnosis of PD with or without agorapohobia who

were drug-free or had not been on any psychotropic medication for 2 weeks prior to the initiation of the study. Patients also were required to have suffered from at least 1 panic attack per week for the previous 3 weeks. Subjects were excluded if the clinical interview revealed a current or past history of major depressive episodes, an affective disorder, organic brain disorders, neurological disorders, or history of drug or alcohol abuse. Patients were prescribed PLZ at doses which would normally be prescribed by Dr. McKenna in the treatment of PD. Patients were started at a dose of 15 mg bid then increased every 3-4 days to a maximum of 45 mg bid or the highest tolerated dose below 45 mg bid. Following completion of the 8 weeks, patients had the option to withdraw from treatment or continue with the medication. Blood samples were taken from patients prior to the initiation of treatment, then at 1, 2, 4, and 8 weeks during treatment for a total of 5 samples per patient. Plasma samples were also obtained from control subjects matched for age and gender. Venous samples were collected using Vacutainers containing EDTA as the anticoagulant. The samples were centrifuged immediately at 1800 g for 10 min, the plasma retained, aliquoted and stored at -80°C until the time of analysis.

2.6 Ethical Considerations

All animal experimentation described in this thesis was approved by the University of Alberta Animal Care Committee in accordance with the

Canadian Council on Animal Care (CCAC) guidelines. All persons participating in the animal portion of the experiments had previously participated in an animal handling course conducted by Health Sciences Laboratory Animal Services.

The clinical study conducted with human volunteers was approved by the University of Alberta Faculty of Medicine Research Ethics Board. All persons participating in the handling of samples had previously participated in a Biohazard Handling course conducted in the Neurochemical Research Unit modeled after the "Universal Precautions" guidelines set out by the Centre for Disease Control/National Institutes of Health in the United States and the Laboratory Centre for Disease Control, Department of National Health and Welfare in Canada.

2.7 Analysis of GABA and ALA in Brain Tissue

A gas chromatographic (GC) assay developed by Wong et al. (1990a) was used for the simultaneous analysis of GABA and ALA. Tissues were prepared for analysis as follows: a 225 μl aliquot of homogenate (in 5 volumes ddH_2O) was vortexed with 25 μl 1N $HClO_4$ containing 100 mg% disodium EDTA and 0.05 mM ascorbic acid, then centrifuged at 10,000 x g for 20 min to remove precipitated protein. A portion (25 μl) of clear supernatant was then used in the analysis. Norleucine (0.25 μg) was added as an internal standard to the supernatant; this was followed by the addition

of 1 ml of 2.5% w/v potassium carbonate solution. One ml of an isobutylchloroformate solution (5 μl isobutylchloroformate in 1ml of toluene:acetonitrile, 9:1 v/v) was added and these solutions were vortexed for 10 min at room temperature. After centrifuging for 2 min at 2500 rpm, the top (organic) layer was aspirated and discarded. To the bottom (aqueous) phase was added 1.5 ml of sodium phosphate buffer (2 M, pH 5-6) followed by the sequential addition of 2.5 ml of chloroform, 200 μl of dicyclohexylcarbodiimide solution (5 μl in 1 ml chloroform) and 200 μl of pentafluorophenol solution (5 μl in 1 ml chloroform). This mixture was vortexed for 15 min at room temperature and centrifuged briefly. The top (aqueous) layer was aspirated and discarded. The bottom chloroform layer was then evaporated to dryness under a stream of nitrogen at 60°C. The residue was reconstituted in 300 μl of toluene then briefly washed with 0.5 ml of ddH_2O. A 1 μl aliquot of the toluene layer was used for GC analysis. Chromatographic separation was accomplished using the following automatic temperature program: initial temperature 100°C for 0.5 min, increasing to 200°C at a rate of 25°C/min; after remaining at 200°C for 0.5 min, the temperature increased at a rate of 3°C/min to a final temperature of 230°C. The chromatographic column used was a fused silica capillary column, cross-linked 5% phenylmethylsilicone phase, 0.31mm I.D. x 25m,

1.03 μm film thickness (Hewlett Packard, Palo Alto, CA, U.S.A.). The assay procedure is summarized in Figure 5.

In plasma samples, an interfering peak with GABA prevented the quantification of GABA with this assay, although it was suitable for analysis of ALA. The assay protocol for plasma ALA was the same as described above, using 25 μl of plasma in place of supernatant and following biohazard safety precautions.

A standard (calibration) curve was prepared with each assay run to permit analysis of the amino acids of interest in the sample. The curve was constructed by adding known, varying amounts of authentic standards and a fixed amount of internal standard (same amount as added to the samples) to a series of tubes and running these tubes in parallel with the sample tubes.

2.8 Analysis of Amino Acids by HPLC

An HPLC assay with fluorescence detection allowing for the simultaneous quantification of ALA, GABA, glutamate, glutamine and glycine was employed in the analysis of rat brain tissues (Sloley et al., 1992). The mobile phase used in this assay consisted of 2700 ml NaH_2PO_4 (0.04 M), 800ml MeOH, 100 ml acetonitrile, and 70 ml tetrahydrofuran. The mixture was brought to pH 6.2 with 10N NaOH, then filtered and degassed under vacuum with 0.2 μm filters. The internal standard used in this assay was homoserine. A 100 μl portion of brain tissue, previously homogenized in 5

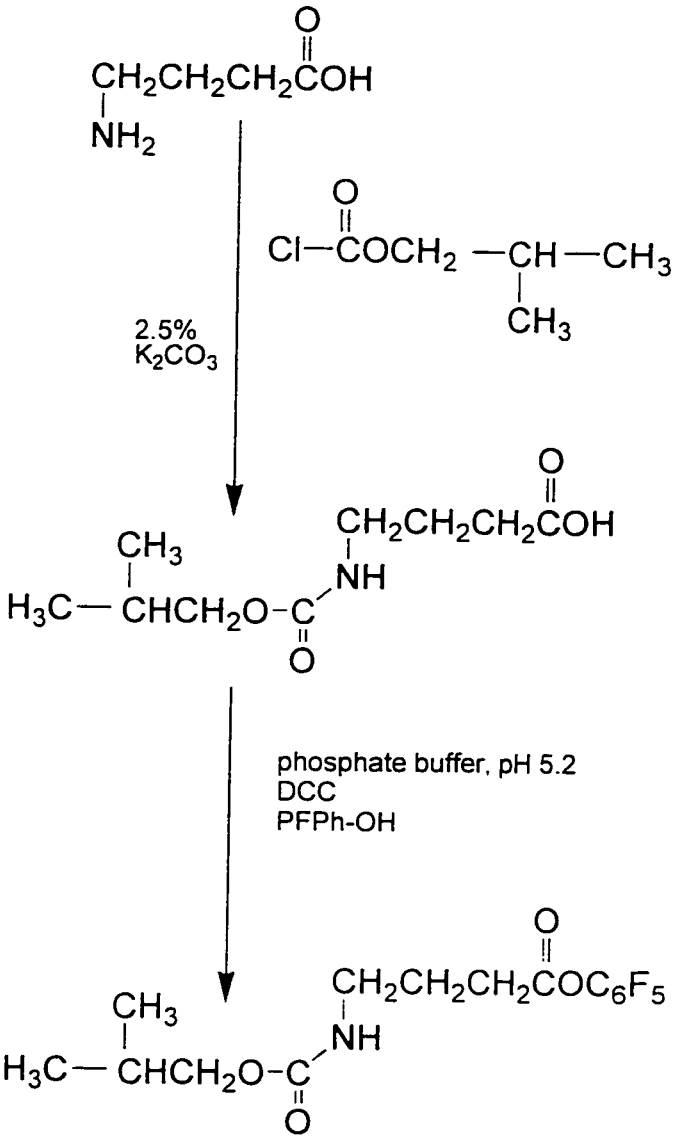


Figure 5: Schematic of assay for analysis of GABA and other aliphatic amino acids (taken from the thesis of James Wong, 1990a).

volumes of ddH_2O, was rehomogenized with 400 μl MeOH containing the internal standard. The homogenate was centrifuged and 20 μl of supernatant was retained for analysis. To the 20 μl sample, 20 μl of Fluoraldehyde OPA reagent (containing o-phthalaldehyde and mercaptoethanol in a borate buffer) was added and derivatization was allowed to occur for 30 sec. (See Figure 6 for a schematic of the reaction.) In an alkaline medium *o*-phthaldialdehyde reacts with primary amines to form highly flourescent compounds. Sodium acetate buffer (100 μl) was then added and the mixture transferred to an HPLC vial and injected and allowed to run for 60 min. Chromatographic separation was accomplished on a Spherisorb 5 ODS 2 column, 250x4.6mm, 5 μm particle size (Phenomenex) protected by a μBondapak C_{18} guard column. Excitation wavelength was set at 224 nm and emission wavelength at 455 nm.

As with the gas chromatographic assay, a standard (calibration) curve was prepared with each assay run to permit analysis of the quantity of amino acids of interest in the sample. The curve was constructed by adding known, varying amounts of authentic standard and a fixed amount of internal standard (same amount as added to the samples) to a series of tubes and running these tubes in parallel with the sample tubes.

2.9 Analysis of GABA in Plasma

Although GABA has been measured in plasma by Petty and coworkers (Petty et al., 1992), there is a paucity of information provided about the assay procedure used. The gas chromatographic and HPLC procedures usually used in our laboratories for brain tissue and microdialysis were unsatisfactory for plasma measurements because of interfering peaks. Therefore, it was necessary to develop a new assay for GABA for the purposes of conducting the plasma work.

Sample handling was as follows: 150 µl of 4N $HClO_4$ (containing 0.5 mM ascorbic acid and 100 mg% EDTA) was added to 1.35 ml of plasma on ice. The mixture was immediately vortexed then centrifuged for 2 min in the microfuge. The supernatant was immediately transferred to a clean microfuge tube and 0.9 ml of supernatant was used in the assay.

To the 0.9 ml of supernatant, standard or blank was added 500 µl 25% K_2CO_3. The tubes were then vortexed, briefly centrifuged and decanted to clean tubes containing 2.5 ml 2.5% of the liquid ion-pairing agent di(2-ethylphenyl) phosphate (DEHPA) in chloroform, vortexed and centrifuged 5 min. The aqueous layer was pipetted to a microfuge tube and centrifuged for 2 min; 100 µl were then transferred to insert vials and used in the automated system.

The analysis of GABA in plasma was accomplished with high pressure liquid chromatography with fluorescence detection (see Figure 6). The mobile phase consisted of 670 ml 0.2M NaH_2PO_4, 555 ml MeOH, and 30 ml tetrathydrofuran, brought to pH 6.2 with 10N NaOH. The mixture was filtered and degassed under vacuum with 0.2 μm filters. No internal standard was used in this assay. A calibration curve (0-200 ng GABA) was prepared in parallel with each assay, using 0.4 N $HClO_4$ with EDTA and ascorbic acid, and run to permit analysis of the quantity of GABA in the samples. Prior to injection, sample, standard, or blank (10 μl) was taken up and 10 μl of Fluoraldehyde OPA reagent (containing o-phthalaldehyde and mercapto-ethanol in a borate buffer) added to it. The sample was then held in the loop for 1.5 min before being injected on to the column. Column conditions and flow rate are outlined in Section 2.2.2.

2.10 Analysis of ALA-T

ALA-T activity was measured using a continuous spectrophotometric assay adapted from Hφrder and Rej (1983). ALA-T catalyses the transamination reaction of ALA and α-ketoglutarate during which pyruvate and glutamate are formed. Pyruvate is further metabolized by lactate dehydrogenase (LDH) to lactate, with the concurrent conversion of

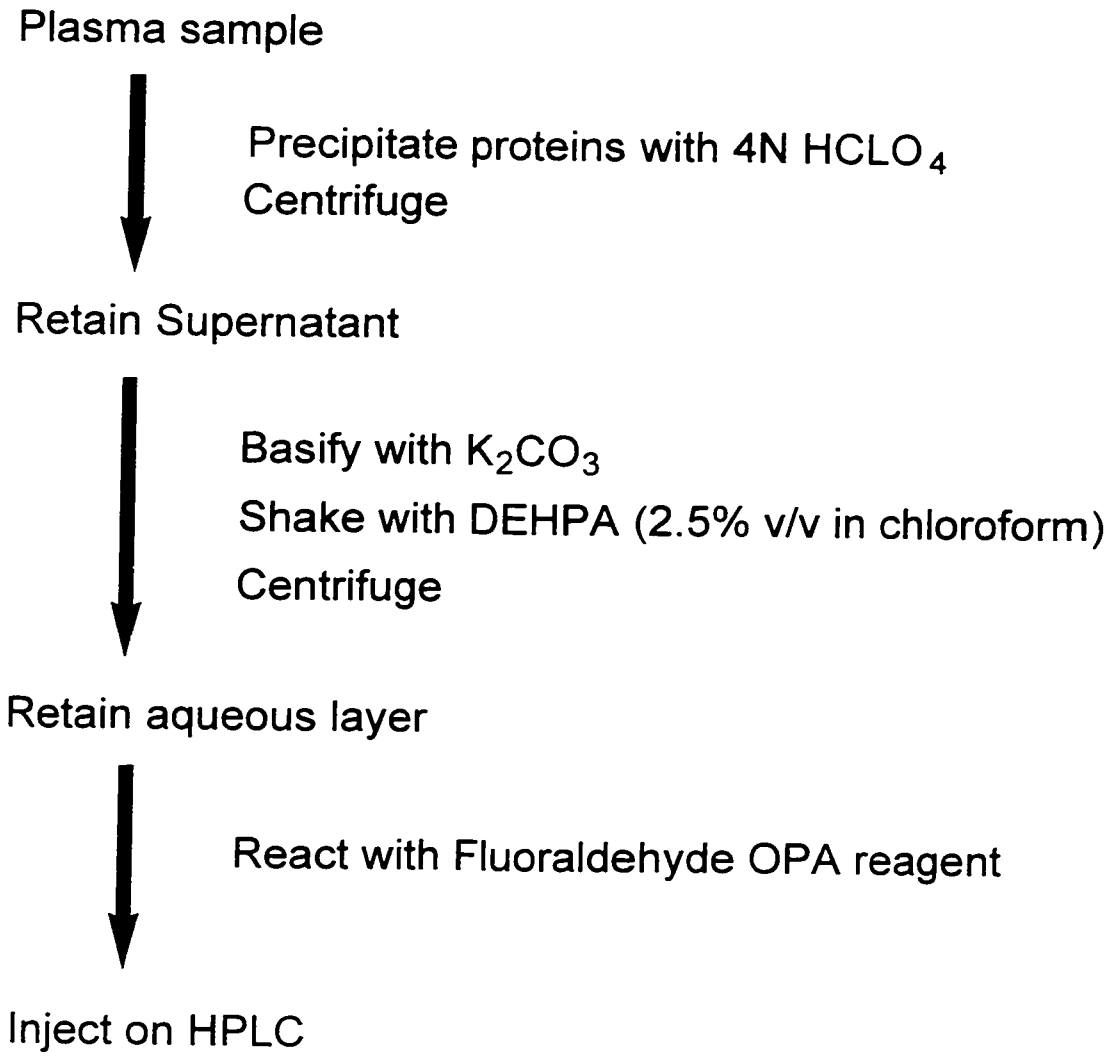


Figure 6: Analysis of plasma GABA by HPLC.

NADH to NAD. The decrease in absorbance at 340 nm due to the decrease in NADH is an indirect measure of the rate of metabolism of ALA by ALA-T. Compositions of the working reagents were as follows: the NADH/PLP/LDH solution was obtained by dissolving 5.1 mg NADH in 16 ml Tris (0.1 M, pH 7.5) with 2 ml PLP (28.8 μM) and 2 ml LDH (0.27 μg). The substrate solution consisted of 10 ml NADH/PLP/ LDH with 10 ml L-ALA (10 mM) in Tris. The blank solution consisted of 10 ml NADH/PLP/LDH solution and 10 ml Tris. For analysis in brain tissue, a homogenate of tissue in 5 volumes dH_2O was diluted 1:9 with Tris buffer. Substrate solution (2 ml) was added to the sample glass cuvette and 2 ml of blank solution was added to blank glass cuvette with 200 μl of homogenate. Both cuvettes were allowed to come to 37°C in the spectrophotometer before adding 200 μl of 2-oxoglutarate (180 mM) to each cuvette and mixing briefly. Cuvettes were scanned for 30 min at 340 nm.

2.11 Analysis of MAO-A and MAO-B

Measurement of activity levels of MAO-A and MAO-B was done using a radiochemical assay protocol adapted from Lyles and Callingham (1982). Homogenate (source of MAO) is incubated with radiolabelled substrates. Some radiolabelled amine is converted to aldehyde (and/or acid, or alcohol). Unreacted amine is protonated with addition of HCl that also terminates the reaction. Organic solvent is added and the radioactive metabolites move into

the organic phase while the amine does not. Radioactivity is then measured in a sample of the solvent. The reaction is carried out at 37 °C (body temperature) and at pH 7.8.

Tissues were homogenized initially in 5 volumes of ice-cold distilled water. Homogenate was diluted in 16 volumes of 0.2M potassium phosphate buffer (pH 7.8) for a total dilution of 80 ml per gram of tissue. Assays were carried out in triplicate in glass tubes on ice. Radioactive substrate (50 µl) was added to 50 µl of homogenate in buffer. The substrates used in this reaction were ^{3}H- or ^{14}C- 5-HT for MAO-A and ^{14}C- PEA for MAO-B. HCl (10 µl of 3M) was immediately added to the blank tubes containing drug-naive tissue homogenate. A gentle stream of oxygen was briefly directed into the tubes which were then stoppered and incubated in a 37 °C water bath for 10 min. The tubes were then removed to ice water and 3M HCl (10 µl) immediately added. One ml of ethyl acetate/toluene (1:1 v/v, water-saturated) was added to each tube and the mixture briefly vortexed twice. The samples were centrifuged in a Sorval centrifuge at 1600 rpm for approximately 30 sec and 700 µl of the organic layer was pipetted into vials with 4 ml scintillation fluid. Radioactivity was measured in a liquid scintillation spectrometer.

2.12 Analysis of GABA-Transaminase

GABA-transaminase (GABA-T) activity was measured using a procedure that was a modification of the one developed by Sterri and Fonnum (1978) (see Figure 7). Homogenate (source of GABA-T) was incubated with radiolabelled GABA. The assay involved a two-step reaction: first, GABA-T converted GABA and oxoglutarate to succinic semialdehyde and glutamate; second, the radiolabelled succinic semialdehyde was converted to succinate by succinic semialdehyde dehydrogenase and NAD. Radiolabelled succinate was then extracted with a liquid anion exchanger and the amount of radioactivity extracted was measured using a liquid scintillation counter. Radiolabelled glutamate formed in the first reaction was prevented from forming new radiolabelled GABA by the addition of a sulphydryl reagent, dithiothreitol.

Tissues were initially homogenized in 5 volumes of distilled water. Homogenate (25 μl) was then diluted with 75 μl of ice-cold homogenizing medium. Composition of the homogenizing medium was as follows: 50 ml of glycerol, 2.5 ml Triton X100 (13%), 2.5 ml of 10 mM glutathione, 250 μl of 1 mM pyridoxal-5-phosphate, 8.15 ml of 143 mM K_2HPO_4, 25 ml of 10 mM EDTA, and 161.6 ml of dH_2O. Composition of the incubation medium was as follows: 2.4 μl of [^{3}H]-GABA (30 Ci/mmol), 18 μl of 100 mM GABA, 40 μl of 50 mM α-ketoglutarate, 40 μl of 10mM nicotinamide adenosine dinucleotide,

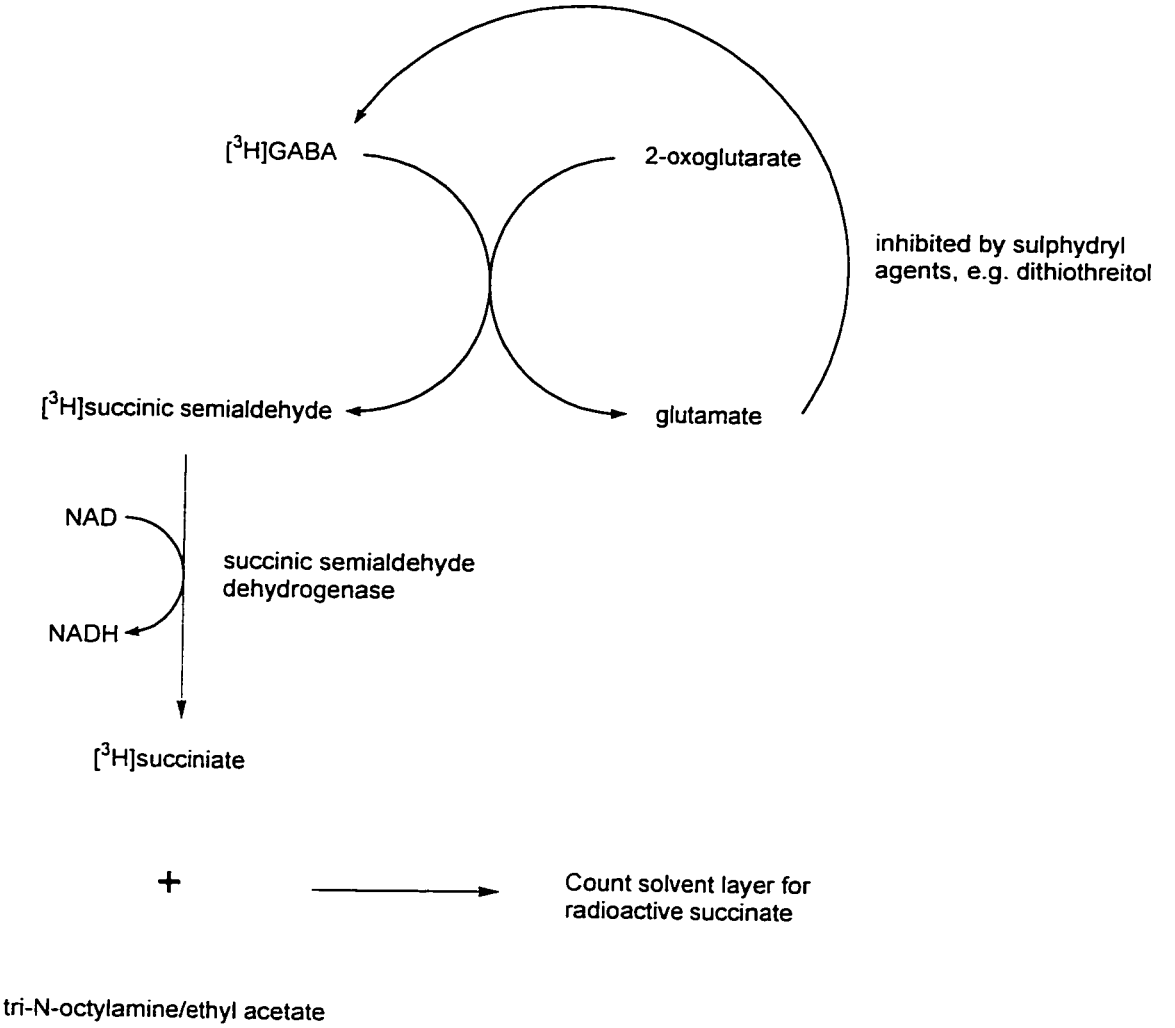


Figure 7: Schematic of the GABA-T assay.

40 µl of 10 mM 2-aminoethylisothio-uronium bromide, 160 µl of distilled water and 100 µl of 1 M Tris buffer (pH= 7.9). Homogenate (10 µl) (distilled water to blanks) and 19 µl of incubation medium were added to 1.5 ml microfuge tubes placed on ice. After 5 min, 5 µl of ^{3}H GABA stock (consisting of 2.4 µl of ^{3}H GABA, 18 µl of 100 mM GABA and 63.6 µl of dH_2O) was added to each tube. The tubes were incubated in a 37 °C water bath for 30 min. Samples were removed to ice and 100 µl of tri-n-octylamine (TOA) was added. The mixture was vortexed briefly then centrifuged at 1,000 x g for 2 min. 35 µl of the top layer was carefully drawn off and added to a counting vial containing 4 ml of scintillation fluid. Radioactivity was measured in a liquid scintillation spectrometer.

2.13 Analysis of ^{36}Chloride Uptake

Measurement of $^{36}Cl^-$ uptake in cerebral cortex from rats treated for 21 days with PLZ (15 mg/kg), DMI (10 mg/kg) or vehicle was completed in Dr. S. Dunn's lab (Department of Pharmacology) in collaboration with Dr. Martin Davies. GABA receptor-mediated $^{36}Cl^-$ uptake has been used to measure the effects of various mediators of the $GABA_A$ receptor (Luu et al., 1987; Heninger et al., 1990; Wood and Davies, 1991; Kang and Miller, 1991). Synaptoneurosomes were prepared from rat cerebral cortex according to a procedure by Schwartz et al. (1985) with minor modifications (Wood and

Davies, 1989; Wood and Davies, 1991). Tissue (800 mg) was homogenized in 7 ml of buffer solution containing 20 mM Hepes-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, and 2.5 mM $CaCl_2$ (pH 7.4). Homogenate was transferred to a 40 ml tube and diluted with 30 ml of ice-cold buffer, then re-filtered by gentle suction through 2 layers of fluorocarbon mesh (160 μm pore size) and one layer of nylon mesh (52 μm pore size). The final filtrate was then filtered by gentle suction through a Millipore filter with 10 μm pores. The filtrate was centrifuged (1000 g for 15 min) and the resulting pellet was suspended in buffer in a manner that provided a final protein concentration of 5 mg/ml. An aliquot of 0.4 ml (2 mg of protein) was preincubated in 0.1 ml of buffer at 30°C for 20 min before 1 μCi of $^{36}Cl^-$ (16.4 mCi/mg specific activity) was added. $^{36}Cl^-$ flux was terminated 5 sec later with the addition of 5 ml ice-cold buffer containing 100 μM picrotoxin and the mixture was filtered through glass-fiber filters treated with 0.5% polyethylene-imine. The filters were washed twice with 5 ml of cold buffer, placed in scintillation vials and soaked in water (2 ml) for 20 min prior to the addition of scintillation cocktail (10 ml), and counting in a liquid scintillation counter.

2.14 Behavioural Analyses of Anxiety: The Elevated Plus Maze

Standardized procedures and analyses have been established for the elevated plus-maze model of anxiety (see Pellow et al., 1985). The testing

room was quiet and dimly lit. Data were collected by a single observer who sat quietly, one meter behind one of the closed arms of the maze. Rats were placed individually in the center of the apparatus and observed for 5 min. The observer measured time spent in the closed arms, time spent in the open arms, and number of entries into closed and open arms, with an entry defined as all four paws in the arm. Open-arm activity was quantified as the amount of time spent in the open arms relative to total amount of time spent in any arm (open/total x 100). Open-arm entries were similarly calculated using the number of entries into the open arms over the total number of entries into either open or closed arms. The maze was cleaned after each rat was tested.

2.15 Synthesis of PEH

PEH was synthesized by Dr. E. E. Knaus, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. Phenylacetaldehyde hydrazone was prepared according to a method reported by Pross and Sternhell (1970) (see Figure 8). Phenylacetaldehyde (Aldrich, > 90% purity) was distilled immediately before use (bp 70°C, 1 mm). A solution of phenylacetaldehyde (12 g, 0.1 mol) and hydrazine monohydrate (2.5 g, 0.05 mol) in ethanol (50 ml) was heated at reflux for 1 h. The reaction mixture was allowed to cool to 25°C. Water (100 ml) was then added, followed by extraction with chloroform (3 x25 ml) and washing the combined chloroform

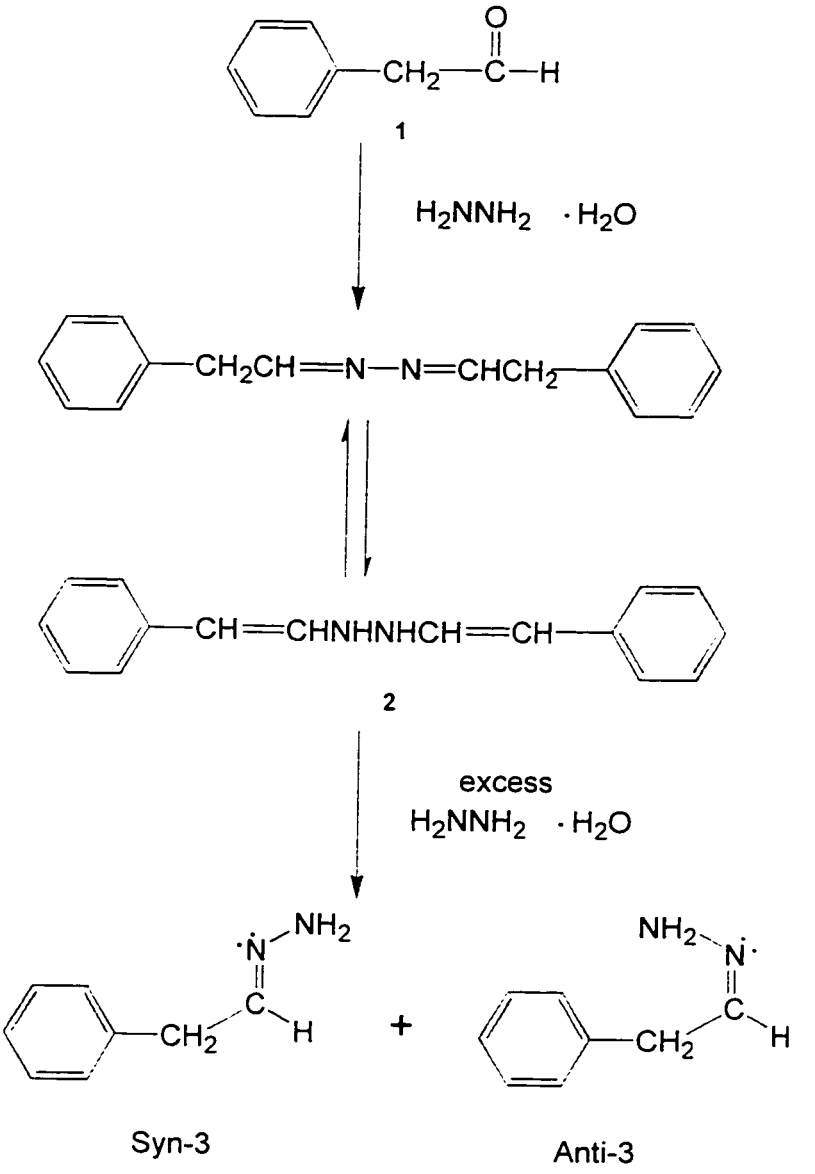


Figure 8: Preparation of Phenylethylidene hydrazine (PEH). Reaction of phenyl-acetaldehyde (1) with hydrazine hydrate yielded the azine (2), which was reacted with excess hydrazine hydrate to produce PEH (79% yield) shown by ^{1}H NMR spectrometry to exist as a mixture of the two isomers *syn-3* and *anti-3* in a ratio of 67:33 at 25 °C (from Dr. E.E. Knaus).

extracts with water (50 ml). The chloroform layer was dried (anhydrous K_2CO_3), and the solvent removed *in vacuo* to produce the impure azine, which was purified by recrystallization from ethanol (2.4 g, 10% yield, mp 60 °C). A solution of the azine in ethanol (10 ml) was added to hydrazine monohydrate (20 ml), and this mixture was heated at 100°C for 1 h prior to cooling to 25°C followed by the addition of water (20 ml). The azine mixture was extracted with chloroform (3 x 20 ml), and the combined chloroform solution was washed with water (25 ml). The chloroform layer was dried down (anhydrous K_2CO_3), and the solvent removed *in vacuo* to produce phenylacetaldehyde hydrazone as an oil (0.9 g, 79% yield) which was stored at -78°C. The ^1H NMR spectrum for phenylacetaldehyde hydrazone indicated that it existed as a mixture of *syn-3* and *anti-3* isomers (ratio of 67:33) at 25 °C.

2.16 Statistical Analyses

All data were analyzed using between-groups, 1-way analysis of variance (ANOVA). When an overall ANOVA reached statistical significance ($p<0.05$), pairwise comparisons of means were run (Tukey HSD tests, $\alpha=0.05$). The clinical study data were also analyzed using repeated measures ANOVA and, when overall significance reached $p<0.05$, Newman

Keuls post-hoc comparisons were run to establish significance between pairs of means.

3 Results

3.1 The Effect of PLZ on GABA and its Precursors, Glutamate and Glutamine

3.1.1 Acute PLZ Time Course

Levels of GABA, glutamate and glutamine were measured in hypothalamus at 1, 3, 6 and 12 h post-injection of PLZ (15 mg/kg) i.p. Hypothalamus was chosen because of its high concentration of GABA, and its ease of accessibility from whole brain, thus minimizing psotmprtem changes in GABA levels. GABA levels were significantly higher than controls at 3 h and 6 h post-injection but by 12 h no longer differed significantly from control values. Glutamine levels were significantly lower than controls at 3 and 6 h whereas glutamate levels were significantly lower only at the 3 h time interval. By 12 h there were no significant differences between treatment and control groups (Figure 9).

A pattern of effects similar to those observed in hypothalamus was obtained in whole brain following administration of PLZ (15 mg/kg) i.p., although the time course was slightly different. GABA levels were significantly elevated at all 4 time intervals (1, 3, 6 and 12 h) post-injection, as has been observed by others in our laboratories (Baker et al., 1991; McKenna et al., 1991). Both glutamine and glutamate levels were lower

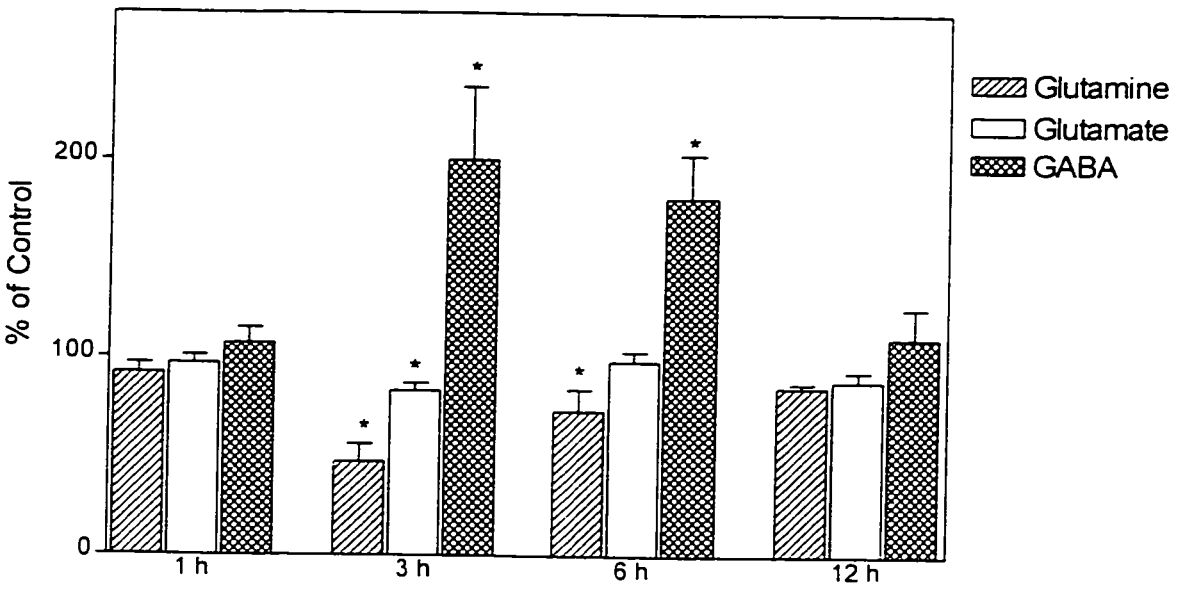


Figure 9: Hypothalamic levels of glutamine, glutamate and GABA following administration of PLZ (15 mg/kg) at 1, 3, 6 or 12 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. Mean control values were as follows: glutamine = 645.0 ± 76.0 μg/g of tissue, glutamate = 975.4 ± 67.8 μg/g, and GABA = 380.0 ± 56.1 μg/g . * denotes significant difference ($p<0.05$) from control values.

following PLZ administration, but glutamine was affected at an earlier time interval than glutamate. Glutamine levels were significantly lower than controls at 3 and 6 h post-injection and glutamate levels significantly lower at 6 and 12 h post-injection (Figure 10).

3.1.2 Acute PLZ Dose Response in Hypothalamus

The i.p. administration of PLZ at 5, 10, 15 and 30 mg/kg and sacrifice of the rats 3 h later revealed that, of the doses evaluated, GABA levels were significantly higher than controls in hypothalamus when the dose was 10 mg/kg or more. Glutamine levels were affected at all four doses examined, while glutamate levels appeared to be significantly decreased only at the 15 and 30 mg/kg doses in this experiment (Figure 11).

3.1.3 Chronic PLZ Administration

Following chronic (14 day) administration of PLZ at 15 mg/kg by osmotic pump, a pattern similar to that seen with acute PLZ administration was observed in whole brain tissue. GABA levels were higher than controls (564.3 μg/g compared to 216.7 μg/g for control values, significant at $p<0.05$), and glutamate and glutamine levels were lower than controls (256.5 μg/g glutamine compared to 483.4 μg/g control values, significant at $p<0.05$; 924.9 μg/g glutamate compared to 1263.3 μg/g for control values, significant at $p<0.05$).

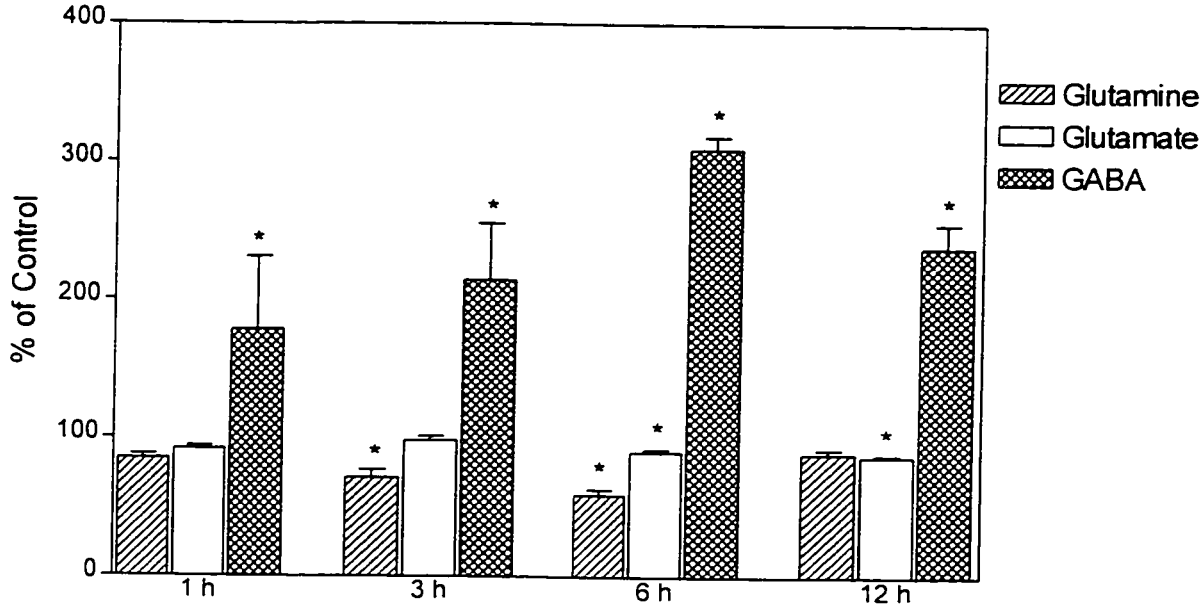


Figure 10: Whole brain levels of glutamine, glutamate and GABA following administration of PLZ (15 mg/kg) at 1, 3, 6 or 12 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. Mean control values were as follows: glutamine = 310.3.0 ± 44.7 μg/g of tissue, glutamate = 1406.9 ± 25.53 μg/g, and GABA = 229.0 ± 12.9 μg/g . * denotes significant difference ($p<0.05$) from control values.

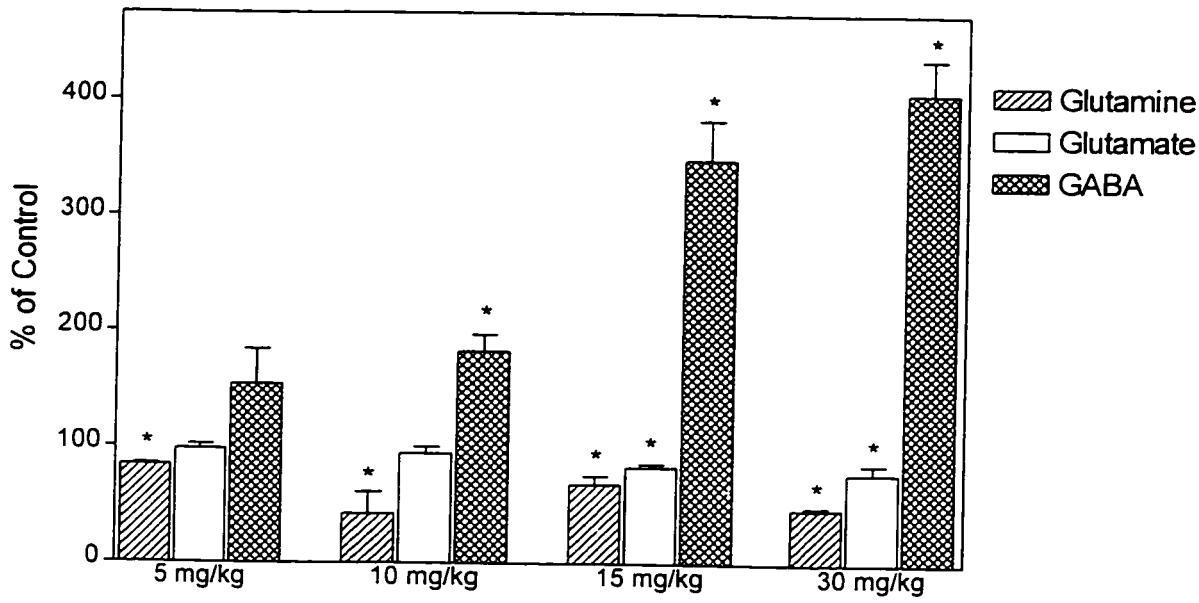


Figure 11: Hypothalamic levels of glutamine, glutamate and GABA following PLZ administration using a 5, 10, 15 or 30 mg/kg dose and a 3 h time interval post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

3.2 The Effect of PLZ on ALA levels: Acute and Chronic Administration

Both acute (3 h) and chronic (14 days) administration of PLZ (15 mg/kg) significantly elevated ALA levels in rat brain as shown in Figure 12. ALA levels inthe treated groups were more than double those of control values.

3.3 The Effect of PLZ on GABA-T and ALA-T

A dose-response study of PLZ at 3 h with 5, 10, 15 or 30 mg/kg i.p. in whole brain revealed that PLZ significantly inhibited GABA-T at doses of 10 mg/kg or more and inhibited ALA-T levels significantly at 15 mg/kg or more (Figure 13).

3.4 The Effect of PLZ on MAO-A and MAO-B

An acute dose-response study in whole brain revealed that both MAO-A and MAO-B were significantly inhibited at 3 h post-injection of PLZ at 5, 10, 15 or 30 mg/kg i.p. MAO-A levels were inhibited to a greater extent than MAO-B levels at the lower doses (Figure 14).

Chronic administration of PLZ (14 days, 15 mg/kg/day) also revealed significant inhibition of MAO-A and -B in whole brain. Inhibition of both MAO-A and -B was greater than 95%, measured as percent inhibition compared to control values (98.4% $\pm$ 0.3 for MAO-A and 99.4% $\pm$ 0.3 for MAO-B).

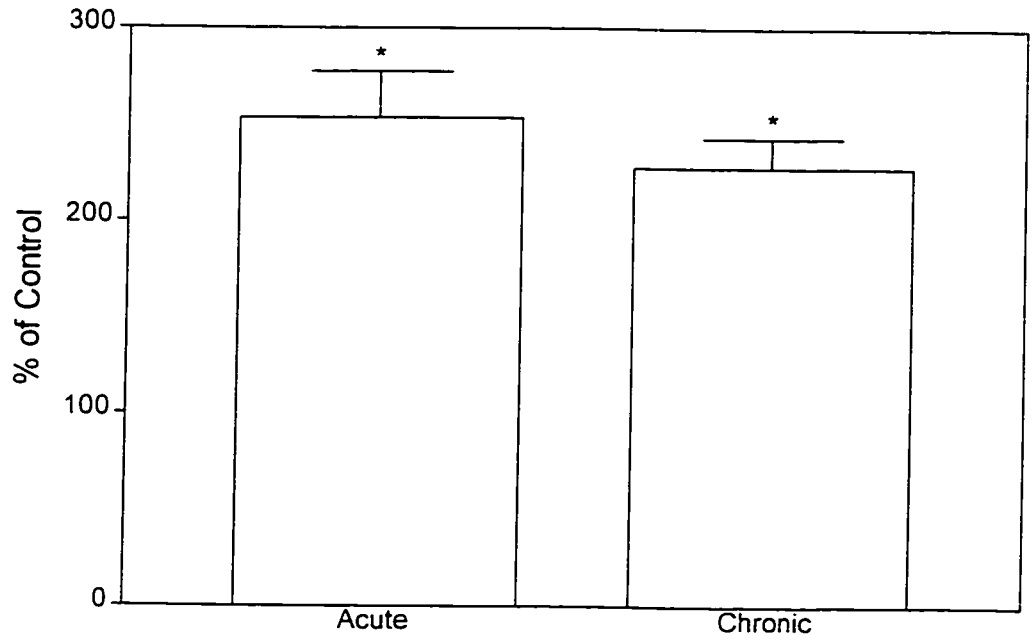


Figure 12: ALA levels in whole brain following administration of PLZ (15 mg/kg i.p.). Acute levels were at 3 h post-injection and chronic levels were after 14 days administration of PLZ by osmotic pump. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. Vehicle control values for acute and chronic ALA were 70.4 ± 7.8 and 77.1 ± 8.8 μg/g of tissue respectively. * denotes significant difference ($p<0.05$) from control values.

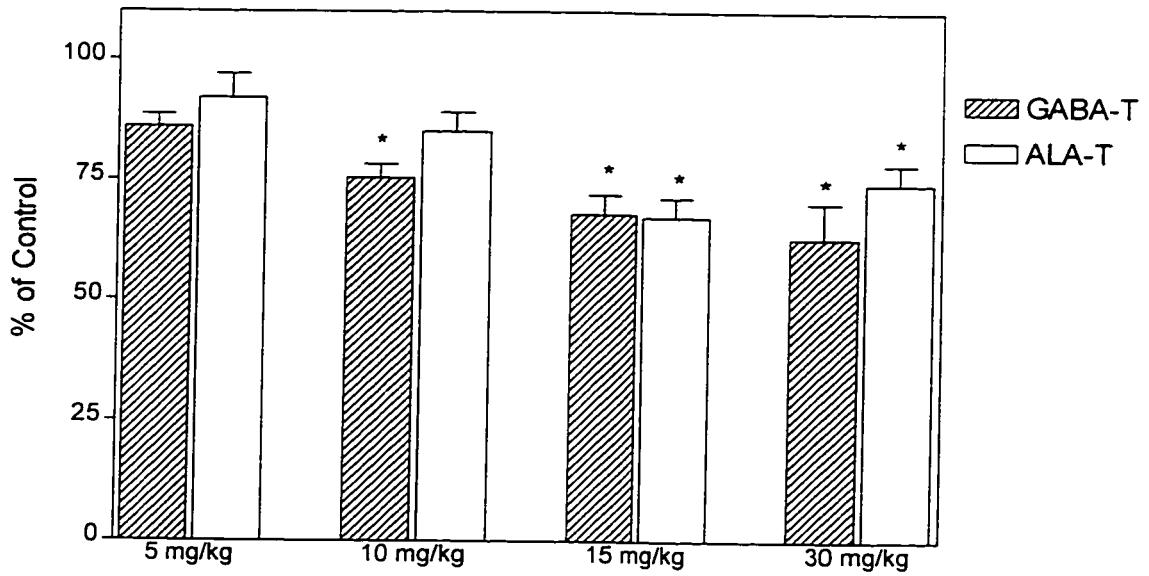


Figure 13: Inhibition of GABA-T and ALA-T in whole brain following administration of PLZ at a 5, 10, 15 or 30 mg/kg dose and a 3 h time interval post-injection. Values are expressed as % of control ± SEM compared to vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

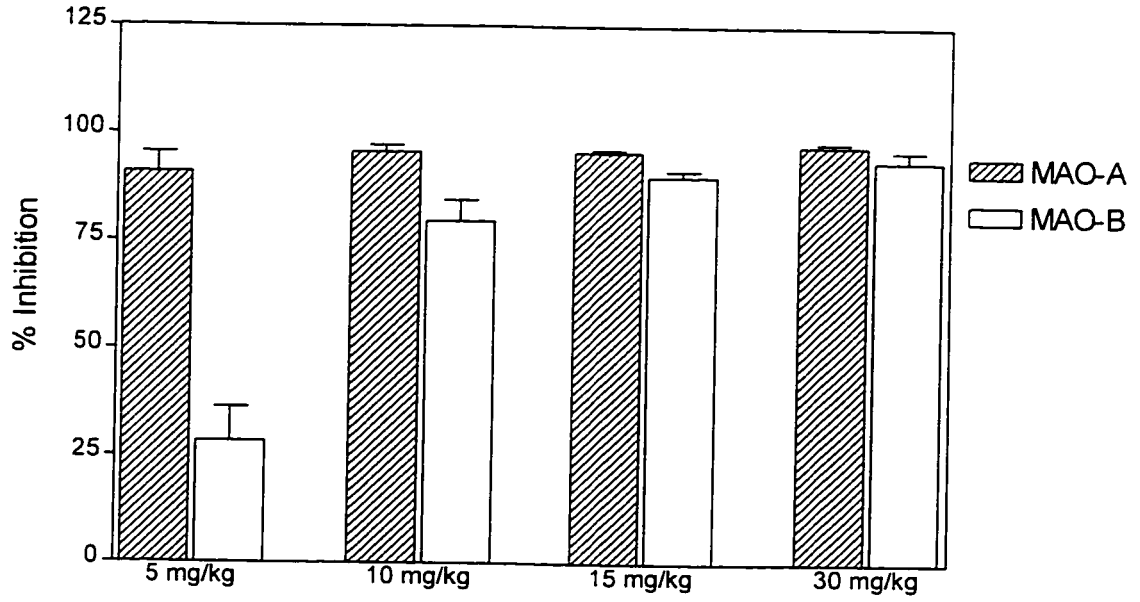


Figure 14: Inhibition of MAO-A and MAO-B following administration of PLZ at a 5, 10, 15 or 30 mg/kg dose and a 3 h time interval post-injection. Values are expressed as % inhibition ± SEM compared to vehicle-treated animals. All levels were statistically significant at $p<0.05$ compared to control values.

3.5 Effect of Pretreatment with TCP on the Actions of PLZ in Hypothalamus

Because PLZ is a substrate for MAO (see Clineschmidt and Horita, 1969a and b; Baker et al., 1992; Popov and Mathies, 1969), inhibiting MAO prior to the administration of PLZ should affect the metabolism of PLZ. The prior administration of TCP, another nonspecific irreversible MAOI, has been shown to block the PLZ-induced elevation of GABA (Popov and Mathies, 1969; Todd and Baker, 1995) and ALA (Todd and Baker, 1995). To determine if pretreatment with TCP would block the effects of PLZ on glutamine and glutamate levels, animals were pretreated with TCP (5 mg/kg i.p.) or vehicle followed one h later with PLZ (15 or 30 mg/kg i.p.) and decapitated 3 h later. Levels of amino acids were measured in hypothalamus.

The vehicle-PLZ treated group showed the same pattern of an increase in GABA and decrease in glutamine and glutamate levels, with the higher dose of PLZ showing a greater effect. TCP blocked the effects of both doses of PLZ on the amino acids (Figure 15). TCP had previously been shown to have no GABA-elevating effects when administered alone (Todd, 1994).

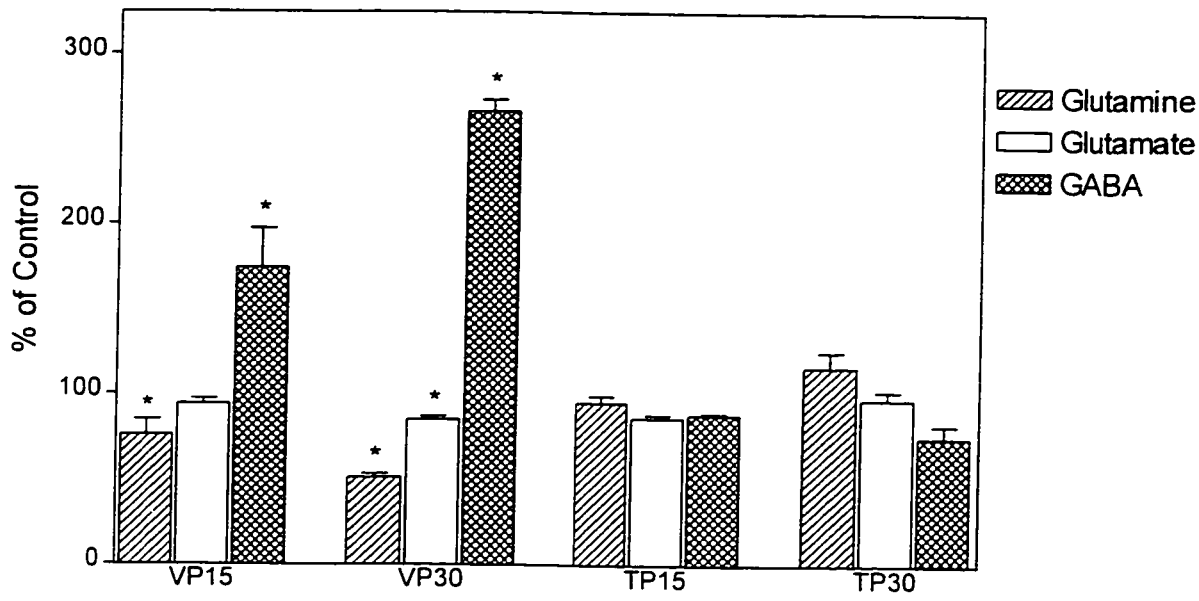


Figure 15: Hypothalamic levels of glutamine, glutamate and GABA following pretreatment with Veh or TCP (5 mg/kg) followed 1 h later by a dose of PLZ of 15 or 30 mg/kg i.p. Rats were killed 3 h after the second injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

3.6 Effect of PLZ on Cl^- Uptake

GABA-stimulated $^{36}Cl^-$ uptake was examined following chronic (21 day) administration of PLZ (15 mg/kg/day) or desipramine (DMI) (10 mg/kg/day) via osmotic pumps. Levels of GABA and activities of GABA-T, MAO-A and -B in whole brain of these rats are listed in Table 2. As expected, PLZ, but not DMI, had significant effects on GABA levels and on activities of GABA-T and MAO-A and -B. However, no significant differences were seen between either drug-treated group and controls with regard to $^{36}Cl^-$ uptake measured in cortex.

3.7 Clinical Study: Chronic PLZ Treatment of Patients with Panic Disorder

3.7.1 HPLC Assay for GABA in Plasma

Calibration curves which were run in parallel with the plasma samples in each assay were linear, and r^2 values > .99 were consistently obtained. Typical HPLC traces are shown in Figures 16 -19. The limit of detection of the assay was less than 10 pg "on column" with a signal to noise ratio of 3:1. The reproducibility of the assay was adequate, with mean between-run coefficients of variation of 10.97% (25 ng), (n=8). The within-run coefficient of variation was 1.85% (25 ng). The recovery of GABA was determined by comparing spiked 0.4N $HClO_4$ which was carried through the DEHPA clean-up to GABA samples spiked in 0.4N $HClO_4$; the mean recovery was 94.4%

	GABA	MAO-A	MAO-B	GABA-T
	μg/gm	% Inhibition		
PLZ (15 mg/kg)	492.1* (45.3)	99.6* (0.3)	99.9* (0.2)	32.2* (4.3)
DMI (10 mg/kg)	293.3 (16.6)	4.7 (5.3)	9.2 (3.3)	4.5 (2.1)
VEH	261.7 (33.24)			

Table 2: Levels of GABA expressed as μg/g of tissue and MAO-A, MAO-B and GABA-T activities expressed as % inhibition compared to controls following administration of PLZ (15 mg/kg/day), DMI (10 mg/kg/day) or vehicle for 21 days using osmotic pumps. SEM in brackets. * denotes significant difference ($p<0.05$) from control values.

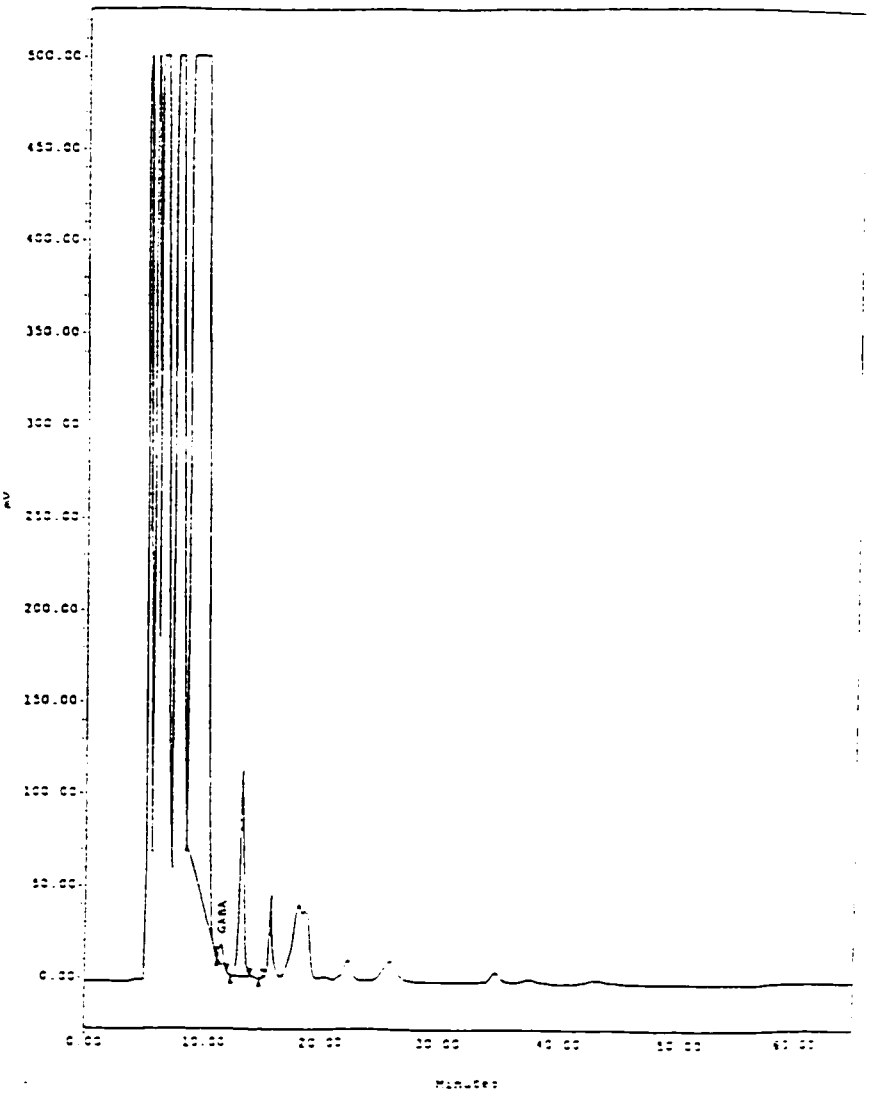


Figure 16: 50 ng standard of GABA in 0.4N perchloric acid.

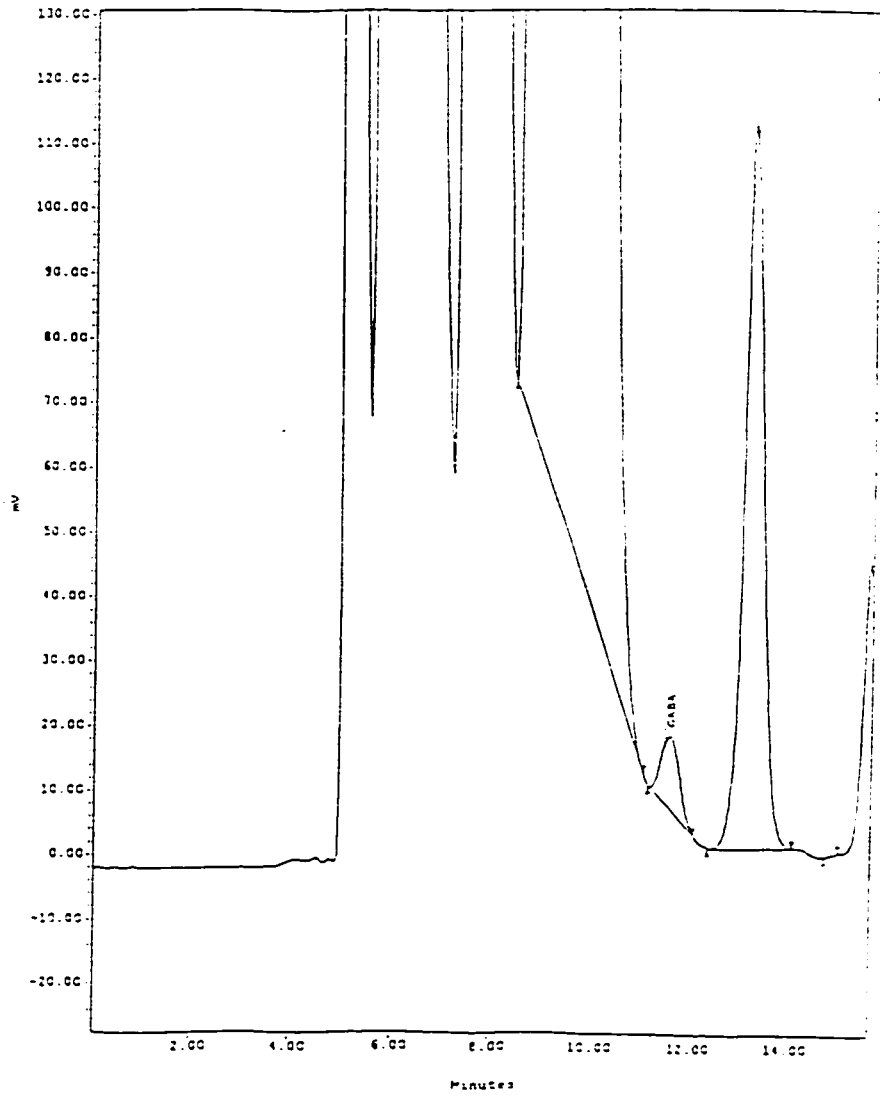


Figure 17: 50 ng standard of GABA in 0.4N perchloric acid (magnified).

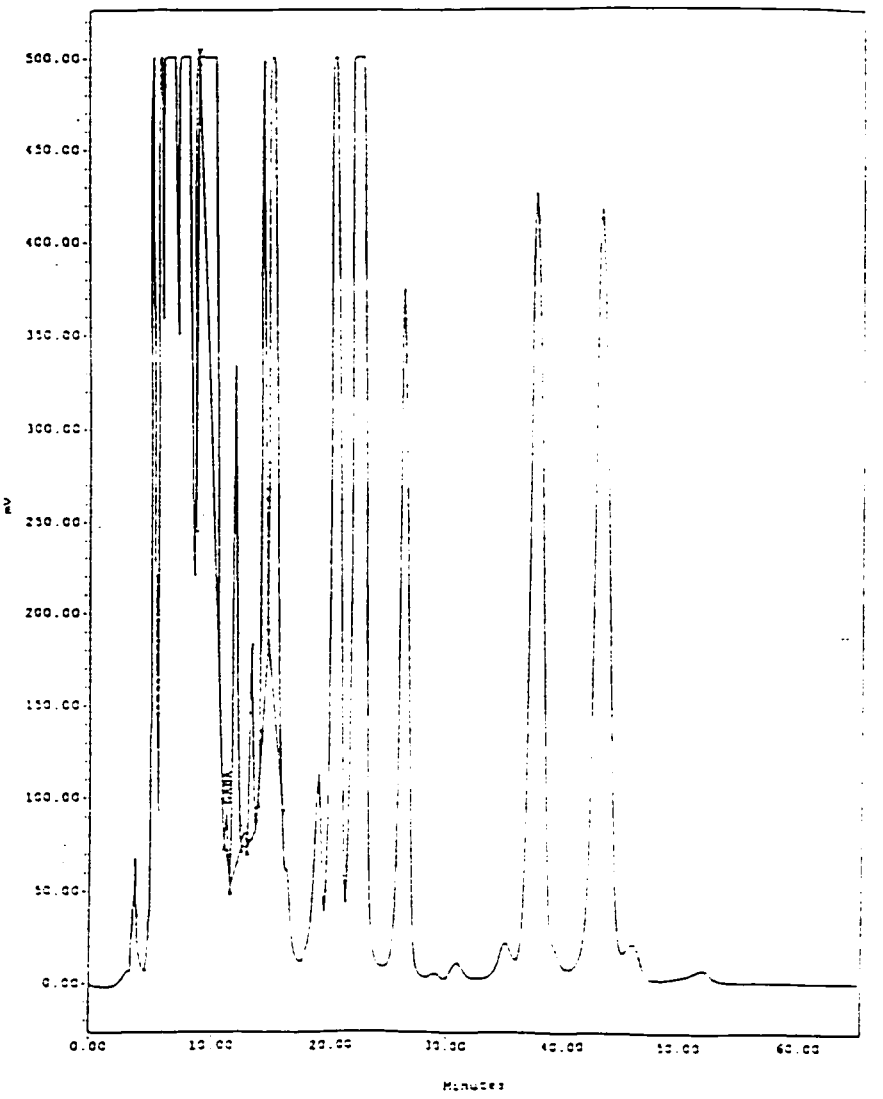


Figure 18: GABA in plasma HPLC trace, clinical baseline sample.

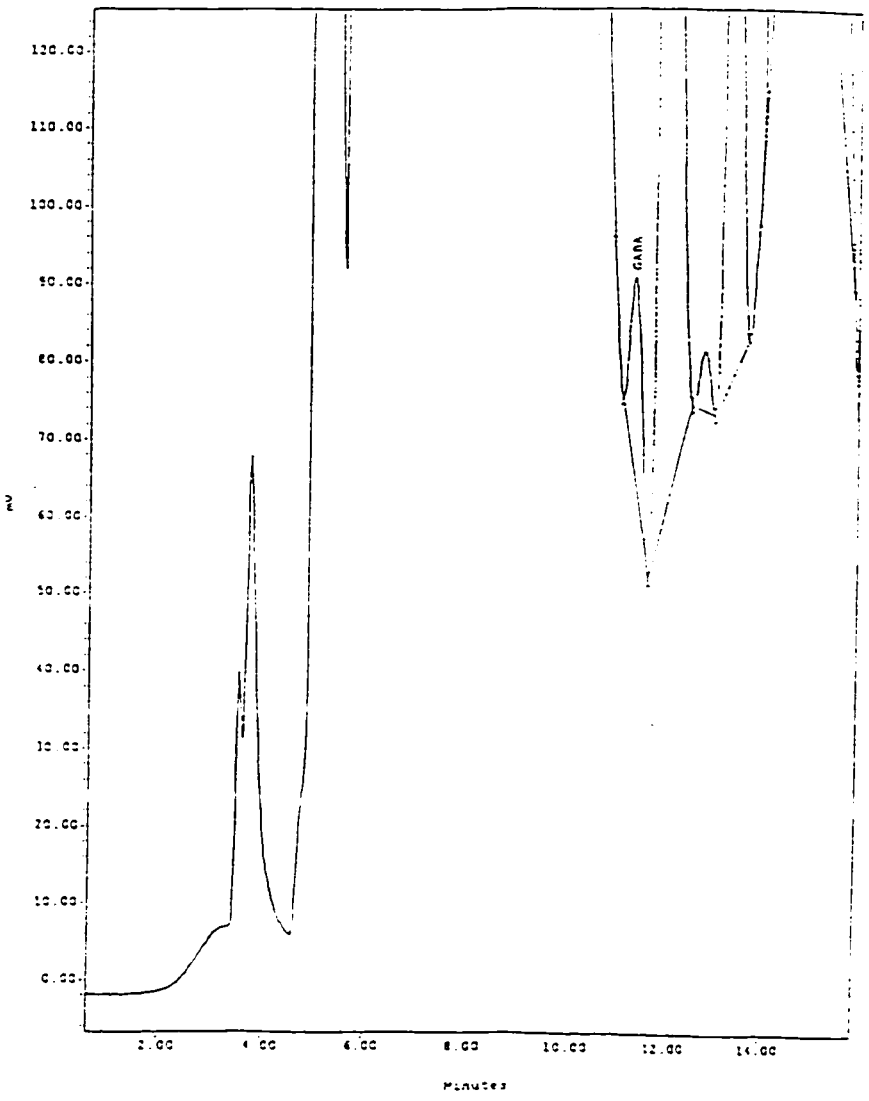


Figure 19: GABA in plasma HPLC trace, clinical baseline sample (magnified).

(n=5).

3.7.2 Clinical Results

Plasma levels of GABA and ALA were determined using blood samples taken from panic disorder patients at baseline prior to treatment, then at 1, 2, 4 and 8 weeks during treatment with PLZ. Control GABA and ALA levels were similar to those reported by others (Petty et al., 1992; Sturman and Applegarth, 1985). Changes in ALA levels failed to reach significance at $p<0.05$ (Figure 20). GABA levels in plasma were significantly higher in patients at 8 weeks of treatment compared to their baseline levels ($p<0.05$) (Figure 21).

3.8 Effects of PLZ and N^2AcPLZ in the Elevated Plus Maze

Rats in a "low dose" condition received either 5.1 mg/kg of PLZ i.p. or an equivalent volume of vehicle solution. PLZ at a dose of 5.1 mg/kg should not significantly affect GABA levels. Rats in a "high dose" condition received either 15 mg/kg PLZ i.p., an equimolar dose of N^2AcPLZ (19.6 mg/kg i.p.), or equivalent volume of vehicle solution. The time course for this acute study was approximately 2.5 h.

3.8.1 Neurochemical Results

High-dose PLZ produced a significant elevation in whole brain levels of GABA compared to vehicle controls, whereas N^2AcPLZ and the low-dose

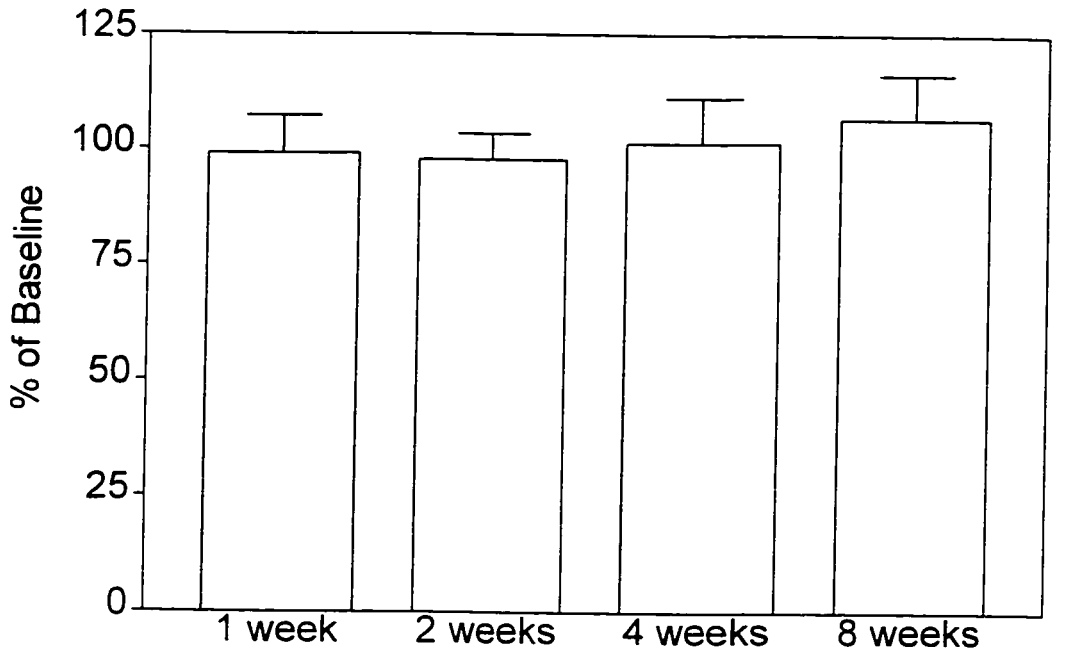


Figure 20: ALA levels in plasma of panic disorder patients (N=10) expressed as % of baseline. Patient samples were taken at baseline (prior to initiation of treatment) and 1, 2, 4, and 8 weeks during PLZ treatment.

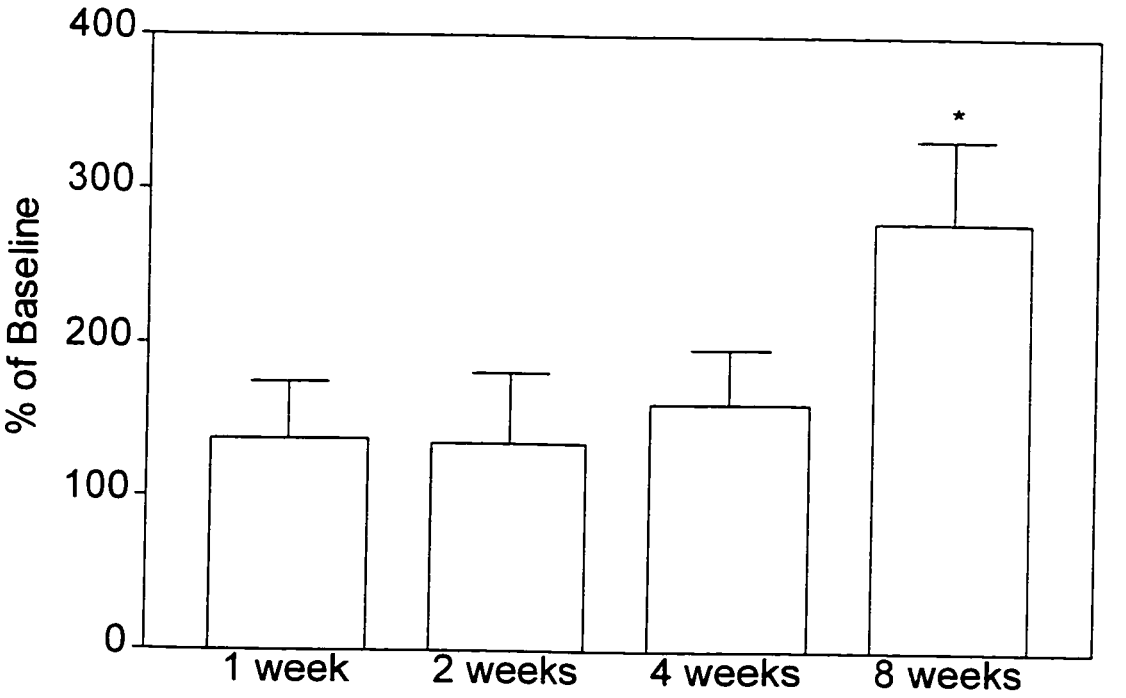


Figure 21: GABA levels in plasma of panic disorder patients (N=10) expressed as % of baseline. Patient samples were taken at baseline (prior to initiation of treatment) and 1, 2, 4, and 8 weeks during PLZ treatment. * denotes significant difference ($p<0.05$) from baseline.

PLZ condition did not (Figure 22). All three treatment groups produced significant inhibition of MAO-A and MAO-B (Figure 23).

3.8.2 Behavioral Results

The high-dose PLZ produced a substantial elevation in the percentage of time rats spent in the open arms compared to vehicle control and N^2AcPLZ conditions (Figure 24). The same pattern was seen in the percentage of open- arm entries, with the high-dose PLZ-treated animals showing a higher percent-age of open-arm entries than either N^2AcPLZ or control groups (Figure 25). No significant differences in general activity were observed among groups, as indicated by total number of arm entries (Figure 26). The low dose of PLZ did not produce a significant effect on either measure of anxiolysis or on the measure of general activity (total arm entries).

3.9 Effect of PEH on GABA and its Precursors, Glutamate and Glutamine.

3.9.1 Time Course of the Effects of PEH in Whole Brain

An acute time study was conducted in which animals were treated with vehicle, PLZ (30 mg/kg, i.p.) or PEH (30 mg/kg, i.p.) and levels of GABA, glutamate and glutamine were measured in whole brain at 1, 3, 6 or 12 h post-injection. GABA levels were higher and glutamine and glutamate

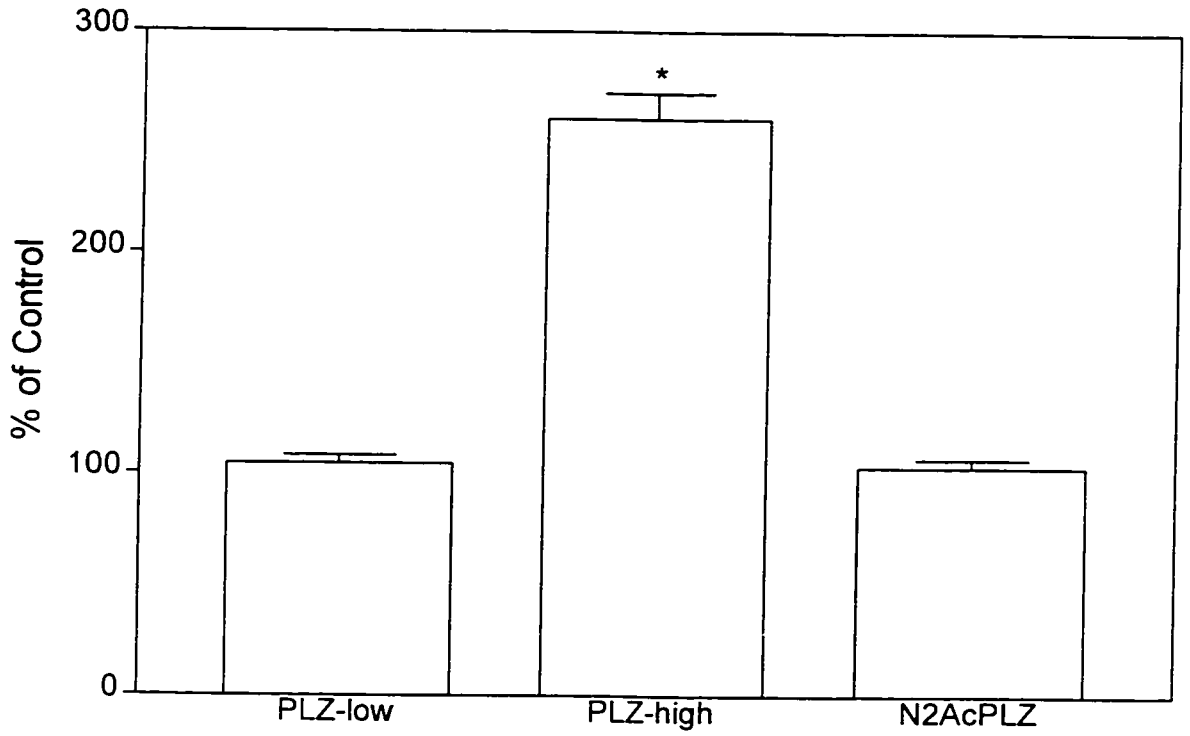


Figure 22: GABA levels in whole brain following administration of low-dose PLZ (5.1 mg/kg), high-dose PLZ (15 mg/kg i.p.), or N^2AcPLZ (19.6 mg/kg) at approximately 2.5 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. Control values were 216.7 ± 8.03 ng/g of tissue. * denotes significant difference ($p<0.05$) from control values.

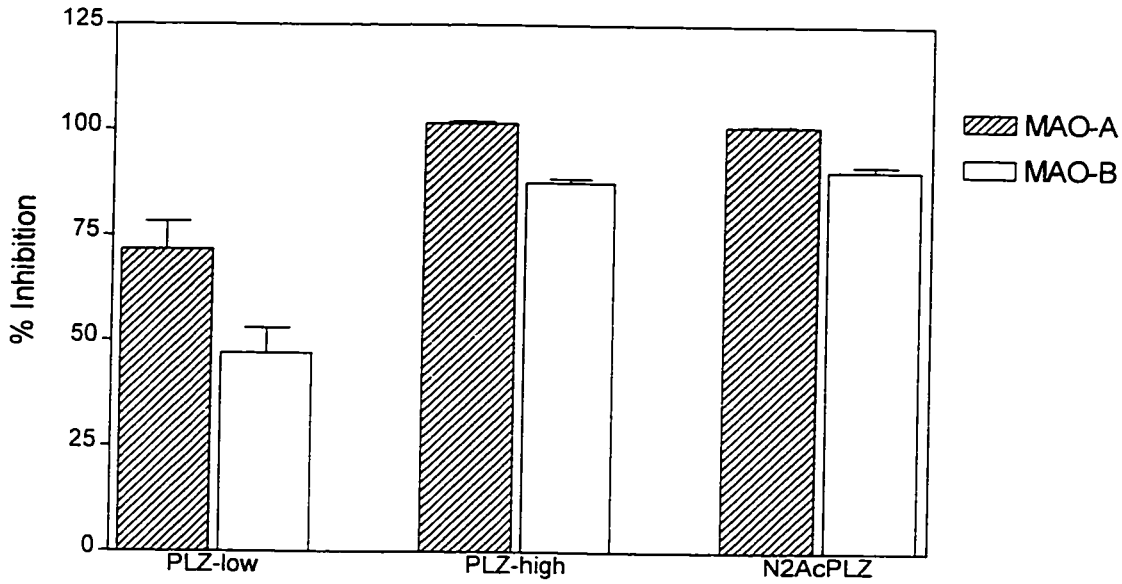


Figure 23: Inhibition of MAO-A and MAO-B following administration of PLZ (low= 5.1 mg/kg, high = 15 mg/kg), or N^2AcPLZ (19.6 mg/kg) and a 3 h time interval post-injection. Values are expressed as % inhibition ± SEM compared to vehicle-treated animals. All levels were statistically significant at $p<0.05$ compared to control values.

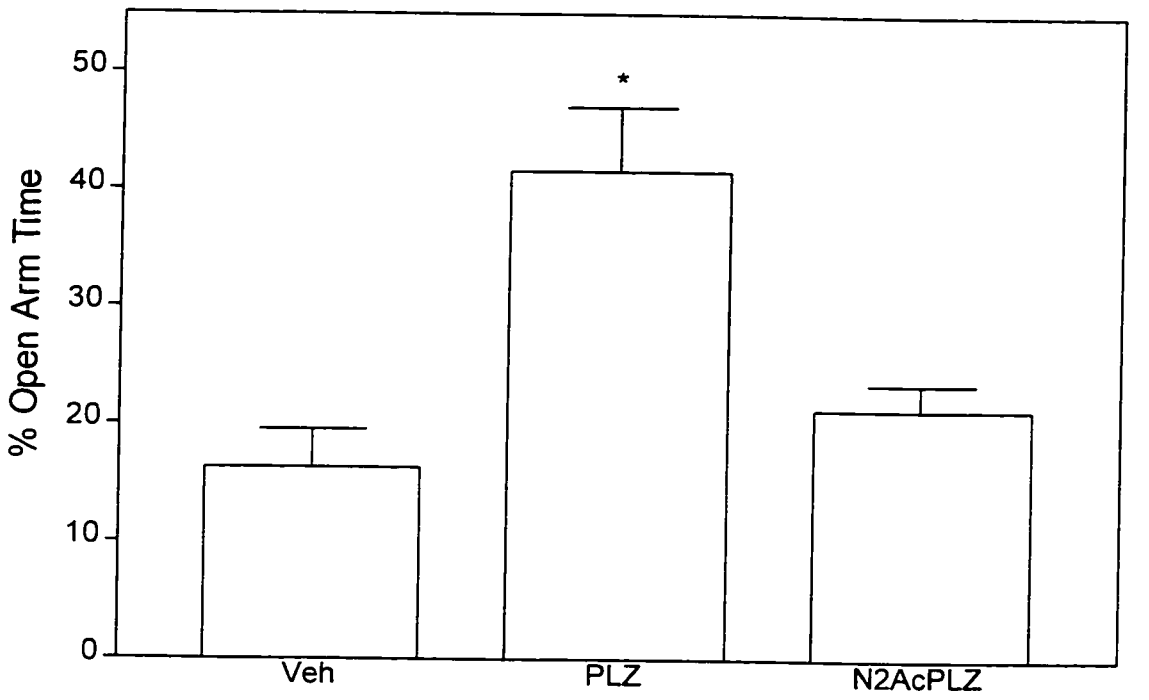


Figure 24: Time spent on open arms by groups of rats treated with PLZ (15 mg/kg), N^2AcPLZ (19.6 mg/kg) or vehicle. Values are expressed as the mean % open arm time (time spent on open arms / time spent on both open and closed arms x 100) ± SEM. * denotes significant difference ($p<0.05$) from control values.

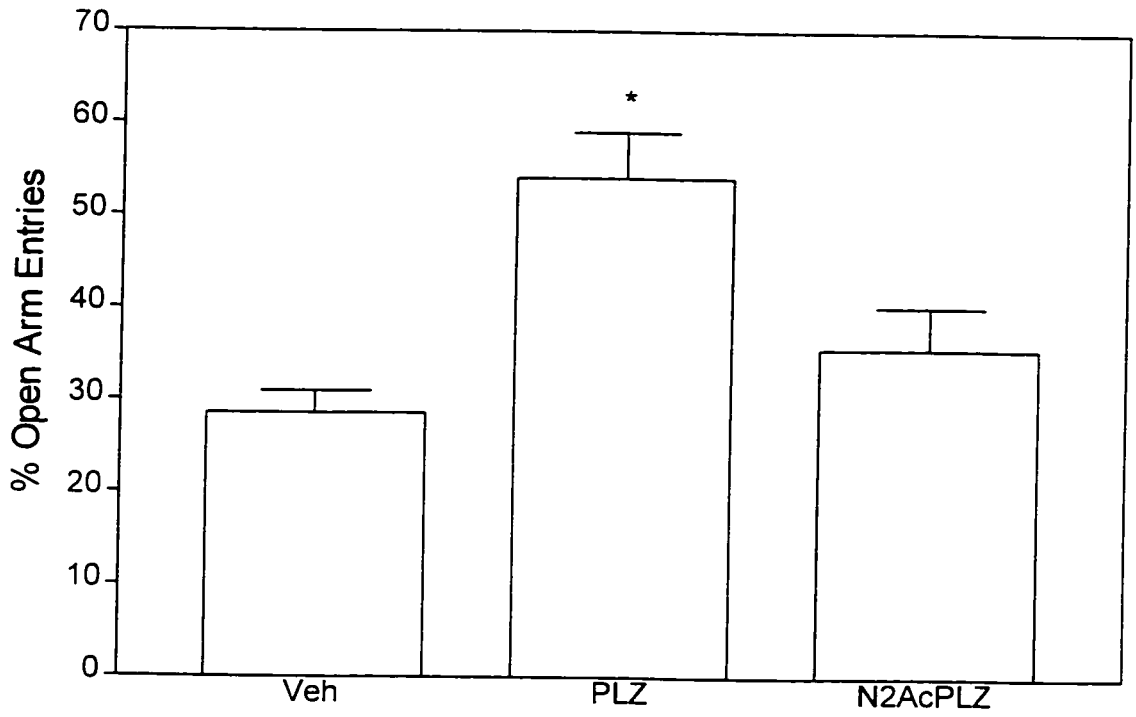


Figure 25: Entries made onto the open arms by groups of rats treated with PLZ (15 mg/kg), N^2AcPLZ (19.6 mg/kg) or vehicle. Values are expressed as the mean % open arm entries (entries made onto open arms / entries made onto both open and closed arms x 100) ± SEM. * denotes significant difference ($p<0.05$) from control values.

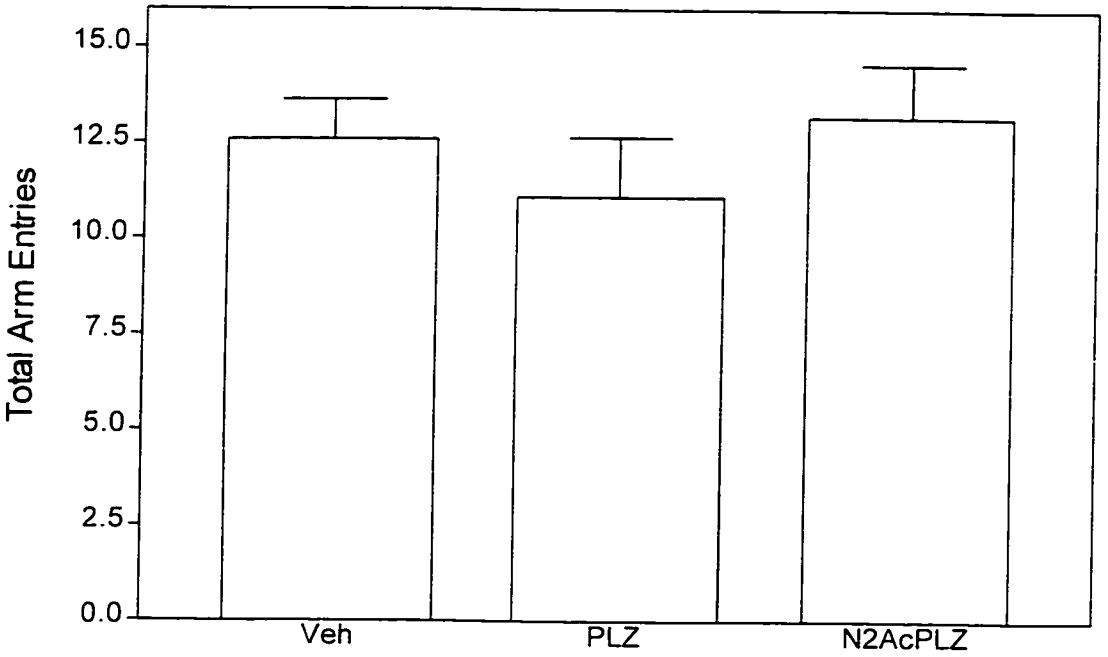


Figure 26: Total number of entries made onto both open and closed arms by groups of rats treated with PLZ (15 mg/kg), N^2AcPLZ (19.6 mg/kg) or vehicle. Values are expressed as the mean ± SEM.

significantly lower in the PLZ-treated animals as expected from our previous studies. Interestingly, this same pattern was also observed in the PEH-treated animals. GABA levels were elevated, and glutamine and glutamate levels decreased with PEH treatment (Figures 27-29).

3.9.2 Studies on PEH in Whole Brain

The i.p. administration of PEH at 5, 10, 15 and 30 mg/kg and a 3 h time interval revealed that GABA levels were significantly higher than controls when the dose was 15 mg/kg or higher. Glutamine levels were significantly lower at all administered doses while glutamate levels were significantly lower than controls only at 30 mg/kg (Figure 30).

3.9.3 TCP-pretreatment and Effects of PEH in Whole Brain

To determine if pretreatment with TCP would block the effects of PEH on GABA, glutamine and glutamate levels, a study was conducted that was similar to the TCP-PLZ experiment described previously in Section 3.5. Animals were pretreated with TCP (5 mg kg-1 i.p.) or vehicle followed one h later with PEH (30 mg kg-1 i.p.) and decapitated 3 h later. Levels were measured in whole brain.

Both the vehicle-PEH treated group and the TCP-PEH treated groups showed an increase in GABA and decrease in glutamine and glutamate levels.

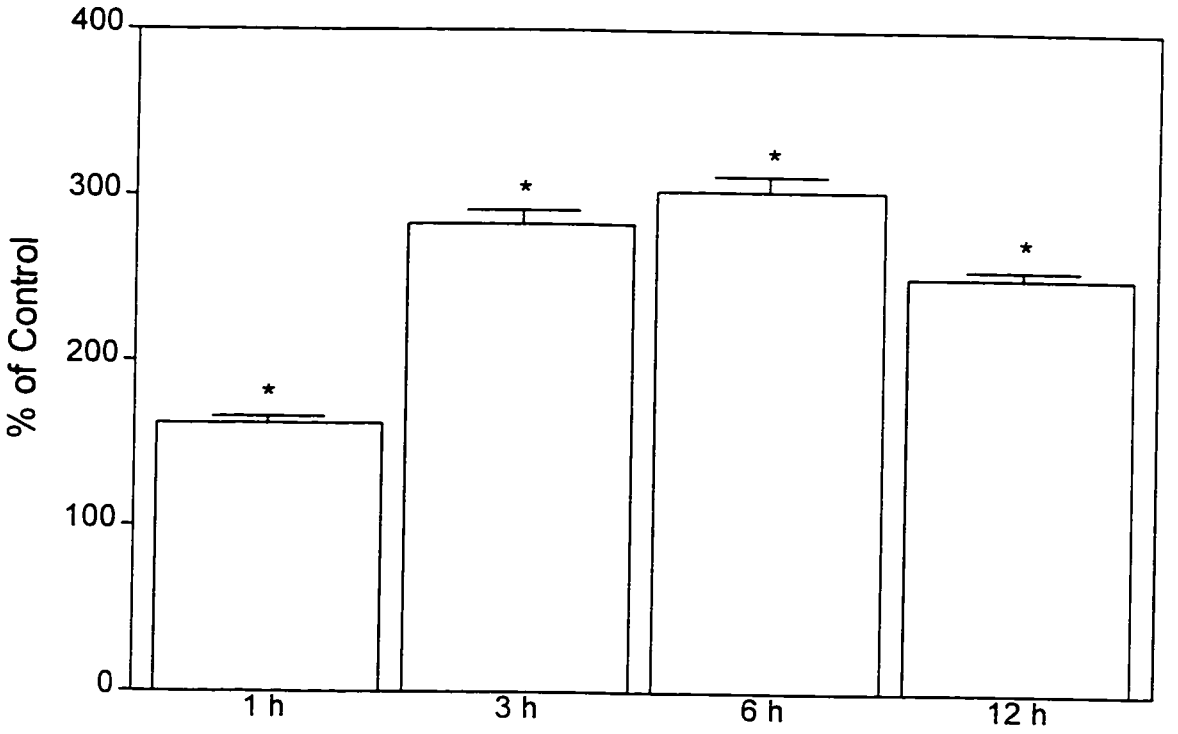


Figure 27: GABA levels in rat whole brain following administration of PEH (30 mg/kg, i.p.) at 1, 3, 6, or 12 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals at each time interval. * denotes significant difference ($p<0.05$) from control values.

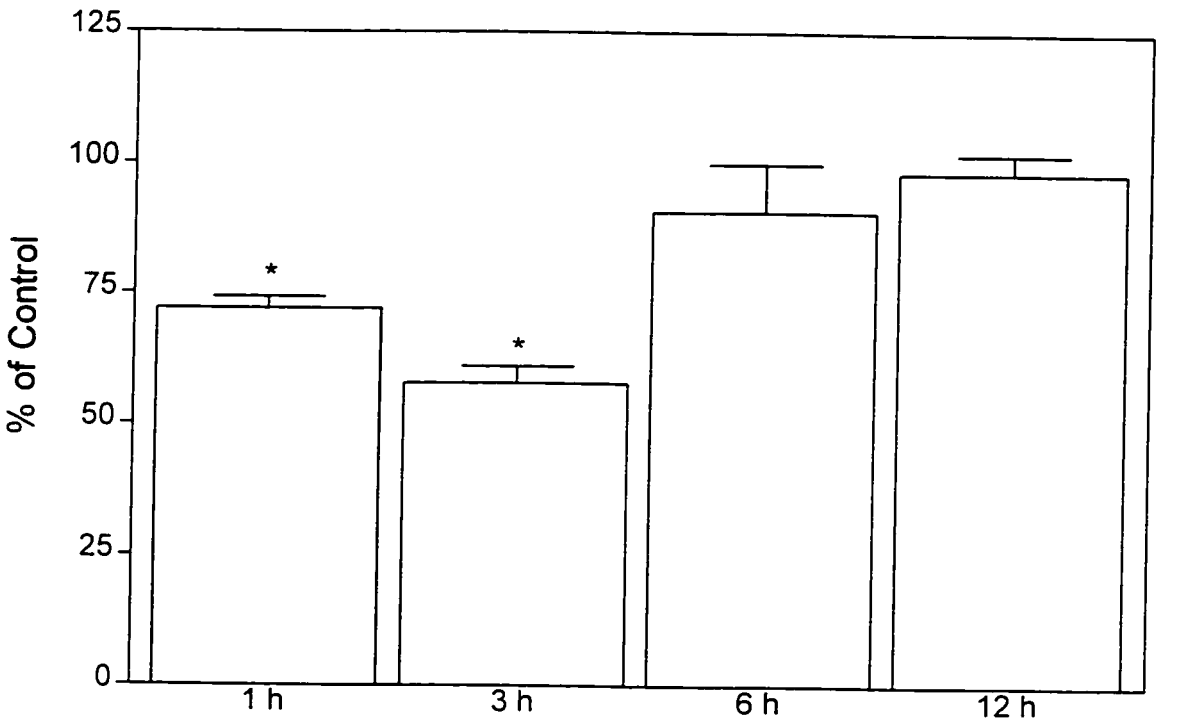


Figure 28: Glutamine levels in rat whole brain following administration of PEH (30 mg/kg, i.p.) at 1, 3, 6, or 12 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

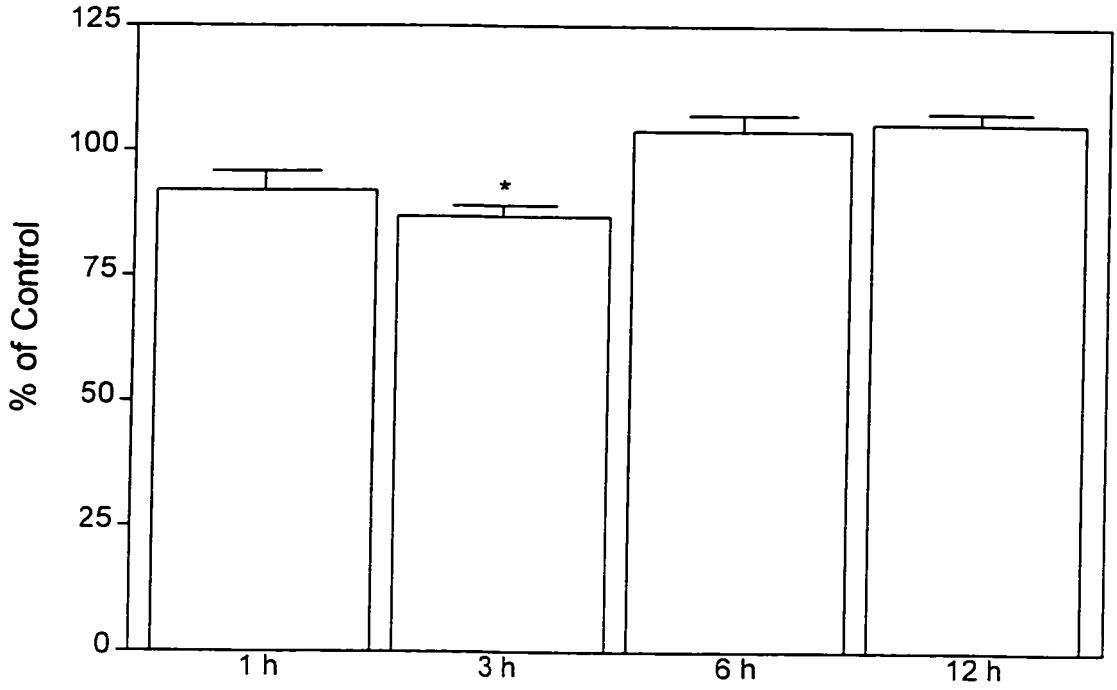


Figure 29: Glutamate levels in rat whole brain following administration of PEH (30 mg/kg, i.p.) at 1, 3, 6, or 12 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

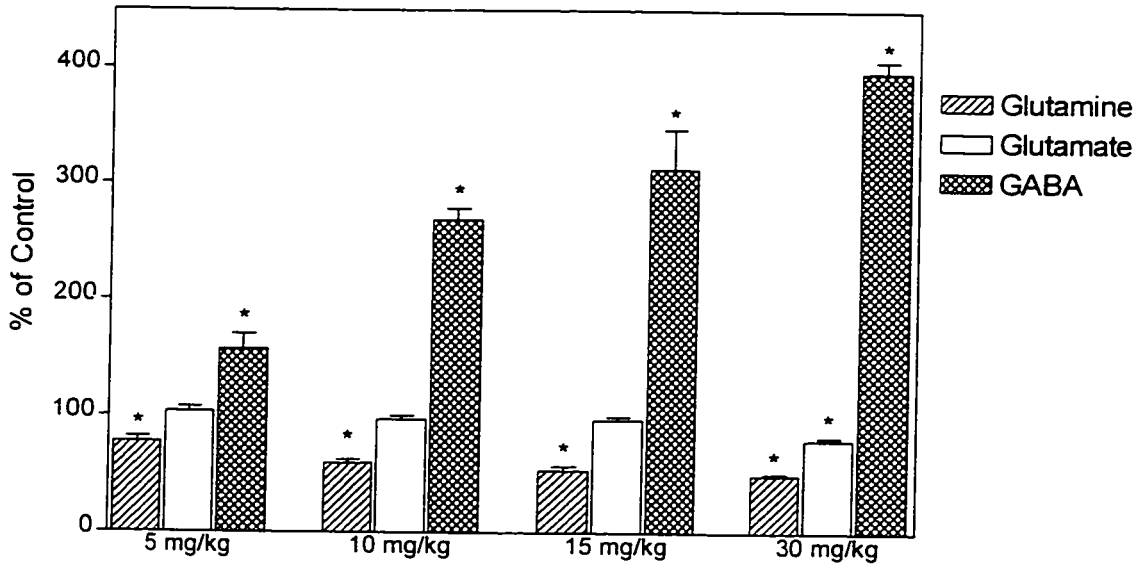


Figure 30: Glutamine, glutamate and GABA levels in rat whole brain following administration of PEH at 5, 10, 15 or 30 mg/kg i.p. at 3 h post-injection. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. Control values were as follows: glutamine = 802.6 ± 101.34 μg/g of tissue, glutamate = 1349.4 ± 97.7 μg/g of tissue, GABA = 254.83 ± 13.8 μg/g of tissue. * denotes significant difference ($p<0.05$) from control values.

TCP did not block the significant effects of PEH on GABA and glutamine (Figure 31).

3.10 Effect of PEH on ALA levels

A 12 h time course revealed that ALA levels were significantly elevated following administration of PEH (30 mg/kg i.p.) (Figure 32), an effect similar to that observed following PLZ administration.

A dose-response study indicated that ALA levels were elevated following i.p. administration of PEH at the 10, 15 and 30 mg/kg doses at 3 h (Figure 33). Pretreatment with TCP (5 mg/kg) did not significantly alter the ALA-elevating effect of PEH (30 mg/kg) (Veh= 76.1± 4.0, Veh-PEH= 219.7± 4.3, TCP-PEH= 241.3± 12.3).

3.11 Effect of PEH on GABA-T and ALA-T

A dose-response study of PEH i.p. at 3 h with 5, 10, 15 or 30 mg/kg revealed that PEH significantly inhibited GABA-T at doses of 10 mg/kg or higher and inhibited ALA-T levels significantly at 15 mg/kg or higher (Figure 34), similar to the effects seen with PLZ treatment. The time course of the inhibition is shown in Figure 35.

3.12 Effect of PEH on MAO-A and MAO-B

In contrast to PLZ, PEH does not appear to significantly affect MAO-A and-B. A dose-response study of PEH at 5, 10, 15 and 30 mg/kg suggests

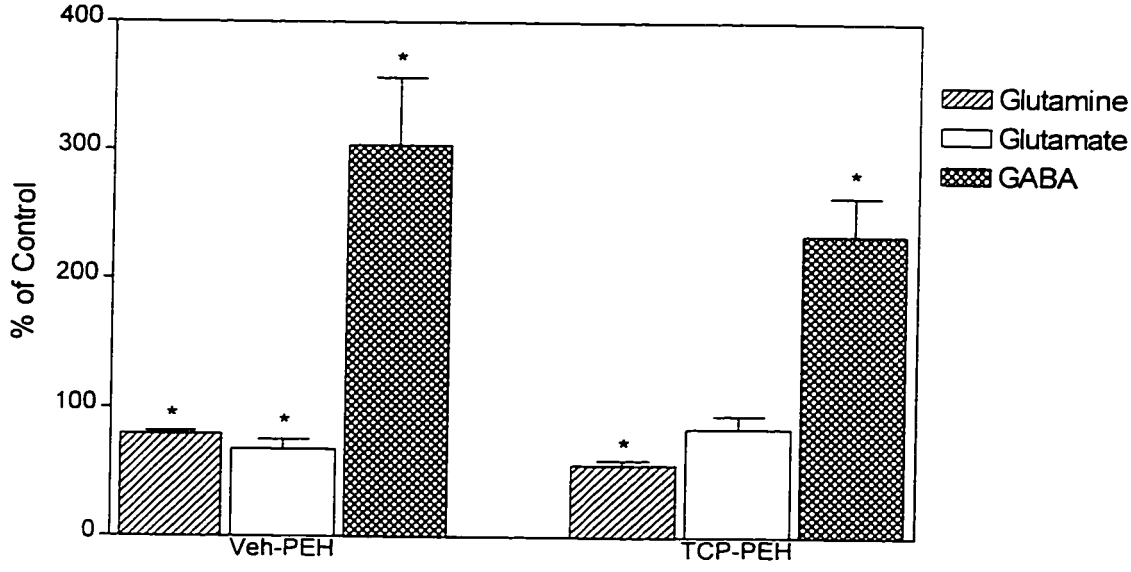


Figure 31: Glutamine, glutamate and GABA levels in rat whole brain following administration of PEH (30 mg/kg, i.p.) after pretreatment with vehicle (Veh-PEH) or TCP (5 mg/kg, i.p.) (TCP-PEH). Rats were killed at 3 h after injection of PEH. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. Control values were as follows: glutamine = 786.9 ± 97.7 μg/g of tissue, glutamate = 1279.4 ± 91.9 μg/g of tissue, GABA = 342.46 ± 41.44 μg/g of tissue. * denotes significant difference ($p<0.05$) from control values.

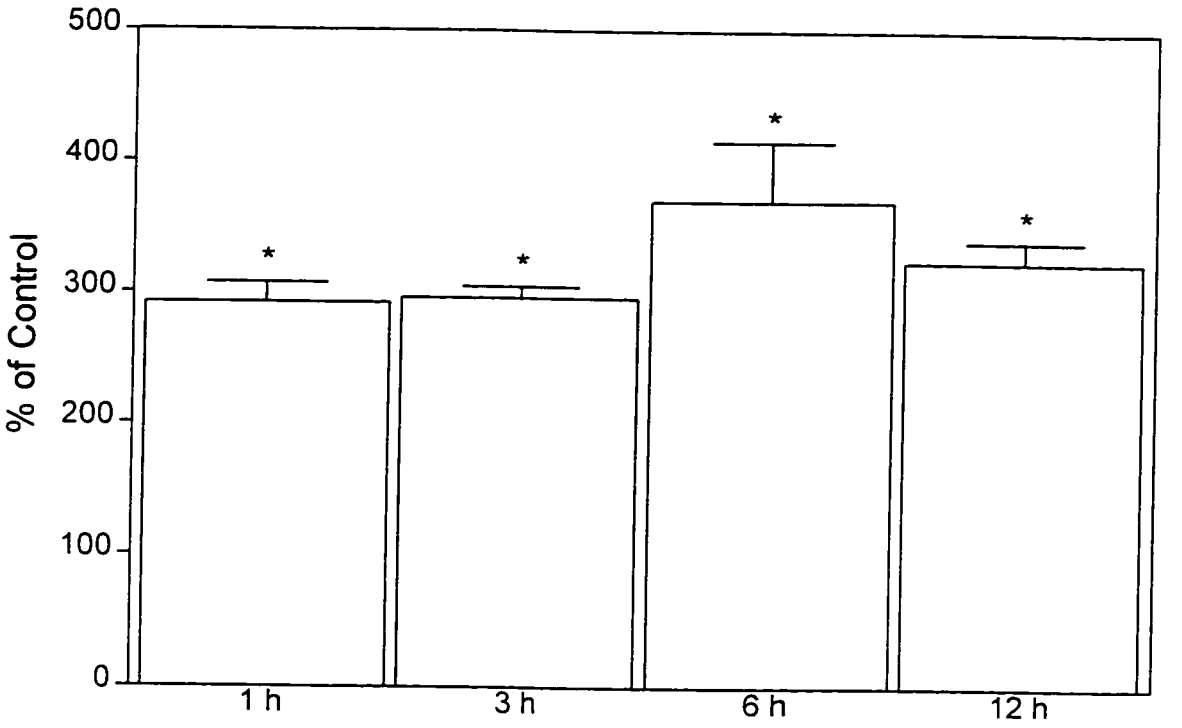


Figure 32: ALA levels in rat whole brain 1, 3, 6 or 12 h following injection of PEH (30 mg/kg i.p.). Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

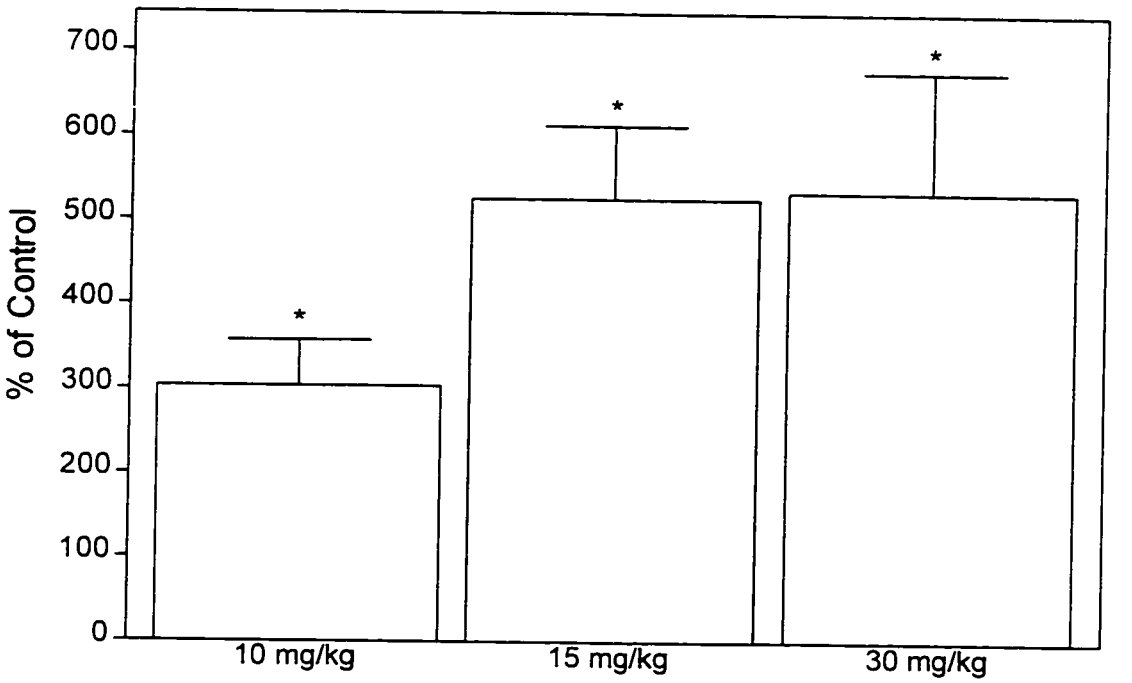


Figure 33: ALA levels in rat whole brain at 3 h following injection of PEH at 5, 10, 15 or 30 mg/kg i.p. Values represent means ± SEM and are expressed as % of control values in vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

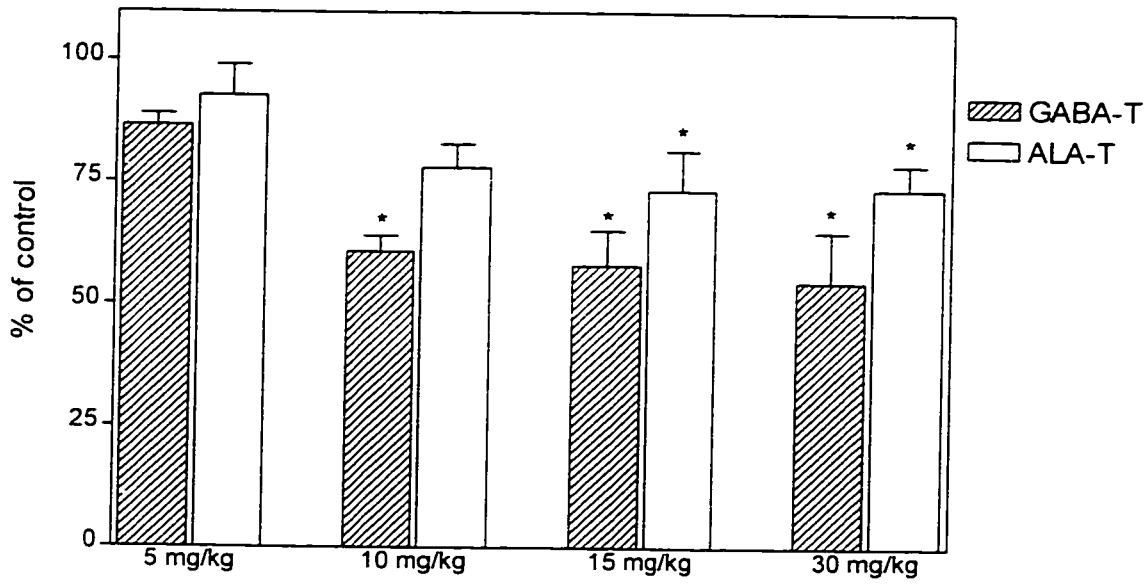


Figure 34: Inhibition of rat whole brain GABA-T and ALA-T following administration of PEH at a 5, 10, 15 or 30 mg/kg dose and a 3 h time interval post-injection. Values are expressed as % of control ± SEM compared to vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

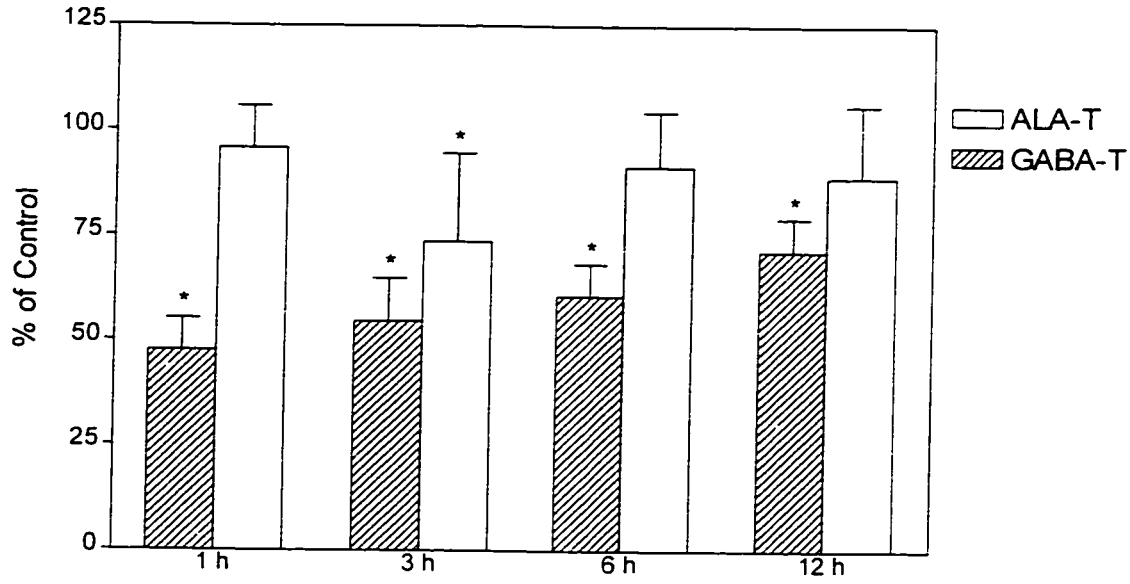


Figure 35: Inhibition of rat whole brain GABA-T and ALA-T 1, 3, 6 or 12 h following administration of PEH, 30 mg/kg. Values are expressed as % of control ± SEM compared to vehicle-treated animals. * denotes significant difference ($p<0.05$) from control values.

that PEH is a weak inhibitor of MAO-A and MAO-B (Table 3). An acute time course study using PEH at 30 mg/kg i.p. revealed the MAO inhibition is short-lived (Table 4).

3.13 Attempts to Develop an Assay Procedure for PEH

In attempts to develop an assay for PEH, derivatization with pentafluorobenzenesulfonyl chloride (PFBSC) or pentafluorobenzoyl chloride (PFBC) under aqueous conditions were first investigated. Derivatization with these reagents has been used successfully in our laboratory in the past for analysis of PLZ, PEA and TCP (Hampson et al., 1984a and b; Baker et al., 1986b; Baker et al., 1986a; Aspeslet et al., 1992). Derivatization with PFBSC resulted in production of interference peaks. Further investigations using derivatization with PFBC and altering oven temperature conditions determined that PEH eluted as two major peaks. An internal standard was then chosen, benzylamine, and the assay was conducted using control tissue spiked with PEH. However linear calibration curves were not attainable. Several attempts were made to clean up the assay by precipitating proteins, altering methods of taking samples to dryness, adding wash steps, and changing extraction solvents and amount of reconstituting solvent, but the calibration curves remained poor. The assay was thus abandoned due to inconsistency, low sensitivity and interfering peaks.

mg/kg	PEH		PLZ	
	MAO-A	MAO-B	MAO-A	MAO-B
[5]	0.00 ± 0.2	0.03 ± 0.4	90.9 ± 4.6	28.4 ± 7.9
[10]	7.9 ± 0.9	5.1 ± 2.6	95.6 ± 1.6	79.6 ± 4.9
[15]	10.3 ± 4.9	1.7 ± 0.5	94.6 ± 0.5	90.1 ± 1.3
[30]	25.4 ± 7.6	21.4 ± 7.1	97.2 ± .8	93.64 ± 2.4

Table 3: Inhibition of rat whole brain MAO-A and MAO-B following administration of 5, 10, 15 or 30 mg/kg of PEH at 3 h post-injection. Values are expressed as % inhibition ± SEM compared to vehicle-treated animals.

Hours	PEH		PLZ	
	MAO-A	MAO-B	MAO-A	MAO-B
1	23.2 ± 0.6	0.8 ± 0.4	100.0 ± 1.2	70.6 ± 2.6
3	25.4 ± 7.6	21.4 ± 7.1	99.3 ± 0.5	82.9 ± 2.9
6	1.1 ± 0.7	2.3 ± 0.2	97.7 ± 0.8	83.3 ± 0.2
12	0.6 ± 0.4	1.6 ± 1.0	98.9 ± 0.8	90.1 ± 2.6

Table 4: Inhibition of rat whole brain MAO-A and MAO-B following administration of 30 mg/kg of PEH at 1, 3, 6 or 12 h post-injection. Values are expressed as % inhibition ± SEM compared to vehicle-treated animals. PLZ (30 mg/kg) values taken from Paslawski et al. unpublished data.

Pentafluoropropionic anhydride (PFPA) has been used successfully in our laboratory for the derivatization of PEA, tryptamine and TCP after acetylation of these amines (Calverley et al., 1980; Baker et al., 1982c) and so derivatization PFPA was attempted with PEH. Acetylation with acetic anhydride under basic aqueous conditions followed by extraction with ethyl acetate and derivatization with PFPA was compared to extraction only (i.e. no acetylation) followed by reaction with PFPA. Under both conditions a diderivative appeared with PEH, however extraction alone was determined to result in better recovery than acetylation and extraction. Derivatization times and temperatures were adjusted to determine the most favorable conditions. Partitioning between borate buffer and organic solvents, compared to taking to dryness under nitrogen after reaction with PFPA, did not significantly alter results. The derivatives had poor sensitivity and the calibration curves were not linear. Acetylation with trichloroacetic anhydride in place of acetic anhydride was also attempted and abandoned due to the presence of interfering peaks.

An attempt was made to add a fluorescent tag, OPA, as in the HPLC technique described in this thesis, but this was unsuccessful. An attempt to form a dansyl derivative of PEH was also unsuccessful, as was an attempt to form a derivative with fluorescamine.

Basification of samples followed by extraction with organic solvent and injection of the underivatized PEH on a gas chromatograph equipped with a capillary column and a nitrogen-phosphorus detector resulted in two peaks, but the method did not produce a linear response. Further attempts at analysis of PEH were thus abandoned.

4 Discussion

4.1 The Effect of PLZ on GABA and its Precursors, Glutamate and Glutamine

PLZ has been shown to cause significant increases in brain levels of GABA (Popov and Mathies, 1969; Perry et al., 1981; Baker et al., 1991; McKenna et al., 1991). In the results reported in this thesis, examination of the effect of PLZ on GABA metabolism was extended to two precursors in the GABA shunt, glutamate and glutamine. In addition to an elevation in GABA levels, both hypothalamic levels and whole brain levels of glutamate and glutamine were decreased following PLZ administration (Paslawski et al., 1995 and other results described in this thesis). These effects appeared to be both time- and dose-dependent. The findings with GABA are in keeping with the results reported by Baker et al (1991). PLZ affected glutamine levels at lower drug doses than those required to affect a change in either glutamate or GABA levels, suggesting that glutamine is more sensitive to the effects of PLZ than either GABA or glutamate. The time-interval required to cause a change in glutamine levels was intermediate to that required to produce a change in GABA and glutamate levels (3 h for glutamine as compared to 1 h for GABA and 6 -12 h for glutamate at 15 mg/kg PLZ). Significant decreases in glutamate levels only occurred at the

later time intervals, between 6 and 12 h, using 15 mg/kg of PLZ. At a higher dose of PLZ (30 mg/kg), a significant decrease in glutamate could be observed at the 3 h post-injection time interval as well.

It is interesting that vigabatrin, a GABA-T inhibiting anticonvulsant which markedly increases GABA levels in brain, has also been reported to cause short-acting decreases in brain levels of glutamate and glutamine in mouse brain (Bernasconi et al., 1988). Chronic PLZ administration (21 days) revealed the same pattern of an elevation of GABA and decreases in glutamine and glutamate levels as was observed in the acute studies with PLZ. This finding with GABA after chronic administration of PLZ is in agreement with the results of McManus et al., (1992) but to my knowledge, no one has looked at glutamate and glutamine levels under chronic conditions with PLZ previously.

As discussed in the introduction of this thesis, GABA is formed in the GABA shunt (McGeer and McGeer, 1989). Upon release from the neuron, GABA can be taken up by both the neurons and the surrounding glial cells. Glutamate is formed in the glial cells by the action of GABA-T on GABA. GABA cannot then be reformed in glial cells because these cells lack GAD. Glutamine synthetase, which is present only in the glia (Peng et al., 1993; Martinez-Hernandez et al., 1977), acts on glutamate to form glutamine which can then be transported back to the nerve ending where glutaminase

converts glutamine to glutamate (a major route for synthesis of glutamate; Timmerman and Westerink, 1997). It is possible that PLZ exerts an effect on the GABAergic system by altering activity of glutaminase and/or glutamine synthetase.

Collins et al. (1994) reported that the anticonvulsant valproic acid, which increases brain levels of GABA, increases glutaminase and decreases glutamine synthetase activities in primary cultures of rat brain astrocytes. The relationship among the effects of PLZ on GABA, glutamine, glutamate and ALA are made more complicated by reports that glutamine serves as a precursor for GABA (Paulsen et al., 1988), glutamate (Timmerman and Westerink, 1997) and ALA (Yudkoff et al., 1988) and that ALA can act as an inhibitor of glutamine synthetase (Tate and Meister, 1971). Researchers in the Neurochemical Research Unit are now investigating the action of ALA on glutamine synthetase activity.

4.2 The Effect of PLZ on ALA levels: Acute and Chronic Administration

Both acute (3 h) and chronic (21 days) administration of PLZ (15 mg/kg) elevated ALA levels significantly. This increase is similar to that observed in GABA levels with PLZ administration and is in keeping with the results reported by other researchers (Wong et al., 1990b; McKenna et al., 1991). As discussed in the next section, PLZ inhibits ALA-T, but as with GABA and GABA-T, this inhibition does not seem to fully explain the marked

elevation in ALA following PLZ administration. ALA and lactate are metabolically related (refer to Figure 3) such that an increase in the formation of ALA from pyruvate could conceivably decrease the formation of lactate from pyruvate, resulting in decreased lactate levels. In this regard, it is of interest that lactate infusion can precipitate a panic attack in some patients (Liebowitz et al., 1984; Shear, 1986; Coupland and Nutt, 1995) and results in increases in brain levels of lactate (Dager et al., 1994). It is tempting to speculate that PLZ, which has been shown to be an effective anti-panic drug, may decrease brain lactate levels by an indirect action through ALA, although this has never been investigated to my knowledge.

The role of ALA in the CNS or its possible involvement in the etiology of anxiety disorders is not yet well addressed in the anxiety disorders literature, despite its metabolic relationship to lactate. ALA, like glycine, has been shown to be a co-agonist at NMDA excitatory amino acid receptors, although it has a weaker effect than glycine (Thomson, 1989) and is considered to be an inhibitory neurotransmitter (Cooper et al., 1991). Interestingly, levels of ALA have been shown to increase during seizure activity (Chapman et al., 1977). Westergaard and colleagues (1995) have shown that ALA can act as an amino group donor in the synthesis of glutamate from α-ketoglutarate, but not at a rate sufficient to sustain GABA synthesis in GABAergic neurons. As mentioned above, ALA has also been

reported to act as an inhibitor of glutamine synthetase (Tate and Meister, 1971).

Although PLZ has been shown here to affect brain levels of GABA, ALA, glutamate and glutamine, the current findings do not, as discussed above, indicate whether these changes are primarily glial or neuronal. Using *in vivo* microdialysis, Parent et al. (1998) have demonstrated that PLZ administered i.p. at doses similar to those described in this thesis, causes a marked increase in extracellular levels of GABA and ALA. It has been suggested that it is possible to determine whether GABA released in such *in vivo* microdialysis is primarily neuronal or glial in nature by infusing tetrodotoxin or high K^+ or using microdialysis medium low in Ca^{2+} (Westerink and de Vries, 1989; Jolkkonen et al., 1992; Shirokawa and Ogawa, 1992; Campbell et al., 1993; Qume et al., 1995; Sayin et al., 1993). However, Timmerman and Westerink (1997) have recently reviewed such studies which have utilized these conditions and found many inconsistencies and controversies, and concluded that the origin of the extracellular GABA detected by microdialysis cannot conclusively be demonstrated by these measures.

4.3 The Effect of PLZ on GABA-T and ALA-T

PLZ significantly inhibited the activity of both GABA-T and ALA-T in a dose-dependent manner, with GABA-T being statistically significantly

inhibited at a lower dose (10 mg/kg) than ALA-T (15 mg/kg) using a 3 h post-injection time interval. Again, these observations are in keeping with those found by other researchers (Baker and Martin, 1989; McManus et al., 1992; Popov and Mathies, 1969). It is assumed that GABA-T inhibition plays a role in the elevation of GABA caused by PLZ administration (Popov and Mathies, 1969; Perry and Hansen, 1973; McManus, 1992) but it may not account fully for the degree of GABA elevation, since GABA levels increase 2-4 fold when GABA-T has been inhibited 50% or less (Popov and Mathies, 1969; McKenna et al., 1994). However, kinetic studies of GABA-T activity should be conducted to determine the extent to which inhibition of this enzyme affects the elevation of GABA. Because PLZ also inhibits a number of other amino acid transferases, including ALA-T and tyrosine amino transferase, it has been suggested that PLZ exerts a general inhibitory effect on transferases (Dyck and Dewar, 1986); however McManus et al. (1992) reported that doses of PLZ that markedly elevate GABA and ALA had no significant effect on levels of other transaminase substrates, i.e. leucine, isoleucine and valine.

Experiments in this thesis demonstrated that PLZ administration elevates GABA levels and inhibits GABA-T. Inhibition of GABA-T alone should cause an increase in GABA and α-ketoglutarate and a decrease in

glutamate. An increase in α-ketoglutarate could also cause an increase in the NADH-dependent formation of glutamate (refer to Figure 3). and therefore glutamate levels may not appear to be affected to the same degree as glutamine. If there is increasing metabolism via the NADH-dependent pathway from α-ketoglutarate to glutamate. there could be a corresponding increase in the formation of ALA from pyruvate (refer to Figure 3). Experiments reported here show a corresponding elevation in ALA levels following PLZ administration. An increase in the formation of ALA from pyruvate could conceivably decrease the formation of lactate from pyruvate. resulting in decreasing lactate levels. ALA has been reported to inhibit glutamine synthetase but not glutaminase (Tate and Meister. 1971). so increasing ALA levels could correspond to decreasing glutamine levels. which were also observed in experiments reported in this thesis.

The commonly accepted concept of the glutamate-glutamine or GABA-glutamine cycle implies glial uptake of the extracellular glutamate or GABA with subsequent conversion to glutamine. Glutamine in turn is exported to neurons and converted back to glutamate or GABA (Westergaard et al., 1995). However, the conversion of glutamate or GABA into pyruvate and lactate *in vitro* has also been shown (Hassel and Sonnewald, 1995). Lactate in turn may be converted to glutamate, creating a glutamate/GABA-lactate cycle (Hassel and Sonnewald, 1995). This is an

interesting cycle in light of the observation that increasing lactate levels can precipitate panic attacks in some individuals (see Cowley and Arana, 1990).

4.4 The Effect of PLZ on MAO-A and MAO-B

PLZ is classified as an irreversible nonselective MAOI, and while its MAO-inhibiting properties were not the focus of this thesis, measurement of inhibition of these enzymes acted as a control to ensure that adequate PLZ was entering the brain following administration. MAO-A and MAO-B were statistically significantly inhibited at all doses administered in the acute dose-response experiment, with MAO-A activity affected to a greater extent than MAO-B at the lowest dose of 5 mg/kg. Inhibition of both MAO-A and -B was also significant following chronic administration of PLZ. These results are comparable to those found by Baker et al. (1991).

4.5 PLZ and Pretreatment with TCP: Effects on Amino Acids in Hypothalamus

Pretreatment with TCP had been shown previously to block the GABA-elevating effects of PLZ (Popov and Mathies, 1969; Todd and Baker, 1995). Because PLZ is both an inhibitor of MAO as well as a substrate for MAO (Clineschmidt and Horita, 1969a and b), the blockade by TCP of PLZ's GABA-elevating effect suggests that a metabolite of PLZ, formed by the action of MAO on PLZ, is contributing the elevation of brain GABA levels. As

reported here, in addition to blocking the elevation of GABA, pretreatment with TCP also blocked the glutamine- and glutamate-decreasing effects of PLZ. This finding suggests that a metabolite of PLZ may also be responsible for the effects of PLZ on these precursors to GABA.

4.6 Effect of PLZ on $^{36}Cl^-$ Uptake

The effects of mediators of the $GABA_A$ receptor as measured by $^{36}Cl^-$ uptake in synaptoneurosomes have been somewhat inconsistent. Chronically administered tricyclic antidepressant/ antipanic agents IMI and DMI reportedly decreased $^{36}Cl^-$ uptake in synaptoneurosomes (Fernandez-Teruel et al., 1989), as did the BZD anxiolytics, alprazolam and diazepam (Lopez et al., 1990), while amitriptyline was found to increase $^{36}Cl^-$ uptake (Malatynska et al., 1991).

Chronically administered PLZ had previously failed to demonstrate a change in $GABA_A$ receptor regulation as demonstrated by ^{3}H-muscimol binding (McKenna et al., 1994) or ^{3}H-flunitrazepam binding (Todd et al., 1995), despite a significant elevation of GABA levels in the brain. The chronic evaluation of GABA-stimulated $^{36}Cl^-$ uptake in rat cortical synaptoneurosomes described in this thesis was conducted to determine if this measure of $GABA_A$ receptor activity might detect a change following PLZ administration. No significant effect of PLZ was observed following 21 days

of administration, even though GABA levels were significantly elevated and MAO-A, MAO-B and GABA-T activities were inhibited. It has been suggested that $^{36}Cl^-$ uptake may lack the sensitivity to detect discrete changes in the $GABA_A$ receptor (Ngur et al., 1990) such as may be occurring with PLZ administration (Susan Dunn, personal communication). Recent studies indicate that BZDs, which often cause no change in BZD receptor number, as determined by radioligand binding studies, do affect changes in mRNA expression for isoforms of the $GABA_A$ receptor subunits (O'Donovan et al., 1992; Tanay et al., 1996). Given these findings, it has been proposed that the effects of PLZ on the $GABA_A$ receptor might better be determined by examination of expression of mRNAs of isoforms of subunits of the $GABA_A$ receptor. Findings by Tanay et al. (1996) indicate that PLZ does indeed have effects on the expression of mRNA (see Tanay et al., 1996 for details of effects). Interestingly, Lai et al. (1997) determined that chronic (21 day) PLZ admin-istration did not affect steady-state mRNA levels of GAD or GABA-T, but mRNA levels for the GABA-transporter, GAT-1, were significantly increased.

4.7 Clinical Study: Chronic PLZ Treatment of Patients with Panic Disorder

4.7.1 HPLC Assay for GABA in Plasma

Various methods of sample clean-up were attempted during development of this assay, including precipitation of proteins with acetonitrile or methanol, as well as filtering through 0.2 μm Nalgene filters and Centricon Micron 30 (mw-30,000) microconcentrators. Precipitation of proteins with perchloric acid followed by basification and washing with an ion-pairing reagent gave an easy and inexpensive clean-up. Scrupulous attention had to be paid to the HPLC system itself to maintain the integrity of the column resolution. Although GABA eluted in 11.7 min, a run time of 60 min was needed to wash the column. The HPLC system was washed with 100% methanol every 75 injections and the guard column changed. It was also necessary to prime the needle wash with 100% methanol every 75 samples. The assay to allow for the detection and quantitation of GABA in plasma samples used a slightly altered mobile phase compared to the mobile phase employed in the analysis of amino acids in brain tissue, although the resulting pH remained a constant 6.2. Sample handling differed markedly from the assay protocol for tissue. With tissue, internal standard was added to homogenate with 400 μl MeOH and supernatant was retained for analysis. The assay for GABA in plasma used no internal standard, and proteins were

precipitated prior to centrifugation to obtain supernatant. A second clean-up of the supernatant, consisting of shaking with the liquid ion-pairing reagent DEHPA, was performed prior to derivatization. This step resulted in a considerable clean-up without resulting in significant losses of GABA. A larger aliquot of plasma supernatant than of tissue supernatant was used for HPLC analysis (100 μl compared to 20 μl). The derivatization procedure was also altered slightly, by adding a smaller amount of derivatizing reagent and allowing the reaction to occur for a longer time period (10 μl for 90 sec compared to 20 μl for 30 sec). These adjustments resulted in a sharper peak, increased sensitivity and a reduced retention time. No interfering peak was observed as had occurred in the other GC and HPLC assays which are routinely applied to brain samples in our laboratory.

4.7.2 Clinical Study

Blood tests are currently in use to assist in the diagnosis of a number of centrally controlled disorders including Cushing's disease, hypoglycemia and hypothyroidism (Petty, 1994). Peripheral biochemical or physiological markers of central nervous system disorders, while not replacing the clinical diagnosis, can conceivably assist in making an accurate differential diagnosis. Low plasma levels of GABA have been shown to be associated with mood disorders, with approximately 40% of patients with MDD having lower than normal levels of plasma GABA (Petty et al., 1992; Petty, 1994).

Additionally, Petty and colleagues have established that in normal subjects plasma GABA levels are stable across time (see Petty, 1994). While the exact relationship between plasma GABA and brain GABA activity in humans is not yet known, Loscher and Schmidt (1984) determined that GABA levels in human plasma correspond to levels in CSF. As well, evidence suggests that in laboratory animals, plasma GABA correlates with brain GABA (Petty and Kramer, 1992). These observations, in conjunction with data supporting a GABAergic hypothesis of anxiety disorders (see Lydiard et al., 1996), suggest that plasma GABA could be a potential marker for anxiety disorders.

Plasma levels of ALA and GABA were not significantly different between control subjects and panic disorder patients at baseline (i.e. prior to treatment with PLZ). This finding with GABA is in accordance with the finding by Goddard et al. (1996) who also found no significant differences in plasma levels of GABA between untreated patients with panic disorder and control subjects. However, a significant increase was observed in GABA levels when comparing individual baseline values to values obtained after 8 weeks of treatment with PLZ. This increase in GABA following PLZ treatment is perhaps not unexpected given the effect of PLZ on GABA levels in brain tissue, and the observation that increases in central GABA concentrations, measured in dogs and rats, are reflected in plasma GABA levels (Ferkany et

al., 1979; Loscher, 1979). These results suggest that the measurement of GABA in plasma may be useful to monitor the central effects of PLZ.

It is interesting that Malcolm and colleagues (1994) reported decreased levels of PLP, a cofactor in GABA metabolism, in plasma from patients being treated with PLZ while Lydiard and colleagues (1989) reported no effect of PLZ on plasma PLP. In the latter study, however, 9 out of 16 patients had below normal levels of PLP at baseline. Lydiard's study suggests the possibility that subsets exist within the treatment populations.

Clearly, an expanded study with larger sample sizes is warranted to determine the significance of the trends observed in the experiment discussed in this thesis. This is particularly true for the comparison between plasma levels of GABA and ALA in the control subjects and the panic disorder patients at baseline. A future study should use a larger population of subjects matched for age and sex. However, the principal reason for conducting the study described in this thesis was to determine if PLZ did cause changes in plasma levels of GABA and/or ALA within individual subjects.

The lack of effect of PLZ on plasma ALA levels is somewhat surprising given the increase in GABA levels. However, in contrast to the situation in brain where GABA levels were considerably higher than those of ALA, plasma levels of ALA are orders of magnitude greater than those of

GABA. ALA is also involved in a number of general metabolic processes in the body, including gluconeogenesis (Davis and Granner, 1997), and plasma levels of ALA may well reflect ALA which is independent of the ALA-T catabolic pathway.

4.8 Effects of PLZ and N^2AcPLZ in the Elevated Plus Maze

Animal models of anxiety have typically been validated pharmacologically based on their sensitivity to acute BZD or barbiturate anxiolytics (Treit, 1985) and have been used both to identify novel anxiolytics and to study the mechanism of action of their anxiolytic effect (see Treit and Menard, 1998). The predictive ability of these models has been suggested to be somewhat limited given that these models have been reported to be relatively insensitive to some TCAs and MAOIs that have been shown to be effective in the treatment of human anxiety disorders (Nutt and Glue, 1989; Sheehan, 1986). Interestingly, the negative findings regarding the antidepressant anxiolytics are not restricted to the elevated plus-maze, since these same negative results have been observed in other animal models of anxiety, including the conflict test (Commissaris et al., 1995) and the shock-probe burying test (Treit et al., 1981; Beardslee et al., 1990). The failure of antidepressants to display an anxiolytic effect in these animal models is puzzling given that the anxiolytic effects of the BZDs, on which many models are based, are commonly attributed to their facilitatory effects on GABA

transmission in the CNS (Breslow et al., 1989; Green, 1991). As well, other GABA-mimetic agents, including the anti-convulsant vigabatrin and the antiepileptic gabapentin, have been reported to have an acute anxiolytic effect in the elevated plus-maze (Corbett et al., 1991; Sayin et al., 1992; Sherif et al., 1994; Singh et al., 1996).

The study reported in this thesis is the first report of an anxiolytic effect of PLZ in the elevated plus-maze. Results from the neurochemical analyses of PLZ and its acetylated metabolite, N^2AcPLZ, are in keeping with those reported elsewhere in this thesis and in the study by McKenna and colleagues (1991). All three treatment groups, low-dose PLZ, high-dose PLZ and high-dose N^2AcPLZ, resulted in inhibition of MAO-A and -B activity, but only the high-dose PLZ condition produced a significant elevation of GABA levels. Behavioral analyses revealed that while none of the treatment groups differed significantly on the measure of general activity, the high-dose PLZ had an anxiolytic effect in both measures of anxiolysis (namely, % open arm entries and % open arm time). The low-dose PLZ and N^2AcPLZ (neither of which resulted in elevation of GABA levels) did not produce significant effects on either measure of anxiolysis. These results strongly suggest that the anxiolytic effects of PLZ are associated with the drug's effects on GABA in the CNS.

There are a number of possible explanations of why animal models of anxiety have previously failed to find an effect with PLZ and other antidepressants. Unless the behavioral studies provide neurochemical data to support their findings, i.e. GABA levels, it is difficult to verify the pharmacological efficacy of their treatments. Previous studies may have failed to show a treatment effect due to inadequate treatment parameters. The drug doses used, for example, may have been too small, or the time period prior to evaluation may have been inappropriate. As well, the drug being evaluated may not have produced neurochemical effects that would affect or correspond to the behaviors targeted by the model, or the drug may have altered the animal's perception of the test stimulus (Green, 1991; Commissaris and Fontana, 1991). Further to this, the anxiolytic effects of the antidepressants targeted by the model may be masked or even antagonized by other effects of the drugs, such as facilitation of 5-HT transmission (Paslawski et al., 1996). Unlike GABA facilitation, which is generally anxiolytic in animal models, increased 5-HT transmission is generally anxiogenic (Pellow et al., 1987; Treit et al., 1993). The effect of N^2AcPLZ in this experiment is interesting in light of this observation, since N^2AcPLZ is known to significantly elevate 5-HT at the dose used (McKenna et al., 1991), yet it was found to have no anxiogenic effect. Clinical evidence suggests that various anxiolytics are more effective in the treatment of some forms of

anxiety than others (Lydiard et al., 1996). The extent to which various animal models are representative of these different forms of anxiety may be reflected in the ability of a drug to produce an anxiolytic effect in a particular model (Paslawski et al., 1996).

4.9 Effect of PEH on GABA and its Precursors, Glutamate and Glutamine

PEH is a putative metabolite of PLZ that is hypothesized to be formed initially by the action of MAO in the metabolism of PLZ to PAA (Tipton, 1972) (refer to Figure 1). Acute time- and dose-response studies revealed that in PEH resembles PLZ in its effect on GABA, glutamine levels and glutamate in whole brain. In the acute time course experiment, GABA levels were comparable to levels observed following PLZ administration. Dose-response data indicated that PEH significantly elevated GABA at all doses examined (5, 10, 15 and 30 mg/ kg). Glutamine levels were significantly decreased at all doses of PEH, again comparable to the effect observed with PLZ; however this effect occurred over a shorter period of time with PEH than with PLZ. Glutamate levels were significantly decreased only with the highest dose of PEH examined (30 mg/kg). TCP pretreatment did not block the effects of PEH on glutamine and GABA, indicating that the actions of PEH are not affected by MAO inhibition, i.e. MAO is not likely metabolizing PEH to another active metabolite that is responsible for the effects observed.

4.10 Effect of PEH on ALA levels

Dose-response data reflected a pattern of increasing ALA levels with increasing doses of PEH, and time-course data revealed PEH to have a potent and long-lasting effect on ALA levels, again similar to the effects observed following PLZ administration.

4.11 Effect of PEH on ALA-T and GABA-T

Dose-response effects of PEH on ALA-T and GABA-T activity were found to parallel those observed with PLZ administration. GABA-T activity was statistically inhibited with 10 mg/kg PEH at 3 h post-injection, while ALA-T activity was significantly inhibited with 15 mg/kg PEH or higher. The effect on GABA-T activity appeared to be both time- and dose-dependent as a higher dose of PEH (30 mg/kg) significantly inhibited GABA-T at 1 h post-injection.

4.12 Effect of PEH on MAO-A and MAO-B

PEH and PLZ differ markedly with respect to their effects on MAO-A and -B. Acute time-course and dose-response studies indicate that maximal inhibition of MAO-A and -B by PEH is only approximately 25% in whole brain at the highest dose examined (30 mg/kg, 1 to 3 h) while a 30 mg/kg dose of PLZ inhibits MAO-A by 98-100% and MAO-B by 70-85% for the same time interval.

These preliminary experiments provide convincing initial evidence that PEH could be an active metabolite of PLZ responsible, at least in part, for the GABA-elevating effects of PLZ. PEH elevates GABA and ALA levels significantly, and inhibits GABA-T and ALA-T with an effect comparable to that of PLZ. Pretreatment of animals with TCP blocked PLZ's effects on GABA and its precursors, suggesting that a metabolite of PLZ may be responsible for the effects observed with PLZ administration. TCP pretreatment failed to block the GABA-elevating and glutamine-decreasing effects of PEH, providing evidence that MAO is not acting on PEH to produce another active substance that may be responsible for these effects. Since GABAergic effects appear to be important in anxiolytic effects of drugs (Bourin et al., 1998; Treit and Menard, 1998), then PEH has the potential to be an anxiolytic/antipanic drug.

PLZ is a potent nonselective inhibitor of MAO-A and -B enzymes, an effect that is believed to be important in the antidepressant efficacy of PLZ (Murphy et al., 1987). PEH differs from PLZ with respect to its MAO-inhibiting properties, being only a weak inhibitor of MAO-A and -B at the times and doses examined. If MAO inhibition is critical to the antidepressant efficacy of PLZ, then PEH would not be expected to be a useful antidepressant drug. However, it is also likely that PEH would lack some of the side-effects and

complications of PLZ which result from the MAO-inhibiting actions of PLZ (see Kennedy and Joffe, 1989).

Since PEH differs primarily from PLZ in that a double bond has been inserted between the α carbon and the N^1 nitrogen, it appears that the $-CH_2-NH-$ portion of the molecule is much more important for the MAO-inhibiting properties than for the GABA-elevating properties of PLZ.

4.13 PEH Assay Development

No satisfactory assay procedure was developed for PEH. The extraction and assay procedures (HPLC and GC) attempted resulted in interference peaks and/or the PEH formed diderivatives with low sensitivity and/or nonlinear responses.

PEH lacks the hydrogen on the N^1 position of PLZ, which may account for the poor response with aqueous acetylation followed by PFPA since under aqueous conditions, the N^1 position of PLZ is preferentially acetylated (Mozayani et al., 1988). Rao et al. (1987) showed that pentafluorobenzylation occurred at both positions (N^1 and N^2) of PLZ, but only the N^2 position would be available for derivatization in PEH; unfortunately, the resultant derivative showed low sensitivity and nonlinearity of response. Because of these initial difficulties in analysis, detailed mass spectrometric studies on the structures of the derivatives were not

conducted. The formation of two peaks when underivatized PEH was put on the gas chromatograph suggests that PEH may be heat-labile and that future studies on assay development should concentrate on procedures such as HPLC where the compound is not exposed to high temperatures. Thus at this time, we are unable to say with complete certainty that PEH is a metabolite of PLZ. However, the neurochemical findings in this thesis indicate that PEH, regardless of whether or not it is a metabolite of PLZ, is a very useful pharmacological tool and has potential as an anti-anxiety agent.

5 Conclusions

1. In addition to elevating GABA in brain tissue, PLZ also decreases glutamate and glutamine in a time- and dose-dependent manner.
2. As with PLZ's GABA-elevating effect, the effects of PLZ on glutamine and glutamate are blocked by prior inhibition of MAO. This finding suggests that the effects of PLZ on all of these components of the GABA shunt are produced, at least in part, by a metabolite of PLZ formed by the action of MAO on the parent drug.
3. Chronic administration of PLZ did not significantly alter $^{36}Cl^-$ uptake in synaptoneurosomes, a measure believed to reflect $GABA_A$ receptor activity. This lack of effect may be due to a lack of sensitivity of the technique, i.e. an inability to detect discrete changes in the receptor.
4. A novel assay procedure was developed for analysis of GABA in plasma. The method was sensitive and reproducible and resulted in high recoveries of GABA.
5. The plasma samples from panic disorder patients undergoing treatment with PLZ showed no significant differences in ALA during treatment, nor were significant differences observed between treatment and control groups with regard to either GABA or ALA. However, GABA levels in these patients increased significantly by 8 weeks of treatment when

compared to their pretreatment values. These results suggest that the measurement of GABA in plasma may be a useful means of monitoring the central effects of PLZ.

6. PLZ, at a dose which elevated brain GABA levels, had an anxiolytic effect in the elevated plus-maze, an animal model of anxiety. Its acetylated metabolite, N^2AcPLZ, which inhibits MAO but has no GABA-elevating properties, did not have an anxiolytic effect in this model.
7. Experiments with PEH, a putative metabolite of PLZ, showed that this compound elevated brain GABA and ALA levels in a time- and dose-dependent manner. As well, PEH decreased levels of glutamate and glutamine. It also inhibited GABA-T and ALA-T activities, similar to the effects observed with PLZ administration. These results support the hypothesis that PEH is an active metabolite of PLZ and the effects of PEH on the GABA shunt suggest that PEH could have anxiolytic/antipanic efficacy and should be studied further as a drug in its own right.
8. The effects observed with PEH on GABA and glutamine were not blocked by pretreatment with TCP, suggesting that PEH is not converted to an active metabolite by MAO.
9. PEH differs from PLZ in that it does not significantly inhibit MAO-A and MAO-B, suggesting that PEH would lack the antidepressant effects of PLZ

but would also lack some of the side-effects and interactions of its putative parent compound.

The experiments reported here provide further insight into the mechanisms of action of the antidepressant/antipanic agent PLZ as well as increasing our understanding of techniques that may be useful in examining the effects of PLZ. Finally, experiments with a putative metabolite of PLZ indicate that it is a very useful pharmacological tool with potential therapeutic efficacy as an anxiolytic/antipanic drug.

6 Possible Future Studies

6.1 Neuronal versus Glial Effects of PLZ and PEH

As described earlier in this thesis, GABA is present in both neurons and glia and, while some steps in its catabolism occur in neurons, others occur in glia. The results reported in this thesis do not indicate if the effects on GABA, ALA, glutamate and/or glutamine occur primarily in neurons or glia. In future, it would be useful to examine this localization, although as indicated by a recent review on the study of microdialysis (Timmerman and Westerink, 1997), such studies are fraught with difficulty.

6.2 Glutaminase and Glutamine Synthetase Activity

Glutamine synthetase is responsible for the conversion of glutamate to glutamine while glutaminase catabolizes glutamine to form glutamate, which can then be catabolized to GABA. As indicated in this thesis, PLZ has an effect on glutamate and glutamine levels. Studies on the effects of PLZ on glutaminase and glutamine synthetase activity may provide important clues to the mechanisms of action of PLZ.

6.3 Lactate

As discussed earlier, ALA is related metabolically to pyruvate and lactate which are transamination products, and panic attacks have been

demonstrated to be precipitated by i.v. administration of lactate to human subjects (Liebowitz et al., 1984; Shear, 1986). Because PLZ has been demonstrated to elevate ALA levels, examination of lactate following PLZ administration would contribute to our understanding of the effects of PLZ in the citric acid cycle and the interactions between the citric acid cycle and the GABA shunt.

6.4 PEH

Two geometric isomers of PEH are possible (see Figure 7), and it would be worthwhile in future to separate them and test them individually for neurochemical and pharmacological properties.

Attempts in this thesis to develop an assay procedure for PEH were unsuccessful, so it cannot yet be stated unequivocally that PEH is a metabolite of PLZ. In order to increase sensitivity and decrease interference from other substances in tissue, it may be necessary to develop a GC-MS or HPLC-MS assay for PEH. A comparison of the brain levels of PEH and PLZ using such techniques may give important insights into why there are small differences in the potency and time effects of these two compounds with regard to their effects on GABA, ALA, glutamate and glutamine.

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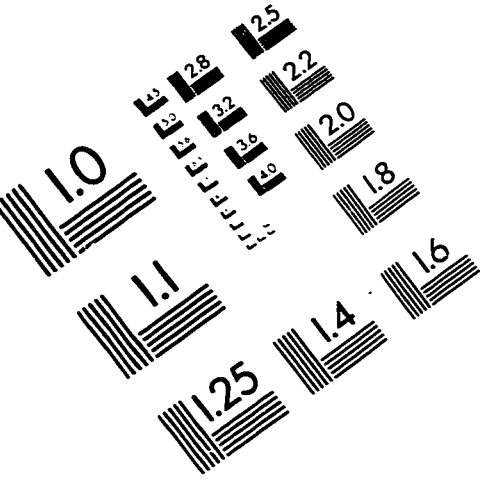
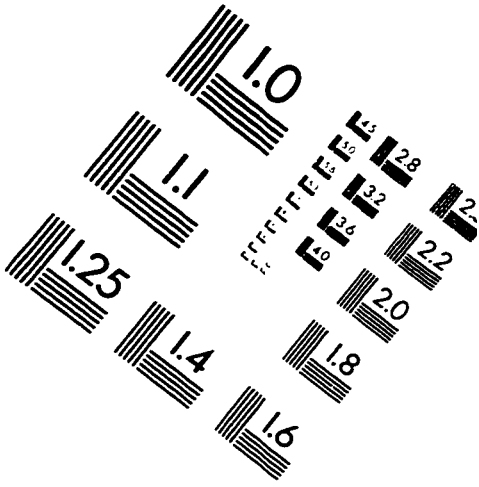
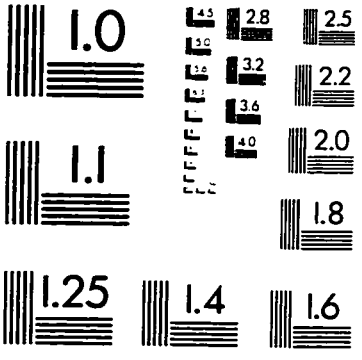
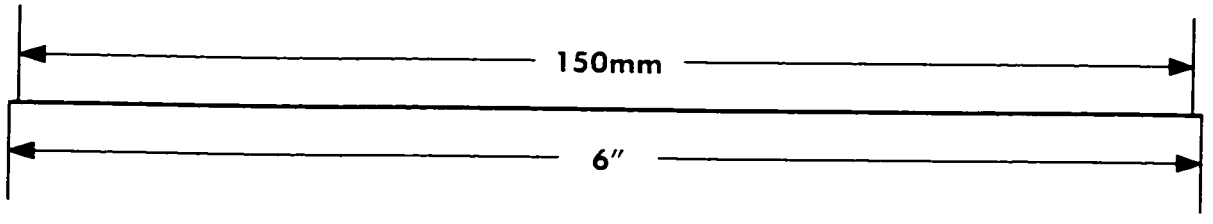
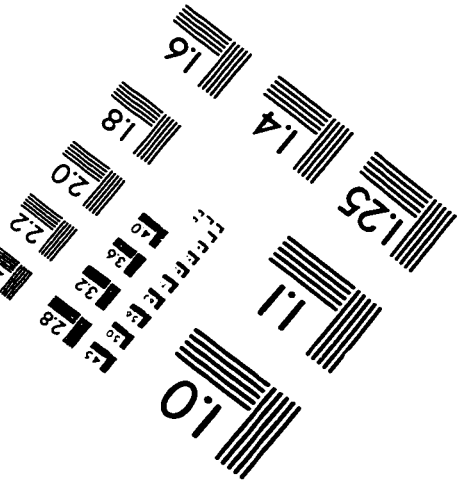
IMAGE EVALUATION TEST TARGET (QA-3)

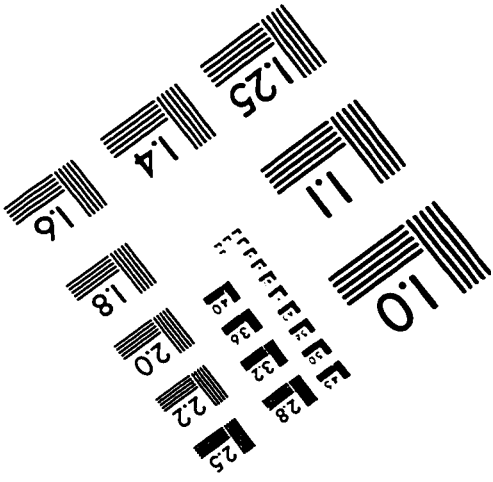












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