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Profile of CL 218,872 a non-benzodiazepine anxiolytic of the triazolopyridazine class.

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PROFILE OF CL 218,872, A NON-BENZODIAZEPINE

ANXIOLYTIC OF THE TRIAZOLOPYRIDAZINE CLASS

A Dissertation Presented

By

JOHN FRANCIS MCELROY

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

September   1984

Department of Psychology

PROFILE OF CL 218,872, A NON-BENZODIAZEPINE

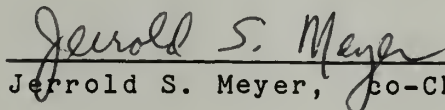ANXIOLYTIC OF THE TRIAZOLOPYRIDAZINE CLASS

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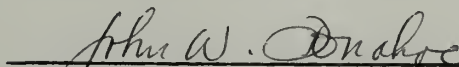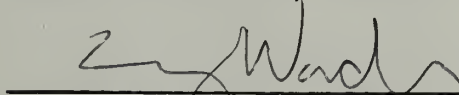
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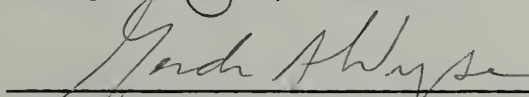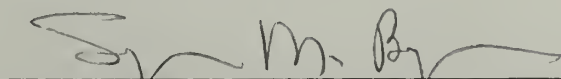
JOHN FRANCIS MCELROY

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DEDICATION

Four years ago I discovered on my desk a journal article concerning some new non-benzodiazepine anxiolytic compound. Several weeks later, unbeknownst to me, a package containing one gram of this substance arrived in the laboratory. Hint! Hint! Until that time I had never heard of CL 218,872. As it turns out, however, that journal article and that complimentary sample of drug were the inspiration for a Doctoral dissertation. I still have that journal article with the name Feldman scrawled across the upper right-hand corner.

As we make the transition from young naive graduate students to aged know-it-all graduate students (just ask any senior student), we tend to forget (or repress) those early years of insecurity and obscurity, and all too often we forget those persons responsible for making the transition possible. Bob Feldman was the principle architect of my overall development as a graduate student. While delicately catering to my stubborn independence, he somehow managed to control the maverick in me. At times Bob was more a father than an advisor to me. As such, we scuffled a bit. Yet for each scuffle, there were a thousand good times. Those are the times I will remember.

For these reasons, and in part for his continued

commitment to higher education for 35 years, I would like
to dedicate this Doctoral dissertation to my good friend
Dr. Robert S. Feldman. Thank you Bob.

ACKNOWLEDGEMENT

I would like to thank my wife Meryl for enduring the past six years. There were many joys and many frustrations, but we survived. You listened to my neverending promises of 'just one more semester'. Would you believe me now if I said 'just one more semester'. "Really! Just one more semester!"

Special thanks are also extended to my mother and to my brother Paul for unconditional financial support throughout this ordeal.

I would also like to thank Dr. Jerrold S. Meyer for taking a chance with me. It is not often that a faculty member accepts a new 4th year graduate student. Although we worked together for only a year an a half, it was one of the most productive times of my life. At last I may have learned to write.

I would finally like to thank the remainder of my committee members; Dr. George N. Wade for his patience with me this past year, and also for his unqualified support and funding while I finished writing my dissertation. Drs. John W. Donahoe and Gordon A. Wyse for gratiously serving as members of my dissertation committee, and for helpful comments and suggestions. John and Gordy are probably the most frequently solicited faculty members to serve on thesis and dissertation committees in the Physiological area. Your efforts do not go unappreciated.

ABSTRACT

PROFILE OF CL 218,872, A NON-BENZODIAZEPINE

ANXIOLYTIC OF THE TRIAZOLOPYRIDAZINE CLASS

(September, 1984)

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There exist in brain at least two pharmacologically
distinct types among benzodiazepine (BDZ) receptors. Type I
receptors show a high affinity for both BDZs and triazolo-
pyridazines (TPZ), a class of non-BDZ anxiolytic compounds.
Type II receptors show a high affinity for BDZs, but a low
affinity for TPZs. Because TPZs have a selective affinity
for Type I BDZ receptors, they afford a unique opportunity
to examine whether various BDZ actions are mediated via
this BDZ receptor subtype.

In experiment 1 rats were trained to discriminate the
BDZ chlordiazepoxide (CDP) from saline. The TPZ CL
218,872 generalized to the CDP discriminative stimulus, an
effect antagonized by the concurrent admisistration of
pentylenetetrazol or amphetamine, but not by bicuculline or
strychnine. These results indicate that the discriminative
stimulus and anticonvulsant effects produced by BDZs may be
mediated via activation of Type I BDZ receptors.

In the next experiment acute injection of CDP decreased the turnover rate in brain of serotonin (5-HT), but not that of catecholamines (CA), while chronic CDP treatment decreased the turnover rates of both transmitters. In contrast, acutely or chronically administered CL 218,872 did not alter baseline 5-HT or CA turnover rates. These results indicate that stimulation of Type I receptors is not sufficient to decrease 5-HT and CA turnover rates, and more importantly question the long held belief that BDZs exert their anxiolytic and depressant effects via reductions in 5-HT and CA turnover respectively.

In the last experiment a low dose of CDP attenuated the increase in serum corticosterone (CS) produced by exposure to a novel environment plus sound stimulation. Somewhat higher doses elevated CS levels in unstressed rats. Parallel results were obtained after central drug injection CL 218,872, however, did not lower the CS elevation produced by stress, and also failed to alter baseline CS levels in unstressed rats. Furthermore, the CDP effect in stressed rats was fully prevented by PK 11195, a receptor antagonist specific for peripheral-type BDZ receptors, but not by Ro 15-1788, an antagonist specific for central-type BDZ receptors. Taken together, these results implicate peripheral-type BDZ receptors in brain as the target sites through which BDZs influence CS secretion.

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GENERAL INTRODUCTION

Since the introduction of chlordiazepoxide (Librium)
and diazepam (Valium) in the early 1960's, the 1,4-
benozdiazepines (BDZs) have been the most widely prescribed
drugs in medicine. BDZ sales peaked between 1973 and 1975,
with approximately 85 million prescriptions filled each
year. Diazepam alone accounted for fully two-thirds of
these sales. Although BDZs continue to account for roughly
80 percent of all minor tranquilizer and 50 percent of all
psychoactive drug prescriptions filled annually (Harvey,
1980; Rickels, 1980), the overall use of sedative-hypnotic-
anxiolytics has declined dramatically since 1975, with
diazepam sales down from 62 million prescriptions (three
billion pills) per year to "only" 45 and 31 million
prescriptions filled in 1978 and 1981 respectively (Colen,
1982). This 50 percent drop in prescription drug use has
been attributed to better education of both doctors and the
general public about the dangers of some drugs, and of
possible alternative non-drug therapy.

The most characteristic effect that the BDZs produce

1

at the smallest pharmacologically active doses is antianxiety and disinhibition of certain behaviors. In several animal models, the type of behavior that originally has a high frequency of occurrence but that is subsequently suppressed by environmental- or experimenter-induced manipulations seems to be sensitive to the pharmacological effects of these compounds (Geller and Seifter, 1960; Zbinden and Randall, 1967; Margules and Stein, 1968). For several qualitatively different conditions which meet this description, BDZs appear to have a general disinhibitory action which can be measured as a partial or complete restoration of responding to presuppression levels.

In addition to their use in the clinical treatment of anxiety, various members of this series are also used for their anticonvulsant, muscle relaxant, and sedative-hypnotic effects. In addition to chlordiazepoxide and diazepam, oxazepam (Serax; introduced in 1964) and clorazepate (introduced in 1977) are prescribed primarily for their antianxiety effects (Warner, 1965; Shader and Greenblatt, 1977). Flurazepam (Dalmane; introduced in 1970) was the first BDZ in the U.S. indicated specifically for the treatment of insomnia, and has subsequently accounted for fully half of all sleeping pill prescriptions (Smith, 1979). Although flurazepam is still the most widely

prescribed sedative-hypnotic, temazepam and triazolam were
introduced as hypnotic agents in 1981, and at least five
other BDZ derivatives (quazepam, brotizolam, estazolam,
midazolam, and lormetazepam) are currently under investi-
gation as sleep-inducing medications (Greenblatt et al.,
1982).

Despite the clinical popularity of BDZs, their site
and molecular mechanisms of action remain somewhat unclear.
"Mechanisms" is used in the plural because the actions of a
psychotropic drug may be expressed on multiple levels,
ranging from behavioral studies to receptor binding assays.
Understanding the mechanism of action of a psychotropic
drug comprises the knowledge of a long chain of events
initiated by the recognition of its specific receptor
molecule in the central nervous system (CNS) by the drug.
Binding of the drug may either 'activate' the receptor
(i.e., alter its conformation in such a way that it will
induce further changes in adjacent structures) or
'inactivate' the receptor (i.e., alter its conformation in
such a way that it cannot be activated by other endogenous
or exogenous molecules that are otherwise capable of
activating it). The activated receptor induces, and the
inactivated receptor prevents, changes in other subcellular
elements of the target cell which regulate its

characteristic activity (e.g., ionophores, enzymes). The altered activity of target cells modifies the interaction of neuron populations in one or more areas of the CNS and results directly in somatic drug effects, and, in an essentially unknown way, in psychotropic effects.

Benzodiazepine Receptors

Perhaps the most significant advance made in understanding the molecular basis of BDZ action was the simultaneous discoveries in 1977 by Squires and Braestrup, and by Mohler and Okada of a specific receptor site for these compounds in brain tissue. In these classic studies, it was demonstrated that tritiated diazepam binds to brain membranes with high affinity. This parameter refers to the attraction of the drug for its receptor and is usually expressed as the dissociation constant (Kd; the drug concentration at which 50 percent of the receptors are occupied; the lower the Kd value, the greater the affinity). For ^{3}H-diazepam, the Kd value is extremely low (2.6-3.6 nM) compared to its affinity for serum albumin (10 uM) (Mohler and Okada, 1977). Binding was further characterized as stereospecific, reversible, and saturable, with maximal binding (18 pmol per g original tissue) occurring

at a concentration of 44 nM (Squires and Braestrup, 1977).

Another characteristic feature of brain BDZ receptors is their specificity. The ^{3}H-diazepam binding site was highly selective for BDZs, with 21 clinically active BDZs displacing ^{3}H-diazepam binding with IC50 values in the range of 3 X 10^{-7} M (Squires and Braestrup, 1977). IC50 refers to the concentration causing 50 percent inhibition of specific ^{3}H-diazepam binding. There was a high degree of correlation between the ability of 26 BDZs to displace ^{3}H-diazepam binding in brain and their activity in a number of predictive behavioral tests including anticonflict, muscle relaxant, and anticonvulsant tests (Braestrup and Squires, 1978). In marked contrast to the situation using pharmacologically active BDZs, clinically inactive BDZs and more than 200 non-BDZ compounds (representing 22 distinct pharmacological classes, as well as 14 presumed CNS neurotransmitters, and more than 100 peptides) exhibited low affinity (Ki > 0.1 mM) for 3H-diazepam binding sites (Squires and Braestrup, 1977; Braestrup and Squires, 1977; Bosman et al., 1977). Taken together, these findings strongly indicate that the pharmacological and clinical effects of the BDZs in vivo are mediated through these central BDZ binding sites.

BDZ receptors are widely and unevenly distributed

within the CNS, with very high concentrations identified in frontal and occipital cortex, hippocampus, and cerebellar cortex. Intermediate densities were found in the corpus striatum, globus pallidus, hypothalamus, and retina, while lowest binding occurred in corpus callosum, pons-medulla, and spinal cord (Braestrup and Squires, 1977; Mohler and Okada, 1977; Mackerer et al., 1978; Speth et al., 1978). Subcellular distribution studies revealed that ^{3}H-diazepam binding was mainly localized in the synaptic membrane fraction (Mohler and Okada, 1977; Mackerer et al., 1978). Studies in 'nervous' mutant mice (Lippa et al., 1979), kainic acid lesioned rats (Sperk et al., 1979), and humans with Huntington's disease (Reisine et al., 1979) have further confirmed that BDZ receptors are located on neuronal structures. Conflicting reports, however, exist concerning the identification of BDZ receptors on glial cells as well (Braestrup et al., 1978; Henn and Henke, 1978; Dudai et al., 1979).

High-affinity BDZ binding has also been identified in a number of peripheral tissues including kidney, liver, and lung (Braestrup and Squires, 1978), muscle (Williamson et al., 1978), peritoneal mast cells (Taniguchi et al., 1980), blood platelets (Wang et al., 1980), pineal gland (Weissman et al., 1983), anterior pituitary gland (Schoemaker et al.,

1983), adrenal cortex (Benavides et al., 1983), and most recently in brown adipose tissue (Hirsch, 1984). Peripheral BDZ binding sites are pharmacologically distinct from the previously mentioned brain binding sites in that peripheral receptors show a high affinity for the clinically inactive BDZ Ro 5-4864 (chlordiazepam, differing from diazepam by only a single p-chloro substitution), and a low affinity for the clinically potent BDZ clonazepam. Exactly the opposite holds true for brain-type BDZ receptors. The nonneuronal sites do not show binding regulation by GABA or by any other agents described below which affect the neuronal BDZ binding sites (Tallman, 1980).

With the development of tritiated Ro 5-4864, peripheral-type BDZ receptors were recently identified in brain (Marangos et al., 1982; Schoemaker et al., 1983). The highest concentration was measured in olfactory bulb, intermediate concentrations were found in hypothalamus, cerebellum, and brainstem, and the lowest concentrations were observed in cortex, striatum, and hippocampus. A comparison of the central- and peripheral-type BDZ receptor density shows that ^{3}H-Ro 5-4864 binding density amounts to about 10-15 percent of that of the central-type receptor in the cortex and hippocampus, and 60 percent in the olfactory

bulb and cerebellum. The brainstem and pituitary respectively contain 1.5 and 30 times as many peripheral-type as central-type recognition sites (Schoemaker et al., 1983). An examination of subcellular distribution revealed that these peripheral-type BDZ receptors were mainly located in the nuclear fraction. Thus, the regional and subcellular distribution of peripheral-type BDZ receptors in brain is quite different from that of classical central-type BDZ receptors. Although some evidence has implicated peripheral-type BDZ receptors in the regulation of prolactin release (Grandison, 1981) and in experimentally-induced hypertension (Regan et al., 1981), the physio-logical significance of this unique type among BDZ receptors remains to be firmly established.

Benzodiazepine - GABA Interactions

Communication between nerve cells is made possible by the release of neurotransmitters, at least 20 of which have already been identified. Hence, drugs that modify behavior have invariably also been found to modify the biosynthesis, release, reuptake, or metabolism of neurotransmitters. Early studies on the interactions between the BDZs and brain neurotransmitters demonstrated that BDZs decreased

the turnover rates of acetylcholine, serotonin, dopamine, and norepinephrine within the same dose range that produces their therapeutic actions (for reviews, see Feldman and Quenzer, 1984; Costa and Greengard, 1975). This effect occurred with all neurotransmitters studied, and the direction of change was identical in all cases, suggesting that the decreased turnover of neurotransmitters might not be a primary effect; rather these alterations might be occurring secondary to a more general CNS depression.

Therefore, it is not surprising that more recent investigations have focused on BDZ interactions with the inhibitory neurotransmitters glycine and gamma aminobutyric acid (GABA). In one of the original neurotransmitter receptor binding studies, Snyder and Enna (1975) demonstrated that BDZs could inhibit tritiated strychnine binding to glycine receptors in rat spinal cord membrane prepartions with relative potencies that were highly correlated with their relative potencies as anxiolytics and muscle relaxants. However, the theory that BDZs exert their therapeutic effects by directly activating glycine receptors soon became untenable when it was found that 1) BDZs failed to enhance postsynaptic inhibition in the spinal cord, a glycine mediated phenomenon (Curtis et al., 1976), and 2) the potency of BDZs to inhibit glycine

receptors soon became untenable when it was found that 1) BDZs failed to enhance postsynaptic inhibition in the spinal cord, a glycine mediated phenomenon (Curtis et al., 1976), and 2) the potency of BDZs to inhibit glycine receptor binding was 1000 fold lower than the affinity of BDZs to bind to their own receptors.

In trying to assign a particular neurotransmitter system as the primary site of action of BDZs, overwhelming evidence would seem to implicate GABA for such a role. As is true for other neurotransmitter systems, BDZs decrease GABA turnover in the brain (Bertilson et al., 1977). Unlike the case with other systems, however, a wealth of electrophysiological and biochemical data identified GABAergic synapses as the primary site of action of the BDZs (for reviews, see Gallager, 1983; Tallman et al., 1980; Gallager et al., 1980; Olsen, 1981).

Electrophysiological studies

Using electrophysiological techniques to measure spontaneous electrical activity, evoked responses, or single-cell neuronal activity, several investigators have concluded that at least some of the actions of BDZs result from a specific interaction with GABA. Schmidt et al. (1967) were the first to observe the potentiation by

diazepam of presynaptic inhibition in the cat spinal cord, and they suggested that this might contribute to the central muscle relaxant effect of this drug; postsynaptic inhibition in the spinal cord was found to be unaffected by diazepam. The significance of these findings for the mechanism of action of BDZs was not recognized for several years, first, because at that time the phenomenon of presynaptic inhibition was still a matter of debate among physiologists and, second, because the transmitter mediating this type of inhibition was not known.

When in the early seventies increasing evidence indicated the role of GABA in the axo-axonal synapses that mediate presynaptic inhibition of primary afferent endings, Polc et al. (1974) reinvestigated the effects of diazepam on the cat spinal cord. Diazepam increased the amplitude and duration of the dorsal root potential, which is the expression of the synaptically-induced depolarization of primary afferent endings, and enhanced the presynaptic inhibition of the monosynaptic excitation of spinal motoneurons (ventral root reflex). GABA antagonists such as picrotoxin and bicuculline antagonized this diazepam action. After depletion of endogenous GABA by the synthesis inhibitor thiosemicarbazide, presynaptic inhibition was abolished and could no longer be restored by

BDZs. By itself diazepam did not produce a depolarization of primary afferents, which means that BDZs have no GABA-mimetic action.

In order to establish whether this BDZ action was unique to presynaptic inhibition in the spinal cord, or could be extended to all GABA-mediated neurotransmission, Polc and Haefely (1976) studied the effect of diazepam on dorsal column nuclei, a site where GABA has been shown to mediate both presynaptic and postsynaptic inhibition. Diazepam potentiated both types of synaptic inhibition (recorded in the cuneate nucleus). As already seen in the spinal cord, BDZs and GABA antagonists were mutually antagonistic in their effects on synaptic inhibition, and again the depletion of endogenous GABA by thiosemicarbazide abolished any effect of diazepam. The results in the cuneate nucleus, therefore, strongly suggested that these drugs might similarly affect all GABAergic synapses in the CNS, and for the first time it was considered possible that the whole pharmacology of the BDZs could be explained by a facilitation of post synaptic GABA receptors.

Further evidence for this notion was provided by Costa and his coworkers using an elegant GABAergic system in the cerebellum. The cerebellum consists of two parts: the cerebellar nuclei where connections to the various parts of

the CNS coordinate muscle movements, and the cerebellar
cortex which exerts an inhibitory influence on the
cerebellar nuclei through the release of GABA from Purkinje
cell axons. The activity of Purkinje cells is regulated by
two excitatory inputs to the cerebellar cortex (the
climbing and mossy fibers), and by a neuronal network
within the cerebellar cortex which inhibits the activity of
Purkinje cells through the release of GABA from inter-
neurons.

The net activity of Purkinje cells, the sole output of
the cerebellar cortex, appears to be related to their
content of cyclic GMP which increases or decreases in
relation to the changes in the excitatory and inhibitory
input to the Purkinje cells (Costa et al., 1975). When
GABA receptors located on Purkinje cells are activated, the
cyclic GMP content of these cells decreases; when they are
inhibited the cyclic GMP content increases. Thus, the
decrease of the cyclic GMP content of cerebellar cortex is
a good index of the state of activation of GABA receptors.
Biggio et al. (1977) showed that diazepam, similar to the
direct GABA receptor agonist muscimol, lowers the cyclic
GMP content of cerebellum by activating GABA receptors.
Furthermore, diazepam antagonized the increase in cerebel-
lar cyclic GMP elicited by isoniazid, an inhibitor of GABA

synthesis.   Diazepam is active when the   cerebellar GABA
content   is decreased by up to about 30 percent.   However,
when this decrease is greater than 30 percent, diazepam
cannot   counteract   the increase in cerebellar cyclic GMP
elicited   by isoniazid (Guidotti, 1978).   In   contrast,
muscimol   completely   counteracts this action of isoniazid
even when the cerebellar content of GABA is reduced by more
than 30 percent.   This suggests that the action of diazepam
on cerebellar GABA receptors depends on the amount of GABA
that is stored in the synapses that impinge on Purkinje
cells.   This finding implies that either GABA is released
by diazepam or that diazepam increases the affinity for
GABA of the postsynaptic GABA receptors.   The   former of
these two possibilities was quickly ruled out when it was
demonstrated that diazepam had no effect on either the in
vitro (MacDonald et al., 1978) or the in vivo (Geller et
al., 1978) release of GABA.   Thus, from electrophysio-
logical studies, alterations in postsynaptic responses to
GABA appeared the most likely mechanism by which BDZs
facilitate GABAergic neurotransmission.

Receptor binding studies

    Although   electrophysiological   experiments   had
indicated an interaction between BDZs and GABA, the first

direct demonstration of the nature of this interaction came soon after the discovery of the brain BDZ receptor in 1977. The application of sensitive receptor-ligand binding assays with compounds of high specific activity has made possible the investigation of postsynaptic mechanisms at the subcellular level. It was originally thought that the BDZ binding site in mammalian brain did not have any relationship to known transmitters (Braestrup et al., 1977). Subsequent studies using lower ligand concentrations, however, revealed an intimate interaction between BDZs and GABA (for reviews, see Ticku, 1983, and Guidotti et al., 1983).

The development of an in vitro biochemical assay for studying brain GABA receptors made it possible to examine the influence of various drugs (notably BDZs, barbiturates, and convulsants) on this site (Enna and Snyder, 1975). For this assay, membranes prepared from brain tissues are incubated with ^{3}H-GABA, and under the appropriate conditions, the number of GABA receptors in the tissue can be quantified by measuring the amount of isotope bound. When added to the incubation mixture, drugs capable of interacting with this receptor site will inhibit the binding of ^{3}H-GABA. The potency of a drug to inhibit ^{3}H-GABA binding may be taken as an index of its potency to

activate, or inhibit, the receptor site in vivo.

A major breakthrough in the understanding of the intimate interaction that exists between BDZs and GABA receptors occurred following an attempt to solubilize the GABA receptor (Enna and Snyder, 1975). Usually the recognition sites for transmitters are solubilized when the membranes are treated with the nonionic detergent Triton X-100. This procedure disrupts the membrane and washes away any water-soluble proteins that might be adhering to the lipid membrane. Therefore, such a treatment reduces the number of receptors (Bmax) for these transmitters, but leaves their receptor affinity (Kd) either unchanged or slightly decreased. In contrast to the usual situation, when brain membranes were incubated with a low concentration of Triton X-100, the amount of ^{3}H-GABA bound to membranes increased dramatically. Scatchard analysis indicated that both the Bmax and the Kd were significantly increased in Triton-treated tissue. In fact, treatment with Triton X-100 revealed the presence of a second GABA receptor site with an affinity for the neurotransmitter some ten times greater than the site observed in tissue not treated with the detergent (Enna and Snyder, 1977). This finding was interpreted as indicating that, on or near the GABA receptor recognition site, there is a substance (or

substances) that normally masks the higher affinity site. Removal of this substance leads to a more sensitive receptor since a higher affinity site will be activated by a lower concentration of ligand. Guidotti et al. (1979) found that, if membranes are incubated with BDZs rather than Triton, the same phenomenon occurs. Thus, without BDZ treatment, only a single, low affinity (210 nM) GABA receptor binding site can be detected in brain membranes. If the tissue is preincubated with increasing concentrations of diazepam, however, a second, higher affinity site becomes evidenced. At maximal concentrations of diazepam this high affinity site has approximately ten-fold greater attraction for GABA than the low affinity component. These authors reported similar effects with other BDZs such as nitrazepam, flunitrazepam, and clonazepam.

To verify the possibility that untreated GABA receptors contained an endogenous inhibitor of the high-affinity GABA receptor, Costa et al. (1978) prepared two types of crude synaptic membranes from rat cerebral and cerebellar cortex. Their Type B membranes were freshly prepared and showed the characteristic low affinity GABA binding. Type A membranes were obtained by further treating Type B membranes with Triton X-100, such that they demonstrated both high and low affinity GABA binding. When

aliquots of supernatant buffer obtained by centrifuging a
suspension of Type B membranes were added to Type A
membranes, high affinity ^{3}H-GABA binding to Type A
membranes was inhibited. A 15,000 dalton protein was
identified following 500-fold purification of the Type B
supernatant. Addition of 0.33 ug of this protein to Type A
membranes completely abolished high affinity GABA binding.
In other words, there appears to be an endogenous protein
inhibitor of high affinity GABA binding that can be removed
following treatment with Triton X-100. When extracted from
non-treated membranes (Type B) and added to Triton-treated
membranes with unmasked high affinity GABA receptors (Type
A), the endogenous inhibitor remasks these high affinity
GABA recognition sites. This endogenous inhibitor was
termed GABA-modulin.

These authors further demonstrated that diazepam can
reverse the effect of GABA-modulin on Type A membranes.
Similarly, GABA-modulin was shown to inhibit ^{3}H-diazepam
binding to Type A membranes. From these results it was
proposed that BDZs compete with GABA-modulin for the high
affinity GABA recognition site. These findings were quite
provocative, and represented a breakthrough in understan-
ding the mechanism of action of BDZs. It was surmised that
BDZ administration results in the displacement of GABA-

modulin, revealing a higher affinity GABA receptor site, making the postsynaptic membrane more sensitive to activation by GABA. In this way, BDZs can potentiate GABAergic activity, which may in turn influence the activity of other neurotransmitter systems (Guidotti et al., 1979).

The effects of various drugs on labelled BDZ binding in brain were being simultaneously investigated. Using cortical membranes, the in vitro addition of GABA or GABA agonists such as muscimol enhanced the binding of ^{3}H-diazepam (Tallman et al., 1979; Williams and Risley, 1979). Other amino acid transmitters including glycine, glutamate, and aspartate did not affect ^{3}H-diazepam binding (Tallman et al., 1978), indicating that this effect was specific to GABA. It was further established that the enhanced binding of BDZs observed after the addition of GABA was due to an increase in the affinity of the BDZ receptor for its ligand without altering receptor number (Tallman et al., 1978). In vivo pretreatment of animals with the GABA catabolic inhibitor amino oxyacetic acid (AOAA) resulted in an increase in specific ^{3}H-diazepam binding. Pronounced increases in BDZ binding were also observed after pretreatment of animals with the GABA analogs muscimol, baclofen, and gamma-butyrolactone (Gallager et al., 1978). The enhancement of BDZ binding by either GABA or muscimol was

blocked by the in vitro addition of the GABA antagonist
bicuculline (Tallman et al., 1978). Taken together with
results from GABA receptor binding studies, it is apparent
that BDZ binding can be altered by GABA and GABAergic
compounds, and similarly GABA binding can be modulated by
BDZs.

Soon after the GABAergic modulation of BDZ binding was
described, it became clear that a number of other compounds
were capable of enhancing the affinity of the BDZ receptor.
Prominent among these were several physiologically relevant
anions, including chloride, iodide, and thiocyanate (Costa
et al., 1979). These results were not surprising, however,
since it was already well established that the GABA
receptor is closely linked with a chloride ion channel.
When a GABA molecule is associated with its receptor site,
chloride gates (ionophores) open up, allowing chloride ions
to freely diffuse across the cell membrane according to the
concentration gradient. The consequence of such an
association, whether hyperpolarization of postsynaptic
membranes or depolarization of presynaptic membranes, is
always reduced effectiveness of sodium excitatory
conductance (inhibition). Thus, the above data are consis-
tent with the notion that the GABA/BDZ receptor complex is
associated with a chloride ionophore.

The fourth and last component of this putative supra-molecular GABA complex is a picrotoxin/barbiturate binding site characterized by high affinity ^{3}H-dihydropicrotoxin (DHP) binding. An analogue of picrotoxin, DHP is also a potent convulsant and GABA antagonist. Although neither GABA nor BDZs bind to the site labelled by DHP, this site appears to be an important locus of action for a number of centrally active depressant and convulsant drugs (Olsen, 1981).

The binding of DHP is inhibited in a stereospecific manner by barbiturates, particularly those with anesthetic and anticonvulsant properties (Willow and Johnston, 1981). DHP binding is similarly inhibited by diphenylhydantoin and ethanol (Ticku and Davis, 1981), as well as by etazolate and cartazolate, several new non-BDZ anxiolytic compounds of the pyrazolopyridine class (Williams and Risley, 1979).

Evidence exists that the pharmacological effects of barbiturates, ethanol, diphenylhydantoin, and the pyrazolo-pyridines may be mediated through GABAergic mechanisms, since each of these drugs increases the number of detectable GABA receptors and potentiates the GABAergic modulation of BDZ binding site affinity. The ability of these agents to enhance GABA binding was blocked by either picrotoxin or DHP (Supavilai and Karobath, 1980; Olsen,

1981; Ticku, 1983; Greenberg et al., 1984). The in vivo significance of this picrotoxin/barbiturate recognition site remains to be determined.

Thus, it is currently believed that BDZs, anticonvulsant and anesthetic barbiturates, alcohol, diphenylhydantoin, certain convulsants, and selected non-BDZ anxiolytics exert at least some of their pharmacological effects through an interaction at a four-site supramolecular complex. Binding sites for DHP, GABA, and BDZs show a similar subcellular and brain regional distribution (Ticku et al., 1978), further supporting such a possibility. This molecular complex is comprised of a GABA recognition site, a BDZ and GABA-modulin recognition site, a picrotoxin and barbiturate recognition site, and an associated chloride ionophore. Although it is not fully clear how interactions at these sites mediate one or another of the pharmacological actions ascribed to these drugs, a number of hypothetical models of this receptor complex have been advanced in an effort to better integrate existing data (Feldman and Quenzer, 1984; Guidotti et al., 1983; Gallager et al., 1980). According to these proposals, the GABA recognition site is believed to be functionally linked to the chloride ionophore, and a conformational change resulting from an interaction between

GABA and its receptor is proposed to regulate the opening of this ion channel. BDZs and GABA-modulin compete for a satellite receptor attached to the GABA receptor. Neither of these substances directly activates the chloride ionophore; rather they are believed either to facilitate (BDZs) or to hinder (GABA-modulin) the activation of the chloride ionophore by GABA. The final ligand binding site is shared by picrotoxin and barbiturates, and is believed to be closely linked to the chloride conductance channel at a site distal from the GABA receptor. The final transducer of this 250- 350,000 dalton supramolecular complex is the chloride ion, and this ion is regulated by what appears a rather complicated set of interactions between endogenous and exogenous chemicals at these various recognition sites.

Endogenous Benzodiazepine Receptor Ligands

By analogy to the search for and the eventual identification of naturally occurring opioid compounds (enkephalins, endorphins, dynorphins), the discovery of a specific BDZ receptor in brain has prompted the quest for a similar endogenous ligand of the BDZ receptor. Such a ligand would be expected to bind with high affinity at physiological concentrations to BDZ receptors and would also be expected

to display "BDZ-like" pharmacological actions in vivo. Using receptor binding methodology to assay tissue extracts for the ability to inhibit labelled BDZ binding, a fair number of putative endogenous ligands for the BDZ receptor have been identified (for reviews, see Guidotti et al., 1983; Hamon and Soubrie, 1983). The ultimate goal in seeking endogenous ligands for drug receptors is a better understanding of the natural mechanisms underlying the drug mediated physiological effects. For example, endogenous "BDZ-like" substances would be expected to have anxiolytic, anticonvulsant, and muscle relaxant effects. Identification of these natural compounds and characterization of their interaction with the BDZ receptor should, therefore, provide insights that concern the mechanisms governing these neurophysiological processes.

GABA-modulin

The first compound to be isolated from brain that competitively inhibited ^{3}H-diazepam binding from cortical membranes was the aforementioned GABA-modulin. This water soluble, basic, heat stable protein having a 15,000 dalton molecular weight was first reported to inhibit GABA binding, and also to prevent the increase in BDZ binding produced by GABA (Guidotti et al., 1978). These results

led Guidotti et al. (1979) to propose that GABA-modulin might be the endogenous ligand for the BDZ recognition site. However, GABA-modulin was extremely difficult to purify due to its rapid degradation of GABA-modulin by proteolytic enzymes, and secondly because of problems encountered removing GABA itself from the crude extract. For this reason, GABA-modulin could not be fully characterized, and sufficient quantities for pharmacological and physiological testing were not obtainable. Thus the "BDZ-like" effects of this substance could not be assessed.

However, Guidotti et al. (1983) eventually succeeded in isolating and characterizing GABA-modulin. It has now been established that purified GABA-modulin contains 126-131 amino acids (none of which is GABA) and has a molecular weight of 16,500 daltons. Purified GABA-modulin blocked high affinity ^{3}H-GABA binding to synaptic membranes, and prevented GABA-stimulated diazepam binding (Guidotti et al., 1982). The presence of a large number of serine and threonine amino acid residues that are preferential phosphate acceptors, led Wise, Guidotti, and Costa (1983) to investigate whether GABA-modulin could be phosphorylated and whether phosphorylation would modify the biological activity of this protein. Results showed that

GABA-modulin could be phosphorylated by both a cAMP-dependent protein kinase and various calcium- and calmodulin-dependent kinases. The cAMP-dependent phosphorylation, but not that catalysed by calcium-dependent kinases, abolished the capacity of GABA-modulin to mask the high affinity binding sites for GABA in brain membranes. It was further shown that the enzymes necessary for phosphorylation are present in vivo in close proximity to the endogenous GABA-modulin in synaptic membranes.

Based on these findings, Guidotti et al. (1983) and Wise et al. (1983) now postulate that GABA-modulin functions in GABA receptors as a coupling protein. In their view, GABA-modulin is not normally phosphorylated. When the BDZ recognition site is occupied by an anxiolytic BDZ the protein is phosphorylated and the high affinity recognition sites of the GABA receptor are unmasked. Thus, GABA-modulin is no longer envisioned as an endogenous ligand for the BDZ receptor; rather this protein is considered a coupling mechanism by which BDZ recognition sites modify GABA receptor mechanisms.

Purines

Using an acetone extraction procedure, Marangos et al. (1978) isolated two non-peptidergic fractions from bovine

brain that inhibited ^{3}H-diazepam binding. Further analysis identified these two fractions as the purines inosine and hypoxanthine. The affinity of inosine and hypoxanthine for the BDZ receptor was approximately 20,000 times lower than that of the BDZs, with the Ki values for inosine and hypoxanthine 836 and 982 uM respectively. These compounds did not bind to opiate, beta-adrenergic, muscarinic cholinergic, or GABA receptors, indicating that purinergic binding is specific for the BDZ receptor (Osano and Spector, 1979). Although inosine and hypoxanthine possessed weak anticonvulsant effects, the concentrations required were extremely high (150 ug i.c.v. and 1 g/kg i.p.) (Marangos et al., 1981).

When viewed in the context of other receptor-ligand systems (with binding potencies in the nanomolar range), the extremely low potency of the purines as inhibitors of diazepam binding seems to preclude their being physiological modulators of BDZ receptors in vivo. However, it was argued by Marangos et al. (1980) that following electrical or chemical depolarization of brain tissue, the concentrations of inosine and hypoxanthine rise dramatically. As further support for their position, they cite the demonstration by Lippa et al. (1979) that only 15-20 percent BDZ receptor occupancy is necessary either to

produce conflict avoidance in the rat, or to fully antago-
nize convulsions in mice produced by pentylenetetrazol.
These arguments notwithstanding, with the concentration of
these compounds in brain estimated to be between 20-60 uM
(and Ki values for diazepam inhibition close to 1 mM), it
seems improbable that BDZ receptors could be occupied by
these compounds at concentrations sufficient to produce any
meaningful effects. As suggested by Karobath (1983), with
binding inhibition evidenced only at concentrations close
to their limits of solubility in aqueous solutions, in
order for the purines still to be considered as endogenous
ligands for the BDZ recognition site, it would first be
necessary to propose some alternative hypothesis such as
compartmentalization with high concentrations at GABAergic
synapses.

Nicotinamide

Using an extraction procedure similar to that employed
for inosine and hypoxanthine, Mohler et al. (1979) isolated
nicotinamide from bovine and rat brain. This naturally
occurring compound similarly inhibited ^{3}H-diazepam binding.
The IC_{50}, however, was extremely high (3.9 mM). Although
nicotinamide displayed "BDZ-like" properties as a muscle
relaxant and anticonvulsant, and showed "BDZ-like" effects

in the rat conflict test, the extremely low concentration
of nicotinamide in brain (0.1 mM) (Mohler et al., 1979) is
difficult to reconcile with a physiologically significant
action. Therefore, for reasons similar to those already
expressed for the purines, nicotinamide can probably be
excluded from consideration as an in vivo BDZ ligand.

Indoles

Squires et al. (1979) reported that tryptophan
displaces ^{3}H-diazepam binding with a potency comparable to
that of the purines and nicotinamide. This prompted
Marangos et al. (1981) to investigate the effects of a
dozen tryptophan derivatives on diazepam binding. Of those
compounds tested, melatonin and its brain metabolite N-
acetyl-5-methoxy kynurenamine (AMK) were found to be the
most potent, with Ki values of 415 and 49 uM respectively.
Therefore, melatonin and AMK were two- and twenty-fold more
potent than the purines or tryptophan as inhibitors of BDZ
binding. However, the concentration of melatonin is only
20 uM in pineal and 1.5 uM in hypothalamus (Marangos et
al., 1981), effectively eliminating melatonin from
contention as the endogenous BDZ-like substance in brain.
It is of interest to note, however, that of all the
putative endogenous BDZ ligands tested to date (including

AMK and the purines), only melatonin was a more potent inhibitor of peripheral-type BDZ binding than of central-type BDZ binding. Whether melatonin serves as a natural ligand for the peripheral-type BDZ receptor, however, remains to be determined. Since the concentrations of AMK in pineal and in brain have not been determined, judgement concerning the physiological relevance of this compound cannot be assessed.

Miscellaneous compounds

Additional low molecular weight compounds that displace BDZ binding have been isolated from brain and urine, but have not been identified (Clow et al., 1983; Massotti et al., 1981; Colello et al., 1978). Before these substances can be considered as possible endogenous "BDZ-like" compounds, it first must be demonstrated that they exist in brain, and secondly it must be shown that they were not formed during the extraction procedure. Such caution is merited in light of the recent demonstration that the putative endogenous BDZ-ligand isolated from rat, bovine, and human brain by Braestrup et al. (1980), beta-carboline-3-carboxylate ethyl ester, was indeed an artifact of their extraction procedure (Karobath, 1983)..

Although still in its early stages, the evidence

summarized above indicates that endogenous "BDZ-like" compounds do exist in the CNS. However, in order that a putative endogenous ligand for the BDZ receptor be established as a true neurotransmitter or neuromodulator, the following criteria have to be satisfied: 1) The compound should competitively inhibit BDZ binding and be present in vivo at concentrations that are consistent with the binding potency. 2) It should mimic the neurophysiologic and biochemical effects of BDZs. 3) Administration of the compound to animals should produce "BDZ-like" pharmacological and behavioral effects. 4) Nervous tissue should be able to metabolize, store, and release the compound in question. 5) The receptor binding inhibition should be specific for BDZs. Although each of compounds just described meet some of these criteria, the evidence to date argues that the natural ligand for the BDZ receptor still remains to be identified.

Exogenous Benzodiazepine Receptor Ligands

Stimulation of BDZ recognition sites by various ligands can elicit opposite types of pharmacological responses. The study of the pharmacological profiles of these various ligands makes it possible to distinguish

three classes of compounds. The first group comprises the classical BDZs and three chemically unrelated compounds, zopiclone (Wickstrom and Giercksky, 1980), CL 218,872 (Lippa et al., 1979), and CGS 9896 (Gee and Yamamura, 1982). Binding of this group of ligands produces the typical BDZ profile, including anticonvulsant, muscle relaxant, and anticonflict effects. The term _agonist_ has been applied to members of this group.

The second group of BDZ receptor ligands similarly displaces BDZs from specific binding sites, yet members of this group display a pharmacological profile opposite to that of the BDZs. Consequently, these so called _inverse agonists_ exhibit proconvulsant, convulsant, and anxiogenic properties. However, in conjunction with an _agonist_, the pharmacological effects of both compounds are attenuated (for reviews, see Boast et al., 1983; Braestrup et al., 1983, 1984). So far this group is restricted to beta-carboline-3-carboxylates such as the methyl-, ethyl-, and propyl-esters (BCCM, BCCE, and BCCP), the methylamide (BCCMA), and 6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM).

The third class of exogenous BDZ receptor ligands is comprised of compounds that display no pharmacological actions of their own, except at very high doses, yet

interaction with this recognition site.

MK-801

A dibenzocycloheptenimine derivative MK-801 (Merck) is a potent anxiolytic and anticonvulsant in rats (Clineschmidt et al., 1982), without profound sedative and muscle relaxant effects (Goldberg et al., 1983). MK-801 had no effects on ^{3}H-diazepam or ^{3}H-flunitrazepam binding up to 1 uM (Goldberg et al., 1983).

CL 218,872

It was originally believed that there existed in brain only a single homogeneous class of central-type BDZ binding sites. In both rat and human, Scatchard analysis of equilibrium binding yielded straight lines with no tendency to resolve into more than one component (Braestrup and Squires, 1977). Hill analysis of displacement curves for 15 BDZs yielded Hill coefficients near unity (Speth et al., 1978). Such coefficients are generally interpreted to indicate a single binding site as well as the lack of cooperativity between binding sites. Further support for the presence of a single class of central-type BDZ binding sites was provided by the demonstration that thermal

inactivation of binding sites (preincubation of membrane preparations at 60°C in Tris HCl buffer) was monophasic (Squires et al., 1979).

However, more recent evidence, together with a reevaluation of some existing data, now indicates that there exist two distinct types (or subtypes) among central-type BDZ receptors. For example, while the ability of BDZs to displace ^{3}H-diazepam binding was highly correlated with their anticonflict (Lippa et al., 1978) and anticonvulsant (pentylenetetrazol-induced; Paul et al., 1979) potencies, only 15-20 percent of ^{3}H-diazepam binding had to be displaced to observe these effects. When heat inactivation of labeled BDZ binding sites is carried out in sodium phosphate buffer rather than Tris HCl, binding sites disappear in a biphasic manner with half-lives of approximately 10 and 70 minutes at 60°C (Squires et al., 1979). In addition, approximately 40 percent of the total number of ^{3}H-flunitrazepam binding sites were found to be GABA-independent (not protected by GABA or muscimol against thermal inactivation) (Klepner et al., 1979). Consistent with the latter finding, about 50 percent of the BDZ receptors are not coupled to chloride ionophores (Klepner et al., 1979).

Perhaps the single most significant advance in the

concept of BDZ receptor heterogeneity was the development of the triazolopyridazines (TPZs). TPZs were the first non-BDZ compounds found that bind to central-type BDZ receptors, both in vivo and in vitro, with a potency comparable to that of the BDZs (Squires et al., 1979). TPZs, however, have an extremely low affinity for peripheral-type BDZ receptors, both in brain and in kidney (Marangos et al., 1981). This unique class of compounds interacts with central-type BDZ receptors with Hill coefficients significantly less than unity (0.5 - 0.7; Squires et al., 1979), indicating the existence of more than one BDZ receptor site. Hofstee analysis of the displacement of labeled diazepam binding by TPZs yielded curvilinear plots which could be resolved into two components (Lippa et al., 1979), again indicating the existence of more than one receptor type. The high-affinity sites for TPZs were designated as Type I receptors, while the low-affinity sites were designated as Type II receptors. Squires et al. (1979) and Klepner et al. (1979) further characterized the Type I receptor as independent of GABA receptors and chloride ionophores. Type II receptors, on the other hand, were coupled to GABA receptors and/or chloride ionophores.

Unlike the situation using BDZs, the potency of CL

218,872 to displace ^{3}H-diazepam binding, as well as its calculated Hill coefficient, varied as a function of brain region (Morelli et al., 1981; Klepner et al., 1979). CL 218,872 was found to be most potent in cerebellum, less potent in thalamus, frontal cortex, globus pallidus, and substantia nigra, and least potent in putamen, hippocampus, and hypothalamus (Morelli et al., 1981). Hofstee analysis revealed that these differences were not due to any differences in the affinity of CL 218,872 for the two receptor types, but rather to differences in the relative density of Type I and Type II BDZ receptors in each brain region (Lippa et al., 1980). They reported that approximately 90 percent of the BDZ binding sites in cerebellum are Type I receptors, whereas only 40 percent of the BDZ binding sites in hippocampus are Type I receptors.

These results confirmed previous reports of multiple types among central-type BDZ receptors and firmly demonstrated the existence of two pharmacologically distinct central-type BDZ binding sites. Both BDZs and TPZs have a high affinity for Type I receptors, whereas only BDZs have a high affinity for Type II receptors. Equally important, however, was the demonstration that the prototypical TPZ, CL 218,872, similar to the BDZs, increased punished responding in a conflict situation and

protected against pentylenetetrazol-induced convulsions (Lippa et al., 1979), pharmacological properties which are highly predictive of anxiolytic activity. This finding further strengthened the notion that antianxiety activity and affinity to the BDZ receptor are closely related. However, unlike the BDZs, CL 218,872 was purportedly devoid (at anxiolytic doses) of depressant side effects associated with the BDZs (i.e., sedation, ataxia) and was very weak in its ability to inhibit the convulsions produced by bicuculline, isoniazid, or strychnine (Lippa et al., 1979).

In addition to further confirming the existence of heterogeneous forms of BDZ receptors, the TPZs present a unique opportunity to further clarify the molecular basis by which BDZs induce their various behavioral and physiological actions. As such, the TPZs may represent a new probe for selectively investigating the neural mechanisms that underlie anxiety and its alleviation. Specifically, it may be possible to infer that any similar actions produced by both BDZs and TPZs (at appropriate doses) result from activation of Type I BDZ receptors. Alternatively, any actions produced by BDZs, but not by TPZs, may be attributable to Type II BDZ receptor stimulation.

Furthermore, since the TPZs may ultimately be of great

value in the clinical treatment of anxiety disorders due to their lack of depressant side effects, it is important to learn more about the behavioral, neurochemical, and endocrine actions of these compounds. Indeed pharmacologists are always seeking drugs with enhanced specificity. It is therefore important to learn more about the similarities and differences between the actions of BDZs and this novel class of compounds, so as to better assess their potential for clinical application.

Therefore, the present series of investigations examined the ability of the prototypical TPZ, CL 218,872, as compared to the BDZ, chlordiazepoxide, to:

1) induce a discriminative stimulus complex similar to that produced by chlordiazepoxide. The potencies at which BDZs generalize to the discriminative cue elicited by chlordiazepoxide correlate well with the potencies of their therapeutic effects (Colpaert et al., 1976). For this reason Lal and Sherman (1982) have suggested that the BDZ-saline drug discrimination paradigm may represent an animal test predictive of anxiolytic activity.

2) alter serotonin and catecholamine turnover rates. It has been proposed that BDZs exert their antianxiety effects by reduction of serotonin activity, and their depressant effects by reduction of catecholamine

activity (Wise et al., 1972). According to this hypothesis one might expect CL 218,872 to decrease serotonin turnover, yet have no effect on catecholamine turnover.

3) prevent stress-induced elevations in serum cortico-sterone. Pretreatment with BDZs or with other agents possessing antianxiety activity, such as meprobamate or barbiturates, has been reported to block or reduce the elevation in circulating corticosterone (a glucocorticoid hormone secreted from the cortex of the adrenal glands) produced by experimenter-induced stress. These and other findings have led Lahti and Barsuhn (1974), and LeFur et al. (1979) to propose that the corticosterone stress test could be used to screen for potential antianxiety agents. Accordingly, one might expect CL 218,872 to block stress-induced elevations in corticosterone concentrations.

Finally, since the above behavioral, neurochemical, and endocrine actions produced by BDZs have each been considered either predictive of, or responsible for, the anxiolytic potential of this class of drugs, CL 218,872 presents an opportunity either to confirm and extend or to refute the validity of such measures as indicators of a drugs' clinical (anxiety reducing) potential.

C H A P T E R I I

EXPERIMENT I

GENERALIZATION BETWEEN CHLORDIAZEPOXIDE- AND

CL 218,872-ELICITED DISCRIMINATIVE CUES

Introduction

Several approaches have been used to establish the
pharmacological identities of BDZs. One such approach has
been the use of drug discriminative paradigms. Drug
discrimination learning requires the drug to produce a
perceivable change in some aspect of the animal's internal
environment. By producing this interoceptive cue, the drug
versus saline injections can then be applied as distinct
discriminative stimuli, thus making it possible to train
animals to perform one type of behavior for reinforcement
following a drug injection and to perform a different, but
topographically similar behavior following injection of
drug vehicle.

In the majority of drug discrimination studies, the
animal is required to either make opposite turns in a T-
maze (Overton, 1978) or opposite choices in a two-lever
test chamber (Colpaert et al., 1976). Different, but
equivalent responses have also been trained in other test

procedures, such as the Lashley jumping stand (Brown et al., 1968) and choices between side compartments in a three-chambered apparatus (Schechter and Rosecrans, 1973). The incentive for the correct response in these studies has either been food reward or escape from shock. When acquisition of such response differentiation is reliably established, the drug is said to produce a discriminative stimulus. At this point, stimulus generalization and antagonism experiments can be employed in an attempt to establish the biochemical mechanisms underlying elicitation of the discriminative stimulus (drug cue).

The BDZs have been extensively investigated in drug discrimination procedures. For example, chlordiazepoxide (CDP) is able to produce a discriminative stimulus in rats (Colpaert et al., 1976; Colpaert, 1977; Brown et al., 1968; Overton, 1979). It was found that the potencies at which other BDZs generalize to the CDP cue (ability of the test compound to produce responding on the CDP appropriate lever) correlate well with their ataxic and anticonvulsant (pentylenetetrazol-induced) effects (Colpaert et al., 1976). Furthermore, only clinically effective BDZs and barbiturates generalized with the standard CDP treatment. Other drugs such as chlorpromazine, haloperidol, thalidomide, and baclofen reliably produced saline lever

selection, even at doses that reduced responding by as much as 80 percent (Colpaert et al., 1976). Taken together, these results demonstrate that the discriminative stimulus produced by BDZs might be relevant to the clinical effects of this class of drugs, and secondly that the discriminative properties constitute a pharmacologically highly specific phenomenon. It has even been suggested that the BDZ-saline drug discrimination paradigm may represent an animal test predictive of anxiolytic activity (Lal and Sherman, 1982).

It has been reported that CL 218,872, a synthetic non-BDZ compound in the triazolopyridazine class selectively displaces 3H-diazepam from its binding sites with a potency intermediate between that of diazepam and CDP (Squires et al., 1979). Similar to the BDZs, CL 218,872 increases punished responding in a conflict situation and protects against pentylenetetrazol-induced convulsions, pharmacological properties that are highly predictive of anxiolytic activity (Lippa et al., 1979). However, unlike the BDZs, CL 218,872 was relatively devoid of depressant side effects and was very weak in its ability to inhibit the convulsions produced by bicuculline and strychnine (Klepner et al., 1979).

The purpose of the present experiment was to compare

further CDP and CL 218,872, structurally distinct, yet pharmacologically similar compounds. Specifically, the aim of this study was to train rats to discriminate between CDP and saline in a two-lever operant task, and to investigate whether or not CL 218,872 would produce a CDP-like discriminative cue. Last, in an effort to further evaluate the specificity of the discriminative complex, convulsant (pentylenetetrazol, strychnine, and bicuculline) and non-convulsant (amphetamine) CNS stimulants were tested for possible antagonism of any CDP stimulus generalization to CL 218,872.

It was previously reported that pentylenetetrazol failed to antagonize the discriminative cue produced by a 5 mg/kg dose of CDP (Colpaert, 1977). Using a modified drug discrimination procedure originally described by Overton (1979), it was recently shown (J.F. McElroy, unpublished data) that in rats initially trained to discriminate 5 mg/kg CDP from saline and retrained to discriminate a lower dose of training drug (3 mg/kg), pentylenetetrazol successfully blocked the CDP discriminative stimulus. Thus, in order to increase the sensitivity of this behavioral test paradigm, all generalization tests in the present experiment were conducted following retraining using this reduced dose of training drug.

Methods and Procedure

Animals

The subjects used in this experiment were 40 male albino rats obtained from the Holtzman Co. (Madison, WI). Each rat weighed between 250 and 350 g at the start of the experiment and was singly housed in a temperature controlled room (approximately 22oC) with a 12 hr light/12 hr dark cycle (lights on at 1900 hr). Throughout the study, all rats had free access to tap water, while availability to standard Purina Rodent Chow pellets was restricted to a daily 4 hr period following testing. All training and testing were done during the dark cycle, 1 hr prior to daily feeding, Monday through Friday of each week.

Apparatus

The behavioral apparatus consisted of standard operant chambers (Skinner boxes) housed in light-proof, sound-attenuated, and fan-ventilated chambers. Each operant chamber was equipped with two levers, one on either side of a centrally placed dipper, which delivered 0.1 ml of a liquid reinforcement for three seconds. The reinforcement mixture was 25% Liquid Similac, 25% condensed milk, and 50%

tap water. Standard electromechanical programming and cumulative counters were used to record and control behavior.

Drugs

CDP (Roche), strychnine, amphetamine, and pentylene-tetrazol (Sigma) were dissolved in physiological saline, whereas bicuculline (Sigma) and CL 218,872 (Lederle) were suspended in a control solution of 20 percent propylene glycol in water. All injections were given intraperitoneally (i.p.) in a volume of 2 ml/kg body weight.

Procedure

Following habituation to the experimental chamber and preliminary shaping, the animals were trained to alternate between response levers on a schedule of continuous reinforcement (only one lever was reinforced for 5 min, then the other, for a total of 30 min). Once lever pressing was well established, the reinforcement contingency was increased incrementally to a fixed ratio 10 (FR 10) schedule of reinforcement, while maintaining the lever alternation.

Next, animals were trained to discriminate between 5 mg/kg CDP and physiological saline. Half of the rats were

randomly assigned the left lever as "CDP-correct" and the right lever as "saline-correct", while the lever assignment was reversed for the remaining animals. Every tenth response on the "CDP-correct" lever led to reinforcement on days when animals were given CDP, whereas the opposite lever was reinforced followed saline injections. Daily saline or CDP treatments, each given 30 min prior to the beginning of testing, were given according to the following two weekly alternating sequences: CDP-saline-saline-CDP-CDP, and saline-CDP-CDP-saline-saline. Discrimination sessions, 10 min in duration, were continued until each animal reached the performance criterion of no more than three incorrect responses before the first reinforcement on 9 out of 10 consecutive sessions.

After each animal attained this initial training criterion, the dosage of CDP was reduced to 3 mg/kg and discrimination training was resumed until each animal again attained the performance criterion of three or fewer errors prior to the initial reinforcement on 9 out of 10 consecutive days.

Following retraining using 3 mg/kg CDP, a test session was conducted on Friday of each week. On Monday through Thursday, training sessions in the order of saline-CDP-CDP-saline were continued for the purpose of providing

appropriate baseline data and to ensure that discrimination was intact. If any animal failed to demonstrate reliable discrimination (each day with three or fewer errors), testing using that animal was postponed and discrimination training continued until this performance criterion was attained. For the stimulus generalization experiments, the training injection was replaced by an injection of CL 218,872 or by a different dose of the training drug. In the stimulus antagonism tests, a CNS stimulant or convulsant was administered concurrently with a dose of CL 218,872. During test sessions, 10 min in duration, the lever on which the rat first totalled 10 responses was reinforced and subsequent FR 10 reinforcement was made contingent upon pressing this "selected" lever.

Data analysis

The accuracy of lever selection for each animal was computed as the percentage of responses on the CDP-correct lever at the occurrence of the first reinforcement. For example, if a rat responded three times to the saline lever before making 10 responses on the CDP lever, the cue detection score for that animal would be 10/13 or 77 percent; if a rat made one response on the CDP lever and 10 responses on the saline lever the score would be 1/11 or

9.1 percent. Thus, high scores indicated preference for the CDP lever and low scores indicated preference for the saline lever. For each generalization or antagonism test treatment, 7-10 animals, randomly selected from the pool of animals available for testing, were assigned to each dose group. Thus, for each test treatment, no animal received more than one drug dose.

Results

Baseline data

All rats reliably learned to discriminate 5 mg/kg CDP from saline, requiring a median number of 27 training sessions (including the 10 criterion sessions) in order to meet the performance criterion of fewer than three errors per session on 9 out of 10 consecutive sessions. When the training dosage of CDP was reduced to 3 mg/kg, discrimination was easily maintained, with a median number of 11 additional training sessions (including the 10 criterion sessions) required to reattain the performance criterion.

On the standard saline and CDP discrimination sessions conducted Monday through Thursday of each week following the retraining period, all animals reliably selected the injection appropriate lever after either saline or CDP

treatment. Incorrect lever selection occurred rarely and each rat reached a highly significant level (one-tailed; Binomial-test; p < 0.001) of correct lever selection. The median percent-correct lever selection value was 100 percent for each animal following either CDP or saline administration.

Stimulus generalization and antagonism experiments

The data from the stimulus generalization experiments with CDP and CL 218,872 are summarized in Figure 1. The training dose of CDP (3 mg/kg) generalized to lower doses of CDP (0.5 to 2.0 mg/kg) in a dose dependent fashion. CDP produced a dose related increase in responding, with the response rate following 2.0 and 3.0 mg/kg significantly above saline (control) levels. CL 218,872 (0.5 to 10.0 mg/kg) produced CDP lever selection in a dose related manner and therefore generalized to the standard 3 mg/kg CDP treatment. However, only three of the 10 rats tested with the largest dose of CL 218,872 (10 mg/kg) responded sufficiently (10 times to either lever) to indicate a lever preference. The rest were mostly inactive during the test session. Whereas CL 218,872 (0.5 to 5.0 mg/kg) failed to significantly alter total responding, the highest dose tested (10 mg/kg) suppressed responding nearly completely.
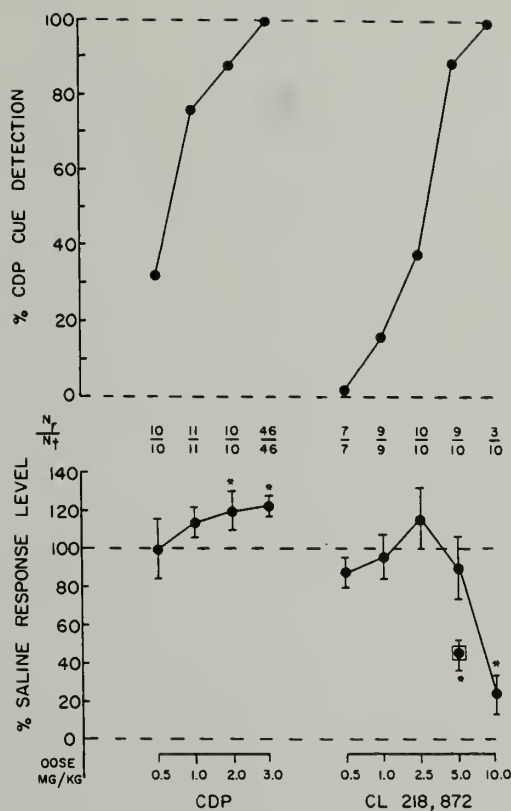
Fig. 1. Stimulus generalization between
CDP and CL 218,872. Percent CDP cue
detection refers to the percentage of
responses emitted on the CDP lever at
the occurrence of the first reinforce-
ment. Nr/Nt represents the number of
rats responding sufficiently to demon-
strate lever selection, out of the total
number of rats tested. The response
level indicates the mean (± S.E.) number
of responses for 10 min is expressed as
a percentage of the previous control
performance. The squared data point
indicates the mean number of responses
for 10 previously drug-naive rats. The
asterisks denote values that differ
significantly (Wilcoxon test; p < 0.05)
from the corresponding saline values.

In marked contrast to the effect of CL 218,872 on responding in CDP trained animals (90.1 % of control levels), 5 mg/kg CL 218,872 significantly depressed responding (46.9 percent of control) in drug naive rats.

The results of the stimulus antagonism experiments are shown in Figure 2. Following concomitant treatment with CL 218,872 (5 mg/kg) and either bicuculline (1.0 to 4.0 mg/kg) or strychnine (0.5 to 2.0 mg/kg), the animals continued to select the CDP lever, thus demonstrating that the generalization of CL 218,872 to the standard CDP treatment had not been antagonized. However, the concurrent administration of CL 218,872 (5.0 mg/kg) and either pentylenetetrazol (5.0 to 20.0 mg/kg) or amphetamine (0.5 to 2.0 mg/kg) caused the rats to select the saline lever in a dose dependent fashion, indicating that perception of the CDP cue, otherwise evident after a 5 mg/kg injection of CL 218,872, had been antagonized. When administered alone, each of these stimulants reliably produced saline lever selection (Binomial test; $p < 0.005$). The response rate following the acute administration of CL 218,872 (5.0 mg/kg) was not significantly altered by the concurrent administration of any of these CNS stimulants.

Discussion
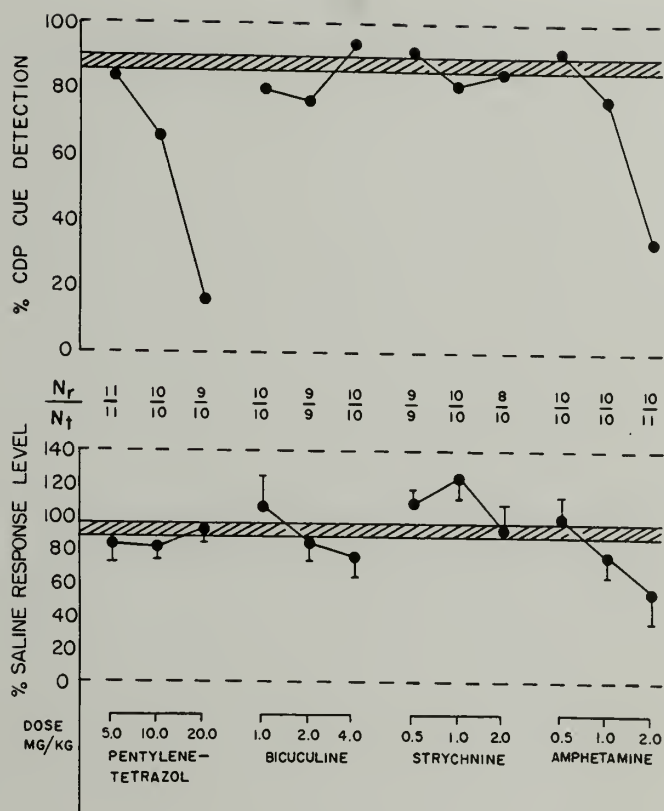
Fig. 2. Effects of pentylenetetrazol, bicuculline, strychnine, and amphetamine on the generalization of CL 218,872 (5 mg/kg) to the CDP discriminative stimulus. The ordinates and Nr/Nt are similar to those in Fig. 1. The dashed areas represent the cue detection and response values in test sessions with 5 mg/kg CL 218,872 only.

The results of this study demonstrate that rats previously trained to discriminate 5 mg/kg CDP from saline can easily discriminate a dose as low as 3 mg/kg. We have also clearly shown that CL 218,872, a synthetic non-BDZ ligand for the BDZ receptor, produces CDP lever selection in a dose related manner and thus generalizes to the standard CDP treatment. It is further shown that cue detection following injection of CL 218,872 is independent of the rate of responding since generalization occurs at a dose (5 mg/kg) that does not significantly alter the rate of lever pressing. The demonstration that 10 mg/kg CL 218,872 virtually abolished responding is at odds with a previous report that a much larger dose of CL 218,872 (223 mg/kg) is required to reduce locomotor activity by 50 percent (Lippa et. al. 1979). However, this discrepancy may be due to methodological differences since their drug to test interval was 60 min and they administered CL 218,872 orally. Furthermore, motoric activity as estimated by lever pressing for a milk reinforcement might not be equivalent to locomotor activity as measured by an activity meter (Animex).

It is well known that BDZs produce an initial general depression or sedation, usually shown by a decrease in unpunished responding or exploratory behavior, an effect

which disappears after a few BDZ treatments (Zbinden and Randall, 1967; Margules and Stein, 1968). In the present experiment CL 218,872 (5 mg/kg) significantly depressed responding in previously drug-naive rats (shown by the squared data point in Figure 1), whereas this same dose did not affect responding in animals that had been repeatedly injected with CDP. These data suggest that cross tolerance to the initial sedative effect of CL 218,872 may have developed between CDP and CL 218,872. However, clarification of this issue will require a more complete investigation.

CL 218,872 potently antagonizes the convulsions produced by pentylenetetrazol, but unlike the BDZs is very weak in its ability to inhibit the convulsions produced by bicuculline or strychnine (Lippa et al., 1979). Consistent with this specificity, the data reveal that the generalization of the CDP discriminative stimulus to CL 218,872 is antagonized by pentylenetetrazol and amphetamine, but not by bicuculline or strychnine. This suggests that stimulus generalization is not associated with a general anticonvulsant activity but rather is associated with a specific antipentylenetetrazol effect. This mutual antagonism between pentylenetetrazol and CL 218,872 may indicate that the same neuronal mechanism subserves both the antipen-

tylenetetrazol and discriminative stimulus properties of
the BDZs.

It has been proposed that two biochemically and
pharmacologically distinct types exist among central type
BDZ receptors (Klepner et al., 1979). Type I receptors are
GABA-independent, display a high affinity for both BDZs and
TPZs, and are presumed to mediate the anxiolytic and
antipentylenetetrazol actions of these drugs. Type II
receptors are GABA-dependent, show a high affinity for BDZs
but a low affinity for TPZs, and are believed to mediate
the depressant side effects associated with the BDZs. In
the interest of establishing whether BDZ discriminative
cues can be traced to one receptor type or the other, the
present data reveal that CL 218,872 (5 mg/kg) produced a
discriminative cue without sedation, at doses similar to
those that produce anticonflict and antipentylenetetrazol
effects (Lippa et al., 1979b). The present demonstration
of stimulus generalization in the absence of sedation
suggests that the discriminative stimulus properties of
BDZs may be mediated via stimulation of Type I BDZ
receptors. The demonstration that stimulus generalization
is antagonized by pentylenetetrazol but not by the GABA
receptor blocker bicuculline further supports such a
notion. It may be that the anxiolytic, antipentylenetetra-

zol, and discriminative stimulus properties of BDZs are mediated via activation of Type I BDZ receptors, whereas the sedative and ataxic effects of BDZs are mediated through activation of Type II BDZ receptors.

Since several neurotransmitter systems have been postulated to be associated with the pharmacological actions of the BDZs (for reviews, see Feldman and Quenzer, 1984;Costa and Greengard, 1975), it is difficult to establish exactly which neurotransmitter is responsible for the discriminative stimulus properties of this class of drugs. However, the catecholamine agonist amphetamine, but neither strychnine nor bicuculline (receptor blockers for glycine and GABA respectively, Curtis et al., 1970; Young and Snyder, 1973) antagonized the stimulus generalization of CDP to CL 218,872. This might suggest that either norepinephrine or dopamine is involved in eliciting the BDZ discriminative stimulus. The ability of pentylenetetrazol to antagonize stimulus generalization does little to clarify this issue as acetylcholine (Rastogi et al., 1979), norepinephrine (Mason and Corcoran, 1978) and GABA (Johnston and Mitchell, 1971) have been implicated in the convulsant action of pentylenetetrazol. Clarification of this issue will require additional research.

CHAPTER III

EXPERIMENT II

A COMPARISON BETWEEN CHLORDIAZEPOXIDE AND CL 218,872

ON LOCOMOTOR ACTIVITY IN RATS

Introduction

It has been firmly established that acute treatment
with benzodiazepines (BDZs) at clinically effective doses
produces a general depression or sedation, usually shown by
a decrease in unpunished responding, exploratory behavior,
or locomotor activity (Geller and Seifter, 1960; Zbinden
and Randall, 1967; Margules and Stein, 1968; Quenzer et
al., 1974). A number of non-BDZ compounds that bind
potently to the BDZ receptor and display a pharmacological
profile similar to that of the BDZs have recently been
introduced (for reviews, see Goldberg et al., 1983; Chapter
I). One such compound, the triazolopyridazine derivative
CL 218,872, reportedly produces anticonflict and
anticonvulsant effects in rats at relatively low doses
(0.75 to 6.0 mg/kg p.o.), whereas ataxic and sedative
effects only become apparent at much larger doses (ED_{50} for
sedation = 223 mg/kg p.o.).

It was demonstrated in the previous chapter (Chapter

II), however, that CL 218,872 (5 mg/kg i.p.) decreased by 50 percent the rate of lever pressing for a milk reinforcement in an operant task, an effect that appeared to be prevented by chronic pretreatment with chlordiaz-epoxide (CDP). The obvious discrepancy between the results reported in Chapter II and those reported by Lippa et al. (1979) may easily be a consequence of methodological differences between the two experiments, however, because motoric activity as estimated by lever pressing might not be equivalent to locomotor activity as measured in an activity chamber (Animex). Thus the present experiment aimed to resolve this discrepancy by evaluating the effects of CL 218,872 on locomotor activity as measured in an activity chamber. For comparative purposes, the effects of CDP were similarly evaluated. Moreover, in order to verify the finding in Chapter II that CDP conferred a cross-tolerance to the sedative effect of CL 218,872, additional rats were treated with either CDP or CL 218,872 prior to behavioral testing with these drugs.

Methods and Procedure

Animals

The subjects were 60 male albino rats obtained from

the Holtzman Co. (Madison, WI). The rats weighed 250-300 g throughout the experiment and were housed in a temperature controlled room (approximately 22 °C) with a 12 hr light/12 hr dark cycle (lights on at 1900 hr). Throughout the study, all rats had free access to tap water and standard Purina Rodent Chow pellets. All testing was done in a quiet darkened room between 0800 and 1100 hr.

Apparatus

The testing apparatus was a cylinder 62 cm in diameter and 42 cm deep with a mesh floor (Lehigh Valley Activity Box, model No. 145-03). Counters provided a record of locomotor activity as each animal interrupted 12 light beams that were detected by photocells equally spaced 2.5 cm above the floor along the perimeter of the testing apparatus.

Procedure

Animals were randomly assigned to one of 12 groups (N = 5). Following habituation to the testing apparatus (10 min per day for six consecutive days), six groups of rats were injected with drug vehicle on day seven, and then with either CDP or CL 218,872 (2.5, 5, or 10 mg/kg) on day eight. The remaining six groups were similarly treated

except that each rat was injected on days one through six with CDP or CL 218,872 (10 mg/kg), or with drug vehicle. CL 218,872 (Lederle) was suspended in a solution of 20 percent propylene glycol in water, and CDP hydrochloride (Roche) was dissolved in physiological saline. All injections were administered i.p. in a volume of 2 ml/ kg of body weight. On drug pretreatment days (one through six), injections were given 30 min after the habituation session, whereas on drug test days (seven and eight) injections were given 30 min prior to the 10 min test session.

Data analysis

The locomotor activity for each rat was determined by the number of light beam interruptions following drug treatment on day eight, and was expressed as a percentage of the locomotor activity on day seven (control performance). All data were subjected to t-tests and to analysis of variance (ANOVA) as appropriate. Results occurring with a chance probability of less than .05 were considered statistically significant.

Results

The effects of acute CDP and CL 218,872 administration on locomotor activity are shown in Fig. 3. The mean ($\pm$ 1 S.E.) number of activity counts during the 10 min control session (day seven) was 619 $\pm$ 37. There was no difference in responding between saline and propylene glycol treated rats (two-tailed; Mann Whitney test; $p > 0.20$). Compared to vehicle performance, each dose of CL 218,872 (2.5, 5, and 10 mg/kg) significantly reduced locomotor activity (two-tailed; Wilcoxon test; $p < 0.05$;). The dose response pattern following CDP administration was less consistent, with the smallest dose (2.5 mg/kg) increasing, and the largest dose (10 mg/kg) decreasing locomotor activity ($p < 0.05$). The intermediate dose of CDP (5 mg/kg) produced a non-significant response suppression ($p > 0.10$). An overall ANOVA revealed no Drug main effect ($F = 2.22$; df = 1, 24; $p = 0.18$), a highly significant Dose main effect ($F = 20.98$; df = 2, 24; $p < 0.001$), and a significant Drug X Dose interaction ($F = 4.35$; df = 2, 24, $p < 0.05$).

From an examination of the dose-response relationship for each compound, the 10 mg/kg dose was adopted as the challenge dose in the tolerance and cross-tolerance experiment (Fig. 4). The mean ($\pm$ 1 S.E.) number of activity counts during the 10 min control session (day seven) was 690 $\pm$ 30. Repeated measures ANOVA of day one
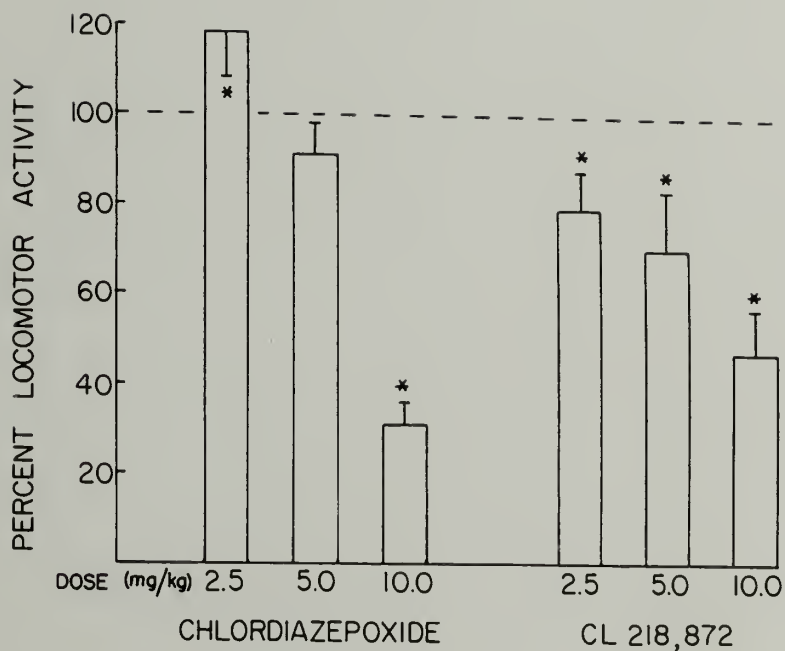
Fig. 3. Spontaneous locomotor activity in rats measured 30 min after acute administration of CDP or CL 218,872 at doses of 2.5, 5, and 10 mg/kg i.p. Data represent the mean ($\pm$ 1 S.E.) for six rats per group, and is expressed as a percentage of the previous (day seven) vehicle performance. The asterisks denote values that differ significantly (two-tailed, $p < 0.05$, Wilcoxon test) from the day seven vehicle performance.
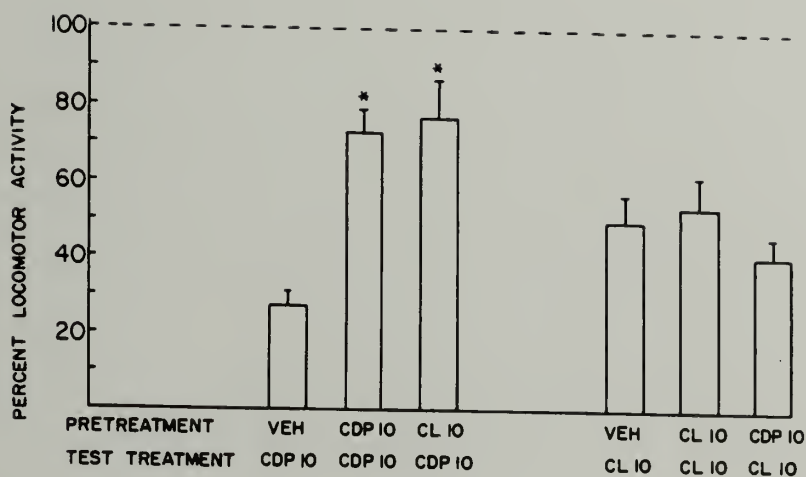
Fig. 4. The effect of CDP, CL 218,872, or vehicle pretreatment on the suppression of spontaneous locomotor activity produced by a test dose of CDP or CL 218,872. The test treatment (10 mg/kg i.p.) was given 30 min before testing and 48 hr after six daily pretreatments (10 mg/kg i.p.). Data represent the mean ($\pm$ 1 S.E.) for six rats per group, and are expressed as a percentage of the previous (day seven) vehicle performance. The asterisks denote values that differ significantly (two-tailed, $p < 0.05$, Wilcoxon test) from the corresponding vehicle pretretment group.

through day seven activity counts revealed no Pretretment
($F = 0.25$; $df = 2$, 27; $p = 0.59$), and Day ($F = 1.33$; $df =$
6, 162, $p = 0.24$) main effects, and no Pretreatment X Day
interaction ($F = 0.72$; $df = 12$, 162, $p = 0.73$). Thus, the
six daily post-training drug injections had no cumulative
effect on baseline locomotor activity. Analysis of
variance of day eight scores expressed as a percentage of
the day seven control performance revealed no Test
Treatment main effect ($F = 2.66$, $df = 1$, 24, $p = 0.16$), but
a reliable Pretreatment main effect ($F = 6.19$, $df = 2$, 24,
$p < 0.01$), and a highly significant Pretreatment X Test
Treatment interaction ($F = 7.48$, $df = 2$, 24, $p < 0.005$).
Post-hoc testing indicated that the suppression of
locomotor activity produced by CDP (10 mg/kg) was clearly
attenuated by six daily pretreatments of either CDP or CL
218,872 at the same dosage (Fisher's Least Significant
Difference Test; $p < 0.05$). In marked contrast to the
situation using CDP, however, the response suppression
produced by CL 218,872 (10 mg/kg) was not significantly
altered by CDP or CL 218,872 pretreatment ($p > 0.05$).

Discussion

Consistent with previous reports (Sansone, 1979;

Herberg and Williams, 1983), acute administration of CDP produced a dose-dependent effect on locomotor activity. A low dose of CDP (2.5 mg/kg) increased activity, whereas a relatively high dose (10 mg/kg) decreased activity. A single challenge injection of CL 218,872 (2.5, 5, and 10 mg/kg) significantly reduced locomotor activity, a finding in agreement with the earlier demonstration in Chapter II that CL 218,872 (5 mg/kg) reliably suppressed lever responding in an operant task. The route of drug administration and the drug to test interval were the same in both experiments. On the other hand, the present report is at variance with the report by Lippa et al. (1979) that a 223 mg/kg dose of CL 218,872 was reqiured to reduce locomotor activity by 50 percent. It is possible, however, that this 20-fold disparity in drug potency may still be due to methodological differences since their drug to test interval was 60 min and they administered CL 218,872 by the oral route.

Pretreatment with CDP or CL 218,872 (10 mg/kg once daily for six days) produced tolerance and cross-tolerance respectively to the sedative effects of a single injection of CDP. Hoogland et al. (1966) demonstrated that chronic CDP treatment (100 mg/kg for five days) decreased the blood concentration of subsequently administered ^{14}C-CDP,

suggesting that the tolerance reported here and in many previous experiments (Margules and Stein, 1968; Cook and Sepinwall, 1975; Sansone, 1979) may be a result of increased hepatic metabolism of the drug. Although chronic pretreatment with ethanol similarly decresed the blood concentration of CDP (Sellers and Busto, 1982), it cannot be established from the present data whether the CL 218,872-induced cross-tolerance to the sedative action of CDP is enzymatic, cellular, or behavioral in nature.

In marked contrast to the situation using a challenge dose of CDP, chronic CDP or CL 218,872 pretreatment did not attenuate the activity-suppressant action of acute CL 218,872 administration. The previous experiment (Chapter II) suggested that CDP pretreatment (three to four injections per week for 10 weeks) conferred a cross-tolerance for the sedative effects of CL 218,872, a finding not supported by the present data. Whether the development of tolerance and cross-tolerance to the sedative effects of CL 218,872 is dependent upon the number and/or duration of pretreatments remains to be established.

However, the demonstration of asymmetry in the development of tolerance and cross-tolerance is not without precedent. For example, morphine pretreated mice showed little or no cross-tolerance to either etorphine or

methadone analgesia, whereas either etorphine or methadone pretreated mice were highly cross-tolerant to morphine (Lange et al., 1980; Neil, 1982). Similarly, fenfluramine-tolerant rats were cross-tolerant to the anorectic effects of quipazine, but quipazine-tolerant animals were not cross-tolerant to fenfluramine (Rowland et al., 1982). Although the precise mechanisms responsible for such differences in the development of tolerance and cross-tolerance cannot be determined from the present data, Neil (1982) has suggested that this complex phenomenon of asymmetry in cross-tolerance may be due to receptor heterogeneity at the single cell level. That is, neurons desensitized to compound A become subsensitive to compound B, whereas cells desensitized to B remain sensitized to A. Just such an asymmetry in acute desensitization in rat cortical neurons was reported for methionine-enkephalin and morphine (Williams and Zieglgansberger, 1981). An alternative possibility is that the asymmetry is due to differential metabolic processes about which, unfortunately, one can only speculate.

C H A P T E R I V

EXPERIMENT III

EFFECTS OF CHLORDIAZEPOXIDE AND CL 218,872 ON

SEROTONIN AND NOREPINEPHRINE TURNOVER IN RATS

Introduction

Acute treatment with benzodiazepines (BDZs) at
clinically effective doses produces a general depression or
sedation, usually shown by a decrease in unpunished
responding (Geller and Seifter, 1960; Margules and Stein,
1968), exploratory behavior (Zbinden and Randall, 1967), or
locomotor activity (Quenzer et al., 1974; Chapter III). It
is this general depressant effect upon the CNS that forms
the basis for their clinical use as sedative-hypnotics (for
review, see Greenblatt et al., 1982). In fact, most BDZs
can be prescribed as antianxiety agents at low doses, and
as sedative-hypnotics at higher doses. It is still
controversial, however, whether, or to what extent, the
anxiolytic and sedative effects produced by these drugs can
be dissociated. Support for such a possibility has been
recently advanced by the development of a number of novel
anxiolytic drugs purported to be devoid of the depressant

side effects typically associated with BDZ use (for review, see Goldberg et al., 1983).

Geller and Seifter (1960) devised a technique by which behavioral pharmacologists can comparatively study the anxiolytic and sedative effects of a drug. Briefly, a conflict situation (punished schedule) is systematically alternated with positively reinforced behavior (nonpunished schedule). An increase in frequency of the punished behavior brought about by a drug is interpreted as an antianxiety effect, while a decrease in frequency of the nonpunished behavior is regarded as a sedative effect. This technique offers the important advantage of studying both behavioral effects in the same animal and within the same experimental paradigm, thus reducing the influence of extraneous variables.

Using a modified version of the Geller and Seifter technique, Margules and Stein (1968) found that the depressant and anticonflict effects of BDZs may be dissociated after chronic administration of these agents. When a rat not previously exposed to drugs was given the BDZ oxazepam (20 mg/kg i.p.), unpunished responding was suppressed on the first day of drug administration (sedative effect), whereas punished responding was slightly greater than in control tests (anxiolytic effect). After three to six

daily doses, unpunished responding returned to control levels, indicating that tolerance had developed to the depressant effect of oxazepam whereas increases in punished responding persisted, indicating that tolerance had <u>not</u> developed to the anticonflict effect of the drug.

Many pharmacological agents have been evaluated using this rat conflict-punishment procedure. The ability to restore punishment-suppressed responding has been extended to additional BDZs such as chlordiazepoxide (CDP), bromazepam, flunitrazepam, lorazepam, clonazepam and diazepam, as well as to other non-BDZ anxiolytics such as meprobamate, phenobarbital, amobarbital, and ethanol. A broad variety of non-anxiolytic psychotropic drugs such as chlorpromazine, haloperidol, imipramine, morphine, amphetamine, and iproniazid were all ineffective in this procedure (Cook and Davidson, 1973; Cook and Sepinwall, 1975), indicating that the rat conflict-punishment test is qualitatively selective for clinically effective anxiolytic agents. This test has also provided a good quantitative estimate of the relative clinical potency of a test compound. Specifically, Cook and Davidson (1973) reported a correlation coefficient of + 0.98 between the minimum effective anticonflict dose in rats for six minor tranquilizers and their average daily dose used for

treating psychoneurotic disorders. Anticonflict activity
in rats has been more recently demonstrated for a number
of novel anxiolytics such as CL 218,872 (Lippa et al.,
1979), zopiclone (Sepinwall and Cook, 1980), and buspirone
(Weissmann et al., 1984), further illustrating the utility
of the rat conflict-punishment procedure in predicting a
drug's potential efficacy as a clinical anxiolytic agent.

Despite considerable evidence to suggest that many, if
not all, of the effects of BDZs in the central nervous
system may be explained by their ability to enhance GABA
mediated transmission (for review, see Tallman et al.,
1980), there is also convincing evidence supporting a role
for brain 5-hydroxytryptamine (5-HT, serotonin) in at least
the anticonflict effects produced by BDZs. This evidence
includes studies showing that an inhibitor of 5-HT biosyn-
thesis, p-chlorophenylalanine (PCPA), as well as the 5-HT
receptor antagonists cinanserin and methysergide, can
produce BDZ-like effects in the rat conflict test (Stein et
al., 1973; Cook and Sepinwall, 1975). Moreover, the
immediate 5-HT precursor, 5-hydroxytryptophan (5-HTP),
reversed the anticonflict effects produced by PCPA and
cinanserin (Geller and Blum, 1970; Geller et al., 1974).
Anticonflict effects have also been observed after destruc-
tion of central 5-HT neurons by direct administration of

the neurotoxins 5,6- and 5,7-dihydroxytryptamine into the
brain (Stein et al., 1977; Tye et al., 1977). Furthermore,
intracerebroventricular (i.c.v.) injection of 5-HT counter-
acted the anxiolytic effects of BDZ administration (Wise et
al., 1972). Taken together, these and other experiments
indicate that 5-HT may play a role in the antianxiety
properties of BDZs.

Since the original discovery by Olds and Milner (1954)
of the rewarding properties of brain stimulation, research
findings compiled over the past 20 years have characterized
a brain reward system as catecholaminergic and a brain
punishment system as mainly serotonergic (for review, see
Deakin (1983). Thus, early BDZ research focused primarily
on brain norepinephrine (NE) and 5-HT systems. In an effort
to determine whether the antianxiety and sedative effects
of BDZs could be correlated with changes in turnover rates
of 5-HT and/or NE (Corrodi et al., 1967; Taylor and
Laverty, 1969; Chase et al., 1970), Wise et al. (1972)
administered a single dose or six daily doses of oxazepam
to rats and measured the turnover rates of NE and 5-HT in
the midbrain-hindbrain region. A single injection of
oxazepam (20 mg/kg i.p.) significantly reduced both 5-HT
and NE turnover rates. Rats sacrificed after the sixth
daily dose of oxazepam showed a significant decrease in

the turnover rate of 5-HT, but not of NE. In other words, following chronic administration of this BDZ, tolerance developed to the reduced turnover rate of NE, but not to that of 5-HT. As noted previously, under these same conditions tolerance developed to the behavioral depression induced by oxazepam, but not to the anticonflict effect. From these results Wise et al. (1972) concluded that the depressant effect of BDZs may be associated with reduced NE turnover, and the anticonflict effect with reduced 5-HT turnover.

Using a different BDZ, however, Cook and Sepinwall (1975) were unable to fully replicate the Wise et al. (1972) findings. While producing behavioral effects and 5-HT turnover effects consistent with those produced by oxazepam, CDP (10 mg/kg p.o.) had the exact opposite effects on NE turnover. Specifically, NE turnover was unchanged following acute CDP treatment (at which time unpunished responding was significantly depressed), but was significantly reduced following repeated CDP administration (at which time a marked depression of unpunished responding was no longer evident). Thus, the Cook and Sepinwall (1975) data are inconsistent with the Wise et al. (1972) proposal that the initial sedative effects produced by BDZs are related to decreased NE turnover. It should be pointed

out, however, that the discrepancies obtained between these two studies may be accounted for by methodological differences such as the route of drug administration and the brain region analyzed (Cook and Sepinwall assayed whole brain).

Since the BDZs alter both 5-HT and NE neuronal systems in the same dose range that they produce antianxiety and sedation, it has not been possible to establish conclusively that the anxiolytic and sedative effects of these compounds are mediated by reductions in 5-HT and NE turnover respectively. However, the development of TPZs affords a unique opportunity to further assess the importance of brain 5-HT and NE systems in contributing to the anxiolytic and sedative actions produced by BDZs. Furthermore, because CL 218,872 is a BDZ receptor agonist with a selective affinity for Type I BDZ receptors (Squires et al., 1979), examination of CL 218,872 might further establish the role, if any, of this BDZ receptor subtype in mediating the BDZ effects on 5-HT and NE neurotransmission. To this end, 5-HT and NE turnover in the midbrain-hindbrain region of the rat brain were assessed after acute and chronic administration of CL 218,872, the prototypical triazolopyridazine. For comparative purposes, and in an effort to further resolve the disparity between Wise et al.

(1972) and Cook and Sepinwall (1975), the effects of CDP on 5-HT and NE turnover were similarly evaluated using the brain region and route of drug administration employed by Wise et al. (1972).

The rate of synthesis of catecholamines (CA) and 5-HT in brain was determined following treatment with NSD 1015 (3-hydroxybenzyl hydrazine HCl). NSD 1015 is a powerful inhibitor of aromatic L-amino acid decarboxylase, the enzyme responsible for converting 5-HTP to 5-HT, and dihydroxyphenylalanine (DOPA) to dopamine, which of course can then be converted to NE. Because the rate limiting steps in 5-HT and CA synthesis occur prior to the enzymatic block, the accumulated levels of 5-HTP and DOPA should reflect the rates of 5-HT and CA synthesis (turnover) in experimental vs. control subjects (Pycock and Taberner, 1981). The results of the NSD method agree quite well with those of other methods for estimating the rate of brain biogenic amine synthesis (Carlsson et al., 1972; Curzon, 1981). The NSD method offers the obvious advantage of measuring both 5-HT and CA turnover in the same animal. Furthermore, because brain levels of 5-HTP and DOPA are virtually negligible, and because the accumulation of each of these compounds is linear for up to 30-45 min following decarboxylase inhibition (Carlsson et al., 1972; Carlsson

and Lidquist, 1970), synthesis rates can be determined from a single measurement. The major weakness of the NSD method, however, is that it is unable to differentiate between the rates of DA and NE synthesis in regions where both neurotransmitters are found. As estimated from a rat brain stereotaxic atlas, the midbrain-hindbrain section used in the present experiment probably contains all 5-HT cells from the B1 to B9 nuclei, all NE cells from the A1 to A7 nuclei, and some DA cells from the A8 and A10 nuclei (the rostral dissection traversed directly through the DA containing A8 and A10 nuclei in the ventral and ventro-lateral midbrain tegmentum, while excluding the A9 nucleus). Inclusion of these DA cells was unavoidable in order that our tissue section contain the 5-HT cells located in the B7 and B8 nuclei, which are the source of approximately 80 percent of all forebrain 5-HT terminals (Azmitia, 1978). Furthermore, in order to make valid comparisons to the study by Wise et al. (1972), it was important to use their dissection coordinates. Thus, the relative contribution of DA in the overall measure of CA turnover in the present study cannot be precisely determined, and the values obtained will probably over estimate the actual rate of NE synthesis.

Methods and Procedure

Animals

Adult male albino rats were bred in our laboratory from stock animals obtained from the Holtzman Co. (Madison, WI) and were housed in a temperature controlled room (approximately 22° C) under a 14:10 light-dark cycle (lights on at 0600 hr). Rats were housed in group cages until they weighed 200-225 g, at which time they were removed to single cages. Tap water and standard Purina Rodent Chow pellets were available ad libitum.

Drugs

CL 218,872 (Lederle) was suspended in a solution of 20% propylene glycol in water, whereas CDP (Roche) and NSD 1015 (Sigma) were dissolved in physiological saline. All injections were given intraperitoneally (i.p.) in a volume of 2 ml/kg body weight.

Procedure

Half of the animals were given a single injection of CDP, CL 218,872, or drug vehicle. The remaining animals were given seven daily drug or vehicle injections (days one through six and day eight). The drug dosages are indicated

in each table. Thirty min following the last drug or vehicle injection each animal received NSD 1015 (100 mg/kg), and thirty min after that the rats were decapitated, their brains immediately removed and the midbrain-hindbrain region rapidly dissected on an ice-cold glass plate. A vertical knife cut was made through a plane beginning on the dorsal surface of the brain just rostral to the superior colliculus and ending on the ventral surface just caudal to the mammillary bodies. The posterior transection was made perpendicular to the obex, with the cerebellum discarded. Immediately after dissection, the tissue was weighed, homogenized in ice-cold 0.4 N perchloric acid containing 0.025% ascorbic acid and 0.25% EDTA, and then centrifuged at 30,000 x g for 10 min at 0oC. Supernatants were treated with KOH to adjust the pH to 2.5, and centrifuged at 30,000 x g for 5 min at 0oC to remove the perchlorate by precipitation of the insoluble potassium salt. The resulting extracts were applied to small columns of Dowex 50W-X4, 200-400 mesh, a strong cation-exchange resin (Atack and Magnusson, 1970). Both the 5-HTP and DOPA were eluted in 1.5 ml 0.01 M sodium phosphate buffer. The well established o-phthaldialdehyde (Maickel and Miller, 1966) and potassium ferricyanide (Kehr et al., 1972) fluorometric methods were used to measure 5-

HTP and DOPA respectively. Fluorescence was measured using a Perkin-Elmer #1000 spectrofluorometer using activation and emission wavelengths of 364/480 and 364/500 for 5-HTP and DOPA respectively. Standards were run in parallel with the samples in each assay.

Data analysis

All data (presented as the mean $\pm$ 1 S.E.) were subjected to analysis of variance (ANOVA) followed by individual mean comparisons using Fisher's Least Significant Difference Test (Kirk, 1968) where appropriate. Results occuring with a chance probability of less than .05 were considered statistically significant.

Results

Each tissue sample consisted of one midbrain-hindbrain region (356 $\pm$ 11 mg wet weight) from a single rat. Recoveries of 22.5 ng of 5-HTP and DOPA added to homogenates of single midbrain-hindbrain samples were 69.3 $\pm$ 5.2 and 66.9 $\pm$ 4.7 percent respectively.

The effects of CDP and CL 218,872 on 5-HTP accumulation in rats treated with NSD 1015 can be seen in Table 1. During the first 30 min after administration of

Table 1. Effects of chlordiazepoxide and CL 218,872 administration on 5-HTP accumulation in the midbrain-hindbrain of rats treated with NSD 1015.

Drug	Dose (mg/kg)	Injection Schedule			
		Acute		Chronic	
		ng/g	(% control)	ng/g	(% control)
Chlordiaz-epoxide					
	0	552 ± 23		528 ± 34	
	2.5	529 ± 29	(94)	433 ± 22	(82) *
	5	440 ± 16	(80) *	448 ± 21	(85) *
	10	406 ± 21	(74) *	348 ± 30	(66) *
	20	395 ± 27	(72) *	385 ± 19	(73) *
CL 218,872					
	0	513 ± 39		522 ± 31	
	2.5	490 ± 17	(96)	493 ± 24	(94)
	5	549 ± 33	(107)	506 ± 23	(97)
	10	563 ± 26	(110)	489 ± 18	(94)
	20	451 ± 21	(88)	538 ± 26	(103)

NSD 1015 (100 mg/kg) was given 30 min after the final drug or vehicle treatment and 30 min before the rats were killed. Chronically treated animals received their final drug or vehicle treatment 48 hr after 6 consecutive daily pretreatments at the same dosage. Data represent the mean ± S.E. for 5 rats per group. *significantly different (at least $p < 0.05$) from the corresponding vehicle (0 mg/kg) treated group.

NSD 1015, the mean concentration of 5-HTP in midbrain-hindbrain increased from essentially zero (0.07 ng/g) to 529 ± 17 ng/g. This accumulation of 5-HTP (uncorrected for percent recovery) corresponds to a 5-HT synthesis (turnover) rate of 1058 ng (4.8nmoles)/g tissue/hr. ANOVA revealed highly significant Drug ($F = 19.99$, $df = 1$, 92, $p < 0.0001$) and Dose ($F = 13.83$, $df = 4$, 92, $p < 0.0001$) main effects, but no Pretreatment main effect ($F = 0.75$, $df = 1$, 92, $p = 0.39$). The only significant interaction was that of Drug X Dose ($F = 6.65$, $df = 4$, 92, $p < 0.001$). Post-hoc testing showed that the formation of 5-HTP was not affected by either the number of vehicle injections (one or seven) or the type of vehicle administered (saline or propylene glycol solutions). A single injection of CDP (2.5 to 20 mg/kg) decreased the concentration of 5-HTP, with the 5, 10, and 20 mg/kg doses significantly different from the control (0 mg/kg) group. Despite the appearance of a dose-dependent trend toward diminished 5-HTP concentrations, no statistically significant differences occured between the 5, 10, and 20, mg/kg doses. The decreased accumulation of 5-HTP persisted after chronic CDP administration, with all doses (2.5 to 20 mg/kg) producing reliable differences from the control (0 mg/kg) group. Only the lowest dose tested (2.5 mg/kg), however, was more effective at reducing 5-HTP

formation after seven drug injections than after a single drug injection. In marked contrast to the situation using CDP, neither acute nor chronic administration of CL 218,872 reliably altered the accumulation of 5-HTP after decarboxylase inhibition.

Table 2 shows the effects of CDP and CL 218,872 on DOPA accumulation after treatment with NSD 1015. The concentration of DOPA in midbrain-hindbrain increased from an unmeasurable level (with a detection limit of 0.03 ng/g) to an average value of 149 ± 8 ng/g/30 min. This accumulation of DOPA represents a CA synthesis (turnover) rate of 298 ng (1.48 nmoles/g tissue/hr. ANOVA revealed significant Dose ($F = 3.82$, $df = 4$, 92, $p < 0.01$) and Pretreatment ($F = 7.04$, $df = 1$, 92, $p < 0.01$) main effects, in addition to marginally significant Drug X Dose ($F = 2.51$, $df = 4$, 92, $p < 0.05$) and Drug X Dose X Pretreatment ($F = 2.69$, $df = 4$, 92, $p < 0.05$) interactions. As was the situation with 5-HTP formation, DOPA formation was not affected either by the number of vehicle injections or by the type of vehicle administered. Although a single injection of CDP (2.5 to 20 mg/kg) failed to influence DOPA accumulation in midbrain-hindbrain, chronic CDP administration at the same doses did reduce DOPA concentrations, with all but the largest dose tested (20 mg/kg) significantly

Table 2. Effects of chlordiazepoxide and CL 218,872 administration on DOPA accumulation in the midbrain-hindbrain of rats treated with NSD 1015.

Drug	Dose (mg/kg)	Acute		Chronic	
		ng/g	(% control)	ng/g	(% control)
Chlordiaz-epoxide					
	0	144 ± 12		141 ± 10	
	2.5	135 ± 9	(94)	108 ± 8	(82) *
	5	122 ± 12	(85)	94 ± 9	(68) *
	10	148 ± 9	(103)	107 ± 6	(77) *
	20	132 ± 20	(92)	120 ± 14	(86)
CL 218,872					
	0	149 ± 12		155 ± 13	
	2.5	132 ± 10	(88)	149 ± 11	(96)
	5	146 ± 16	(98)	129 ± 11	(83)
	10	128 ± 8	(86)	172 ± 17	(111)
	20	164 ± 21	(110)	163 ± 14	(105)

NSD 1015 (100 mg/kg) was given 30 min after the final drug or vehicle treatment and 30 min before the rats were killed. Chronically treated animals received their final drug or vehicle treatment 48 hr after 6 consecutive daily pretreatments at the same dosage. Data represent the mean ± S.E. for 5 rats per group. *significantly different (at least $p < 0.05$) from the corresponding vehicle (0 mg/kg) treated group.

different from the control (0 mg/kg) group. Similar to its effect on 5-HTP formation, CL 218,872 (2.5 to 20 mg/kg), whether administered only once or seven times, had no reliable affect on DOPA formation.

Discussion

The present results demonstrate that 5-HT turnover, as measured by the accumulation of 5-HTP after decarboxylase inhibition by NSD 1015, was significantly reduced following both acute (5 to 20 mg/kg) and chronic (2.5 to 20 mg/kg) CDP administration. These results are in agreement with previous reports showing that CDP reduced 5-HT turnover in whole brain (Cook and Sepinwall, 1975), cerebral cortex (File and Vellucci, 1978), and hippocampus (Lister and File, 1983). Moreover, this effect does not appear to be unique to CDP since 5-HT turnover is similarly reduced by other BDZs such as diazepam (Biswas and Carlsson, 1978; Jenner et al., 1975; Chase et al., 1970), clonazepam (Jenner et al., 1975), and oxazepam (Wise et al., 1972), as well as by other non-BDZ anxiolytics such as pentobarbital (Corrodi et al., 1967), and diphenylhydantoin (Jenner et al., 1975). Thus, the 5-HT turnover results reported here are fully consistent with, and further support the

hypothesis first proposed by Wise et al. (1972) that BDZs may be exerting their antianxiety effects by a reduction in 5-HT neurotransmission.

Reports concerning the effects of BDZs on NE turnover, however, have been less consistent. For example, a single injection of oxazepam decreased NE turnover (Wise et al., 1972), whereas NE turnover after a single treatment with CDP was no different from that in control animals (Corrodi et al., 1967; Corrodi et al., 1971; Cook and Sepinwall, 1975). Following repeated administration, however, just the opposite occurred, with oxazepam leaving unchanged (Wise et al., 1972), and CDP decreasing (Cook and Sepinwall, 1975) NE turnover. In the present experiment, acute injection of CDP (2.5 to 20 mg/kg) left CA turnover unchanged from control values, whereas seven daily injections of CDP (2.5 to 10 mg/kg) significantly lowered the CA turnover rate. Thus, the present findings confirm the Cook and Sepinwall (1975) report in whole brain using CDP, and extend their findings to midbrain-hindbrain. Furthermore, this study demonstrates that the discrepancies between Wise et al. (1972) and Cook and Sepinwall (1975) are not due either to the route of drug administration (i.p. vs. p.o.) or to the brain region assayed (midbrain-hindbrain vs. whole brain). Therefore, despite having

similar effects on unpunished responding after acute administration, CDP and oxazepam appear to have very different effects on NE turnover. Taken together with previous reports, our data do not lend support to the proposal by Wise et al. (1972) that, in general, BDZs exert their acute depressant effects by a reduction in NE neurotransmission. Rather it appears that the effects of BDZs on brain NE activity might be specific for the BDZ under consideration. It should be emphasized, however, that our data do not exclude the possibility that at least oxazepam can be producing its depressant effect through a reduction in NE turnover.

As a non-BDZ anxiolytic purported to be devoid of the depressant side effects typically associated with acute BDZ administration (Lippa et al., 1979), CL 218,872 offers an opportunity to further assess the relative importance of brain 5-HT and NE systems in contributing to the anxiolytic and sedative actions produced by BDZs. Consistent with the Wise et al. (1972) proposal, CL 218,872 would be expected to decrease 5-HT turnover, while having no effect on CA turnover. In light of the recent demonstration that CL 218,872 produces a marked reduction in both lever pressing (chapter 2) and locomotor activity (last chapter) under experimental conditions identical to those employed in the

present study, one might alternatively predict that CL 218,872 would reduce CA turnover. In the present experiment CL 218,872 (2.5 to 20 mg/kg), whether administered acutely or chronically, did not alter either 5-HT or CA turnover. These negative results cannot be attributed to the experimental procedure, however, since the results using CDP showed that this procedure can detect changes in both 5-HT and CA metabolism in midbrain-hindbrain. Whether or not the results obtained here are unique to CL 218,872 or can be extended to pharmacologically similar anxiolytic compounds such as zopiclone (Wickstrom and Giercksky, 1980), PK 9084 (LeFur et al., 1981), and buspirone (Weissman et al., 1984) remains to be determined.

Finally, since CL 218,872 is a BDZ agonist with a selective affinity for Type I BDZ receptors (Squires et al., 1979), the failure of CL 218,872 to decrease 5-HT and CA turnover indicates that the alterations in 5-HT and CA metabolism produced by BDZs are probably not mediated via an interaction with Type I BDZ receptors. It is still unknown, however, whether BDZs are influencing biogenic amine neurotransmission through binding to another subtype among BDZ receptors (i.e., central Type II or brain located peripheral type), or alternatively, through an as yet

unidentified neural mechanism.

Taken together, the present findings using CDP and CL 218,872 indicate that behavioral sedation, as measured by a decrease in either unpunished responding or locomotor activity, can be evidenced without a concomitant decrease in CA turnover. These results similarly reveal that anxiolysis, as assessed by anticonflict activity, can be demonstrated in the absence of a parallel reduction in 5-HT turnover. Therefore, the data reported here are inconsistent with and do not support the Wise et al. (1972) hypothesis that reductions in brain 5-HT and NE activity respectively can account for the anticonflict and sedative effects produced by BDZs.

CHAPTER V

EXPERIMENT IV

ATTENUATION OF STRESS-INDUCED CORTICOSTERONE ELEVATIONS BY

ANXIOLYTIC DRUGS: A POSSIBLE ROLE FOR PERIPHERAL-TYPE

BENZODIAZEPINE RECEPTORS IN BRAIN

Introduction

One approach to studying the biochemical basis of anxiety has suggested that anxiety may result from biochemical changes induced by glucocorticoid hormones secreted from the cortex of the adrenal glands. Evidence from clinical studies has indicated that high concentrations of circulating glucocorticoids may increase anxiety-proneness (Persky, 1966), and Warburton (1974) proposed that anxiety-reducing agents such as benzodiazepines (BDZs) may do so by antagonizing the anxiety-inducing actions of glucocorticoids.

The hypothalamic-pituitary-adrenocortical (HPA) axis can be stimulated by a variety of stressful stimuli, including novel situations (File, 1982), sound (Torrellas et al., 1980), handling (Marc and Morselli, 1969), light (File and Peet, 1980), forced swimming (LeFur et al., 1979)

and footshock (Thiebot et al., 1982). These stress-induced glucocorticoid elevations can be blocked or attenuated by pretreatment with relatively low doses of BDZs (above references), or with other agents possessing antianxiety activity such as diphenylhydantoin (Bonnycastle and Bradley, 1959), meprobamate (Lahti and Barsuhn, 1974), and barbiturates (Lahti and Barsuhn, 1975; Norton, 1971; Rerup and Hedner, 1962). However, neither neuroleptics, psycho-stimulants, antidepressants, anticholinergics, alpha- and beta-blockers, serotonin receptor blockers, nor a narcotic analgesic lowered corticosteroid concentrations in stressed rats (LeFur et al., 1979; Lahti and Barsuhn, 1974), illustrating the pharmacological specificity of this action.

Relatively high doses of BDZs themselves elevate glucocorticoid concentrations in unstressed rats (Torrellas et al., 1980; Lahti and Barsuhn, 1975; Mark and Morselli, 1959), an effect reportedly related to the behaviorally depressant action of these drugs (Lahti and Barsuhn, 1975). Although some conflicting results regarding the effects of chronic BDZ treatment on glucocorticoid secretion have been reported, most investigators report that tolerance develops to the BDZ-induced elevation in glucocorticoid concentrations but not to their glucocorticoid attenuating action in

stressed animals (for review, see Torrellas et al., 1980).

The presence of BDZ receptors in the adrenal cortex, anterior pituitary, and other tissues outside of the brain raises questions concerning the site of action of these drugs on the HPA axis. Corticosterone increases produced by a high dose of diazepam could be prevented by blocking pituitary ACTH release (Chabot et al., 1981), indicating that diazepam was not directly stimulating the adrenal cortex in this situation. BDZ inhibition of stress-induced glucocorticoid secretion probably also does not involve an adrenal site of action, inasmuch as diazepam was unable to prevent adrenocortical stimulation by exogenously administered ACTH (Lahti and Barsuhn, 1974). Thus it appears that the effects of BDZs (and probably other similarly acting drugs) on adrenocortical secretion occur secondary to changes in ACTH secretion. However, it is not yet clear whether the BDZ receptors mediating such effects are located in the brain, the pituitary gland, or in a non-neuroendocrine tissue. Thus, one aim of the present research was to determine whether BDZs influence corticosterone secretion through a central (i.e., brain) or peripheral site of action. To this end, serum corticosterone concentrations were measured in sound-stressed and in unstressed rats following systemic or intracerebro-

ventricular (i.c.v.) chlordiazepoxide (CDP) administration.
The second goal was to determine whether the actions of
BDZs and other antianxiety agents on the HPA system could
be extended to the triazolopyridazine CL 218,872. For this
purpose, the effects of acute CL 218,872 administration on
corticosterone concentrations in stressed and unstressed
rats were studied. In all cases where positive results
were obtained with CDP or CL 218,872, the effects of
chronic drug pretreatment were examined to test for the
development of tolerance and/or cross-tolerance. The final
aim of this investigation was to establish whether the
influence of BDZs on corticosterone secretion could be
related to one particular BDZ receptor type by means of
central- (Ro 15-1788) and peripheral- (Ro 5-4864 and PK
11195) type receptor antagonists. A portion of this work
was presented in abstract form (McElroy and Meyer, 1983).

Methods and Procedure

Animals

Adult male albino rats were bred in our laboratory
from stock animals obtained from the Holtzman Co. (Madison,
WI) and were housed in a temperature controlled room
(approximately 22 $^{\circ}$C) under a 14:10 light-dark cycle (lights

on at 0600 hr). Rats were housed in group cages until they weighed 200-250 g, at which time they were removed to single cages and handled daily for at least 5 days prior to testing. Tap water and standard Purina Rodent Chow pellets were available ad libitum.

Drugs

CDP (Roche) was dissolved in physiological saline, whereas CL 218,872 (Lederle), Ro 15-1788 and Ro 5-4864 (Roche), and PK 11195 (Pharmuka) were suspended in a solution of 20% propylene glycol in water. Intraperitoneal (i.p.) injections were given in a volume of 2 ml/kg body weight, while i.c.v. injections were given in a volume of 25 ul.

Cannula implantation

The animals were anesthetized with Equithesin (3 mg/kg, Jensen-Salsbery, Kansas City, MO) and a hole was drilled in the skull 2.1 mm lateral to the sagittal suture and 0.5mm posterior to bregma. Cannula guides were constructed and implanted as described in McElroy et al. (1984). The surgery was performed 48 to 72 hr prior to i.c.v. drug administration. A microinjection unit was used to deliver the injectant through an internal cannula (25

gauge disposable needle) that extended 0.5 to 1.0 mm beyond the tip of the guide cannula. The location of the cannula in the lumen of the lateral ventricle was verified in all animals by examining the stain produced by injecting a 0.1% toluidine blue solution immediately after decapitation.

Procedure

Experiments were conducted between 0800 and 1200 hr to ensure that baseline corticosterone concentrations would be low. Rats (5-9 per group) were injected with drug or control solutions at the time and dosage schedules indicated in each figure and table legend. Sixty min after systemic CDP or CL 218,872 injection, or 15 min following i.c.v. CDP injection, animals were quietly transferred from the animal room (one at a time, at 15 min intervals) to an adjacent room where they were placed singly in an unfamiliar test apparatus. The test apparatus consisted of a standard operant chamber (Skinner box) enclosed in a sound-attenuated, fan-ventilated enclosure. Each rat was exposed to constant sound stimulation (120 dB) produced by a ceiling-mounted Sonalert. Following stress, the animals were quietly removed from the test apparatus and quickly decapitated. Unstressed rats remained in their home cages for 75 min following drug administration, after which time

they were quietly removed from the animal room and quickly decapitated. Trunk blood samples were collected at the time of decapitation, allowed to clot, centrifuged (2700 RPM for 10 min), and the serum either assayed immediately or stored at -40oC for subsequent corticosterone determination by radioimmunoassay (Meyer, 1983).

Data analysis

All data were subjected to analyses of variance (ANOVA) followed by individual mean comparisons using Fischer's Least Significant Difference Test (Kirk, 1968) where appropriate. Results occurring with a chance probability of less than .05 were considered statistically significant.

Results

Fig. 5 illustrates that placement in a novel environment and exposure to sound stimulation elevated blood corticosterone concentrations in a time-dependent fashion ($F = 15.05$, $df = 4$, 35, $p < 0.001$). Post-hoc testing revealed that corticosterone concentrations were significantly increased after 10, 15, or 30 min of exposure to stress. Based on these results, 15 min of sound stimula-

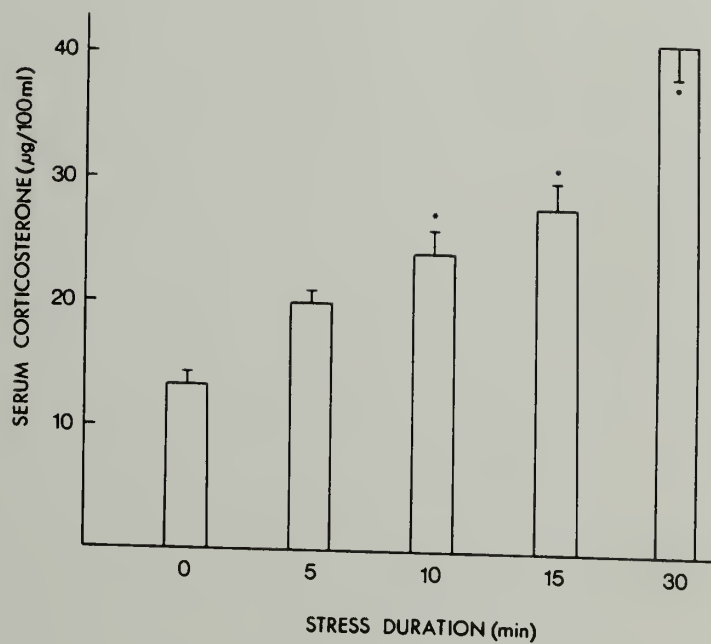Fig. 5. Effect of constant 120 dB sound stimulation on serum corticosterone concentrations in rats. Data represent the mean (± 1 S.E.) for eight rats per group. *Groups that differ significantly (at least $p < 0.05$) from the control (0 min) group.

tion served as the stressor in subsequent experiments.

The effects of different doses of systemically administered CDP on serum corticosterone in sound-stressed and in unstressed rats can be seen in Fig. 6. ANOVA revealed highly significant Stress ($F = 27.08$, $df = 1$, 50, $P < 0.001$) and Dose ($F = 19.32$, $df = 4$, 50, $P < 0.001$) main effects, as well as a Stress x Dose interaction ($F = 3.55$, $df = 4$, 50, $P < 0.05$). In unstressed rats CDP elevated corticosterone values in a dose-dependent fashion with the 10 and 20 mg/kg doses significantly different from the control (0 mg/kg) group. In rats subjected to sound stimulation, 2.5 to 10 mg/kg CDP attenuated the stress-induced elevation in corticosterone, with the 5 mg/kg dose significantly different from the control group. The largest dose of CDP tested (20 mg/kg) produced a nonsignificant enhancement of the corticosterone elevation produced by sound stress.

The effects of i.c.v. CDP administration to sound-stressed and to unstressed rats paralleled those observed after systemic injection (Fig. 7). ANOVA revealed highly significant Stress ($F = 33.85$, $df = 1$, 48, $P < 0.001$) and Dose ($F = 12.60$, $df = 5$, 48, $P < 0.001$) main effects, as well as a Stress x Dose interaction ($F = 2.44$, $df = 5$, 48, $P < 0.05$). A low dose of CDP (5 ug) significantly atte-
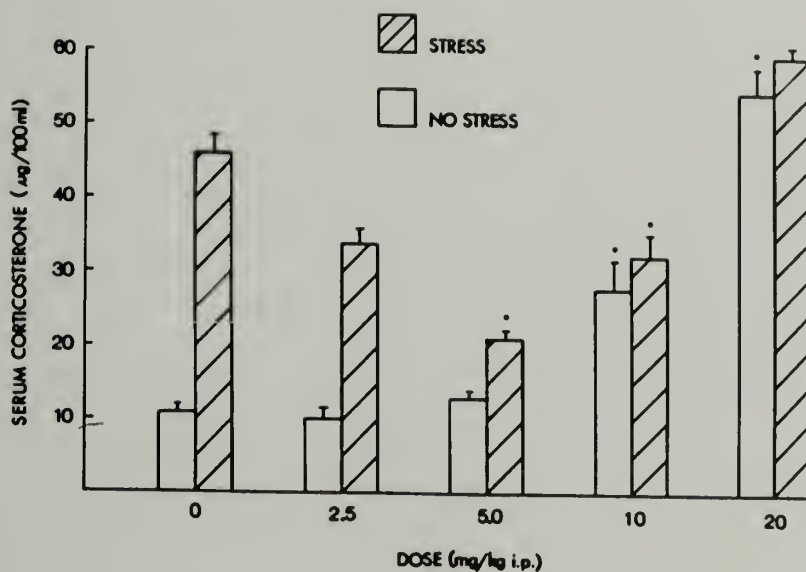
Fig. 6. Effect of various doses of systemically administered chlordiazepoxide (CDP) on serum corticosterone concentrations in sound stressed rats. Animals were killed 75 min after drug or vehicle injection and immediately after 15 min of constant 120 dB sound stimulation. Data represent the mean ($\pm$ 1 S.E.) for six rats per group. *Groups that differ significantly (at least $p <$ 0.05) from the corresponding control (0 mg/kg) group.

Fig. 7. Effect of various doses of i.c.v. administered chlordiazepoxide (CDP) on serum corticosterone concentrations in sound stressed and in unstressed rats. Animals were killed 30 min after drug or vehicle injection and immediately after 15 min of constant 120 dB sound stimulation. Data represent the mean ($\pm$ 1 S.E.) for five rats per group. *Groups that differ significantly (at least p < 0.05) from the corresponding control (0 mg/kg) group.
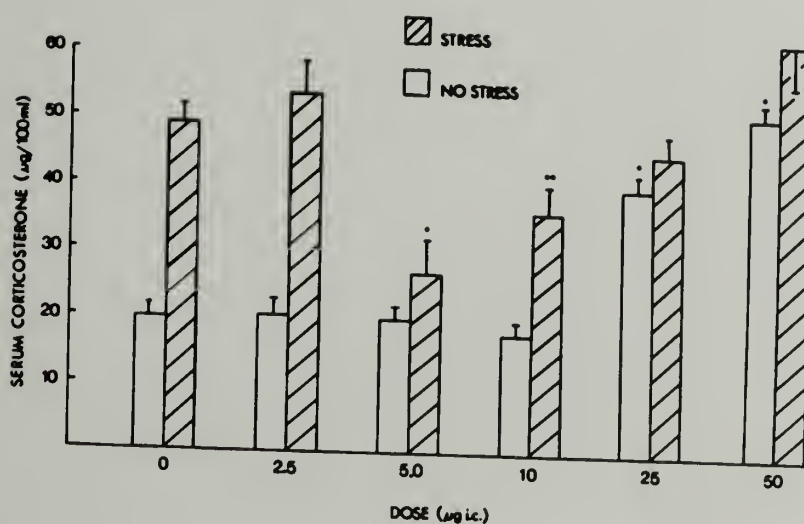
nuated the stress induced elevation in corticosterone, whereas relatively higher doses of CDP (25 and 50 ug) elevated corticosterone concentrations in unstressed rats. As was observed following i.p. administration, the highest dose of CDP tested (50 ug) produced a non-significant enhancement of the corticosterone elevation produced by sound stress.

The effects of different doses of CL 218,872 on circulating corticosterone in stressed and in unstressed rats are presented in Table 3. In marked contrast to the results with CDP, CL 218,872 (2.5 to 20 mg/kg) did not alter the corticosterone elevation produced by sound stress, and also failed to elevate baseline corticosterone concentrations in unstressed rats (F = 0.47, df = 4, 50, P = 0.75 and F = 0.25, df = 4, 50, P = 0.91 for the Dose main effect and Stress x Dose interaction respectively).

Table 4 (leftmost two columns) shows the effects of 15 mg/kg CDP on serum corticosterone in unstressed rats 48 hr after six daily pretreatments with eitherCDP (15 mg/kg) or CL 218,872 (2.5 and 10 mg/kg). ANOVA disclosed highly significant Test Treatment (F = 14.41, df = 1, 64, P < 0.005) and Pretreatment (F = 8.75, df = 3, 64, P < 0.005) main effects, in addition to a Test Treatment x Pretreatment interaction (F = 8.14, df = 3, 64, P < 0.005). A

Table 3. Effect of CL218,872 on serum corticosterone
   concentrations in unstressed and in sound-stressed
   rats.

CL218,872 Treatment (mg/kg)	Serum Corticosterone (ug/100 ml)	
	Unstressed	Sound Stressed
0	13.7 ± 2.6	34.2 ± 2.7
2.5	14.1 ± 3.8	38.9 ± 6.6
5.0	14.4 ± 3.9	32.9 ± 6.5
10	10.7 ± 2.5	36.2 ± 4.6
20	16.1 ± 4.2	40.2 ± 3.5

CL 218,872 was given 1 hr before the rats were stressed and
75 min before the rats were killed. Data represent the
mean ± S.E. for 6 rats per group. No groups were
significantly different (at least $p < 0.05$) from the
corresponding control (0 mg/kg) group.

Table 4. Effect of chlordiazepoxide (CDP) or CL 218,872 pretreatments on A) the serum corticosterone elevation produced by CDP in unstressed rats, and on B) the CDP-induced attenuation of corticosterone in sound-stressed animals.

Pretreatment (mg/kg)	Serum Corticosterone (ug/100 ml)			
	Test Treatment			
	(A) Unstressed		(B) Sound Stressed	
	CDP(15)	vehicle	CDP(5)	vehicle
Vehicle	35.2 ±5.3	11.8 ±1.1	20.1 ±2.5	39.8 ±4.8
CDP (15)	11.3 ±1.0*	12.2 ±0.9	20.0 ±1.9	37.9 ±5.0
CL218,872 (2.5)	19.6 ±4.7*	14.6 ±2.0	25.1 ±4.3	34.1 ±3.4
CL218,872 (10)	12.2 ±1.7*	10.4 ±0.9	27.3 ±4.8	43.2 ±5.1

All test treatments were given 1 hr before the rats were stressed, and 48 hr after 6 consecutive daily pretreatments. Data represent the mean ± S.E. for 9 rats per group. *Significantly different (at least $p < 0.05$) from the corresponding vehicle pretreatment group.

single test injection of CDP (column 1) produced a reliable
corticosterone elevation in vehicle pretreated animals
(from 11.8 to 35.2 ug/100 ml), but not in rats pretreated
with either CDP or CL 218,872. Thus, prior CDP or CL
218,872 treatment produced tolerance and cross-tolerance
respectively to the CDP-induced elevation of corticoste-
rone. In vehicle tested animals (column 2), chronic
pretreatment with CDP, CL 218,872, or vehicle had no effect
on baseline corticosterone levels.

The effects of CDP (5 mg/kg) or CL 218,872 (2.5 and 10
mg/kg) pretreatment on the CDP-induced attenuation of
corticosterone secretion in sound-stressed rats can also be
seen in Table 4 (rightmost two columns). ANOVA revealed a
highly significant Test Treatment main effect ($F = 26.72$,
$df = 1, 64$, $P < 0.001$) but no Pretreatment main effect ($F =
1.12$, $df = 3, 64$, $P = 0.35$) or interaction ($F = 0.92$, $df =
3, 64$, $P = 0.44$). A single test injection of CDP (5 mg/kg)
reliably attenuated the hormone elevation produced by sound
stress (from 39.8 to 20.1 ug/100 ml). This CDP action was
not altered by prior CDP or CL 218,872 treatment, indica-
ting that neither tolerance nor cross-tolerance had
developed to this BDZ action. Corticosterone values in
vehicle tested animals were also not affected by CDP or CL
218,872 pretreatment.

The effects of central- (Ro 15-1788) and peripheral-type (Ro 5-4864 and PK 11195) BDZ receptor antagonists on the ability of CDP (5 mg/kg) to attenuate sound-induced corticosterone elevations are presented in Table 5. One-way ANOVAs disclosed no significant Dose effects for either Ro 15-1788 ($F = 0.43$, df = 4, 20, $P = 0.73$) or Ro 5-4864 ($F = 2.18$, df = 5, 24, $P = 0.09$), although there was a tendency for higher doses of the latter drug to antagonize the corticosterone lowering action of CDP. On the other hand, a significant dose-related blocking of the CDP effect was obtained with PK 11195 ($F = 2.77$, df = 5, 24, $P < 0.05$). Post-hoc testing revealed that both the 1 and 2 mg/kg doses were effective in this regard. When given

alone, neither Ro 15-1788 (5 and 20 mg/kg), Ro 5-4864 (2 and 8 mg/kg), nor PK 11195 (1 and 2 mg/kg) had any effect on basline corticosterone concentrations in unstressed rats (data not shown).

Discussion

It was previously established that novelty and/or sound stimulation can produce an elevation in blood glucocorticoid concentrations, and that BDZ pretreatment

Table 5.   Effect of Ro 15-1788, Ro 5-4864, or Pk 11195 pretreatment on the chlordiazepoxide(CDP)-induced attenuation of serum corticosterone in sound-stressed rats.

Pretreatment (mg/kg)	CDP (mg/kg)	Serum Corticosterone (ug/100 ml)
Vehicle	0	41.6 ± 4.7*
Vehicle	5	23.3 ± 2.7
Ro 15-1788 (5)	5	22.3 ± 2.6
(10)	5	17.5 ± 4.9
(20)	5	19.9 ± 5.2
Ro 5-4864 (1)	5	24.5 ± 6.7
(2)	5	17.6 ± 3.3
(4)	5	33.1 ± 10.4
(8)	5	36.7 ± 6.4
PK 11195 (0.25)	5	24.9 ± 3.6
(0.5)	5	21.6 ± 5.7
(1)	5	42.8 ± 5.8*
(2)	5	38.0 ± 9.4*

CDP was given 1 hr before rats were stressed and 30 min after either Ro 15-1788, Ro 5-4864, or PK 11195 pretreatment.   Data represent the mean ± S.E. for 5 rats per group, except all vehicle pretreated groups were combined (N = 15) for purpose of presentation. *Significantly different (at least $P < 0.05$) from the vehicle-CDP group.

can prevent this action (File, 1982; Torrellas et al., 1980). The present findings are consistent with these reports. Fifteen min. exposure to a novel environment plus sound stimulation produced a three-fold increase in circulating corticosterone. Relatively low doses of CDP (2.5 to 10 mg/kg) attenuated this stress-induced hormone elevation, with the 5 mg/kg dose significantly reducing corticosterone concentrations. In marked contrast to the corticosterone lowering action produced by relatively low doses, the highest dose of CDP tested (20 mg/kg) led to a non-significant enhancement of the stress response. Similar dose-dependent effects of BDZs upon the pituitary-adrenocortical response to stress have previously been reported (Lahti and Barsuhn, 1974; Torrellas et al., 1980).

A single i.p. injection of CDP to rats not subjected to stress was followed by a dose-dependent increase in serum corticosterone concentrations. These results are in general agreement with several other studies (Lahti and Barsuhn, 1975; LeFur et al., 1979), although Krulik and Cerby (1971) reported that subcutaneous administration of CDP did not alter circulating corticosterone concentrations in their animals. Nevertheless, it appears that the overall effect of a given dose of CDP in stressed rats depends on the interaction of two opposing tendencies. At

low doses, the drug acts to antagonize the pituitary-adrenocortical stress response, while intermediate and high doses mask this effect because of their tendency to stimulate adrenocortical secretion even in the absence of an applied stressor.

One aim of the present studies was to determine whether BDZs influence pituitary-adrenocortical activity via a central (i.e., CNS) or peripheral site of action. For example, these drugs could be acting in the hypothalamus, as central type BDZ receptors have been found in this brain region (Mohler and Okada, 1977). On the other hand, the notion that BDZs might be controlling glucocorticoid secretion through a peripheral mechanism is not unreasonable as both the 5-HT releasing drug fenfluramine (McElroy et al., 1984) and the GABA-mimetic drug muscimol (Grandison and Guidotti, 1979) have been found to influence pituitary hormone release via peripheral receptors. Furthermore, the identification of GABA and GABA receptors (Racagni et al., 1982) along with the previously mentioned peripheral-type BDZ receptors (Schoemaker et al., 1983) in the anterior pituitary strengthens the possibility that BDZs could affect the pituitary-adrenocortical system by a direct action at that level. However, we found evidence for a central action of BDZs in that the effects of i.c.v.

administered CDP in both stressed and unstressed rats paralleled those observed following systemic administration. A relatively low dose of CDP (5 ug) attenuated the stress-induced elevation in corticosterone, while somewhat higher doses of CDP (25 and 50 ug) elevated corticosterone concentrations in unstressed animals. This 200-fold increase in potency of centrally- vs. systemically-administered CDP supports the hypothesis of a CNS site of BDZ action, although we cannot completely rule out the possibility that small amounts of the drug reached the pituitary gland via the pituitary portal circulation. Further studies will be necessary to resolve this issue.

Attenuation of stress-induced glucocorticoid responses has been observed with other antianxiety agents such as meprobamate (Lahti and Barsuhn, 1974), diphenylhydantoin (Bonnycastle and Bradley, 1959), and barbiturates (Lahti and Barsuhn, 1974; Norton, 1971). However, neuroleptics, psychostimulants, antidepressants, anticholinergics, alpha- and beta-blockers, 5-HT receptor blockers, and a narcotic analgesic did not reduce circulating corticosterone concentrations in stressed rats (LeFur et al., 1979; Lahti and Barsuhn, 1974), demonstrating the pharmacological specificity of this action. These findings led Lahti and Barsuhn (1974) and LeFur et al. (1979) to propose that the corti-

costeroid stress test could be used to screen for potential antianxiety agents. The present experiment evaluated this hypothesis by examining the effects of CL 218,872, a novel anxiolytic compound of the triazolopyridazine class, on the pituitary-adrenocortical system. CL 218,872 did not attenuate the corticosterone elevation produced by sound stimulation, and also failed to alter baseline corticosterone concentrations in unstressed animals. These results indicate that stress (as measured by pituitary-adrenocortical activation) can be dissociated from anxiety (as measured for example by conflict paradigms), thus challenging the validity of the corticosterone test as a screening procedure for anxiolytic activity. Whether or not the results obtained here are unique to CL 218,872 or can be extended to pharmacologically similar anxiolytic compounds such as zopiclone (Wickstrom and Giercksky, 1980) or PK 9084 (LeFur et al., 1981) remains to be determined.

Previous studies report conflicting effects of prolonged BDZ administration on circulating corticosteroid concentrations. For example, it was reported that tolerance develops to the diazepam-induced rise in serum corticosterone in unstressed rats, but not to the drug's glucocorticoid-attenuating action in stressed rats (Lahti and Barsuhn, 1975). On the other hand, Torrellas et al.

(1980) showed the development of tolerance to both BDZ effects, whereas Marc and Morselli (1969) demonstrated no tolerance to the elevation of corticosterone produced by high doses of diazepam in unstressed rats. In the present experiment, six daily CDP pretreatments produced tolerance to the elevation in plasma corticosterone triggered by acute administration of the drug. In marked contrast, however, chronic drug treatment did not affect the ability of a subsequent CDP injection to antagonize the corticosterone elevation produced by sound stress. Thus, our results are consistent with those of Lahti and Barsuhn (1975). The discrepancies between the various reports cited here are probably a consequence of the numerous differences in experimental conditions (i.e., choice of stresor, choice of BDZ, mode of drug administration, and number of drug treatments), and may simply reflect the extreme lability of the HPA system.

CL 218,872 pretreatment had the same effect on a subsequent challenge injection of CDP as did CDP pretreatment itself. Specifically, six daily injections of CL 218,872 (2.5 and 10 mg/kg) did not impair the ability of a low dose of CDP (5 mg/kg) to attenuate the corticosterone elevation produced by sound stress, whereas this same pretreatment regimen did prevent the enhanced corticosterone

secretion produced by a relatively large dose of CDP (15 mg/kg) in unstressed rats. The latter effect demonstrates cross-tolerance by CL 218,872 to the glucocorticoid eleva- ting action of CDP, and is particularly interesting because this dose of CL 218,872 did not itself affect corticoste- rone concentrations. Chronic CDP (Hoogland et al., 1966) or ethanol (Sellers and Busto, 1982) treatment has been reported to decrease the blood concentration of subsequently administered CDP, indicating that tolerance and cross-tolerance to the effects of CDP can be a result of enhanced hepatic metabolism of the drug. It is not yet clear whether the tolerance and cross-tolerance reported here are enzymatic, cellular, or behavioral in nature.

Overwhelming biochemical, pharmacological, and behavioral evidence suggests that classical brain-specific BDZ receptors mediate at least the anxiolytic and anticon- vulsant actions of this class of drugs (Tallman et al., 1980; Schallek et al., 1979; Braestrup and Squires, 1978). The physiological relevance of peripheral-type BDZ recep- tors, however, is still unclear. The present studies attempted to determine which BDZ receptor type mediates the pituitary-adrenocortical actions of BDZs. To this end, several compounds with well defined interactions with BDZ receptors were evaluated for their effects on the cortico-

sterone response to stress. As mentioned above, CL 218,872 did not produce a BDZ-like action in either stressed or unstressed rats. As this compound is known to be an agonist at central Type I BDZ receptors (Squires et al., 1979), it appears that BDZs are not exerting their pituitary-adrenocortical actions via stimulation of such receptors. Moreover, Ro 15-1788, a BDZ antagonist specific for brain-type BDZ receptors (Mohler and Richards, 1981), failed to prevent the CDP effect in stressed rats. Consistent with these results, brief or prolonged exposure to physiological or psychological stress had little, if any, effect on brain-type BDZ receptor binding (Braestrup et al., 1979; LeFur et al., 1979).

In marked contrast, however, the peripheral-type BDZ receptor antagonist PK 11195 (LeFur et al., 1983a) fully prevented the CDP effect in stressed rats. Our results using Ro 5-4864, another ligand for peripheral-type BDZ receptors (Braestrup and Squires, 1977), were less conclusive as Ro 5-4864 did not block this CDP action but only produced a non-significant trend in that direction. The ineffectiveness of Ro 5-4864 may be due to the mode of administration in that Weissman et al. (1983) recently reported that the potency of this compound is reduced when administered as a suspension (present experiment).

Alternatively, our negative results using Ro 5-4864 may be explained by thermodynamic studies suggesting that PK 11195 is an antagonist, while Ro 5-4864 is a full or partial agonist at peripheral-type binding sites (LeFur et al., 1983a). Nonetheless, the results reported here indicate that CDP is altering corticosterone secretion via binding to peripheral-type BDZ receptors. Taken together with the evidence that CDP is exerting this action at a CNS location, our results implicate peripheral-type receptors in brain as the target sites through which BDZs influence glucocorticoid secretion. This is one of the first physiological roles postulated for peripheral-type BDZ receptors in rat brain.

Bizzi et al. (1983) recently reported that Ro 15-1788 reversed the effect of diazepam on plasma corticosterone in stressed rats, results at odds with those reported here. Aside from numerous methodological differences between the two studies, we cannot reconcile these disparate findings. However unlikely, the possibility exists that CDP and diazepam are exerting their corticosteroid actions through separate sites and/or receptor types. Resolution of this issue will certainly require further investigation.

Evidence for a widespread interaction between BDZs and several hormonal systems has been growing in the last few

years. For example, parallel to their effects on the pituitary-adrenocortical system, BDZs also inhibit stress-induced prolactin secretion (Grandison, 1981a), with this effect similarly persisting even after chronic drug administration (Grandison, 1981b). In contrast to these inhibitory effects on hormone secretion, BDZs stimulate growth hormone release both in vivo (Laakmann et al., 1982) and in vitro (Acs et al., 1984). BDZ regulation of pituitary hormone secretion may in some cases be related to the presence of peripheral-type BDZ receptors in this gland (Schoemaker et al., 1983).

There is also evidence for multiple interactions between BDZs and hormone binding sites and between various hormones and BDZ receptors. Thus, thyrotropin-releasing hormone binding in anterior pituitary, retina, and amygdala was inhibited by micromolar concentrations of CDP and diazepam, but not by clonazepam, Ro 15-1788, B-CCE, or 44 other neuroactive substances tested (Sharif et al., 1983). In other experiments, L-thyroxine (Nagy and Lajtha, 1983) and N-acetyl-5-methoxy kynurenamine, a brain metabolite of melatonin (Marangos et al., 1982), were found to have higher affinities for BDZ receptors than any of the several hundred other compounds that have been tested to date (Braestrup and Squires, 1978; Mackerer et al., 1978;

Marangos et al., 1981). It is interesting to note that of all the endogenous compounds evaluated for their relative potencies at peripheral- vs. central-type BDZ receptors (L-thyroxine not included), only melatonin was a more potent inhibitor of binding at the peripheral-type sites (Marangos et al., 1982). This finding is of particular interest considering the relatively high concentration of these sites observed in the pineal gland (Weissman et al., 1983). Finally, both melatonin (Sugden, 1983) and thyrotropin-releasing hormone (Vogel et al., 1980) possess some pharmacological effects characteristic of BDZs. The above results have led investigators to speculate that one of these hormones, one of their metabolites, or perhaps a structural analog might be an endogenous ligand for BDZ receptors (Nagy and Lajtha, 1983; Holmes and Sugden, 1982; Marangos et al., 1981). Verification of this hypothesis awaits further investigation.

GENERAL SUMMARY

The present series of experiments profiled the non-BDZ anxiolytic compound CL 218,872. Specifically, this investigation examined the ability of CL 218,872, as compared to the BDZ chlordiazepoxide (CDP), to 1) produce a discriminative stimulus complex; 2) alter 5-HT and CA turnover rates in brain; and 3) prevent stress-induced elevations in serum corticosterone. The rationale for these experiments was three-fold. First, because the TPZs may ultimately be of great value in the clinical treatment of anxiety disorders, it was considered important to learn more about the behavioral, neurochemical, and endocrine actions of this class of drugs. Secondly, as agonists selective for Type I BDZ receptors, the TPZs represent a new probe for further clarifying the molecular basis by which BDZs induce their various behavioral and physiological actions. Lastly, since the above behavioral, neurochemical, and endocrine actions produced by the BDZs have each been considered either predictive of, or responsible for, the anxiolytic potential of this class of drugs, CL 218,872 presents an opportunity either to confirm and extend or to refute the validity of such measures as indicators of a

drugs' clinical (anxiety reducing) potential.

In the first experiment rats were trained to discriminate CDP from saline using a two-lever operant procedure. An acute injection of CL 218,872 elicited CDP lever selection in a dose-dependent fashion, demonstrating that CL 218,872 produces a discriminative stimulus similar to that produced by CDP. The ability of a drug to generalize to the CDP discriminative stimulus had previously been restricted to barbiturates and other BDZs, agents with clinical efficacy in the treatment of anxiety disorders. Therefore, the present results are consistent with, and further extend, the notion that the BDZ drug discrimination paradigm can be utilized to screen for potential anxiolytic agents. Furthermore, because CL 218,872 is an agonist selective for Type I BDZ receptors, these results suggest that the discriminative stimulus produced by BDZs may be mediated via an interaction with this BDZ receptor subtype. Finally, the demonstration that 10 mg/kg CL 218,872 virtually abolished responding (as measured by lever pressing for a milk reinforcement) is at odds with a previous report that a much larger dose (223 mg/kg) is required to reduce locomotor activity by 50 percent (Lippa et al., 1979).

The second experiment attempted to resolve this

discrepancy by examining the effects of CL 218,872 on locomotor activity in a conventional activity chamber. Acute thirty min CL 218,872 pretreatment (10 mg/kg i.p.) significantly reduced locomotor activity, a finding in good agreement with that reported in Experiment I, but still at variance with that reported by Lippa et al. (1979). However, it is possible that this 20-fold disparity in drug potency may still be a result of methodological differences since their drug to test interval was 60 min and they administered CL 218,872 by the oral route. The principle finding of this experiment, however, was that CL 218,872 as used in the present series of investigations is not devoid of depressant side effects as was originally reported (Lippa et al., 1979).

The third experiment showed that acute administration of CDP decreased the turnover (synthesis) rate in midbrain-hindbrain of serotonin (5-HT), but not that of catecholamines (CA), whereas chronic CDP treatment decreased the turnover rates of both neurotransmitters. In marked contrast, whether administered acutely or chronically, CL 218,872 did not alter baseline 5-HT or CA turnover rates. These results indicate that stimulation of Type I BDZ receptors is not sufficient to decrease 5-HT and CA turnover rates, and more importantly these results do

not lend support to the Wise et al. (1972) hypothesis suggesting that the anxiolytic and sedative effects produced by BDZs are mediated via reductions in 5-HT and CA turnover respectively.

The final experiment confirmed that a low dose of CDP attenuated the increase in serum corticosterone produced by exposure to a novel environment plus sound stimulation. Somewhat higher doses of CDP elevated corticosterone levels in unstressed rats. Parallel results were obtained after central drug administration. CL 218,872, however, did not attenuate the corticosterone elevation produced by stress, and also failed to alter baseline corticosterone concentrations in unstressed rats. Additionally, the CDP effect in stressed rats was fully prevented by PK 11195, a receptor antagonist specific for peripheral-type BDZ receptors, but not by Ro 15-1788, an antagonist specific for central-type BDZ receptors. Taken together, these findings implicate peripheral-type BDZ receptors in brain as the target sites through which BDZs influence corticosterone secretion. Furthermore, the results using CL 218,872 are inconsistent with the notion that the corticosterone stress-test can be utilized to predict the efficacy of a compound as an anxiolytic agent.

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