A NEUROPHARMACOLOGICAL STUDY OF HOMOSYNAPTIC DEPRESSION OF THE SPINAL STRETCH REFLEX LOOP: THE ACTIONS OF BENZODIAZEPINES

by

M. Frances Davies

Department of Pharmacology and Therapeutics

McGill University

Montreal, Quebec, Canada

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M. Frances Davies
Department of Pharmacology and Therapeutics Ph. D.

A Neuropharmacological Study of Homosynaptic Depression of the Spinal Stretch Reflex Loop: the Actions of Benzodiazepines

ABSTRACT

The actions of benzodiazepines on homosynaptic depression of the spinal stretch reflex were examined in spinal cats. On the biceps semitendinosus pathway (BST), clonazepam enhanced the depression of the second response of the early tetanic rundown (ETR) (10 stimuli at 10, 5, and 2 Hz every 30 seconds) without affecting the first response or the plateau level. This effect was antagonized by an antagonist for the central benzodiazepine receptor, Ro15-1788, and by reducing GABAergic transmission with semicarbazide or bicuculline. On the triceps surae (TS) pathway, clonazepam increased the first response and alleviated the depression of the second response without changing the plateau level. Clonazepam appeared to influence BST homosynaptic depression by lengthening the primary afferent depolarization (PAD). The PAD also underwent depression, therefore later responses in the ETR were unaffected by clonazepam. TS ETRs were not similarly affected because activation of TS afferents did not cause a significant PAD of its own afferents.

Mercaptopropionic acid was shown to be unsuitable as a GABA depleting agent for in vivo experiments in the feline spinal cord.

M. Frances Davies
Département de Pharmacologie et Thérapeutique Ph. D.

Une Etude Neuropharmacologique de la Dépression Homosynaptique de l'Arc Réflexe Extenseur Spinal: les Effets des Benzodiazépines

CONDENSE

Les effets des benzodiazépines sur la dépression homosynaptique du réflexe extenseur spinal furent étudiés chez les chats spinaux. Sur la voie du biceps semitendinosus (BST), le clonazépam augmentait la dépression de la deuxième réponse du début de la fatigue tétanique (DFT) (10 stimuli à 10, 5, et 2 Hz chaque 30 secondes) sans toutefois modifié la première réponse ou le niveau du plateau. Cet effet était contrecarré par un antagoniste du récepteur benzodiazépine central, le Ro15-1788, et par une réduction de la transmission GABAergique au moyen du semicarbazide ou de la bicuculline. Sur la voie du triceps surae (TS), le clonazépam augmentait la première réponse et réduisait la dépression de la seconde réponse sans aucun changement du niveau du plateau. Le clonazépam semblait influencer la dépression homosynaptique du BST par un allongement de la dépolarisation des afférences primaires (DAP). La DAP était également sujette à une dépression, d'où le fait que les réponses tardives du DFT étaient inchangées par le clonazépam. Les DFT du TS n'étaient pas affectés de façon similaire, dû au fait que l'activation des afférences du TS ne causait pas une DAP significative sur ses propres afférentes.

Au cours des expériences in vivo sur la moelle épinière féline, l'acide mercaptopropionique s'était révélé inadéquat comme agent d'épuisement du GABA.

PREFACE

Format of the Thesis

In accordance with the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and with the Thesis Format adopted by the Department of Pharmacology and Therapeutics, McGill University on March 2, 1973, the results in this thesis are presented in a form suitable for publication in a learned journal. Cited in full, section 7 of the Guidelines Concerning Thesis Preparation is as follows:

> 7. MANUSCRIPTS AND AUTHORSHIP
>
> The Candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must still conform to all other requirements explained in this document, and additional material (e.g. experimental data, details of equipment and experimental design) may need to be provided. In any case abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstract, introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted.
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> While the inclusion of manuscripts co-authored by the Candidate and others is not prohibited for a test period, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should also be noted that the task of the External Examiner is much more difficult in such cases.

This thesis is composed of five chapters. Chapter 1, the introduction, presents a review of the literature pertinent to the work presented in this thesis. Chapter 2 has been published in the Canadian Journal of Physiology and Pharmacology, while Chapters 3 and 4 have been submitted for publication to Brain Research. An overall discussion of the results is presented in Chapter 5. Finally, a brief list of the results are presented in the Summary

of Contributions to Original Knowledge.

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LIST OF ABBREVIATIONS

ADP	afterdepolarization potential
AHP	afterhyperpolarization potential
BST	biceps semitendinosus
CNS	central nervous system
DRP	dorsal root potential
DRR	dorsal root reflex
EPSP	excitatory postsynaptic potential
ETR	early tetanic rundown
GABA	gamma-aminobutyric acid
GABA-T	gamma-aminobutyric acid transaminase
GAD	glutamic acid decarboxylase
HRP	horseradish peroxidase
Hz	Hertz
MDG	(2RS,3E)-2-methyl-3,4-didehydroglutamic acid
MPA	mercaptopropionic acid
MSR	monosynaptic response
PAD	primary afferent depolarization
PAH	primary afferent hyperpolarization
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol
TS	triceps surae

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Benzodiazepines are used in the treatment of epilepsy, a disease characterized by excessive uncontrolled neuronal discharge. Despite the abundance of literature on the clinical effect and mechanism of action of benzodiazepines, it is still not known how they have their anticonvulsant action. In general, anticonvulsants are thought to act by pushing the excitation-inhibition balance in favour of enhanced inhibition, and in this way suppress the spread of uncontrolled excitation associated with epilepsy. This shift in balance can be achieved by either increasing the strength of the inhibitory processes or by reducing excitatory forces. Homosynaptic depression, where the efficacy of a synapse is reduced by previous use, represents a phenomenon in which excitatory transmission is self-limiting (Beswick and Evanson, 1957). It is conceivable that an anticonvulsant may shift the excitation-inhibition balance by enhancing homosynaptic depression. This has been shown to be the case for ethosuximide (Čapek and Esplin, 1977b). The aim of this present study was to investigate the effect of benzodiazepines on homosynaptic depression of the monosynaptic stretch reflex in the feline spinal cord.

More specifically, the objectives of this research project were:

1. To characterize the effects of benzodiazepines on homosynaptic depression;
2. to determine whether any benzodiazepine action is mediated by the central benzodiazepine receptor;
3. to investigate whether benzodiazepines affect homosynaptic depression by enhancing inhibitory transmission mediated by GABA;
4. to elucidate how benzodiazepines affect homosynaptic depression.

1.2 The Spinal Monosynaptic Pathway

1.2.1 The Stretch Reflex

The stretch reflex is a spinal monosynaptic reflex which maintains the muscle at a predetermined length (Matthews, 1972). The receptor organ, the muscle spindle, is imbedded in the muscle. When the muscle increases in length the spindle is passively stretched which causes the primary ending of the group Ia afferent to fire. The primary afferents convey this muscle length information to the CNS where it is used in the feedback control of the muscle. One of the primary afferents' principal targets is the alpha-motoneurons in the spinal cord ventral horn with which they make excitatory connections. When activated, the motoneuron causes the shortening of the same muscle or a synergistic muscle from which the primary afferents originated. In this way, the muscle length is corrected and the spindles cease firing.

The stretch, or myotatic, reflex was first described by Liddell and Sherrington (1924, 1925). When they attempted to flex the rigidly extended limb of a decerebrate cat the limb resisted by active muscular contraction of extensor muscles. They described two components, the short latency and relatively intense phasic component and the less intense but longer duration tonic component. The tonic component is determined by the steady stretch of the muscle while the phasic component is activated by movement of the muscle.

1.2.2 Primary Afferents

The structure of Ia primary afferents within the cat spinal cord is reasonably well understood. In the periphery they have large myelinated axons and conduction velocities of about 80 to 100 m/sec. Upon entering the spinal cord from the dorsal root, afferent axons bifurcate sending branches rostrally and caudally in the dorsal funiculus (Réthelyi and Szentagothai, 1973). The

total length of the rostral plus caudal branches is about one centimeter (Brown and Fyffe, 1978) thereby allowing one primary afferent to make contact with widely spaced motoneurons over as much as five spinal segments. From these major branches, single collaterals arise every 100 to 2500 μm, the average being one every millimeter. These collaterals descend to lamina V before branching to form the first set of terminal arborizations which then enter lamina VI. The main collaterals then move towards the region of the motor nuclei where they branch in the sagittal plane to form a terminal arborization. As a result, a continuous column of terminals is formed within the motor nuclei. The collaterals create third to fifth order branches before forming synaptic boutons. In the motor nuclei, there are three types of terminal arborization patterns: isolated boutons at the ends of terminal axons, a string of four or five boutons plus a terminal bouton, or a branched terminal axon with small clusters of boutons.

As Ia primary afferents branch in the spinal cord, the diameter of the collaterals and degree of myelination changes (Iles, 1976). At the point of entry into the spinal cord the axon diameter ranges from 2 to 12 μm with a mean of about 6 μm. The largest major collaterals reaching the ventral horn have axon diameters of about 2.5 μm which, after branching, are further reduced to about 1 μm. Until this point all Ia fibres are myelinated. From there, the terminal branches become very fine (less than 1 μm), the extent of myelination becomes uncertain and boutons begin to appear. Munson and Sypert (1979a) have estimated the conduction velocities of medial gastrocnemius Ia afferent fibres to be 50-60 m/sec in the dorsal funiculus stem axon, 8-19 m/sec in major collateral branches and 0.2-1.0 m/sec in terminal branches. A decrease in conduction velocity would be expected as the axon diameter decreases. The very low conduction velocity in the terminal branches

indicates that the axons are unmyelinated.

There is evidence that collaterals of many fibres run down to the ventral horn in small groups called microbundles (Scheibel and Scheibel, 1969). The axons of Ia, Ib and small unmyelinated fibres are visible in these bundles, with the average number of fibres being about 6. There does not seem to be any overlapping between different microbundles but within each bundle nodular enlargements and small spine-like extensions appear to establish contacts with immediately adjacent fibres. It has been suggested that axo-axonal interactions could occur before the first synapse is reached. The real significance of these findings is still unknown.

1.2.3 Motoneurons

Motoneurons have their cell bodies in lamina IX of the ventral horn and are among the largest neurons in the central nervous system, with a soma diameter of between 30 and 70 μm. By intracellular staining with horse radish peroxidase (HRP), Brown and Fyffe (1981) found that motoneuron dendritic trees stretch extensively through the ventral horn and into the ventral white matter and ventral root filament. They found seven to eighteen primary dendritic trunks giving rise to up to between fourth and sixth order daughter branches. At branch points dendrites usually bifurcate with 5% forming trifurcations. Triceps surae dendrites reach up to 1600 μm in length therefore the total spread in any direction (rostral-caudal, medio-lateral or dorsal-ventral) can be as much as 2 to 3 mm. Despite the tremendous length of the motoneuronal dendrites, electrophysiological studies indicate that their electrotonic lengths are short (Barrett and Crill, 1974), although there is some debate as to the appropriateness of the methods (de Jongh and Kernell, 1982). If we accept short electrotonic length as correct, synapses on distal dendrites are almost as effective as those on or near the soma.

1.2.4 Primary Afferent-Motoneuron Synapse

The density of Ia endings on motoneurons of various sizes seem to be about equal, being about 1% of the total synaptic coverage of motoneurons (Conradi, 1969a). From morphological data, it has been estimated that a triceps surae Ia collateral makes contact with about 50 to 60 TS motoneurons (Brown and Fyffe, 1978) totalling 550 for the entire Ia fibre. Between a single Ia fibre and an alpha-motoneuron, one to ten contacts have been identified with an average of three to four (Ishizuka et al., 1979; Brown and Fyffe, 1981). Mendell and Henneman (1971) have shown by stimulating a single Ia fibre and recording excitatory post synaptic potentials (EPSPs) from many motoneurons, that a single Ia fibre from the medial gastrocnemius sends terminals to nearly all of its 300 alpha-motoneurons and to about 65% of the heteronymous lateral gastrocnemius motoneurons. They also suggested that endings of a single Ia fibre are clustered together on the surface of the motoneuron. Anatomical studies have shown that all boutons originating from the same afferent are closely placed on the same dendrite or make contacts on many dendrites but at the same physical and therefore also the same electronic distance from the soma (Brown and Fyffe, 1981). Mendell and Henneman (1971) found that a given Ia fibre sends its terminals to all parts of the motoneuron since EPSPs elicited by stimulation of one Ia fibre and recorded in different motoneurons varied in their time course. Scott and Mendell (1976) found that the mean EPSP was larger in homonymous rather than heteronymous motoneurons.

1.2.5 Nature of the Transmission

There has been a long standing controversy concerning the nature of transmission between Ia primary afferents and alpha-motoneurons, that is, whether it is a chemical synapse or a low resistance electrical connection. A number of experimental findings did not seem compatible with the idea of

chemical transmission (Burke, 1967). In contrast to the early reports (Coombs et al., 1955), many groups were unable to entirely reverse the intracellularly recorded Ia monosynaptic EPSP even with very high depolarizing currents which reversed the initial parts of other EPSPs (Werman and Carlen, 1976). These data led Werman's group to propose that this was a combined electrical-chemical synapse. This idea was challenged on grounds that a significant and fluctuating synaptic delay was measured, which is incompatible with an electrotonic component at this synapse (Munson and Sypert, 1979b). Finally, using a double barrel micropipette technique, Engberg and Marshall (1979) were able to reverse the initial as well as the later EPSP component at reversal potentials of about 0 to 10 mV positive. Thus, this is now accepted as a chemical synapse.

The synaptic boutons of Ia afferents which synapse on the motoneuronal membrane are large and contain spherical vesicles, scattered throughout the bouton (Conradi, 1969b). On the surface of these boutons, smaller boutons make contact and are thought to be involved in presynaptic inhibition. The opposing postsynaptic membrane is covered with a thick layer of contrast rich material and postsynaptic dense bodies.

The identification of the neurotransmitter has proved to be very difficult, assuming that this is indeed a chemical synapse. Although many candidates have been presented, none has gained wide acceptance (Redman, 1979). Glutamate has been a prime candidate since it is present in large quantities in the ventral horn and depolarizes the motoneurons. However, when iontophoresed onto cultured spinal neurons, the reversal potential for glutamate is much more negative than the EPSP elicited by stimulation of dorsal root ganglion cells in co-culture (Ransom et al., 1977). Possible explanations for this contradictory result are that the iontophoresed

glutamate interacted with non-synaptic glutamate receptors which were different from the subsynaptic receptors or that glutamate is not the excitatory transmitter. Substance P has also been proposed as the neurotransmitter at this synapse but, when it is iontophoresed onto spinal cord cells, the depolarization is too slow in onset for it to be a serious candidate (Otsuka and Konishi, 1976). Moreover, substance P immunoreactivity was detected in small diameter afferents rather than in the Ia fibres (Pickel et al., 1977).

The characteristics of transmission at the Ia afferent-motoneuron synapse has been the subject of many studies over the past twenty years. The EPSPs evoked in motoneurons by stimulation of a single primary afferent were shown to fluctuate in amplitude and, in some cases, transmission failures occurred (Kuno, 1964a). Using the statistical method developed for analysis of synaptic responses at the neuromuscular junction (del Castillo and Katz, 1954), Kuno concluded that monosynaptic transmission in spinal motoneurons occurs in quantal steps. One impulse in a single afferent fibre releases, on the average, only one quantum and the variation in the size of the observed EPSPs can be described by the Poisson distribution. The mean quantum content was later revised from the original estimate of one to between 1 and 15, with an average being between 2 and 3 (Kuno and Miyahara, 1969). This study was criticized because spontaneous unit EPSPs, equivalent to the miniature end-plate potentials, could not be measured directly, since potentials can originate from a variety of synaptic projections. Instead, they were calculated from other data under the assumption of Poisson statistics. Edwards' group (1976a) did not make this assumption and found that neither Poisson nor binomial statistics described the data, and fluctuations were non-quantal in nature. They suggested that transmission occurs in an all-or-none manner and fluctuations result from the combined effect of transmission

transmission failures at several terminals arising from a single fibre. The effect of different stimulation frequencies on charge fluctuations was investigated (Edwards et al. 1976b), but no significant change in probability of failures or in the size of charge transfer was detected. Posttetanic potentiation and facilitation of synaptic potentials result from a decrease in the probability of failure (Hirst et al., 1981). In a subsequent study using more refined techniques (Jack et al., 1981a), EPSPs were shown to fluctuate between different discrete amplitudes, with peak amplitudes that were integer multiples of the increment between successive components. By relating these findings to the anatomical data, this group concluded that each bouton behaves in an all-or-none manner and the probability of failure varies at different boutons arising from the same afferent. They also suggested that the amount of transmitter released resulted in a saturation of the postsynaptic receptors since there was very little variation in the size of a single unit. Compounds such as 4-aminopyridine which increase the monosynaptic response (Jankowska et al., 1977) decreased the probability of failure to release transmitter at each bouton (Jack et al., 1981b). Studies of inhibitory postsynaptic potentials (IPSPs) obtained from goldfish Mauthner cells followed a similar all-or-none relationship (Korn et al., 1982). More importantly, Korn's group was able to demonstrate that the binomial term n was very often equal to the number of presynaptic boutons seen with intracellular staining of the presynaptic fibre, and therefore corresponded to the number of release sites.

Failure of transmission could occur because either the action potential does not invade the terminal or due to failure of the transmitter release process following the depolarization of the terminal (Edwards et al., 1976b). The failure of an action potential to arrive at the terminals might occur because of the low safety factor of conduction at axonal bifurcations and at

sudden enlargements of axonal membrane surface (Ramon et al., 1976). At single terminal axon making en passant synaptic contacts, the axon suddenly broadens to form a bouton and an action potential reaching this point would have to excite a much larger surface area of the membrane to continue its progress along the axon. The nerve terminal may also possess different membrane characteristics than the axon. In the crayfish neuromuscular junction, it appears that nerve terminals are inexcitable and are depolarized by the electrotonic spread of the action potential (Dudel, 1982). While the action potential is conducted into the vertebrate neuromuscular junction terminal, this may not be the case in CNS synapses. The failure of transmitter release in the presence of primary afferent depolarization is discussed more fully later.

The latency of EPSPs produced by activation of single Ia fibres fluctuates from trial to trial (Collatos et al., 1979; Cope and Mendell, 1982a). Also, EPSPs evoked with short latencies are larger in peak amplitude and rise time than those of longer latency. These results suggested that the variability of the transmitter release process from Ia terminal boutons is the most likely source of EPSP fluctations. When distributions of synaptic latency of single spike triggered EPSP at different synaptic connections were compared (Cope and Mendell, 1982b), the distribution differed in standard deviation but the minimum latency was judged to be the same. These variations in distribution could be due to action of different numbers of release sites at different connections. An individual release site may in fact be synonymous with a terminal bouton.

1.3 Primary Afferent Depolarization

Gasser and Graham first detected slow potentials on the cord surface in 1933. Similar potentials were recorded by means of paired electrodes on the dorsal root (Barron and Matthews, 1936), hence the term dorsal root potentials (DRP). These depolarizing potentials had a fast rise time and a slower decay phase, and were produced in the body of the spinal cord and electrotonically conducted back along the primary afferents. Occasionally spikes were seen on the rising phase of the DRP and were labelled dorsal root reflexes (DRR). It is thought that DRR are action potentials that are generated when the depolarization of the primary afferents becomes large enough to bring the membrane potential to threshold (Eccles et al., 1961a). If the DRRs are caused by the antidromic firing of the primary afferent, they should produce a monosynaptic EPSP in the contacted motoneuron. This has been demonstrated (Eccles et al., 1961a) and from the latency it appears the impulses are generated in Ia fibres close to the presynaptic terminals on the motoneurons.

The concept of presynaptic inhibition was first proposed by Frank and Fuortes (1957) to explain the reduction of Ia EPSPs recorded from extensor motoneurons by conditioning volleys in group I afferents from flexor nerves. Eccles' group (1961a,b) was able to relate the depression of the EPSP to the amount of DRR and therefore established the link between the depolarizing potential and presynaptic inhibition. It was found that activation of muscle receptors, similar to what occurs during movement, also led to presynaptic inhibition of afferent fibres (Devenandan et al., 1965) hence establishing that this phenomenon is not an artifact due to the electrical stimulation of peripheral nerves.

The presynaptic depolarization detected by various methods i.e. by intracellular recording from primary afferents, with pairs of gross electrodes on the cord surface or from dorsal roots, or the inhibition of the

monosynaptic response, has the same time course (Eccles et al., 1963c) . In general, the latency is 3 to 5 ms, although recent studies have lowered this estimate of the minimal latency to between 1.7 and 2.0 ms (Jankowska et al., 1981), with a rising time of 19 to 25 ms and a total duration of up to 200 ms. This characteristic time course may be due to the repetitive bombardment of the interneurons causing depolarization of the presynaptic fibres. Interneurons which show this pattern of firing have been observed in the intermediate nucleus (Eccles et al., 1962b). Alternatively, the transmitter could be deactivated slowly. It has been suggested that no other action of group I afferents continues beyond 25 ms (Eccles et al., 1961b).

1.3.1 The Mechanism of PAD

EPSP depression occurred at stimulus strengths which activated the Ia-Ib fibres with only a negligible additional effect when group II and III afferents were activated (Eccles et al., 1961b). Group I muscle afferents effectively produce presynaptic inhibition of all flexor reflex afferents, but flexor reflex afferents do no presynaptically inhibit group I afferents (Eccles et al., 1962b). Volleys from cutaneous and group II and III fibres from muscle produce little or no depolarization of group I muscle afferents. Group Ia fibres receive presynaptic inhibition almost exclusively from group Ia afferents from flexor muscles (Eccles et al., 1962b).

Two major hypothesis have been proposed to explain the cause of the DRP. The first, put forth by Barron and Matthews in 1938 suggested that the DRP was caused by ionic changes around the fine terminals of afferent fibres due to conduction and transmission. A buildup of K^+ ions in the extracellular space was considered the best candidate. The other hypothesis, which has become dominant in recent years, was first proposed by Eccles and colleagues

(1962b). According to this group, the DRP arises through axo-axonic synapses located near the terminal boutons, which upon stimulation, release their transmitter and depolarize the afferent terminals. An action potential invading this already depolarized terminal is diminished and would not be able to release as much transmitter as it would when normally polarized and hence the resulting EPSP would be smaller.

There is a growing body of evidence supporting the second hypothesis as the major cause of DRP in group I afferents. Intracellular recording from primary afferent fibres has shown that impulses in the impaled fibre do not produce a PAD within that fibre (Eccles and Krnjevic, 1959). Any changes in the ionic composition of the extracellular fluid due to activation of one axon would be expected to affect that axon more than any other fibre, therefore this does not support the concept of Barron and Matthews. In the frog spinal cord, low frequency stimulation (1 to 10 Hz) of the dorsal root caused a decremental summation of the extracellular K^+ concentration while the DRPs remained constant or decreased (Nicoll, 1979). The lack of parallelism between them indicates that they are not causally related. It has also been demonstrated (Rudomin et al., 1982) that the PAD of group Ia fibres produced by stimulation of group I muscle afferents is not due to K^+ buildup and hence probably a result of activation of a specific neural pathway. Other groups (Sykova and Vyklický, 1978) have suggested that a small proportion of the DRP may be attributable to an increase in extracellular K^+. Blocking synaptic transmission with magnesium does not completely abolish depolarization of amphibian afferents evoked by stimulation of an adjacent dorsal root (Nicoll, 1979) and the magnitude of this picrotoxin resistant depolarization correlated well with changes in the extracellular K^+ concentration. Krnjevic and Morris (1975) have postulated that the DRP has two components, an early sharp GABAergic component and a later, somewhat slower

and smaller component due to the rise in the extracellular K^+ concentration.

While most studies favour the idea that PAD results in presynaptic inhibition some studies have found, using sensitive methods for detecting postsynaptic changes, that conditioning stimuli produced postsynaptic changes in the motoneuron (Cook and Cangiano, 1972; Werman and Carlen, 1976; Sypert et al., 1980). This suggests that both pre- and postsynaptic inhibition can be evoked by the standard conditioning stimuli used to generate presynaptic inhibition, although the relative importance of each to normal functioning is not known.

1.3.2 Pharmacology of the PAD

The original investigation of the pharmacology of this phenomenon was carried out by Eccles group (1963a). Drugs which act upon cholinergic synapses had no action on presynaptic inhibition. They found that pentobarbital sodium increased the DRP, the surface P wave and presynaptic inhibition. Picrotoxin depressed presynaptic inhibition and could antagonize the actions of pentobarbital. Topically applied GABA and 3-aminopropanesulphonic acid depressed DRP and P waves but increased DRR, which indicates that the primary afferent fibres had been depolarized. In the spinal cord, bicuculline and picrotoxin selectively antagonized the GABA induced depolarization (Barker and Nicoll, 1973). Consistently, the excitability of primary afferents was increased by systemically or topically applied GABA or 3-aminopropanesulphonic acid. This effect was antagonized by bicuculline and picrotoxin but not by semicarbazide (Čapek and Esplin, 1982). From the order of relative potencies of iontophoretically administered agonists the GABA receptors on Ia terminations appear to be similar to the receptors responsible for hyperpolarizing spinal interneurons (Curtis et al., 1982).

From the depolarizing action of GABA on primary afferents (Feltz and Rasminsky, 1974; Sastry, 1979b; Čapek and Esplin, 1982; Curtis et al., 1982) and the demonstration of GABAergic terminals apparently making axo-axonic synapses with Ia primary afferents (McLaughlin et al., 1975), it has been inferred that GABA may be released by the last interneuron in the PAD pathway. Alternatively, Levy and Anderson (1972) have proposed that GABA inhibits a tonically active inhibitory pathway which in turn modulates a tonic presynaptic depolarization.

1.3.3 Ionic Conductance Mechanism

It has been demonstrated in dorsal root ganglion cells that the GABA response was associated with an increase in membrane conductance, and had a reversal potential of -33 mV which was found to be related to the log of extracellular chloride concentration (Nishi et al., 1974). Gallagher et al. (1978) showed by intracellular injection of various anions into the dorsal root cells, that GABA only increased the permeability of anions whose hydrated radius was smaller than BrO_3^-. In most CNS neurons, GABA causes hyperpolarization and hence inhibition by opening chloride channels and allowing chloride ions to enter the neuron. However, GABA depolarizes primary afferents and if an increased chloride conductance was responsible for PAD then the chloride equilibrium potential of the primary affferent would have to be more positive than the resting membrane potential.

1.3.4 Modulation of Transmitter Release by PAD

The exact mechanism by which PAD reduces transmitter release is not clear although many hypothesis have been put forth. Based on the work of Takeuchi and Takeuchi (1962) in the squid, Eccles (1964) suggested that a reduction in the size of the presynaptic action potential by superposition on a PAD would

reduce the amount of transmitter released. However in view of the findings of Edwards and co-workers (1976a) which supported the concept of an all or none activation of separate boutons, it is not a simple task to extrapolate from the results obtained in invertebrates. Kato and Kuba (1980) postulated that GABA increases the chloride conductance of the presynaptic terminal and thereby reduces the amplitude of the action potential by its shunting effect. GABA can also shorten the calcium component on the action potential of the dorsal root ganglia soma but this action is not blocked by bicuculline (Dunlap and Fischbach, 1978). It is possible that PAD causes conduction failure at branch points by shunting the membrane so that fewer boutons would be activated or cause a graded reduction of release from single boutons or a combination of both.

The characteristics of both the action potential and PAD are changed if an action potential is evoked in an afferent fiber which is experiencing depolarization (Eccles et al., 1963c). Whereas the normal action potential was followed by an after-depolarization (ADP) and a later after-hyperpolarization, when superimposed on the DRP, the ADP was reduced in size and even reversed if it occurred at the time of the PAD summit. The PAD does not fully recover at all intervals after the interpolated action potential. Therefore a considerable proportion of the PAD either survives the propagation or is rebuilt afterword. This group concluded that the depolarizing transmitter is still present at the terminal, either due to slow inactivation or by continuous release of transmitter. They also argued that when superimposed on the PAD, the spike potential is reduced by an amount approximately equivalent to the depolarization.

1.3.5 Repetitive Activation of PAD

When the conditioning and testing volleys are applied to the same afferent fibres, the second DRP changes as a function of stimulus interval (Eccles et al., 1963b). In the case of stimulation of BST, facilitation was seen at intervals less than 20 ms but depression at longer intervals. This depression of the DRP reached a maximum at 50 ms and returned to control levels at about 500 ms. The supression could be due to homosynaptic depression in the primary afferents which caused the DRP, or elements after the primary afferent synapse in the inhibitory pathway might also be sensitive to repetitive stimulation. Interestingly, the DRR is more sensitive to repetitive stimulation than DRP for unknown reasons (Eccles et al., 1961a). As the frequency of stimulation was increased, the DRR declined until at 2 to 5 Hz the reflex disappeared and required about 10 seconds of rest to fully recover.

1.3.6 Primary Afferent Hyperpolarization

Primary afferents can also be hyperpolarized under some conditions, which is detected as a positive DRP (Mendell and Wall, 1964; Mendell, 1972). In general, primary afferent hyperpolarization (PAH) is produced by activation of fine high threshold fibres such as group III and C fibres. When fibres of a larger diameter are fired, the larger the fibre diameter, the greater the negative component of the DRP. The largest Ia fibres seem to produce a purely negative DRP. The duration of PAH in response to a single shock sufficient to activate group III afferents is of the order of 100 ms. Mendell (1972) demonstrated that PAH as well as PAD are picrotoxin sensitive.

Just as PAD has been associated with an increase in excitability and hence a lessening of transmitter release, PAH has been linked to a decrease in excitability (Mendell, 1972). When a single shock of group III intensity was

applied to the TS nerve which evoked a negative-positive DRP, the excitability of group I fibres of the semitendinosus nerve increased during the negative phase of the DRP and decreased during the positive phase. At 20 Hz high intensity stimulation of the TS nerve causes a sustained PAH (Mendell, 1972).

1.4 Repetitive Stimulation

1.4.1 Repetitive Stimulation of the Spinal MSR

When peripheral nerves are repetitively stimulated, the resulting MSR does not stay at a constant level, but is either facilitated or depressed depending on the frequency of stimulation. Lloyd and Wilson (1957) proposed that the reflex depression could be divided into two parts, that occurring at frequencies higher than 10 Hz was of postsynaptic origin whereas the depression at lower frequencies was due to presynaptic events. Between 60 and 100 Hz, monosynaptic reflex responses display temporal summation which can be described by an exponential decaying to 1/e in 4 ms (Lloyd, 1957a). At frequencies between 60 and 10 Hz, the inability of all motoneurons to follow the afferent firing rate is probably the major factor in depression, although presynaptic events may also contribute (Lloyd, 1957b). Antidromic activation of motoneurons has shown that the phase of depressed excitability of motoneurons is usually over within 100 ms, although there have been cases where complete recovery of TS motoneurons was only seen after 170 to 200 ms (Brooks et al., 1950a).

It is likely that low frequency depression is a phenomenon intrinsic to group Ia fibres with no involvement of group II or III afferents. Lloyd and Wilson (1957) found that even the smallest afferent input volleys produced an enduring depression in the second supramaximally stimulated response. The

degree of depression grew in parallel as the conditioning volley size was increased until the volley size reached 60-80% of its maximum. With subsequent increases in stimulus strength, the degree of depression remained relatively constant. Since group Ia afferents have the lowest threshold and almost all are activated when 60% of the group I afferents are excited (Rall, 1955), it would appear that there is also no influence from Ib fibres. When the first of two pulses was so small that virtually no reflex response was elicited, the supramaximal second volley also showed depression but not as much as when the conditioning volley was maximal for group I fibres (Brooks et al., 1950b). In this situation, the subthreshold conditioning stimulus causes a depression in transmission capabilities of only a few fibres. When the supramaximal test stimulus is applied, the previously unstimulated afferents produce a mixture of full and reduced size EPSPs. A supramaximal conditioning stimulus reaches almost all terminals and therefore more depression of the composite test MSR is seen.

Lloyd and Wilson (1957) could not find a difference in the pattern of depression caused by a two pulse paradigm and the steady state depression achieved by long trains of pulses over a large range of frequencies. They also could not detect any difference in depression between flexors and extensors under steady state conditions.

When the entire TS nerve was subjected to repetitive stimulation, the MSR did not show as much depression as occurred when afferent stimulation was restricted to the medial gastrocnemius branch (Lloyd and Wilson, 1957). In this case stimulation of the lateral gastrocnemius alone produced no MSR. From this it would seem that there may be an interaction between these two branches in which prior activation of lateral gastrocnemius sets up a series of events which contribute to the depression of medial gastrocnemius but alone would not be strong enough to fire any motoneurons.

1.4.2 Mechanism of Homosynaptic Depression

The underlying cause of homosynaptic depression in the spinal monosynaptic pathway is not known. From the work of Lloyd and Wilson (1957) it becomes apparent that changes in postsynaptic properties are not a major factor, but that events in the Ia primary afferents as resulting from previous stimulation cause their terminals to release less transmitter in total. Decandia's group (1967a,b) advanced the hypothesis that homosynaptic depression was mainly caused by depolarization of the Ia afferent terminals by the same mechanism as presynaptic inhibition. This was discounted by Čapek and Esplin (1977a) who showed that homosynaptic depression was still present when GABAergic transmission was reduced by administration of semicarbazide, a GABA synthesis blocker. There are many possible causes for a reduction in transmitter release within primary afferents. Either each terminal is always invaded by the action potential, but some process which occurs after the arrival of the depolarizing wave is depressed or the action potential does not reach every terminal each time. When EPSPs elicited by stimulation of a single afferent were recorded (Collins and Mendell, 1981) they found that EPSP declined over time especially in the first 5 to 20 stimuli, then reached a constant level. This group was unable to determine whether the depression of EPSP amplitude was due to conduction failure at branch points or lack of terminal invasion or due to a depression of the amount of transmitter released at individual synaptic sites.

Evidence is accumulating suggesting that failure of the action potential to arrive at the terminal is a common occurence. Silent synapses have been found (Lüscher et al., 1982) that were inactive for long periods of time. These investigators felt that a failure in transmission in the terminal

arborizations had occurred since they were not aware of CNS synapses which have null transmitter release probabilities for long periods of time, while branch point block can definitely occur. In lobster, high frequency stimulation causes selective conduction block at branch points, first in larger daughter branches then later in smaller branches (Grossman et al., 1979b). A 2 to 3 mM increase in extracellular K^+ ion concentration was sufficient to produce block of conduction into both branches (Grossman et al., 1979a). It has been shown that with very modest rates of stimulation of peripheral nerves (less than 10 Hz) an increase in the extracellular K^+ can be detected in the cuneate nucleus and the spinal dorsal horn which parallels changes in extracellularly recorded focal potentials (Krnjevic and Morris, 1975). This rise in extracellular K^+ does not significantly affect the grossly measured membrane potential of primary afferents or spinal neurons (Lothman and Somjen, 1975). However, it is possible that small local increases in K^+ may cause a failure of invasion of the fine terminations thereby significantly hindering transmitter release. Changes in spike pattern conducive to conduction block, such as reduction of spike amplitude, decrease in hyperpolarization and a marked decrease in the effective membrane resistance was also seen where the axon diameter abruptly changes as is the case of the synaptic boutons (Spira et al., 1976). In the goldfish Mauthner cell, the IPSP undergoes depression during high frequency stimulation but every synaptic bouton appears to continue functioning as an independent all or none releasing unit with just a lower probability of release (Korn et al., 1982). This is in keeping with the occurrence of conduction block at branch points or axonal enlargements.

In the case of depression of release, there are many steps which may be involved. With stimulation, the amount of transmitter available for release could be decreased and subsequent impulses would release less transmitter than

the first. Curtis and Eccles (1960) postulated that facilitation and depression of subsequent EPSPs was determined by a balance of two opposed processes within the terminal: a mobilization of the available transmitter, and a depletion of the transmitter. However, Kuno (1964b) demonstrated that the size of the second EPSPs after failures was in the same range as when no failures occurred in the first response. He felt that the mechanisms of both potentiation and depression are intrinsic to the terminals but are independent of the amount of transmitter released by the first impulse. The concept of transmitter depletion as a cause of homosynaptic depression has been used as the basis for the model of Čapek and Esplin (1977a), which assumes that the pool of the transmitter available for release is depleted. Clearly, the operational definition of this pool may include factors other than the transmitter itself. It is possible that restoration of the available transmitter stores occurs rapidly enough to maintain an optimal level of transmitter release but that some step in the release mechanism is still sub-optimal when the next impulse reaches the terminal. It has long been known that an influx of calcium ions is essential for transmitter release (Katz and Miledi, 1967). Calcium influx is known to contribute to the action potential in group I muscle afferents terminals (Sastry, 1979a) and is decreased by the application of verapamil, manganese or cobalt.

The gill withdrawal reflex pathway in Aplysia undergoes habituation or a decline in reflex responsiveness when the stimulus is repeatedly presented at low frequencies. Although this phenomenon occurs at lower frequencies than homosynaptic depression of the mammalian stretch reflex (1-0.01 Hz), this discrepancy may be due to slower metabolic functioning rate at the lower body temperature and not to a difference in the underlying mechanisms. On gross examination these two processes appear to be similar in that the amplitude of

the MSR of the stretch reflex and the amplitude of the EPSP recorded from a single motoneuron decline rapidly for the first two or three responses then reach a constant plateau level within five pulses (Byrne, 1982). In Aplysia, the magnitude of synaptic depression is relatively insensitive to the quantity of transmitter released by the previous impulse (Castellucci and Kandel, 1976). It is more likely that a reduction in the amount of calcium entering the terminal is responsible for the reduction in the size of the EPSP (Klein et al., 1980). Tillotson (1979) has shown that an increase in intracellular calcium causes an inactivation of calcium channel and therefore less calcium enters the terminal with each depolarization.

1.5 Differences in the BST and TS Monosynaptic Pathways

The biceps-semitendinosus (BST) and triceps surae (TS) muscle groups have radically different functions. The TS muscle group, made up of the medial and lateral gastrocnemius and the soleus muscles extend the ankle and as such resist the force of gravity. The BST muscles are knee flexors and, to some extent, hip extensors. It is likely that their neuronal circuitry is specialized to allow the optimal functioning of the particular muscles. Documented differences in the anatomical structure of afferents of these muscles are rare because most studies using intracellular staining of individual fibres have concentrated on TS afferents (Brown and Fyffe, 1978, 1981; Burke et al., 1979). In only one study (Ishizuka et al., 1979) were afferents of both origins examined and it was found that TS afferents terminate in the dorsal lateral area of lamina IX whereas BST afferent terminals are located in the ventral portion of lamina IX. The same problem exists for the motoneuron literature. The only demonstrated difference in the alpha-motoneuron innervating these two muscle groups is that TS motoneuron dendrites radiate equally in all directions whereas flexor motoneurons such as

those of BST are oriented mainly in the cord's longitudinal axis (Scheibel and Scheibel, 1969).

In the spinal preparation, there is a lack of inhibitory supraspinal control. Under these conditions, spinal interneurons (Wall, 1967) and flexor motoneurons are hyperactive, whereas extensor motoneurons are not (Jankowska et al., 1967).

When the reversal potential of Ia EPSPs in alpha-motoneurons of various leg muscles was determined there did not seem to be any differences between those from the TS and BST (Flatman et al., 1982). However, reversal was much easier to obtain in motoneurons of the deep peroneal group than all others tested. With only the small sample of motoneurons (total of 22) and the indication that diversity exists, it is impossible to exclude the possibility that differences between EPSP characteristcs in TS and BST motoneurons also exist.

1.5.1 Differences Seen with Repetitive Stimulation

Flexor and extensor reflexes do display different patterns when repetetively stimulated (Fuortes and Hubel, 1956). Firing of TS motoneurons was easier to evoke with high rather than low frequencies of orthodromic stimulation, whereas flexor motoneurons responded to each shock when the stimulation frequency was low but responded only initially to stimuli delivered at high frequencies. Following a single stimulus to a flexor, the test response was greater than the first response at stimulus intervals less than 15 ms but was considerably decreased later. In extensors, the early facilitation lasted longer but the late depression was less pronounced or absent.

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1.5.2 Differences in PAD

Eccles group (1962a) measured the P waves recorded on the surface of the spinal cord and elicited by stimulating various muscle afferents with a brief train. They found that at the L7 segment, stimulation of the BST produced a large P wave while stimulation of the TS produced a very small or sometimes no P wave. Flexor muscle groups such as BST and deep peroneal are more effective in producing DRR than extensors which, even at cord temperatures as low as 30^{o}, were very small or nonexistent (Eccles et al., 1961a). The changes of excitability of primary afferent were also found to correlate with the depolarization of these fibres (Eccles et al., 1962a). A conditioning stimulus to BST increased the excitability of knee flexors and extensors and ankle extensors but a conditioning stimulation to TS did not significantly change the excitability of any muscle afferents. Decandia's group (1967a) found that conditioning stimulation, consisting of a brief tetani to the lateral gastrocnemius, did cause an increase in excitability and hence a presynaptic inhibitory action on medial gastrocnemius Ia afferent terminals that lasted up to 400 ms. However, a single conditioning pulse did not cause any PAD, even in their barbiturate anaesthetized cats.

In these two nerves, different types of afferents are responsible for the production of PAD. In TS, a maximal group I volley (at 2.1 times threshold) evoked a very small DRP. The DRP was doubled when the stimulus was maximal for group II and was greatly increased for a maximal group III volley. In BST, group I volleys were relatively more effective and group III relatively less. In general, group I afferent volleys from flexor muscles produced larger DRP than those from extensors (Eccles et al., 1962a).

There is a difference in the relative proportions of PAD and PAH between muscle afferents (Eccles et al., 1962a). Stimulation of BST tended to develop PAH rather than PAD whereas TS produced mixed effects in response to single

shocks of sufficient strength to stimulate all myelinated fibres. However, since it is known that PAD in muscle group I afferents is evoked mainly by muscle group I volleys, it would seem that the high threshold reflex afferents produce mainly PAH in BST group I afferents and a mixture of PAH and PAD in TS.

1.6 GABA

GABA is one of the major inhibitory transmitters of the CNS, being found in all regions in an uneven distribution pattern (Fahn and Côté, 1968). GABAergic neurons are usually small interneurons although some in the cerebellum and basal ganglia have long processes extending into other structures.

In the spinal cord, the levels of GABA are quite low compared to those in the brain (Graham et al., 1967). In the lumbar area, the highest level of GABA is found in the dorsal horn, intermediate levels in laminae IV and VII and quite low levels in the ventral horn (Otsuka and Konishi, 1976). This distribution parallels that of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) (McLaughlin et al., 1975). GAD is a better marker for GABAergic neurons than GABA itself, since GABA may be released and taken up into other structures (Fonnum, 1975). Antibodies to purified GAD have been raised and are used extensively in immunocytochemical studies to locate GABAergic neurons (Wood et al., 1976; Ribak et al., 1981). GAD containing somata were found in all regions of the spinal cord except in the motoneuron pools within lamina IX (Barber et al., 1982). There are, however, GABAergic terminals which may be presynaptic to Ia primary afferents and motoneuronal dendrites and somata in lamina IX (McLaughlin et al., 1975) but their somata probably lie in lamina VII, VIII and X (Barber et al., 1982). In the ventral

horn, GAD-positive terminals form axo-axonal synapses with larger terminals, which are in turn presynaptic to motoneuron somata as well as forming axo-dendritic and axo-somatic synapses with motoneurons (Ribak et al., 1981).

1.6.1 GABA Metabolism

In its major metabolic pathway, GABA is synthesized in the cytoplasm from glutamate, by the enzyme glutamic acid decarboxylase (GAD E.C.4.1.1.15). This is the rate limiting enzyme under normal conditions (Roberts and Kuriyama, 1968). Since glutamate is derived from glucose via the tricarboxylic acid cycle, GABA metabolism is intimately related to the energy metabolism of the cell. In fact when GABA is broken down by its major catabolic enzyme, the carbon backbone is returned to this cycle.

GAD requires a cofactor, pyridoxal-5'-phosphate, to be bound to it for activity. Originally, it was assumed that this is a loose bond, since in vivo GAD is very susceptible to anti-vitamin B_6 agents such as hydrazines (Baxter, 1969). It was then shown that pyridoxal phosphate is tightly bound to GAD in the absence of glutamate, but glutamate promotes the dissociation of pyridoxal phosphate from the enzyme (Miller et al. 1978). As glutamate is the substrate of GAD, the constant contact would tend to cause a slow release of pyridoxal phosphate from the active site. Therefore the enzyme must be constantly resupplied with the cofactor for reassociation. In the presence of anti-vitamin B_6 agents or hydrazines, the source of pyridoxal phosphate is reduced and this results in a rapid decrease in the rate of formation of GABA.

GABA synthesis by GAD is regulated by many compounds. Divalent cations, especially zinc, are very potent inhibitors while monovalent cations show no activity (Wu and Roberts, 1974). Carboxylic acids such as alpha-ketoglutarate, fumarate, aspartate and glutarate are strong inhibitors. GABA is a very weak inhibitor indicating that end-product inhibition is not an important

regulatory mechanism for GAD.

Release of GABA from neuronal elements was shown to require calcium but the release from glia was not calcium dependent (Jaffé and Cuello, 1981). The regulation of GABA release is presented later in the text.

The extracellular action of GABA is terminated by uptake into neurons and glia (Henn, 1971). The two saturable GABA uptake systems, the high affinity and low affinity, are both sodium dependent (Bennett et al., 1973; Battistin et al., 1969).

The principal catabolic enzyme that degrades GABA, 4-aminobutyrate transaminase or GABA-T, is present in various tissues with the highest activites being found in the brain, liver, and kidney (Wu, 1976). In brain tissue, GABA-T has been found in both neurons and glial cells with the majority of the activity present in neurons (Chan-Palay et al., 1979). GABA-T is known to be associated with mitochondria (Waksman et al., 1968), in particular, the inner membrane (Schousboe et al. 1977). GABA-T catalyses the conversion of GABA to succinic semialdehyde, which in turn is transformed to succinic acid by semialdehyde dehydrogenase. The amine group is transferred to alpha-ketoglutarate, forming glutamate, thus replenishing the precursor supply for GABA (Baxter, 1976). GABA-T is a typical pyridoxal phosphate dependent reversible transaminase in that the pyridoxal phosphate accepts the amine to become pyridoxamine but the enzyme bound cofactor is restored in the transamination reaction with alpha-ketoglutarate (Metcalfe, 1979).

1.6.2 Inhibition of GABA Synthesis

To study the physiological role played by GAD, various inhibitors can been used. They can be broadly classified into three categories: suicide inhibitors such as (2RS,3E)-2-methyl-3,4-didehydroglutamic acid (MDG)

(Chrystal et al., 1979), competitive inhibitors which are analogs of glutamic acid of which 3-mercaptopropionic acid (MPA) is an example, and those which interfere with the synthesis of pyridoxal phosphate, such as semicarbazide and other hydrazines.

While suicide inhibitors are very attractive agents on theoretical grounds, all known GAD suicide inhibitors, MDG, and fluoromethyl- and difluoromethyl-glutamate, do not cross the blood brain barrier (Jung, personal communication). Therefore they would only be useful in in vitro studies, on cultured neurons or topically applied to the surface of the CNS. This type of inhibitor is also very slow in inactivating enzymes (Chrystal et al., 1979), which is a serious drawback for in vivo electrophysiological experiments.

Of the next most promising group of inhibitors, those that compete with glutamate for the active site, MPA is one of the most potent with a Ki of 1.8 μM (Wu, 1976). It was reasoned that MPA caused short latency convulsions by inhibiting GAD (Lamar, 1970) as the MPA induced reduction of GABA levels were found to correlate with the onset of convulsions (Karlsson et al., 1974). The pattern of decrease of GABA levels was shown to parallel the reduction of presynaptic inhibition in the dorsal column nuclei as assessed by the size of the P wave recorded on the cord surface (Roberts et al., 1978). This supported the contention that the releaseable pool of GABA was depleted by MPA. There are however reasons to believe that MPA is not a selective GAD inhibitor. This is more extensively reviewed in the MPA manuscript.

The hydrazide, semicarbazide, was originally shown to be a convulsant agent by Jenney and Lee in 1951. Killam and Bain (1957) demonstrated that the administration of semicarbazide reduced GABA levels through inhibition of GAD. The underlying mechanism of this enzyme inhibition was shown to be due to a depletion of GAD's cofactor, pyridoxal phosphate, by directly complexing with it to form hydrazones (Holtz and Palm, 1964).

Studies have been conducted to correlate the degree of semicarbazide induced depression of GABA level with a change in a proposed GABA mediated processes. Banna (1973) showed that, in the cat, semicarbazide produced a depression of the surface positive wave and the dorsal column reflex, and reduced the excitability of cuneate terminals induced by conditioning cutaneous nerve stimulation. The time course of these effects correlated with the depletion of GABA in the dorsal column nuclei. All the effects of semicarbazide were antagonized by supplying pyridoxine hydrochloride. In the spinal cat, semicarbazide was shown to reduce DRPs and presynaptic inhibition with a close correlation of the time course of their disappearance with the decline of GABA levels in the lumbar cord (Bell and Anderson, 1972). In their study, excitatory transmission was slightly augmented as the MSR elicited by dorsal root stimulation was increased to about 60% of control, 120 minutes after semicarbazide administration.

Since semicarbazide acts by depleting the cofactor pyridoxal phosphate, any enzyme which requires this coenzyme may be deleteriously affected by the drug. This group of enzymes includes various amino acid decarboxylases, transaminases and phosphorylases (Holtz and Palm, 1964). Indeed, semicarbazide has been shown to inhibit GABA-T although to a much smaller extent than GAD (Abe and Matsuda 1979; Wood and Abrahams, 1971).

1.7 GABA Receptors

1.7.1 Receptor Studies

Many binding studies using labelled GABA were done in the early 1970's (DeFeudis, 1973a), under the assumption that GABA binding was sodium dependent (DeFeudis, 1973b). However, members of Snyder's group (Zukin et al., 1974)

found that even though GABA binding was less in the absence of sodium, the remaining binding could be inhibited by the GABA antagonist, bicuculline, and by the amino acids which where previously shown to mimic the synaptic actions of GABA. Sodium dependent binding was demonstrated to be representative of binding to the GABA uptake site. Therefore it seemed that sodium independent GABA binding represented a specific interaction with the postsynaptic GABA recognition site. In a follow-up study (Enna and Snyder, 1975), the binding of GABA to this site was found to be saturable, have a dissociation constant of 1.2 μM and show regional variations in distribution throughout the CNS. More recent binding studies revealed that there were probably two binding sites for GABA (Enna and Snyder, 1977), one with a K_D of 13 nM and the other of 300 nM, with different association and dissociation rates, and different thermal inactivation rates (Olsen et al., 1981). These two sites were not just interconversions of one molecular species since there were differences in distribution between regions and subcellular fractions of mammalian brain.

GABA is not the only compound that is an agonist for the GABA receptor. In one classification scheme proposed by Meldrum's group (1980), GABA agonists have been classified into four types according to their central actions on bicuculline sensitive postsynaptic inhibition, on the binding of labelled GABA or benzodiazepines to membrane preparations and on their peripheral actions on autonomic ganglia. By these criteria, muscimol is representative of type I since it mimics GABA action on bicuculline sensitive postsynaptic inhibition, binds to the sodium independent GABA binding site and enhances benzodiazepine binding. 3-aminopropanesulphonic acid and isoguvacine are classed as type II because they act similarly to muscimol except they are partial agonists/antagonists for enhancement of the benzodiazepine receptor binding; while type III agonists such as 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) are antagonists of GABA enhancement of the benzodiazepine binding.

Baclofen and 4-hydroxybutyrate are designated as type IV because they do not meet any of the criteria mentioned above yet mimic the ability of GABA to inhibit noradrenaline release. It now appears that type I, II, and III agonists interact with the bicuculline sensitive postsynaptic receptor while baclofen binds to a novel, bicuculline insensitive, presynaptic GABA receptor (Bowery et al., 1980).

1.7.2 Postsynaptic Receptors

Activation of the postsynaptic GABA receptor increases chloride conductance and causes hyperpolarization of most neurons and depolarization of primary afferents (Krnjevic and Schwartz, 1967; Choi and Fischbach, 1981). Until recently it was the only known receptor, but with the discovery of the baclofen sensitive presynaptic receptor, or $GABA_B$ receptor, (Bowery et al., 1980) the bicuculline sensitive postsynaptic receptor is now classed as a $GABA_A$ receptor.

1.7.3 Bicuculline

Bicuculline, an alkaloid isolated from plants of the genera Cordydalis and Dicentra (Manske, 1933) causes convulsant activity in both invertebrates and vertebrates (Olsen et al., 1976). Curtis' group (1970a; 1970b; 1971a; 1971b) provided evidence that bicuculline was a relatively specific GABA antagonist when tested in the feline spinal cord, cerebellum and cerebral cortex. Bicuculline did not alter GABA uptake into rat brain slice or the GABA metabolism (Straughan et al., 1971; Olsen et al., 1976). While some investigators were unable to detect this GABA antagonism (Godfraind et al., 1970) probably for technical reasons (Curtis et al., 1970b), bicuculline appeared to be a competitive antagonist with a K_i in the micromolar range

(Möhler and Okada, 1977a). There are some discrepancies in conclusions arising from binding and neurophysiological studies with regard to the type of antagonism exerted on GABA receptors by bicuculline. In rat superior cervical ganglion (Bowery and Brown, 1974) and lateral olfactory tract (Pickles, 1979), bicuculline cannot be characterized as a simple competitive antagonist. Manipulations of membranes which are known to enhance the affinity of GABA for its receptor do not influence the K_i for bicuculline (Enna and Snyder, 1977). In a very recent study (Jordan et al., 1982), the evidence pointed to two binding sites, a high and low affinity receptor. At 4^{o} C bicuculline is thought to be a competitive inhibitor of [^{3}H]-muscimol at the high affinity site, but a non-competitive inhibitor at the low affinity site. At body temperature, the high affinity site reacts the same way but bicuculline exhibits a mixed competitive/non-competitive inhibiting action at the low affinity site.

Bicuculline's overall specificity and the manner in which it causes convulsant action (Olsen et al., 1976) may be suspect. There are indications that bicuculline may act as a competitve inhibitor of acetylcholinesterase with a K_i value of 65 μM (Svenneby and Roberts, 1973). In fact, iontophoretically applied bicuculline has been shown to enhance the excitation of cells within the rat septal nucleus induced by iontophoretically applied acetylcholine. Bicuculline also inhibits specific [^{3}H]-strychnine binding to postsynaptic glycine receptor sites in rat spinal cord synaptosomal membranes with an inhibition constant of 5 μM, which is similar to that for the GABA receptor (Goldinger and Müller, 1980). Conversely, the supposedly specific glycine blocker, strychnine, and tubocurarine, the acetylcholine nicotinic blocker, antagonize the increase in conductance caused by GABA and muscimol in the olfactory bulb (Scholfield, 1982).

At physiological pH and temperature, bicuculline is unstable and degrades quite rapidly to the less active convulsant bicucine (Welch and Henderson,

1934). This has to be kept in mind when bicuculline is used in experimental situations and it is conceivable that the potency of bicuculline may be underestimated in any given test. The methiodide and methochloride derivatives of bicuculline are much more stable compounds and have been shown to have similar effects as the parent compound (Olsen et al., 1976; Möhler and Okada, 1977a). Unfortunately, they do not penetrate the blood brain barrier and therefore are of limited use for in vivo experiments.

In the spinal cord, bicuculline antagonizes many effects known to be mediated by GABA. This convulsant depressed the segmental dorsal root potentials obtained by stimulation of the ipsilateral hindlimb while enhancing those elicited from supraspinal sites (Benoist et al., 1972). Bicuculline reduced the prolonged, presumably presynaptic inhibition of spinal motoneurons but had no effect on the strychnine-sensitive postsynaptic inhibition (Curtis et al., 1971a). When the excitability of Ia primary afferent terminals were tested by the Wall technique in cat spinal cord, bicuculline suppressed the phasic increase in terminal excitability elicited by prior stimulation of an antagonistic muscle nerve without changing the tonic polarization of the terminals (Levy and Anderson, 1972). This is in contrast to picrotoxin which primarily increased tonic afferent excitability and decreased the phasic depolarizing effect of conditioning stimulation.

1.7.4 Presynaptic Receptors

In addition to the well known ability of GABA to inhibit release of transmitter from primary afferents via a bicuculline sensitive GABA receptor, there is now evidence that GABA acts on neuron terminals to modulate neurotransmitter release. Two separate systems have been identified: a GABA autoreceptor which mediates a negative feedback of GABA release (Mitchell and

Martin, 1978), and a GABA receptor on nerve terminals which modulates the release of transmitters other than GABA (Bowery et al., 1980). The characteristics of each is described below.

The pharmacological profile of GABA autoreceptors seem remarkably similar to the classic GABA receptor. Muscimol, 3-aminopropane sulphonic acid and THIP act as agonists (Mitchell and Martin, 1978; Arbilla et al., 1979), while bicuculline and picrotoxin appear to be antagonists (Mitchell and Martin, 1978). The GABA autoreceptor does seem to differ from the postsynaptic GABA receptor in that it is not functionally coupled to benzodiazepine receptors (Brennan, 1982).

The novel $GABA_B$ receptor which may play a part in the regulation of release of other transmitters such as noradrenaline (Bowery et al., 1980) and various amino acids (Potashner, 1978; Mitchell, 1980a) has a pharmacology quite different from the classical $GABA_A$ receptor. It is insensitive to the majority of GABA-mimetics such as muscimol, 3-aminopropanesulphonic acid or isoguvacine and to the GABA antagonist, bicuculline (Hill and Bowery, 1981). Baclofen (beta-(4-chlorphenyl)-gamma-aminobutyric acid) seems to be a specific agonist for the $GABA_B$ receptor on which it has same affinity as GABA. Unlike the GABA autoreceptor, this presynaptic receptor seems to be coupled to a benzodiazepine receptor, since diazepam facilitates GABA action on [^{3}H]-glutamate release from striatal prisms (Mitchell, 1980b), although in guinea pig vas deferens, benzodiazepine facilitation was not shown (Doble and Turnbull, 1981). Binding of GABA to this receptor is dependent upon divalent cations, such as calcium and magnesium, (Hill and Bowery, 1981). $GABA_B$ receptors have been found in all brain regions and in the spinal cord where they consistently represent one-third of the total GABA binding sites in all tissues tested (Bowery et al., 1982a). Preliminary observations in the rat spinal cord indicate that $GABA_B$ sites are concentrated in lamina I, II, and

III of the dorsal horn (Bowery et al., 1982b).

1.7.5 Baclofen

With the discovery of baclofen as a specific agonist for the $GABA_B$ receptor, it is now possible to determine which effects of GABA are mediated by this receptor. Baclofen has been shown to block excitatory transmission at the Schaeffer collateral-CA1 pyramidal cell synapse, presumably by suppressing the release of excitatory amino acid transmitter (Olpe et al., 1982). In the spinal cord, baclofen selectively reduced monosynaptic excitation by impulses in low threshold (Ia and Ib) afferents, and reduced the primary afferent depolarization of Ia extensor afferent terminals produced by impulses in flexor afferents without altering either their electrical excitability of the terminals or depolarization induced by iontophoretically applied GABA or glutamate (Curtis et al., 1981). These investigators concluded that baclofen reduced the release of both the excitatory transmitter from primary afferents and GABA from the axo-axonic synapses on Ia terminals. Čapek and Esplin (1982) found some decrease in excitability of primary afferents, but the magnitude was not in keeping with the formidable depression of monosynaptic excitatory transmission. Repetitive stimulation of the primary afferents to produce posttetanic potentiation was able to overcome the depressant effects of baclofen, indicating that there was no depletion of excitatory transmitter.

1.8 Actions of GABA

When GABA is applied to nervous tissue the observed response usually declines rapidly in a matter of seconds, a phenomenon termed desensitization. This could be due to a diminishing chloride gradient, a fading of GABA evoked conductance increase (such as inactivation of chloride channels), removal of

GABA from the receptor area by GABA uptake, or inactivation of the receptor (Krnjevic, 1981). On primary afferent fibres, both inactivation of the receptor and neuronal uptake are responsible in part for GABA desensitization (Hackman et al., 1982).

In cultured mouse spinal neurons, a very small amount of GABA (1-10 nM) applied by the pressure ejection technique, excited about 25% of the cells. This was associated with a lowering of threshold for action potential generation but with no change in resting membrane properties (Barker et al., 1980). Neither picrotoxin nor bicuculline blocked this excitatory action of GABA and even lowered the action potential threshold further. The cause of this phenomenon is unknown.

Besides blocking GABA mediated inhibition, bicuculline produced membrane depolarization by blocking a potassium conductance in mouse spinal neurons (Heyer et al., 1982). This convulsant also prolonged calcium dependent action potentials, probably due to the reduction of the potassium conductance that would normally repolarize the neuronal membrane. If bicuculline had a similar action at the presynaptic terminals, the reduction of potassium conductance might increase calcium entry and thus enhance transmitter release. GABA, on the other hand, should increase the potassium conductance and shorten the calcium component of the action potential as has been demonstrated in dorsal root ganglia soma (Dunlap and Fischbach, 1978). They found, however, that this GABA induced decrease was not blocked by 100 μM bicuculline. In a subsequent study (Dunlap, 1981), using cultured chick sensory neurons, GABA produced changes in input resistance (R_{in}) and action potential duration (APD), but only in 10% of the cells were both exhibited. Muscimol just produced a change in R_{in}, while baclofen selectively changed the APD. Bicuculline only blocked muscimol-induced changes in R_{in}. The two responses also differed in properties of desensitization. GABA induced decrease in R_{in} was completely

desensitized within 10 seconds while GABA and baclofen induced decrease in APD persisted until the drug was removed.

There is preliminary evidence that bicuculline and picrotoxin decrease the conductance of gap junctions within the turtle retina, although the mechanism by which this occurs is speculative (Piccolino et al., 1982).

Much of the recent work on the action of GABA at the membrane level has been done on cultured neurons from spinal cord and brain (Macdonald and Barker, 1978), while the biochemical data on the binding properties of the GABA receptor were gathered from studies in which brain membranes from freshly killed animals were used. Hence it is difficult to compare information obtained from these two sources in order to determine which binding site was responsible for GABA's effect on neurons. When the GABA binding characteristics of cultured neurons were compared with those of whole tissues (Frere et al., 1982), two binding sites, both with matching characteristics, were found in the rat cerebral cortex and cultured mouse cortical neurons. There were about six times as many low affinity as high affinity sites. In the case of the spinal cord, only the low affinity binding site, was found in any amount in both adult and cultured tissue. For both tissues, muscimol only displaced binding to the high affinity site. Bicuculline competitively inhibited muscimol binding with an inhibition constant of about 4 μM for brain and 10 μM for spinal cord but only inhibited GABA binding at the low affinity receptor at very high concentrations (115 μM). It would seem, therefore, that the high affinity site represents the classical GABA receptor.

In a companion paper to Frere et al. (1982), the dose dependency of GABA responses and antagonism of these responses by bicuculline on cultured neurons were presented (Nowak et al., 1982). They found that on both spinal and cortical neurons, GABA produced inhibition by increasing the chloride ion

conductance thereby causing membrane hyperpolarization. GABA responses were competitively antagonized by bicuculline with a half maximal inhibitory concentration of about 1 μM. With a Hill constant of one, it appears that one molecule of bicuculline is required to block each GABA receptor. The threshold concentration of GABA required was higher (2 μM) than the K_D of 9 nM of the high affinity GABA binding site. This discrepancy in potency might be due to removal of an endogenous modulator by the detergent used in binding studies. Although no data was presented, this group postulated that the low affinity site represented the presynaptic receptor described by Bowery's group (Bowery et al., 1981).

1.9 Benzodiazepines

1,4 benzodiazepines were originally developed by Hoffmann-LaRoche medicinal chemists in the middle 1950's and were shown to have muscle relaxant, taming, sedative, hypnotic, and anticonvulsant properties in animals (Sternbach, 1973), and potent antianxiety effects in humans (Harris, 1960). After the appropriate toxicological and clinical studies, chlordiazepoxide was marketed under the trade name Librium in 1960, followed by diazepam under the name Valium in 1963.

Clinically, benzodiazepines are used in the treatment of anxiety, insomnia, status epilepticus, in alcohol withdrawal, as a muscle relaxant, and as a premedication for anaesthesia (Harvey, 1980). Although different benzodiazepines are used preferentially for each disorder, i.e., flurazepam as a hypnotic, diazepam for anxiety and clonazepam as an anticonvulsant, all members of the benzodiazepine family would probably be effective in each case.

The original observation that benzodiazepines had a taming effect on animals (Randall et al., 1960) has been extended and expanded. Behavioral studies have shown that benzodiazepines are able to selectively release

behavior previously suppressed by a punishing stimulus, a test which is thought to mimic a state of anxiety (Geller et al., 1962).

The anticonvulsant effect of benzodiazepines seen in animal studies has been confirmed in epileptic patients (Rossi et al., 1973). After acute intravenous injection, clonazepam showed good antiepileptic action on primary and secondary generalized epileptic activity, beneficial action on the propagation of focal epileptic discharges to distant cerebral regions, but a poor effect on focal epileptic activity or propagation to cerebral structures directly connected to the epileptogenic zone. However, the usefulness of benzodiazepines for chronic treatment of epilepsy is of limited value because tolerance develops to their anticonvulsant actions.

1.9.1 Effects of Benzodiazepines on the Nervous System

Electrophysiological studies have uncovered that, in general, benzodiazepines reduce CNS excitability. In the brain, they display a depressant action on unit activity in the cortex (Phillis, 1979), cerebellum (Pieri and Haefely, 1976), hippocampus (Wolf and Haas, 1977) and amygdala (Chou and Wang, 1977). Many investigators have shown that benzodiazepines increase inhibition in the cerebellum (Curtis et al., 1976), sensory motor cortex (Zakusov et al., 1975) and pyramidal tracts (Raabe and Gumnit, 1977). In the hippocampus, the duration of recurrent inhibition was increased by benzodiazepines (Wolf and Haas, 1977) possibly by increasing the rate of firing of the basket interneurons (Lee et al., 1979).

The effect of diazepam have been extensively studied on the spinal cord. Diazepam, at doses of 0.5 to 1.5 mg/kg, had little effect on monosynaptic reflex potentials (Schmidt et al., 1967), posttetanic potentiation, or recurrent and direct inhibition (Schlosser, 1971). Recently, however, it was

found that the late part of recurrent inhibition has a GABAergic component and that it could be enhanced in the presence of benzodiazepines (Polc and Haefely, 1982). Polysynaptic reflexes were found to be unchanged by some investigators (Schmidt et al., 1967) but depressed by most others (Polc et al., 1974, Schlosser, 1971). Diazepam also depressed spontaneous gamma-motoneuron activity (Polc et al., 1974). Applying paired pulses to the dorsal root, Schlosser (1971) demonstrated that synaptic recovery of the monosynaptic pathway was greatly depressed at conditioning intervals between 25 and 400 ms, while the recovery of the motoneuron excitability was not influenced.

From the earliest spinal cord study (Schmidt et al., 1967), it was evident that the most striking effect of diazepam was to greatly enhance the DRP and presynaptic inhibition. The DRP was judged bigger by an increase in maximum amplitude and duration. In fact the amplitude at the half time of the falling phase was always increased more than the maximal amplitude. Schlosser (1971) found that the DRR was also increased. Phase V of DRP is taken as an indication of PAD (Lloyd and McIntyre, 1949). Alternatively, PAD can be assessed by measuring the excitability of the terminals by the Wall's technique (Wall, 1958). In this way, diazepam was shown to increase PAD resulting from a conditioning stimulus. As PAD is thought to have a GABAergic component, it was very interesting that the effect of diazepam on dorsal root potential and presynaptic inhibition was prevented by bicuculline and thiosemicarbazide while aminooxyacetic acid increased its action (Polc et al., 1974). Pretreatment of semicarbazide caused a similar antagonism of the diazepam action on segmental dorsal root reflex (Banna et al., 1974). The short latency dorsal root potentials elicited by stimulation of the pontine and medullary reticular formations were also augmented by diazepam and this effect was antagonized by picrotoxin (Stratten and Barnes, 1971; Polzin and Barnes, 1976). From this data, it was concluded that normal levels of GABA

and a functioning GABA receptor were necessary for the enhancement of the DRP and presynaptic inhibition by diazepam in the spinal cord. However, instances of antagonism of GABA responses by benzodiazepines on vestibular and cerebellar neurons have also been reported (Steiner and Felix, 1976; Gahwiler, 1976).

With the information gained from whole animal experiments it was not possible to determine if benzodiazepines had their effect by potentiating the action of endogenous GABA, by increasing the release or decreasing reuptake of GABA, or by another more complex interaction. No change in [^{3}H]-GABA uptake was detected when chlordiazepoxide was applied to chick spinal cord cultures (Choi et al., 1977). Using the primary afferents' cell body found in the dorsal root ganglia, it was shown that high doses of diazepam had no direct GABAmimetic action and did not change the resting potential, membrane conductance, or excitability (Desarmenien et al., 1980). This study, however, was not able to show that the GABA induced depolarization was enhanced, which was probably due to the high dose of diazepam employed (100 μM).

1.9.2 The Benzodiazepine Receptor

Specific receptor sites for diazepam were found in the rat brain by Squires and Braestrup (1977), and Möhler and Okada (1976). Both groups found that diazepam had a high affinity for the receptor, the apparent affinity constant (K_d) was about 4 nM at 0^o, but at body temperature the K_d was about 6 times greater (Braestrup and Squires, 1978b). A large group of substances, including many neurotransmitters did not have any effect on [^{3}H]-diazepam binding (Braestrup and Squires, 1978a). Equipped with this knowledge, many groups began investigating the benzodiazepine receptor. These receptors were shown to be localized in regions of synaptic contacts (Möhler et al., 1980a).

Diazepam binding sites were also found in non-neuronal tissue such as the kidney, lung and liver (Braestrup and Squires, 1977). These binding sites were quite different than central benzodiazepine receptors in that the pharmacologically almost inactive benzodiazepine, Ro5-4864, displaced diazepam from this site with an IC_{50} of about 4.5 nM, whereas the very potent benzodiazepine, clonazepam, inhibited diazepam binding only at very high concentrations. Flunitrazepam also binds to the peripheral "receptor" (Schoemaker et al., 1981). This peripheral type receptor was also discovered in the brain where there are about one-fourth as many of these binding sites as the central types (Marangos et al., 1982). Looking at diazepam binding in the spinal cord, there appears to be a larger proportion of Ro5-4864 binding sites there than in the brain (Del Zompo et al., 1983). This binding site is not modulated by GABA (Patel and Marangos, 1982). Its regional distribution within the brain is distinct from that of the central type receptor and, subcellularly, the Ro5-4864 binding site is also localized in the nuclear membrane in high densities. By using neuronal and non-neuronal cultures, it was shown that clonazepam preferentially inhibits [^{3}H]-diazepam binding to neuronal tissue whereas Ro5-4864 inhibits the [^{3}H]-diazepam binding in glial enriched cultures (Gallager et al., 1981).

1.9.3 GABA-Benzodiazepine Receptor Interaction

As stated above, a functional link between benzodiazepines and GABA had been established, but the exact nature of this interaction was only elucidated when a relationship between benzodiazepine and GABA receptors was discovered. Tallman's group (1978) was the first to show that the presence of GABA increased the affinity of the [^{3}H]-diazepam binding site for its ligand. This effect was mimicked by the GABA analogue, muscimol and antagonized by the GABA antagonist, bicuculline. GABA-mimetic compounds such as 3-aminosulphonic acid

and isoguvacine were found to be partial agonists, while others such as piperidine-4-sulphonic acid and THIP showed antagonism to the GABA/benzodiazepine complex (Braestrup et al., 1979). It seemed therefore that the GABA receptor site mediated this action, although in a complicated manner. When extensively washed brain tissue membranes were used, GABA increased [^{3}H]-diazepam binding by more than 100% as compared to 15-25% in unwashed preparations (Karobath and Sperk, 1979). Presumably, the washing procedure removed endogenous GABA which would have increased the basal level of diazepam binding and hence diminished the exogenous GABA induced elevation. Alternatively, a factor other than GABA that influences benzodiazepine binding was removed by washing. Marangos and Martino (1981) pointed out that the concentration of GABA required for half maximal enhancement of benzodiazepine binding was in the micromolar range whereas the reported K_D for high and low affinity receptor site ranged from 5 to 70 nM. They concluded from their studies that the high affinity muscimol binding site was not mediating the GABA enhancement effect.

The precise nature of the GABA/benzodiazepine unit is not completely known, but it has been established that the two binding sites are on different proteins because they can be differentially extracted from the membrane (Mazzari et al., 1981). The chloride ionophore is also probably intimately associated with these two receptors (Costa et al., 1979; Fujimota and Okabayashi, 1981). It is probable that the binding site for barbiturates is also located in this complex (Leeb-Lundberg et al., 1980; Olsen and Leeb-Lundberg, 1981).

The close relationship of GABA and benzodiazepine receptors became more evident with the study by Guidotti, Toffano and Costa (1978a). Treatment of brain membranes with Triton X-100 or repeated freezing and thawing, unmasked a

high affinity GABA receptor by removal of an endogenous modulator of GABA binding. This substance, named GABAmodulin, was characterized as a 15,000 dalton polypeptide. When added back to Triton treated membranes, GABAmodulin inhibited high affinity GABA binding and this effect was stereospecifically antagonized by benzodiazepines in a competitive manner. Further, GABAmodulin reduced the apparent affinity of diazepam for its recognition site.

It has been proposed (Guidotti et al., 1978b) that the GABA/benzodiazepine receptor complex consists of the chloride ionophore, a functional GABA receptor with two binding sites for GABA, or alternatively, two conformational states of the same site, one or more benzodiazepine receptors and GABAmodulin. Binding of GABA or other agonists to the low affinity receptor site would uncover the high affinity site in some unknown manner. Subsequent interaction of GABA with the high affinity site would open the ion channel. The closing of the ionophore and the return of the receptor to its resting state may be due to release of GABA from this site in a process involving a conformational change. The reoccupation of the high affinity GABA binding site by GABAmodulin would actually close the ion channel. Benzodiazepines would compete with GABAmodulin for this binding site and hence delay the closing of the ionophore. The overall effect of benzodiazepines would be to prolong the chloride ion flux due to GABA receptor activation. However, analysis of the kinetic processes of the GABA-activated chloride ionophore indicates that diazepam increases the frequency of channel opening with little change in the duration of opening (Study and Barker, 1981).

An alternate proposal of the nature of the GABA/benzodiazepine/anion complex was put forth by Squires (1982) in light of many binding studies. Squires envisages that there are two or more anion recognition sites, two or more GABA receptors and a total of four complexes of these components. The GABA receptors appear to be indirectly coupled to benzodiazepine receptors

through an anion recognition site. Associated with this complex may be a purine-barbiturate picrotoxinin binding site which interacts allosterically with the elements of the complex (Leeb-Lundberg et al., 1980; Olsen and Leeb-Lundberg, 1981).

In the search for a possible non-GABAergic mediated mode of action for benzodiazepines, investigators have tried to find differences in GABA and benzodiazepine receptor distribution. Indeed, in the cerebellum, the benzodiazepine receptor (as shown by [^{3}H]-flunitrazepam binding) are more concentrated in the molecular layer than granule layer, while for the GABA receptor, the distribution is reversed (Unnerstall et al., 1981). In addition, GABA binding was found to be highly concentrated in the ventral thalamus whereas the relative density of benzodiazepine receptors in that same region was small. However, in the same study it was noted that there were fewer benzodiazepine binding sites than sites for GABA, that exogenous GABA increased benzodiazepine binding in all regions, and that this effect was proportional to the regional density of benzodiazepine receptors. Taken together these results suggest that most, if not all, benzodiazepine receptors are coupled to a type of GABA receptor but this benzodiazepine-linked GABA receptor represents a subpopulation of GABA binding sites.

Olsen (1982) has taken a slightly different stand by proposing that the components of the GABA receptor chloride ionophore complex, the GABA receptor, the benzodiazepine receptor, and the picrotoxin-barbiturate receptor can exist either as individual entities, or as a combination of two or three. The different profiles of binding seen between various regions of the brain may in fact be a reflection of different combinations of receptors. He has also suggested that the benzodiazepine receptor exists in two states: a low affinity state which is favoured when either bicuculline or picrotoxin are

bound to their respective sites, and a high affinity state favoured when either GABA or pentobarbital occupy their binding sites. Partial agonists for either the GABA or barbiturate sites produce an intermediate effect by binding to both sites.

There is strong evidence that the release of GABA from central nerve endings is subject to negative feedback control through presynaptic receptors on GABAergic terminals (Mitchell and Martin, 1978). The presynaptic GABA autoreceptor does not seem to be functionally coupled to benzodiazepine receptors since flunitrazepam was not able to change the ability of muscimol and THIP to inhibit K^+ stimulated GABA release (Brennan, 1982). These GABA autoreceptors may represent a portion of the total GABA receptor population which is not distributed in parallel to the benzodiazepine receptors.

1.9.4 Action of Benzodiazepines Unrelated to GABA

The doses of benzodiazepines that are required to produce an enhancement of GABAergic transmission are quite high compared to that needed to have anxiolytic action in humans. Indeed, some benzodiazepines have a different pharmacological profile when used at very low concentrations. Very low concentrations of flurazepam caused depression of excitabiltiy by directly increasing chloride conductance, by elevating spike threshold as well as potentiating responses to GABA (MacDonald and Barker, 1982). On the other hand, high doses of flurazepam have been reported to have central excitatory actions (Rosenberg, 1980). When small amounts of a water soluble benzodiazepine Ro21-3981 were iontophoretically applied to cultured spinal neurons, the excitatory responses to acetylcholine, and to the two amino acid analogues kainate and N-methyl-D-aspartate were reduced (Davies and Polc, 1978).

In mouse spinal cord neurons, Heyer and Macdonald (1982) have correlated

the reduction of calcium-dependent action potential with the sedative-anaesthetic effect of barbiturates. Leslie et al. (1980) showed that chlordiazepoxide reduced the depolarization-induced ^{45}calcium influx into synaptosomes. Chronic dietary chlordiazepoxide administration resulted in the development of tolerance to the inhibition of calcium influx during the same time period that behavioral tolerance developed. These two studies provide evidence that the reduction of calcium influx into the terminals may be responsible for the sedative and anaesthetic effects of benzodiazepines and barbiturates.

There is evidence that not all the effects of benzodiazepines are due to its potentiation of GABAergic transmission. It has been suggested that benzodiazepines potentiate the depressant effect of adenosine on cerebral cortical neurons (Phillis, 1979), possibly by inhibiting its uptake (Mah and Daly, 1976). There is a good correlation between K_i values for the inhibition of [^{3}H]-diazepam binding and the inhibition of adenosine uptake by various adenosine uptake inhibitors (Wu et al., 1981) and, conversely, there is a reasonable correlation between the potencies of various benzodiazepines as adenosine uptake inhibitors to their clinical and pharmacological potencies (Phillis and Wu, 1981). Diazepam has been shown to enhance the efflux of labelled adenosine at the same time depressing the release of acetylcholine (Phillis et al., 1980). However, the benzodiazepine antagonist, Ro15-1788 failed to reverse the inhibition of adenosine uptake by diazepam (Morgan et al., 1983). Caffeine, a methylxanthine, has been shown to antagonize the effects of purines on neuronal adenosine receptors (Phillis et al. 1979). Polo's group (1981a) found that caffeine also partially reversed some of diazepam's actions in behavior tests and in the spinal cord function. At present the exact role that adenosine and its derivatives play in the

mechanism of action of benzodiazepines is not known.

Fairly low doses of clonazepam reduced myoclonus caused by DDT in mice (Chung Hwang and Van Woert, 1979). This antimyoclonic action was counteracted by serotonin receptor blockers but not by bicuculline, suggesting clonazepam had this action by enhancing serotoninergic rather than GABAergic transmission.

1.9.5 Endogenous Ligands

When the benzodiazepine receptor was discovered, investigators naturally tended to feel that since it exists in the body, it should be physiologically relevant to the organism. Hence there should be an endogenous ligand which would either cause effects similar to benzodiazepines if in fact benzodiazepines were agonists, or have opposite effects if benzodiazepines operated as antagonists to this receptor.

Some of the substances that were proposed as endogenous ligands include thromboxane A2 (Ally et al., 1978), nicotinamide (Möhler et al., 1979), purine nucleotides (Asano and Spector, 1979, Skolnick et al., 1980) and various proteins isolated from brain tissue (Davis and Cohen, 1980). The most interesting group of compounds, the beta-carbolines or harmala alkaloids, were isolated from human urine (Braestrup et al., 1980) and were found to inhibit diazepam binding with an IC_{50} in the range of 4-7 nM. The original compound isolated, beta-carboline-3-carboxylic acid ethyl ester (beta-CCE) is unlikely to be the endogenous ligand because the purification procedure was responsible for the formation of the ethyl ester moiety. However, tetra-hydro-beta-carbolines are found in the body and are probably synthesized from 5-hydroxytryptamine as well as from other compounds (Airaksinen and Kari, 1981). In primitive cultures, plants containing beta-carbolines have been used for their psychoactive properties and as anthelmintics (Airaksinen and Kari,

1981). When administered to animals, beta-carbolines (harmaline and harmine) cause a fine generalized tremor, excitation and ataxia (Fuentes and Longo, 1971). Tremorogenic doses are sufficient to produce inhibition of benzodiazepine binding (Robertson, 1980). They are proconvulsant to pentylenetetrazol and antagonize the anticonvulsant action of diazepam (Tenen and Hirsch, 1980). Methyl-beta-carboline-3-carboxylate itself causes dose related clonic/tonic seizures in mice (Jones and Oakley, 1981). This suggests that beta-carbolines have an action opposite to benzodiazepines. Beta-carboline-3-carboxylate has been found to antagonize the action of GABA and benzodiazepines and at high doses produced seizure-like activity in the rat hippocampus (Polc et al., 1981c). The differences between beta-carboline potencies in vivo and in vitro are probably due to their rapid elimination from the brain (Fehske and Müller, 1982).

The specificity of beta-carboline derivatives have come into question since the binding of specific ligands for the muscarinic, opiate, dopamine, and serotonin receptors (quinuclidinylbenzylate, naloxone, spiroperidol, and serotonin, respectively) were inhibited at the same concentrations that were required for inhibition of flunitrazepam binding (Müller et al., 1981).

In binding studies, beta-carbolines have been used to elucidate the properties of the benzodiazepine receptor. The earliest reference to the possible beta-carboline-benzodiazepine receptor interaction states that beta-carboline-3-carboxylate derivatives show mixed type competitive inhibition of [^{3}H]-flunitrazepam binding in rat forebrain and shallow binding inhibition curves (Braestrup et al., 1980). This indicated the presence of more than one type of receptor or negative cooperativity. There is now evidence that beta-carbolines, especially propyl-beta-carboline-3-carboxylate have different affinities for at least two subclasses of the benzodiazepine receptors (Fehske

et al., 1982). However, beta-carboline and benzodiazepines may bind to separate domains on the same receptor protein (Skolnick et al., 1982). This idea is in accordance with most of the available binding data.

1.9.6 Receptor Heterogeneity

The earliest studies of the benzodiazepine receptor suggested that there was only a single receptor population, judging by a Hill coefficient near unity and Scatchard and saturation analysis (Möhler and Okada, 1977b; Squires and Braestrup, 1977; Braestrup and Squires, 1978a). However, it soon became evident that multiple central benzodiazepine receptors might exist (Squires et al., 1979) because a new class of agents, the triazolopyridines, inhibited [^{3}H]-diazepam binding with shallow binding inhibition curves and Hill coefficients below 1. The existence of multiple benzodiazepine receptors was also suggested by thermal inactivation studies (Squires et al., 1979) and by differential binding characteristics of [^{3}H]-diazepam in various brain regions (Klepner et al., 1979). A very recent paper (Chiu et al., 1982) challenged the concept of multiple benzodiazepine receptors. Their kinetic studies of [^{3}H]-flunitrazepam binding indicated that these receptors exist as one homogenous population with two interconvertible conformational states. Benzodiazepine binding to the low affinity conformation facilitates the isomerization of the receptor to a more stable complex. GABA enhancement of the benzodiazepine receptor is primarily due to a reduction in the dissociation of the less tightly bound reversible complex. A follow up study (Chiu and Rosenberg, 1982) showed that some ligands, such as flunitrazepam, are better than others, such as diazepam, in inducing the conversion of the receptors to the higher affinity state. Quast and Mählmann (1982) also concluded that [^{3}H]-flunitrazepam binding kinetics indicated a two step mechanism where, presumably, the flunitrazepam rapidly binds to the receptor and then the

complex slowly isomerizes to a final complex. However, this group suggested that the presence of GABA increased the association rate constant and did not affect the dissociation rate constant. While the evidence presented in these studies are above reproach, there are discrepancies compared to previous work. It is difficult to reconcile the data on beta-carboline and triazalopyridine binding and the physical separation of two proteins which bind benzodiazepines (Sieghart and Karobath, 1980). The binding studies which support the concept of receptor heterogeneity were all routinely performed at 0^{o} to 4^{o} C. When these same in vitro studies were performed at physiological temperatures (Gee and Yamamura, 1982), CL218 872 and propyl-beta-carboline-3-carboxylate lost their ability to discriminate benzodiazepine receptor subtypes in cerebral cortex, hippocampus and pons-medulla but still showed higher affinity for benzodiazepine receptors in the cerebellum. They proposed that distinct cerebellar and non-cerebellar type receptors exist in vivo but the differential affinity of CL218 872 and propyl-beta-carboline-3-carboxylate in the cerebral cortex, hippocampus and pons-medulla is an artifact due to the low temperature. This group suggested that CL218 872 may be a partial agonist for the benzodiazepine receptor (Gee et al., 1983).

Physical separation of the different benzodiazepine receptors has been attempted. Originally, Sieghart and Karobath (1980) isolated two proteins which were labelled by [^{3}H]-flunitrazepam in the presence of ultraviolet light. They estimated that the molecular weight of one protein to be about 51,000 daltons (P51) which seemed to be the sole benzodiazepine binding site in the cerebellum. The other major protein had a molecular weight of 55,000 (P55) and was most apparent in the hippocampal membranes. Membranes prepared from spinal cord only showed [^{3}H]-flunitrazepam binding to the P51 fraction, although the amount of this protein was very small. The binding to both was

inhibited by diazepam (2 μM) and augmented by GABA (10 μM) and this increase was reversed by bicuculline. Binding to these proteins was not inhibited by Ro5-4864, a compound which is thought to selectively inhibit binding to peripheral benzodiazepine binding sites (Braestrup and Squires, 1977). The triazolopyridazine, CL218 872, inhibited binding more strongly to P51 than to P55. Further studies (Supavilai and Karobath, 1980) suggested that the affinity of [^{3}H]-flunitrazepam was higher in hippocampus than in the cerebellum indicating that the P55 binding site exhibits a higher affinity than the P51 binding site. It was found for the hippocampal membranes compared to cerebellar membranes, that a 3.3 times higher concentration of GABA was needed for half maximal stimulation of binding, although maximal stimulation of binding by GABA was comparable in both regions. This would indicate that the GABA recognition site associated with the benzodiazepine sites of the hippocampus and cerebellum have different apparent affinities for GABA. In a subsequent study (Sieghart and Mayer, 1982), additional evidence was presented which supported the idea that P51 was associated with type 1 and P55 with type 2 benzodiazepine receptors.

When binding of [^{3}H]-flunitrazepam was done after solubilization, a complex with an estimated molecular weight of about 200,000 to 250,000 was found (Sherman-Gold and Dudai, 1980). This complex appeared to be a multimer made up of 51K subunits. Binding studies that were performed to investigate the photoaffinity labelling reaction of flunitrazepam, indicate that synaptic membranes contain proteins or protein complexes with four benzodiazepine binding sites in close spatial proximity (Möhler, 1982). Irreversible binding of flunitrazepam leads to conformational changes of the other three sites which result in a decreased affinity of benzodiazepine agonists but not antagonists. Reversible binding of flunitrazepam does not cause this reduction in affinity of the remaining sites (Karobath and Supavilai, 1982).

1.9.7 Benzodiazepine Receptors in the Spinal Cord

Most binding studies have been performed on brain tissue, hence information available on spinal cord receptors is very limited. Sieghart's and Karobath's data (1980) suggests that the majority of spinal benzodiazepine receptors are of the type 1 variety. With radiohistochemical techniques using [^{3}H]-flunitrazepam, it was shown that, in the cervical spinal cord, dense labelling (comparable to that found in the hippocampus) was found in laminae II, III and IV with moderate labelling in laminae V, VII and part of VIII (Young and Kuhar, 1980). In cultures of rat spinal cord, [^{3}H]-flunitrazepam binding was found on many medium sized neurons, presumably interneurons, whereas the presumed large motoneurons were not labelled (Hosli et al., 1980). Glial cells did not show any tendency to bind benzodiazepines. Karobath and Sperk (1979) found that diazepam binding to spinal cord membranes could not be increased by muscimol to the same extent as was found with brain tissue.

1.9.8 Antagonists

In recent years, many substances have been proposed which were thought to antagonize the actions of benzodiazepines in vivo and inhibit specific benzodiazepine binding. A representative of a series of imidazodiazepines, Ro15-1788, was found to be a very potent competitive inhibitor of [^{3}H]-diazepam binding (IC_{50}=2 nM) (Hunkeler et al., 1981) and antagonized the behavioral and neurophysiological effects of benzodiazepines (Polc et al., 1981b). Oral Ro15-1788 was very potent in reversing the anticonvulsant effect of diazepam on pentylenetetrazol-induced seizures (Bonnetti et al., 1982).

In the spinal cord, Ro15-1788 had no intrinsic ability to affect segmental DRPs, polysynaptic ventral root reflexes, Renshaw cell responses to

antidromic ventral root volleys and spontaneous gamma-motoneuron activity, but antagonized the effects of various benzodiazepines on these same responses (Polc et al., 1981b). In rats, Ro15-1788 abolished the midazolam induced decrease of spontaneous multiunit activity in the substantia nigra, raphe nucleus, locus coeruleus and in the CA1 region of the hippocampus (Polc et al., 1981b).

The binding characteristics of Ro15-1788 and clonazepam are very similar in that both interact with the same number of binding sites in various brain regions, the density and distribution of both shown autoradiographically are similar throughout the CNS, and the binding of both are inhibited to a similar extent by various benzodiazepine agonists and antagonists (Möhler and Richards, 1981a). It was also demonstrated autoradiographically, that [^{3}H]-Ro15-1788 binding sites were also found to be indistinquishable from those of [^{3}H]-flunitrazepam in rat brain, cervical spinal cord and retina (Möhler and Richards, 1981b). They differ in that the affinity of the benzodiazepine receptor for [^{3}H]-clonazepam is increased in the presence of GABA, pentobarbitone and SQ20 009, while the affinity for [^{3}H]-Ro15-1788 does not change (Möhler and Richards, 1981a). Agonist and antagonists also differ in other binding characteristics. When agonists bind to one of the four binding sites in the benzodiazepine receptor complex, they induce a conformational change of the neighbouring sites which in turn decreases their affinity for agonists. Antagonists on the other hand, do not cause a conformational change therefore the affinity of all binding sites remains unaltered (Möhler, 1982).

Although most studies have concluded that Ro15-1788 has no intrinsic activity, others have provided evidence that Ro15-1788 is anxiogenic (File et al., 1982a) or have the same type of action as chlordiazepoxide in a behavioral test (File et al., 1982b).

Ro15-1788 may be a partial benzodiazepine agonist since at high doses (50

mg/kg) in rat, it has been demonstrated to have an anticonvulsant effect when tested against pentylenetetrazol and bicuculline induced seizures (Nutt et al., 1982). Thus in this test, Ro15-1788 is one fiftieth as potent as diazepam. In behavioural tests on rats, large amounts of Ro15-1788 also seemed to have a partial agonist effect (Dantzer and Perio, 1982). When Ro15-1788 was applied to isolated cervical sympathetic ganglia, it also augmented GABA induced depolarization at high concentrations although at low concentrations an antagonist action was evident. In contrast, Polc's group (1981b) found no evidence of partial antagonist activity in the cat spinal cord at slightly lower doses (10 mg/kg).

At high doses (10 to 50 mg/kg), Ro15-1788 enhances isoniazid convulsions (Corda et al., 1982), although no satisfactory explanation for this phenomenon was offered. On the other hand, Ro15-1788 reversed the proconvulsant action of beta-carboline-3-carboxylate, another benzodiazepine antagonist (Nutt et al., 1982).

1.9.9 Clonazepam and Diazepam Compared

In many tests, clonazepam is of higher potency than diazepam. Extrapolating from the data of Braestrup and Squires (1978a), clonazepam is 5 to 7 times more potent than diazepam in inhibiting pentylenetetrazol convulsions and electric foot shock-induced fighting in mice, in muscle relaxant effect and in relieving human anxiety. Other investigators (Duka et al., 1979) found clonazepam to be 16.5 and 12 times more effective in inhibiting pentylenetetrazol seizures and picrotoxin seizures respectively.

Large differences in anticonvulsant potencies between diazepam and clonazepam have been noticed. Swinyard and Castellion (1966) found that diazepam was mildly effective against maximum electroshock seizures, whereas

clonazepam showed little activity. In contrast, clonazepam was 200 times more effective than diazepam in increasing the threshold for low frequency electroshock seizures. Whether low frequency electroshock is a good model for clinically relevant epilepsies is uncertain (Krall et al., 1978), but it appears that clonazepam has a different pharmacological profile than diazepam. The only biochemical difference noted in the brain which might explain this action is diazepam's ability to reduce serotonin synthesis, a property not shared by clonazepam (Chung Hwang and Van Woert, 1979).

In receptor studies of rat brain tissue (Sepinwall and Cook, 1980; Braestrup and Squires, 1978a; Skolnick et al., 1980) using [^{3}H]-diazepam as the radioligand, clonazepam was about 4.5 times more potent in inhibiting specific [^{3}H]-diazepam binding. Differences in clonazepam and diazepam binding are more evident when individual brain structures are examined. About 5 times more diazepam than clonazepam was required to inhibit flunitrazepam binding in the hippocampus, whereas 10 times more was required in the cerebellum (Braestrup and Nielsen, 1981).

When more in depth studies of binding characteristics using rat brain membranes were done (Braestrup and Squires, 1978a) it was found that diazepam binding displayed a Hill coefficient of 1.0 whereas clonazepam's was 1.5. This may suggest a slight positive cooperativity between binding sites.

At present, there is no data on the ratio of the type 1 receptor to the total receptor population in the spinal cord, but extrapolation from the medulla suggests that there is a preponderance of type 1 receptors in this area (Braestrup and Nielsen, 1981). Beta-carboline derivatives (propyl-beta-carboline-3-carboxylate) were proposed to be the selective ligand for the type 1 receptor. However, when [^{3}H]-propyl-beta-carboline-3-carboxylate binding was inhibited by these two benzodiazepines, clonazepam appeared to be 10 to 15 times more potent than diazepam in both the hippocampus and cerebellum. This

suggests that the beta-carboline binding site could be involved in differences in clonazepam and diazepam action, although it would be expected that by increasing the quantity of diazepam present by 10 to 15 times, the two drugs should have similar pharmacological actions.

Clonazepam and diazepam differ in their ability to irreversibly bind to the benzodiazepine receptor when exposed to ultraviolet light (Johnson and Yamamura, 1979). Like flunitrazepam, the commonly used irreversibly bound ligand, clonazepam can bind covalently due to a nitro sustituent at the 7 position. Future studies with clonazepam as the irreversible ligand may be useful in examining the central benzodiazepine receptor.

Since the discovery of benzodiazepine receptors, most investigators have tended to use diazepam, flurazepam or flunitrazepam as their radiolabelled ligands and have examined other benzodiazepines ability to inhibit this binding (Asano and Ogasawara, 1980; Braestrup and Squires, 1978b; Lo et al., 1982; Gavish and Snyder, 1980). Very recently, Richards, Möhler and Haefely (1982) have suggested that both flunitrazepam and diazepam label two types of specific binding sites, one the pharmacologically active receptor and the other an inactive "acceptor" found also in peripheral organs (see above), in the olfactory nerve, the glomerular layers of the olfactory bulb, and the ependyma and choroid plexus (Schoemaker et al., 1981). It is felt that clonazepam and the antagonist Ro15-1788 bind only to the centrally active receptor and therefore should be the preferred ligands for binding studies of central benzodiazepine receptors. This insight reduces the usefulness of information gained from binding studies where diazepam and flunitrazepam were employed.

1.10 References

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CHAPTER 2

GABA-MEDIATED RESPONSES ARE NOT SELECTIVELY DEPRESSED BY 3-MERCAPTOPROPIONIC ACID IN THE SPINAL CORD

M.F. Davies, B. Esplin, and R. Čapek

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Abstract

The competitive inhibitor of glutamic acid decarboxylase (GAD), 3-mercaptopropionic acid (MPA), causes depletion of gamma-aminobutyric acid and convulsions. We expected it to be a superior GABA depleting agent for *in vivo* experiments to the frequently used semicarbazide. The dorsal root potentials (DRP) and monosynaptic reflex responses (MSR) evoked by adjacent dorsal root and peripheral nerve stimulation, respectively, were recorded in spinal unanaesthetized cats. MPA (100-200 mg/kg) caused a gradual decrease in DRP, reaching a peak in about 40 min after drug administration. This was usually associated with convulsive activity. Postsynaptic direct inhibition was not substantially affected. However, the MSR was consistently decreased, within 10 min after injection of MPA. It is concluded that MPA is not a suitable tool, at least in the spinal cord, for the selective reduction of GABAergic transmission.

Condensé

L'acide mercaptopropionique (MPA), un inhibiteur de la décarboxylase de l'acide glutamique provoque une déplétion de l'acide gamma-aminobutyrique (GABA) et des crises convulsives. Il nous a semblé que cet agent serait supérieur au semicarbazide pour la déplétion du GABA lors des études in vivo. Les décharges éléctrotoniques recueillies sur la racine dorsale (DR) après une stimulation de la même racine, et les décharges monosynaptiques enregistrées sur la racine ventrale (VR) après la stimulation des nerfs périphériques était étudiées chez le chat non anesthésié. L'application de MPA (100-200 mg/kg) entrainait une diminution graduelle du potentiel de la racine dorsale avec un effet maximal a 40 min. Celle-çi était accompagnée de crises convulsives. L'inhibition postsynaptique directe n'était pas modifiée mais les décharges monosynaptiques étaient toujours diminuées au cours des 10 min suivant l'administration de cet agent chimique. Nous trouvons que le MPA n'est pas un agent approprié pour éliminer sélectivement la transmission GABAergique dans la moelle épinière du chat.

Introduction

3-Mercaptopropionic acid (MPA) is a reversible competitive inhibitor of glutamic acid decarboxylase (GAD) in vitro (Lamar 1970) and in vivo (Adcock and Taberner, 1978) and was used in several studies in vitro and in vivo to inhibit GABA synthesis and deplete GABA stores within the nervous system (Stone and Javid, 1980). It has been repeatedly shown that the administration of MPA to intact rats caused tonic-clonic convulsions coincident with rapid fall in GABA levels in the brain (Karlsson et al., 1974), while other amino acid levels changed only after convulsive activity. A previous electrophysiological investigation suggested that the reduction of depolarization of primary afferents, a presynaptic inhibitory mechanism, in the cuneate nucleus of the rat correlated with the reduction of GABA and GAD activity (Roberts et al., 1978) in this structure.

In the present study, we hoped to demonstrate that MPA selectively suppresses presynaptic inhibition in the spinal cord of the cat, and could be used later as a fast and specific GABA depleting agent for further electrophysiological investigations of anticonvulsants.

Methods

Cats of either sex 2.2-4.0 kg were used. Anaesthesia was induced with ethyl chloride and maintained by diethyl ether until insertion of the tracheal cannula, anemic decerebration, and transection of the spinal cord at the atlanto-occipital junction were completed. The anaesthetic was then discontinued and the animals were artificially respired with air. The cephalic vein was cannulated for administration of drugs. The mean blood pressure from the carotid artery was monitored throughout. Gallamine triethiodide was administered as needed to maintain paralysis. The lumbosacral cord was exposed by laminectomy and covered by mineral oil. The

temperature of the cord was maintained at 35° C.

Monosynaptic spinal reflexes (MSRs) were evoked by supramaximal stimulation of either the biceps semitendinosus (BST) or both branches of the triceps surae (TS) nerves by rectangular pulses of 50 μs duration at 0.5 Hz. The responses were recorded monophasically from either the ipsilateral L7 or S1 ventral root (VR) cut peripherally.

Presynaptic inhibition of the MSR from TS nerve was evoked by conditioning stimulation of the BST nerve ranging from 20 to 100 ms prior to the stimulation of TS. Postsynaptic inhibition of the BST MSR was evoked by a conditioning stimulus applied to the quadriceps nerve from 0.3 to 1.0 ms before the BST stimulation. Dorsal root potentials (DRP), evoked by stimulation of the severed dorsal root, were recorded from a fine filament of the same root.

Usually 10 recorded responses were averaged by a computer. The amplitude of the MSR or the time integral of the DRP were determined by a computer. The nonparametric Wilcoxon signed rank test was used for statistical evaluation.

Results

In all experiments described, 100 mg/kg MPA intravenously was used. This dose of MPA caused an immediate fluctuation in the heart rate, in some cases cardiac arrest and always a rapid rise of blood pressure. Higher doses (up to 200 mg/kg) were found to increase the risk of cardiac arrest with no further increase in effects on studied CNS parameters. Lower than 100 mg/kg doses (35, 50, and 75 mg/kg) had little effect.

The DRP showed a gradual reduction in the area to about 55% of the predrug levels 40 min (Fig. 1) after the drug administration. In the same

time, spontaneous activity was detected in recordings from the ventral root.

MPA caused an instantaneous and significant reduction of the TS MSR at all time periods of testing. The MSR evoked from BST nerve was also affected, although to a lesser degree than the response from TS (Fig. 1). These responses never returned to control levels within the 80-min period of observation. By comparing the effects of MPA on DRP and MSR, it appears that in the cat spinal cord, MPA produces a faster and greater effect on monosynaptic excitatory transmission than on a GABAergic response.

No change in presynaptic inhibition, examined in six experiments, was observed after administration of MPA (Fig. 2). However, a reduction in the unconditioned TS MSRs could have masked an effect of MPA since less inhibition is required to diminish the smaller MSRs (Weakly et al., 1966; Laskey and Esplin, 1974).

The level of postsynaptic inhibition was tested in five experiments (Fig. 3). It was unchanged following MPA administration, regardless of the degree of depression of the unconditioned MSR. No change was detected in two experiments where the unconditioned MSR was practically not diminished by the drug. This would be expected since in postsynaptic inhibition in the spinal cord, glycine, and not GABA, is the neurotransmitter involved. However, as with presynaptic inhibition, the unconditioned BST MSR was usually smaller and could have been easier to inhibit, thereby obscuring a drug induced effect.

Discussion

The exclusion of GABAergic processes by a suitable GABA depleting agent has particular advantages in the studies on either the mechanism of seizures or the actions of anticonvulsants. In the spinal cord of the cat, a selective GABA depleting agent should suppress only presynaptic inhibition without any effect on direct postsynaptic inhibition, and with no direct effect on the

excitation. In this context presynaptic inhibition designates the long latency, picrotoxin- and bicuculline-sensitive, and presumably GABA-mediated inhibition (Davidoff 1981) while recognizing that, at the spinal level, this inhibition may have a postsynaptic component (Cook and Cangiano, 1972; Carlen et al., 1980). Direct postsynaptic inhibition is synonymous with short latency, strychnine-sensitive, and presumably glycine-mediated inhibition (Werman et al., 1968). The usually observed increase in the excitatory phenomena following the application of anti-inhibitory drugs results indirectly from the decrease of inhibition. Semicarbazide has been successfully used for such a purpose (Bell and Anderson, 1972; Banna and Jabbur, 1971; Banna 1973; Banna et al., 1974; Čapek and Esplin, 1977). However, the very slowly developing depression of GABAergic responses is a distinct disadvantage of this drug. In search for a fast acting selective depleting agent, we turned to MPA which produces convulsions after a very short latency period, presumably by inhibition of GAD (Lamar 1970; Sprince et al., 1970; Horton and Meldrum, 1973; Karlsson et al., 1974). MPA has been also reported to stimulate GABA transaminase (Loscher 1979). Such an action could further contribute to depletion of GABA. However, Fan and co-workers (1981) have shown that MPA inhibited the potassium-evoked release of GABA from hippocampal slices <u>in vitro</u> without any detectable change in the metabolism, tissue content, or basal release of GABA, but failed to depress the potassium-evoked release of noradrenalin, 5-hydroxytryptamine, or somatostatin. Such a direct effect on GABA release could better account for the short latency of MPA-induced convulsions than an effect on GABA synthesis with subsequent depletion.

In our experiments, MPA caused a gradual reduction of DRP. This electrotonic manifestation of the primary afferent depolarization (PAD) is

believed to be GABA-mediated, although at least in the amphibian spinal cord, accumulation of extracellular potassium may contribute to the late part of the PAD (Davidoff 1981). The depression of DRP after MPA developed about three to four times faster than that after semicarbazide administration. However, it was much slower than the appearance of convulsive activity in mice, which occurred only 6 min after MPA administration (Loscher 1979). It is likely that this difference could be due to a slower rate of GABA turnover in the spinal cord, suggested by the findings that the GABA turnover decreased from the highest levels in the rostral parts of the brain towards the medulla (Leach and Walker, 1977). Differences in GABA turnover could have also contributed to slower onset of seizure activity in spinal cats. Semicarbazide exhibited similar differences, with tonic convulsions occurring at about 50 min in mice (Maynert and Kaji, 1962) as compared with about 180 min before seizure activity was detected in spinal cats (Bell and Anderson, 1972). Thus our finding is in agreement with the gradual reduction of the P wave, a transient positivity recorded from the dorsal surface of the medulla over the cuneate nucleus analogous to the spinal DRP, which was seen after MPA administration (Roberts et al., 1978).

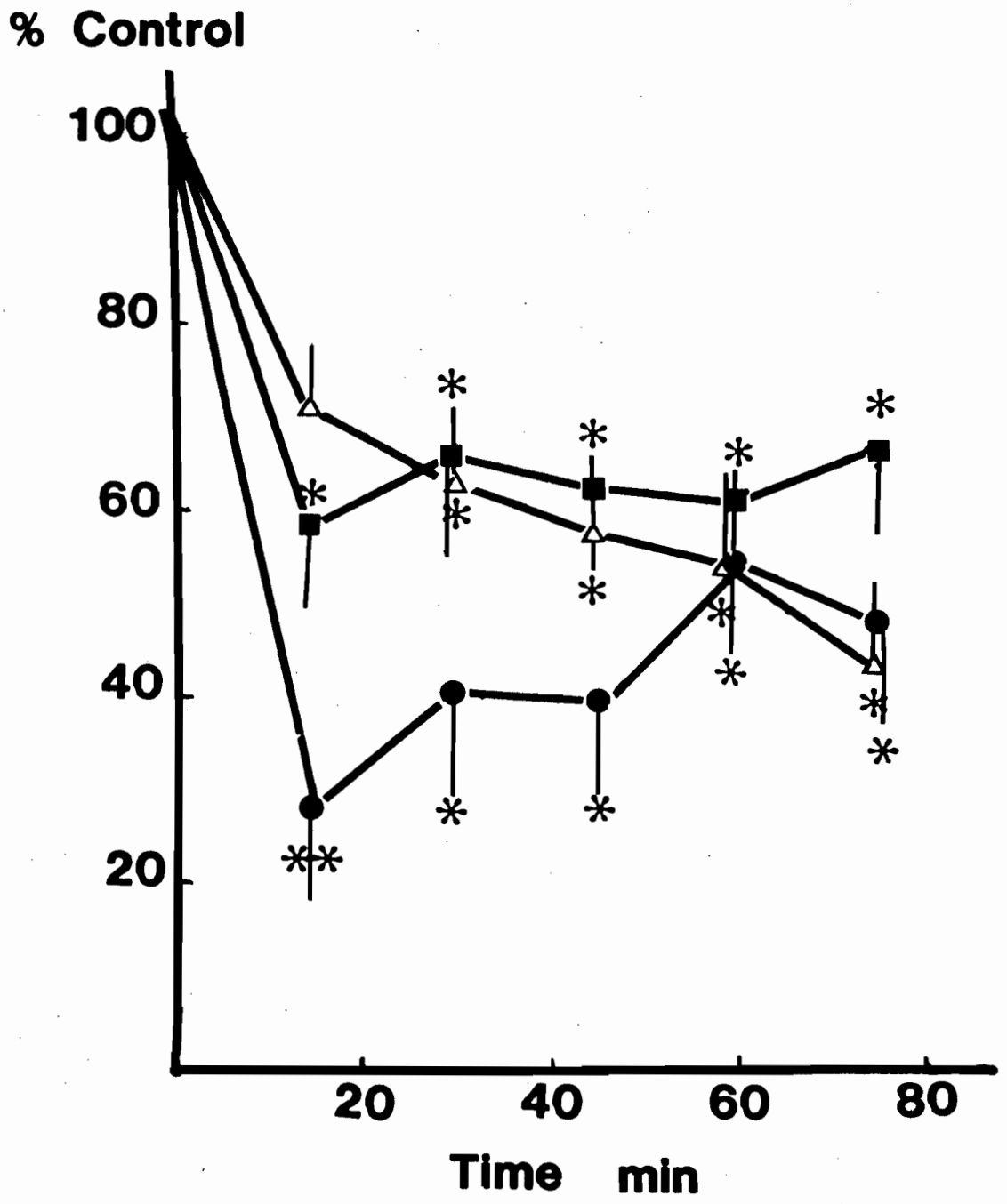
However, the most prominent effect of MPA observed in our experiments was depression of the spinal monosynaptic transmission, which developed faster and was deeper than the reduction of DRPs. Such a profound reduction of MSR seriously hampered the testing of inhibition. Therefore the fact that neither presynaptic nor postsynaptic inhibition were influenced by MPA has to be taken with reservation.

There are multiple factors which may underlie the observed depressant effect of MPA. MPA may cause a general depression of excitable membranes, as the immediate changes in cardiac function suggests. This drug was shown to decrease protein synthesis in isolated nerve endings and mitochondria from rat

cerebral cortex by an impairment of the intracellular energy supply (Rodriguez de Lores Arnaiz et al., 1975). Further, a postsynaptic action of MPA is suggested by its ability to inhibit the binding of aspartate and glutamate to a hydrophobic protein fraction from the rat neocortex (Sabato et al., 1979), which presumably contains glutamate, aspartate, and GABA receptors. Finally, in light of the evidence that the evoked GABA release was depressed by MPA (Fan et al., 1981), it is possible that release of other amino acid neurotransmitters, which may be the excitatory transmitters of the primary afferents, is similarly depressed. However, most of these speculative mechanisms, because of their general and nonspecific nature, are difficult to reconcile with the fact that MPA does produce convulsions. Generalized depression throughout the CNS is not therefore the predominant characteristics of the MPA effects. Any extrapolation of our findings that this drug depressed excitatory transmission at the primary afferents, to other structures, is unwarranted.

Bell and Anderson (1972) have demonstrated that semicarbazide, while leaving the excitatory transmission unaffected, specifically depresses the DRP and presynaptic inhibition with a concomitant depression of GABA concentration in the spinal cord. We conclude that MPA is unsuitable as a GABA depleting agent for <u>in vivo</u> electrophysiological experiments in the spinal cord and consider semicarbazide superior for this purpose.

Fig. 1 Time course of reduction in amplitude of MSR from BST (■), TS (●), and area of DRP (△) after intravenous administration of 100 mg/kg MPA. Each point and vertical line represent the mean ± SEM, respectively, for five to nine animals. Values marked with * are significantly different from control at $p < 0.05$, ** at $p < 0.01$.


% Control
100
80
60
40
20
20
40
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80
Time min

Fig. 2 Presynaptic inhibition before (○) and 10 (●), 30 (■), or 50 (▲), min after administration of MPA (100 mg/kg, i.v.) in a typical experiment. Ordinate: amplitude of the conditioned MSR as fraction of the unconditioned one; the unconditioned response was 65, 57, and 47% of test before the drug administration at the three respective time intervals. Abscissa: time interval between the conditioning stimulus to the BST nerve and the stimulus to the TS nerve.

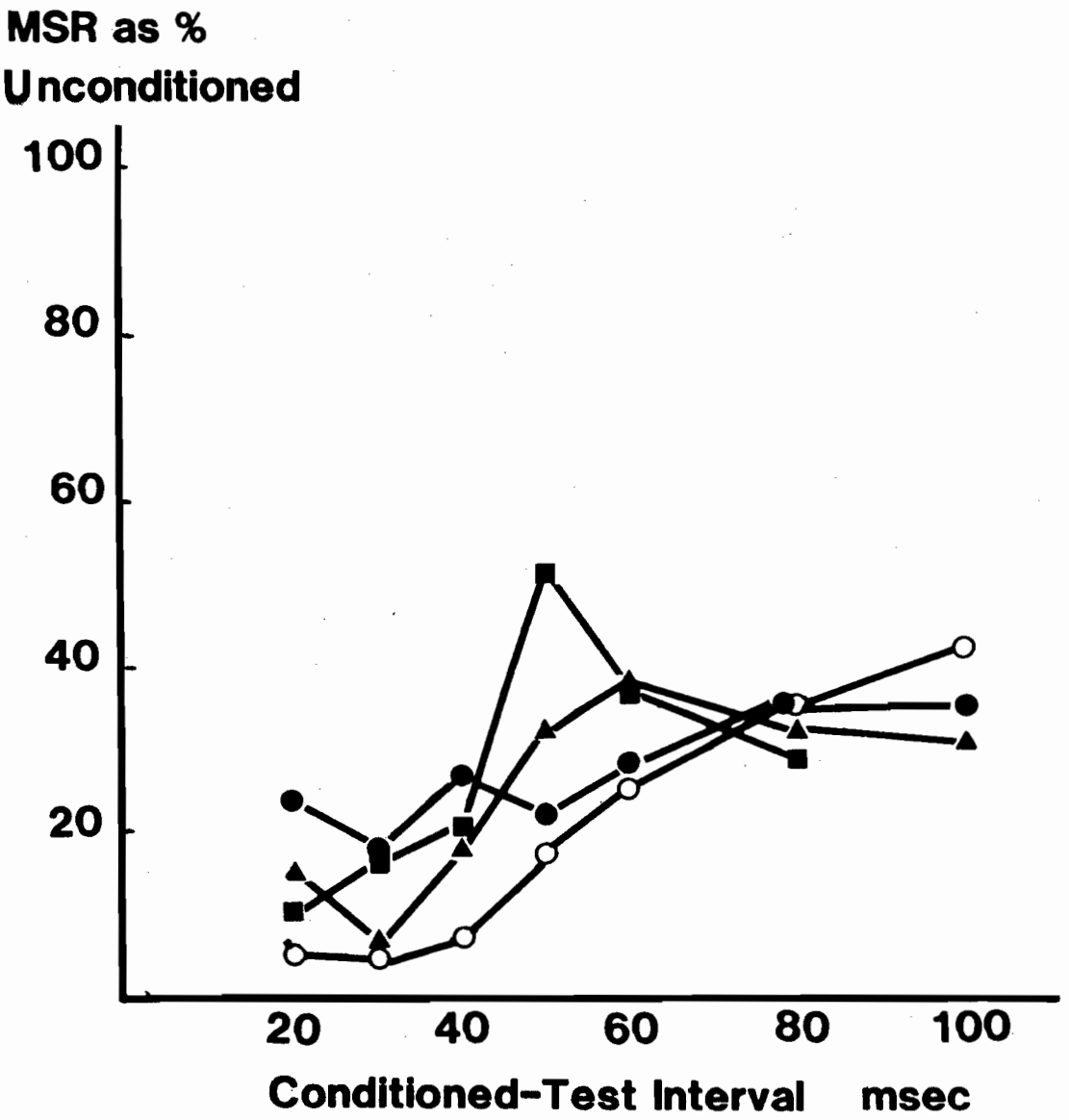
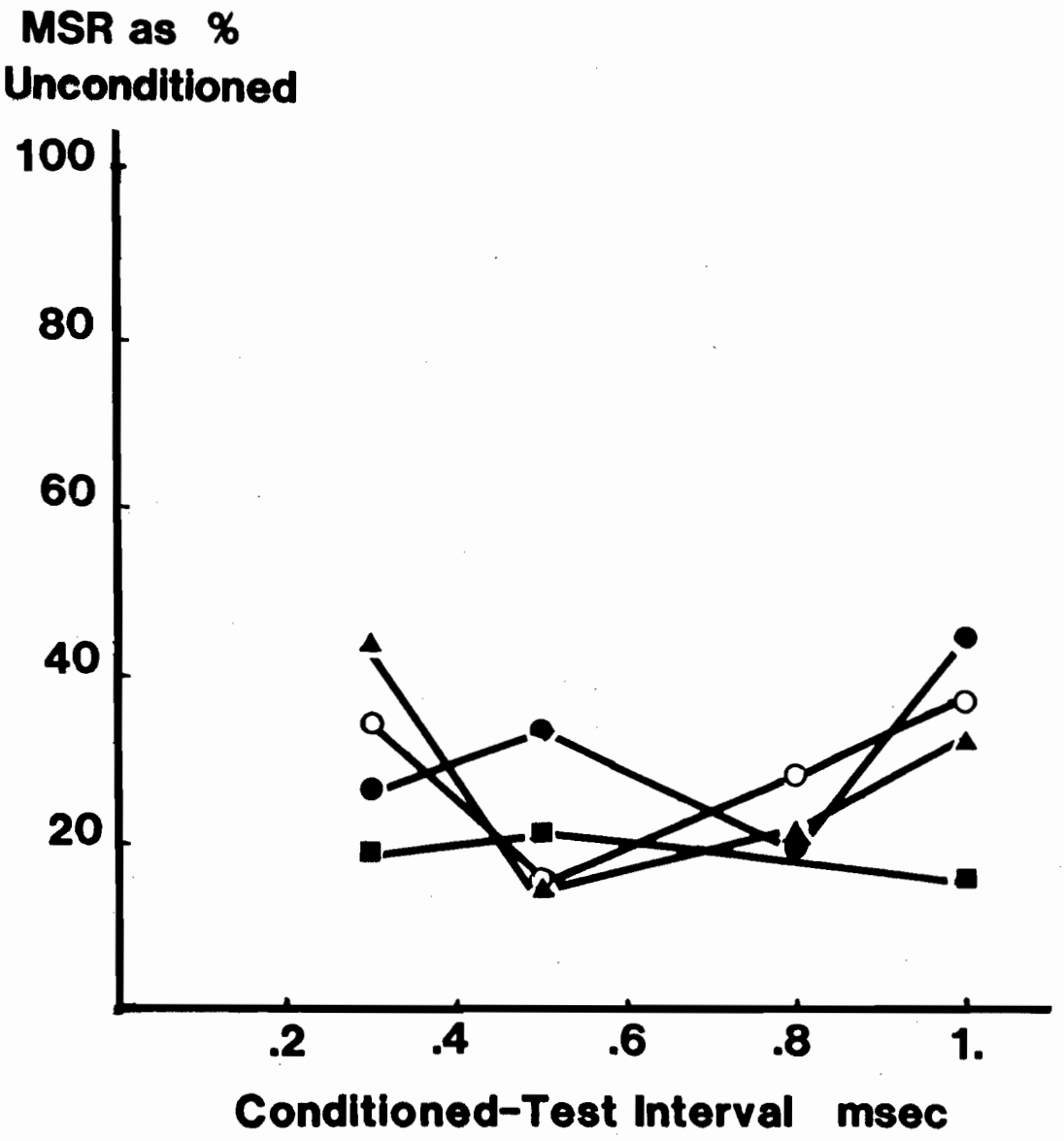

MSR as %
Unconditioned
100
80
60
40
20
20
40
60
80
100
Conditioned-Test Interval msec

Fig. 3 Postsynaptic inhibition before (○) and 10 (●), 30 (■), or 50 (▲) min after administration of MPA (100 mg/kg, i.v.). Amplitude of the BST preceded by quadriceps stimulation at time intervals indicated on the abscissa, expressed as percentage of the test BST MSR.


MSR as %
Unconditioned
100
80
60
40
20
.2
.4
.6
.8
1.
Conditioned-Test Interval msec

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CHAPTER 3

THE EFFECTS OF BENZODIAZEPINES ON SPINAL HOMOSYNAPTIC DEPRESSION

M.F. Davies, B. Esplin, and R. Čapek

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Abstract

Clonazepam (0.5 mg/kg, i.v.) changed the characteristic pattern of exponential decline of the monosynaptic responses (MSR) termed Early Tetanic Rundown (ETR) evoked by trains of 10 stimuli (2, 5 or 10 Hz) applied to either the biceps-semitendinosus (BST) or triceps surae (TS) nerve and recorded from the ventral root in spinal cats. In the case of BST, CLON did not affect the first MSR or the last five MSRs forming the plateau, while the second MSR was markedly depressed especially at higher frequencies tested. TS reacted differently to clonazepam administration in that the first response was increased and the amount of depression of the second response was lessened with no change of the plateau. All effects of clonazepam were reversed by the benzodiazepine antagonist, Ro15-1788 (5 mg/kg, i.v.), which alone had no effect of its own on any parameters, suggesting that the effects of clonazepam were mediated by the central benzodiazepine receptor. Diazepam (1.0 mg/kg, i.v.), caused the same changes in the BST ETR and DRP as clonazepam but increased the plateau instead of the second response from TS. It was also demonstrated that higher threshold afferents tended to lessen the depression of both BST and TS ETRs at higher frequencies of stimulation.

Introduction

Homosynaptic depression of the stretch reflex has been studied for many years (Beswick and Evanson, 1957; Curtis and Eccles, 1960; Čapek and Esplin, 1977a), and can be experimentally elicited by applying a low frequency stimulation to a muscle nerve to produce a series of monosynaptic responses that decline in amplitude within a few pulses and then reach a plateau level. As the excitability of motoneurons usually returns to normal within 100 ms, any depression seen at frequencies lower than 10 Hz is probably presynaptic in origin (Lloyd and Wilson, 1957). Depression of excitatory postsynaptic potentials (EPSPs) with repetitive stimulation of a single Ia afferent has been observed in motoneurons (Kuno, 1964). Since such stimulation would not significantly change the overall activity of the spinal cord or alone cause motoneurons to fire, the decline in EPSP is probably the result of a depression of the transmitter release from the primary afferents and not due to a long lasting depressant influence on the afferent set up by previous activation of other afferents. This however, does not preclude the existence of outside factors which might modify the functioning of the monosynaptic pathway when many more than one afferent are active.

Homosynaptic depression is of interest to neuropharmacologists because it represents a phenomenon whereby excitatory transmission is self-limiting. In the nervous system, excitation is balanced by inhibitory forces which prevent the system from going out of control as occurs in epilepsy. In general, anticonvulsants try to shift this balance in favour of inhibition in an attempt to suppress the uncontrolled excitation associated with epilepsy. Since a reduction in excitatory transmission would have the same kind of effect as an increase in inhibition, anticonvulsant agents may have their action by enhancing homosynaptic depression. Members of our group have previously examined the effect of ethosuximide on this phenomenon (Čapek and

Esplin, 1977b). In this study we investigated the actions of benzodiazepines.

We report that benzodiazepine changes the pattern of the decline of MSRs elicited by short trains of stimuli at moderate frequencies. In these experiments, we have used clonazepam and compared it with diazepam. Investigations reported in this paper were done to ascertain whether both of these benzodiazepines act in a similar fashion on homosynaptic depression, and to establish whether this action is mediated by the central benzodiazepine receptor using the criteria of Richards and co-workers (1982). We have also manipulated the stimulation parameters in an attempt to answer some of the questions about the underlying physiology of the phenomenon. A preliminary report of some of these observations has been presented (Esplin et al., 1983).

Methods

Cats of either sex weighing 2.2-4.0 kg were used. Anaesthesia was induced with ethyl chloride and maintained by diethyl ether until insertion of the tracheal cannula, anemic decerebration and transection of the spinal cord at the atlanto-occipital junction were completed. The anaesthetic was then discontinued and the animals were artificially respired with air. The concentration of carbon dioxide in the air was continually monitored by an infrared analyser and the respiratory volume was adjusted so that the concentration of carbon dioxide in the end-tidal air reached 2.0-2.5%. The cephalic vein was cannulated for administration of drugs. The mean blood pressure from the carotid artery was monitored throughout. Gallamine triethiodide was administered as needed to maintain muscular paralysis. The lumbo-sacral cord was exposed by laminectomy and covered by mineral oil. The temperature of the cord was maintained at 35° C.

The ventral roots of L7 were severed on one side of the cord. The

combined BST and both branches of the TS nerves were dissected in the ipsilateral hindlimb and severed from the muscle.

Dorsal root potentials (DRP) and dorsal root reflexes (DRR), evoked by a single 250 μsec supramaximal stimulus applied to the severed L7 dorsal root, were recorded from a fine filament of the same root contralateral to the side from where the monosynaptic responses were recorded.

Platinum hook electrodes were used for both stimulation and recording. The peripheral muscle nerves were placed on pairs of electrodes and supramaximally stimulated by 10 volts rectangular pulses of 50 μsec duration. Monosynaptic reflex responses were recorded monophasically from the cut L7 ventral root. To elicit the typical pattern of responses called early tetanic rundowns (ETR), trains of 10 pulses of constant voltage were delivered to muscle nerves every 30 seconds. This time interval between trains allowed full recovery of the monosynaptic pathway so that no difference between first and subsequent ETRs could be detected.

Differentially recorded signals were analysed on line by a microcomputer based system. In the case of ETRs, the amplitudes of the MSRs were measured in real time and the responses of 5 trains of 10 responses were averaged and expressed as a fraction of the averaged first response. DRPs were first recorded on a magnetic tape, the average of 10 responses determined, and the time integral of this average was calculated by computer.

Three aspects of the ETR were characterized. The amplitude of the first response reflects the number of the motoneurons that were activated by a single stimulus and gives a general indication of any changes in the excitability of the monosynaptic pathway. The second response was expressed as a percentage of the amplitude of the first response and is taken to indicate the transient phase of depressive changes. A steady state plateau was reached within 5 responses and therefore the last 5 responses in the train

were averaged together and also expressed as percent of the first response. The DRP was also monitored as an estimate of the relative status of GABAergic transmission within the spinal cord.

Statistical significance was tested by means of either the nonparametric Friedman's two-way analysis of variance or by the Wilcoxon signed rank test. In all figures, statistical significance is indicated below the data points, where symbols connected by the brackets are significantly different from each other. All data points are expressed as the mean $\pm$ the standard error.

Clonazepam and diazepam were first dissolved in polyethyleneglycol 400 (10 mg/ml) then the desired amount was added to 5 ml warm saline and injected immediately. A microsuspension of Ro15-1788 was made by adding the desired amount of drug to 5 ml of saline to which a drop of Tween-80 had been added.

Any differences in the pattern of BST and TS ETR was not likely to be due to preparation variation since in an overwhelming majority of experiments, both nerves were used.

Ro15-1788 was generously provided by Dr. W. Haefely of Hoffman-LaRoche, Basel. Clonazepam and diazepam were supplied by Hoffman-LaRoche, Vaudreuil, Quebec.

Results

ETRs elicited by stimulation of either BST or TS were monitored to ascertain whether the drug action was common to both the stretch reflex loop of flexors and of extensors. In the control situation, the rundown pattern of the monosynaptic pathway was characteristic for each nerve. The ETR elicited by stimulation of BST usually reached a plateau level faster and the plateau was higher than that of TS (Fig. 1). When data from ETRs of all 3 frequencies

are summarized in the form in which percent depression of the second response or of the plateau are plotted against the reciprocal of the frequency or the stimulus interval (Fig. 3), the second response and steady state plateau of both pathways displayed more complex relationships than anticipated. The BST second response was less depressed at a stimulus interval of 100 ms than at 200 or 500 ms, although the means of all three were within 10% of each other. The BST plateau was actually lower at 5 Hz than at 10 Hz. With stimulation of TS, both the second response and plateau were lowest at 5 Hz. If recovery of the pathway were only dependent on replenishment of the transmitter, the data displayed in this manner would be expected to more closely follow an exponential or linear increase with an increase of the stimulus interval. The relationship found, suggests the possibility of a facilitating process most prominent at higher frequencies superimposed on a longer lasting depressive phenomenon. In some experiments, the earlier responses of the train were sometimes greater than the first, especially for BST when stimulated at 10 Hz. This was often associated with an unstable preparation which had endured an anoxic period, but some preparations which displayed facilitation of the second response could not be discarded on the grounds that they did not meet the standard of stability.

In the absence of drugs, ETRs were elicited at two stimulation voltages as a crude means of assessing the contribution of higher threshold fibres to the phenomenon of homosynaptic depression. All drug studies were performed with a stimulus strength set at 10 volts since this was a supramaximal stimulus for the monosynaptic response as judged by measurement of the input-output relation. An intensity of 6 volts was chosen as the lower strength stimulus because this was found to be just supramaximal for the BST MSR in most preparations (Table 1). In fact, it was found during analysis that 6 volts produced a slightly submaximal MSR in TS. The depression of the second

response and plateau of both BST and TS were greater at 10 Hz than at lower frequencies (Fig. 2). This change was most pronounced for TS. Thus the higher threshold fibres may be having essentially a facilitating effect on subsequent monosynaptic transmission, an effect which lasts between 100 and 200 ms.

Intravenous administration of 0.5 mg/kg clonazepam reliably increased the area of the DRP by 25% (Table 1). DRR activity was not markedly changed (Fig. 3). The amplitude of the DRP was only slightly increased. Clonazepam profoundly depressed the BST second response especially at stimulus intervals of 100 and 200 ms. The plateau was only slightly decreased at 10 Hz but was not different from control levels at 5 and 2 Hz. In the case of stimulation of TS, the second response was significantly increased at all intervals by approximately 10-15% without a change in the plateau curve. In contrast to BST, where no change in the first response was seen, the amplitude of the first TS MSR in the train was increased 10-20% by clonazepam (Table 1).

To establish whether the effects of clonazepam were mediated by the central benzodiazepine receptor, the benzodiazepine antagonist Ro15-1788 was administered before (Fig. 4) and after (Fig. 5) clonazepam. Given alone, 5.0 mg/kg Ro15-1788 had no effect on blood pressure, or on background neuronal activity within the spinal cord as subjectively appraised from audio monitoring of ventral root activity. DRP area (Table 1) and the DRR activity were unchanged by drug application (Fig. 4) indicating that, at this moderate dose, Ro15-1788 seems to have no intrinsic activity on GABAergic transmission. The second response and plateau of both nerves were unchanged by this antagonist (Fig. 5) and the amplitude of the first MSR showed no change (Table 1). With prior administration of Ro15-1788, clonazepam was devoid of

any of its action on DRP or on the ETRs of BST and TS, but the increase in the first TS MSR was not prevented (Table 1). When clonazepam was administered first, Ro15-1788 reversed all the effects of clonazepam except the increase of the TS first response. However, the relatively short duration of action of Ro15-1788 (Polc et al., 1981) compared to that of clonazepam caused technical difficulties. During the time required to record the ETRs, the effect of Ro15-1788 began to fade. Therefore the antagonistic action of Ro15-1788 is more clearly seen when administered after clonazepam (Fig. 5).

Administration of 1.0 mg/kg diazepam caused the same changes as clonazepam, in all measurements of the BST ETR (Fig. 6), in that the second responses was depressed at high frequencies while the plateau and first response were unaffected, but caused a very different profile in relation to TS. Diazepam did not change the depression of the second response but lessened the homosynaptic depression seen in the plateau. The first MSR of the train was slightly elevated by diazepam, but this increase was not statistically significant (Table 1). As with clonazepam, diazepam increased the area under the DRP curve by about 25%, primarily by increasing its duration.

Discussion

The frequencies of stimulation used in this study were chosen after considering the physiology of the system. To examine presynaptic events in isolation it is necessary to allow the excitability of postsynaptic elements to fully recover. Normally the motoneuronal excitability returns to control values within 100 ms (Lloyd and Wilson 1957), therefore 10 Hz was the highest frequency tested. There is, however, some evidence that complete recovery of TS motoneurons may take as long as 170 to 200 ms (Brooks et al., 1950). If this were the case, then the 10 Hz of the second response and plateau may be lower than would be expected from just presynaptic depression.

The lessening of depression seen with relatively high voltages of stimulation indicates that activation of high threshold afferents change the repetitively evoked synaptic transmission by an action on either Ia primary afferents or motoneurons. If increasing the stimulus intensity from 6 to 10 V caused a larger primary afferent depolarization (PAD) due to activation of group III afferents that would affect subsequent MSRs in a train, then it would be expected that the next MSR would be depressed. In our experiments the opposite occurs, higher voltage stimulation lessens the homosynaptic depression on both nerves, especially for TS. Hyperpolarization of the afferents would be one way to cause the observed effects. Indeed, primary afferent hyperpolarization (PAH) is seen when group III and C fibres are activated by strong stimulation and is associated with a decrease in terminal excitability (Mendell, 1972). It has been suggested that this hyperpolarization is caused by the inhibition of tonically active inhibitory interneurons (Rudomin et al. 1974). This possibility is discussed in the subsequent paper (Davies et al., 1983). These results indicate that both TS and BST Ia primary afferents are influenced by previous activation of high threshold afferents arising from the same muscle group. Since 10 V stimulation was used in all drug studies, it must be kept in mind that there is a facilitating component included in all responses elicited at 10 Hz that is probably due to the previous activation of high threshold afferents. The use of a lower voltage level for the entire study to reduce this type of interference would not eliminate all high threshold influences since no method exists for selectively activating Ia afferents. The almost linear relationship of the relative amplitudes to the stimulus interval seen with 6 V stimulation is closer to the exponential form of the curve of both the second and plateau responses predicted by the model proposed previously (Čapek and Esplin, 1977a).

A similar complex relationship was seen when the phenomenon of habituation was studied in the monosynaptic pathway of Aplysia (Byrne, 1982). When the sensory neuron was stimulated by a train of 10 pulses, the degree of synaptic depression from 0.33 to 0.033 Hz was nearly identical and it appeared that at higher frequencies there was an underlying facilitating process antagonizing the depression. In addition, when presented with a train of pulses at various frequencies, the pathway was able to recover faster if previously stimulated by a high frequency train. Byrne suggested that this facilitating process might be the same as that which promotes a quicker recovery at high frequency and might be related to processes underlying posttetanic potentiation. This data was also inconsistent with the classical depletion model proposed by Liley and North (1953) and Čapek and Esplin (1977a) which would predict that the depression of EPSPs is a monotonic function of stimulus interval. These models are also contradicted by studies in which it has been shown that the magnitude of synaptic depression is relatively insensitive to levels of release (Castellucci and Kandel, 1976). In Aplysia, it is more likely that a change in the amount of calcium entering the terminal is responsible for the depression seen. In depth studies of the action potential in Aplysia sensory neurons in the presence of a blocker of potassium channels have indicated that during homosynaptic depression the calcium influx per impulse becomes smaller and the EPSP decrease declines in parallel (Klein et al., 1980). This would indicate that, at least in Aplysia, the influx of calcium is the most important factor in controlling the amount of transmitter released. Inactivation of the calcium channel has been shown to be a function of the extent of calcium entry (Tillotson, 1979) and may be the underlying process of habituation (Klein, 1981). Changes in potassium conductance does not seem to be involved (Klein et al., 1980).

It is evident that the second response and those of the plateau are

differentially affected by many manipulations. It can be surmised that changes occur in the pathway throughout application of the train of stimuli. Repetitive stimulation of cuneate nucleus at similar frequencies to those employed in this study increased the extracellular potassium concentration in the region of primary afferent terminations within a few stimulations (Krnjevic and Morris, 1975). However Lothman and Somjen (1975) demonstrated that changes in the extracellular potassium did not significantly affect the membrane potential of primary afferents or spinal neurons. Since BST and TS do not react in a similar manner it is probable that depolarization secondary to potassium buildup is not a major influence.

The deepening of depression of the second response without a large change in the first response by a benzodiazepine was originally seen by Schlosser (1971) using a two pulse stimulation paradigm applied to the entire root. He attributed this action of benzodiazepines to their ability to enhance presynaptic inhibition, since motoneuronal excitability was unaffected. In a previous study from our group (Čapek and Esplin, 1977a) it was demonstrated that GABA mediated transmission did not play a large role in homosynaptic depression from the stimulation of nerves from one muscle group, therefore Schlosser's explanation seemed inadequate. The question of the GABA dependence of the benzodiazepine effect is addressed in the subsequent paper (Davies et al., 1983).

The fact that clonazepam did not cause the same effect in TS suggests that not all stretch reflex arcs react in the same manner to clonazepam administration. Therefore the observed depression of the BST second response with clonazepam is probably not due to changes in the basic release kinetics of Ia primary afferents but may be caused by changes external to the terminal. TS primary afferents are known to be influenced by very different types of

inputs than flexors such as BST. Most strikingly, they receive strong presynaptic input from flexors such as the BST, but do not supply such input to other afferents (Eccles et al., 1961).

The observed ability of Ro15-1788 to reverse and prevent the increase of DRP area by clonazepam with no effect of its own, is in agreement with the work of Polc's group (1981) with meclonazepam. Ro15-1788 is postulated to be one of the most specific ligands for the central benzodiazepine receptor (Hunkeler et al., 1981; Richards et al., 1982). On the basis of the specificity of Ro15-1788, it would seem that all the observed actions of clonazepam on MSRs elicited by repetitive stimulation of BST and TS are mediated by the central receptor, except perhaps for the increase in amplitude of the TS first response. While Ro15-1788 alone causes a slight increase which does not reach statistical significance, there is no indication of reversal of the clonazepam induced increase but possibly a further enhancement. The lack of effect of Ro15-1788 on any parameter measured suggests that there is no endogenous ligand normally active in modulating the homosynaptic depression of these two pathways.

The dose of clonazepam used was always 0.5 mg/kg and that of diazepam, 1 mg/kg. These doses were chosen on the basis of their ability to cause the characteristic change of pattern of the BST ETR, and on the knowledge that in many tests, clonazepam is more potent than diazepam. Extrapolating from the data of Braestrup and Squires (1978), 5 to 7 times more diazepam than clonazepam is needed to inhibit pentylenetetrazol convulsions and electric foot shock induced fighting in mice, to produce the same muscle relaxant effect and relieve human anxiety. In receptor studies of rat brain tissue (Sepinwall and Cook, 1980; Braestrup and Squires, 1978; Skolnick et al., 1980), clonazepam was about 4.5 to 15 times more potent in inhibiting specific [^{3}H]-diazepam binding. The differential effects of clonazepam and diazepam on

the TS ETR is interesting because the bulk of the literature tends to treat all benzodiazepines as having qualitatively similar effects within the CNS, except that some, such as clonazepam, do not bind to the purportantly inactive peripheral type of the receptor (Braestrup and Squires, 1977). Peripheral receptors have been found in the CNS and comprise about 10-25% of total number of benzodiazepine binding sites in the brain (Marangos et al., 1982) and 30% in the spinal cord (Del Zompo et al., 1983). However, instances have been reported where clonazepam was much more effective than diazepam than could be predicted on the basis of their respective binding affinities. Swinyard and Castellion (1966) found that clonazepam was 210 times more effective than diazepam in antagonizing seizures elicited by low frequency electroshock while maximal electroshock seizures was suppressed only by diazepam (Krall et al., 1978). These discrepancies would suggest that the classical receptor, as displayed by binding studies, is not involved in some of the observed anticonvulsant effects and cannot be explained by the existing binding literature.

If the data of the effect of diazepam and clonazepam on the TS ETR is expressed as amplitude of the responses in mV, it becomes evident that clonazepam reduces the depression of the second response to a greater extent than does diazepam while the relative changes in plateau are not as significant. The ability of clonazepam to relieve the depression of the second response more than diazepam is probably not due to a greater effect on GABAergic transmission, since both benzodiazepines cause a similar enhancement of the DRP. However the DRP elicited by our techniques may be maximally enhanced at 130% of control and therefore not be a faithful reflection of the status of GABAergic transmission.

From this study, it appears that benzodiazepines cause a unique type of

change in the BST ETR which has not been seen with any other drug tested. Also, the fact that TS reacts with a very different pattern to benzodiazepine administration indicates that any extrapolation from responses due to stimulation of one nerve or the whole dorsal root may be unwarranted.

Fig. 1 Representative ETRs elicited by stimulation of BST and TS at 10 Hz (▲), 5 Hz (◇) and 2 Hz (●) before and after 0.5 mg/kg clonazepam. Amplitude of the first response in the train elicited by stimulation of BST at 10 Hz was 2.07 mV before and 1.81 mV after clonazepam administration; whereas the first response for TS changed from 1.01 mV to 1.08 mV with clonazepam.

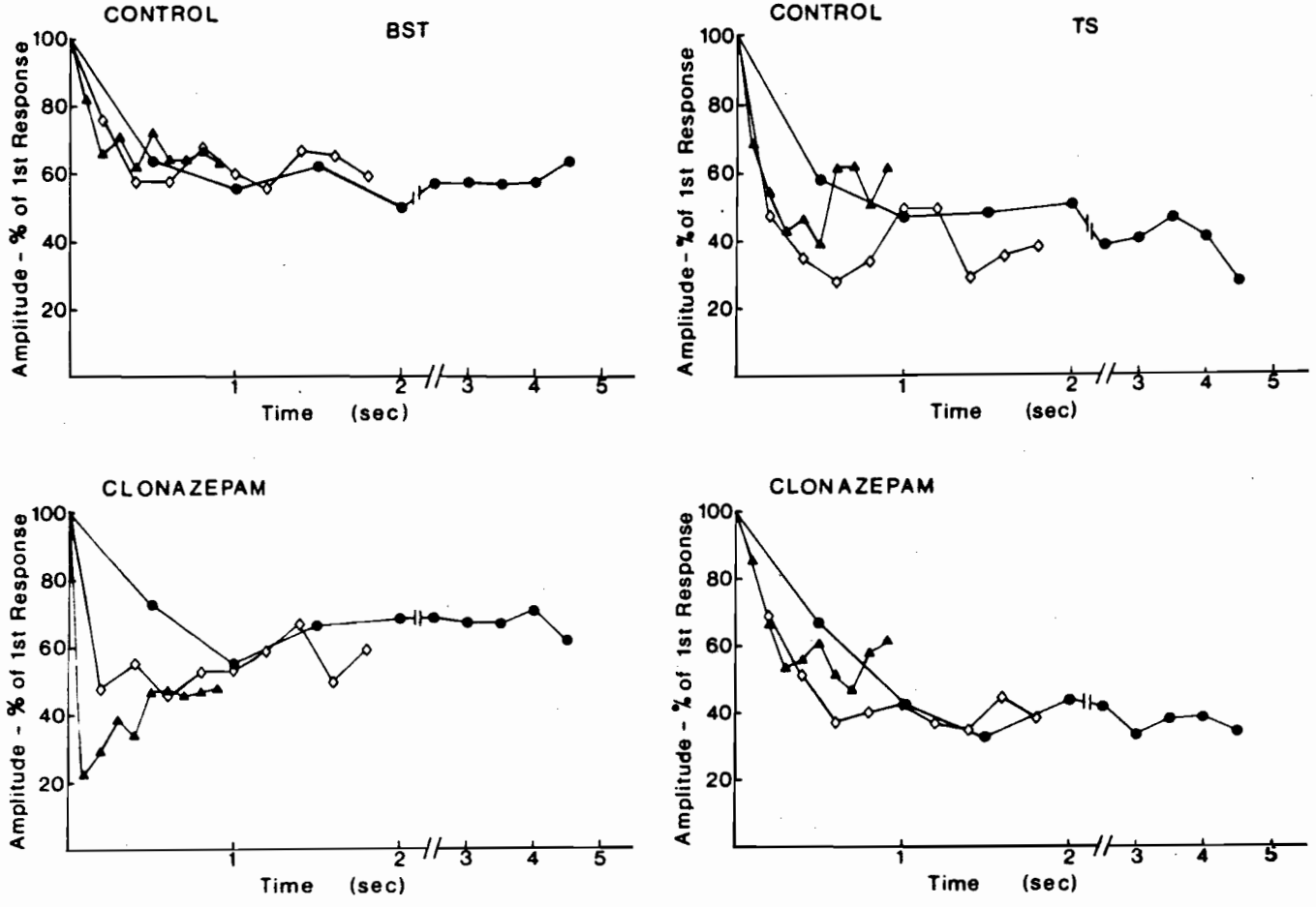
CONTROL
BST
CONTROL
TS
CLONAZEPAM
CLONAZEPAM
Amplitude - % of 1st Response
Time (sec)
100
80
60
40
20
1
2
3
4
5

Fig. 2 The second and plateau responses from the BST (N=14) and TS (N=10) ETRs with the stimulation intensity set at 10 V (○) and 6 V (●). In all figures, statistical significance is indicated below the data points, where symbols connected by the brackets are significantly different from each other. All data points are expressed as the mean ± the standard error.

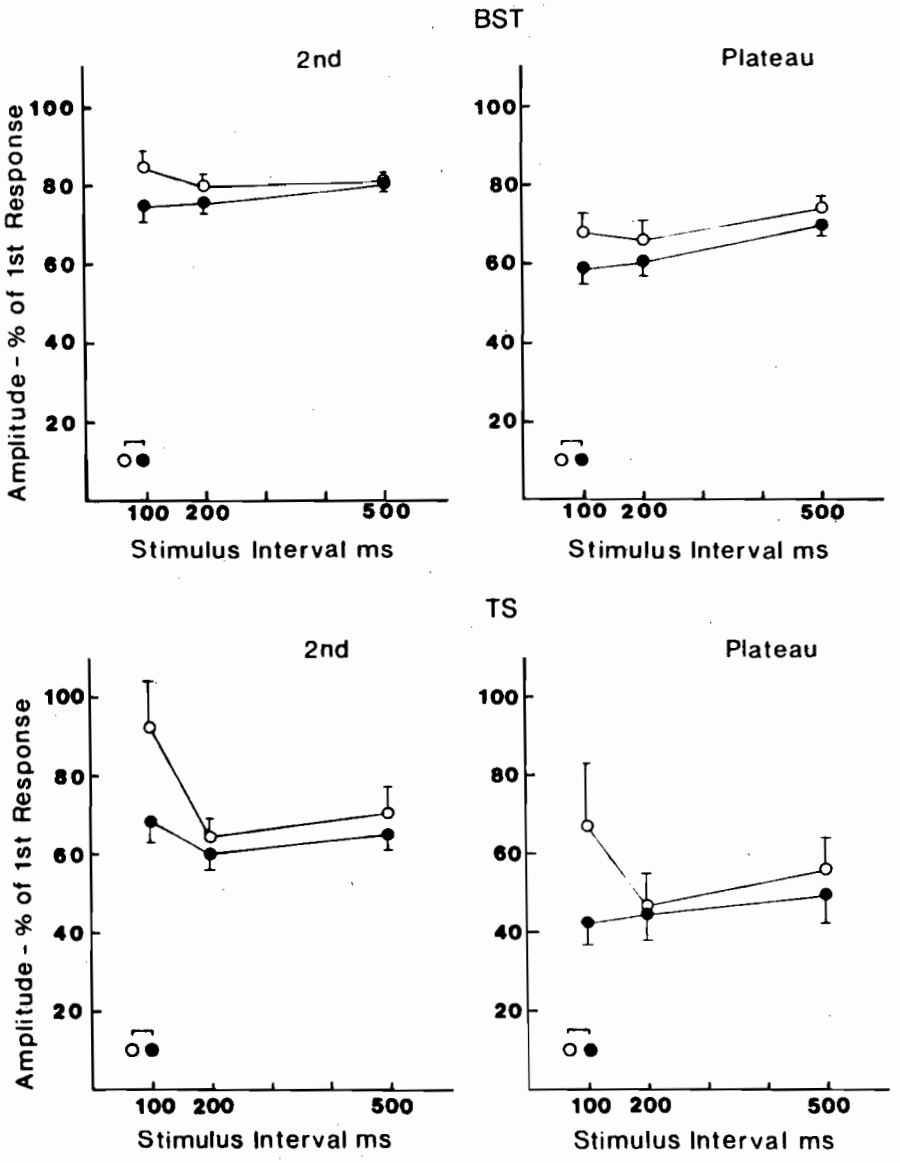
BST
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms
TS
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms

Fig. 3 The second and plateau responses of BST (N=21)and TS (N=14) ETRs under control conditions (○) and after 0.5 mg/kg clonazepam (■). Representative DRPs before and after clonazepam.

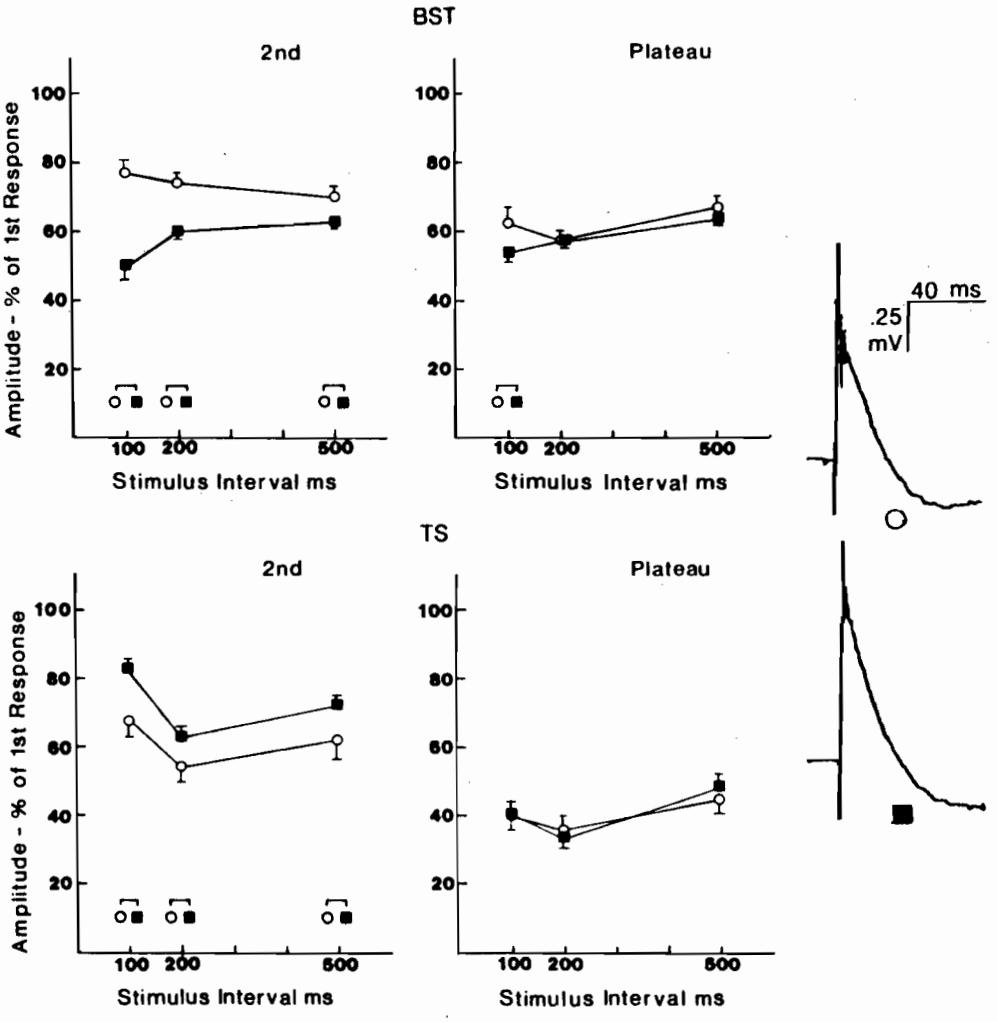
BST
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms
40 ms
.25
mV
TS
2nd
Plateau
Amplitude - % of 1st Response
Stimulus Interval ms

Fig. 4 The second and plateau reponses elicited by stimulation of BST (N=7), and TS (N=7) under control conditions (○), after 5.0 mg/kg Ro15-1788 (□), and followed approximately 30 minutes later by 0.5 mg/kg clonazepam (■). DRPs from a typical experiment.

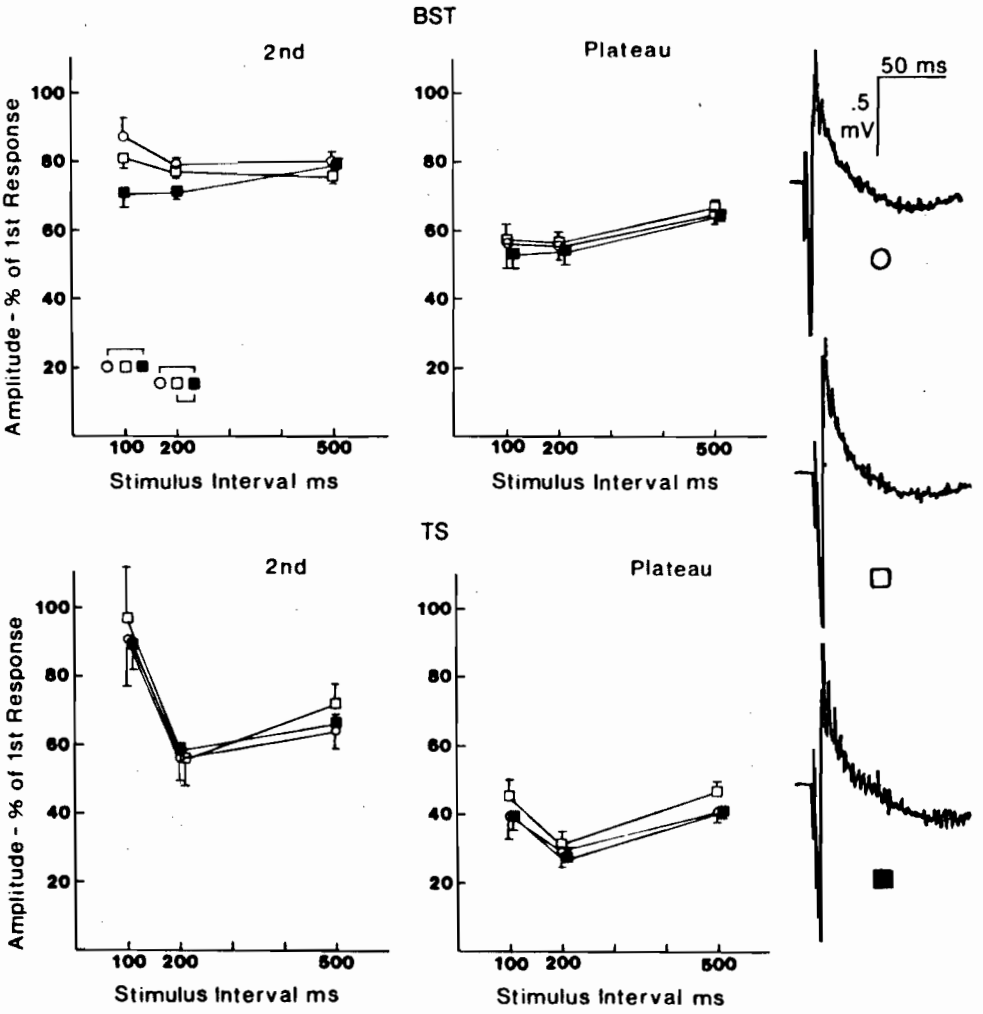
BST
2nd
Plateau
50 ms
.5
mV
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms
TS
2nd
Plateau
Amplitude - % of 1st Response
Stimulus Interval ms

Fig. 5 The second and plateau responses of BST (N=8) and TS (N=7) ETRs under control conditions (○), after administration of 0.5 mg/kg clonazepam (■), and then followed about 30 minutes later by 5.0 mg/kg Ro15-1788 (□). DRPs from a typical experiment.

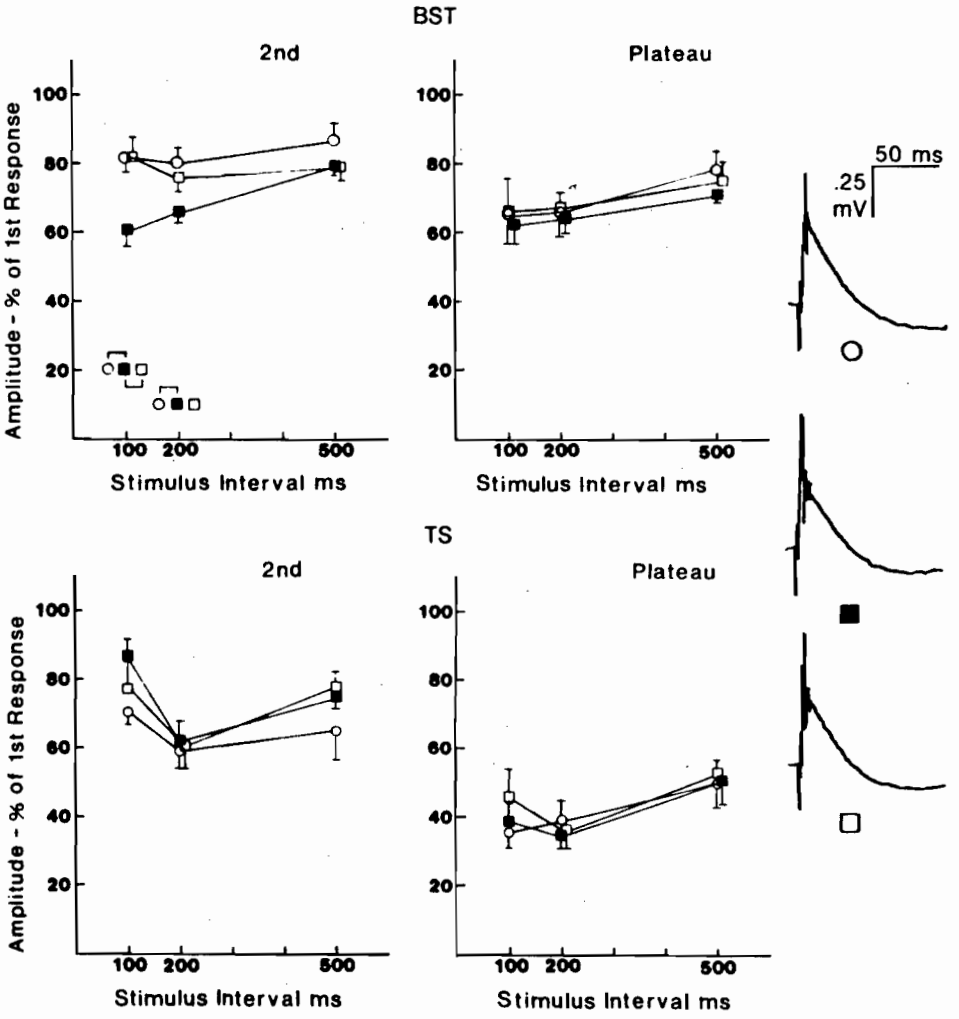
BST
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms
50 ms
.25
mV
TS
2nd
Plateau
Amplitude - % of 1st Response
Stimulus Interval ms

Fig. 6 The second response and plateau taken from BST (N=9) and TS (N=7) ETRs before (○) and after administration of 1.0 mg/kg diazepam (▼). Representative DRPs taken from a typical experiment.

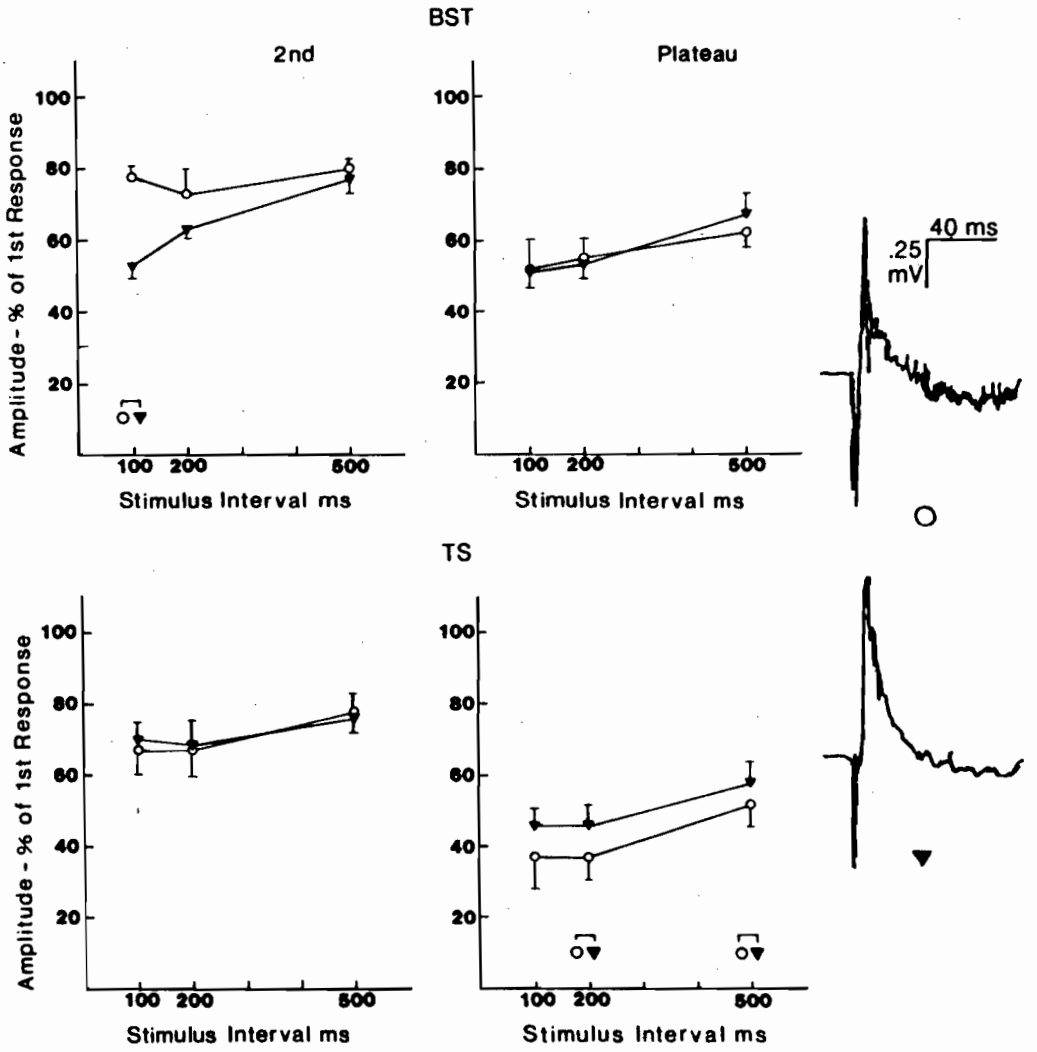
BST
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms
40 ms
.25
mV
TS

Table 1

Summary of the amplitudes of the first MSR in the ETR and the area of the DRP before and after various treatments.

(x ± S.E.M.)

Treatment	Amplitude of First Response		DRP Area
	BST (mV)	TS (mV)	percent of control
Control - 10 V	2.7 ± 0.3	2.1 ± 0.5	-
6 V	2.5 ± 0.3 N=14	1.7 ± 0.5* N=10	-
Control	2.7 ± 0.4	1.8 ± 0.4	
Clon	2.8 ± 0.5 N=21	2.2 ± 0.4* N=14	126.3 ± 6.0*
Control	3.2 ± 0.4	1.4 ± 0.4	
Ro15-1788	3.3 ± 0.5	1.7 ± 0.5	101.1 ± 3.0
R015-1788 + Clon	3.4 ± 0.5 N=7	2.2 ± 0.4* N=7	104.5 ± 4.0
Control	2.9 ± 0.4	1.9 ± 0.7	
Clon	2.9 ± 0.4	2.5 ± 0.7*	131.6 ± 6.8*
Clon + Ro15-1788	2.9 ± 0.4 N=8	2.3 ± 0.6 N=7	110.3 ± 4.6
Control	2.1 ± 0.3	2.1 ± 0.7	
Diazepam	2.2 ± 0.4 N=9	2.2 ± 0.6 N=7	130.6 ± 10.6*

* value is significantly different ($P < 0.05$) than control

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CHAPTER 4

A GABAERGIC COMPONENT IN HOMOSYNAPTIC DEPRESSION IN THE SPINAL MONOSYNAPTIC PATHWAY: A REQUIREMENT FOR BENZODIAZEPINE ACTION

M. F. Davies, B. Esplin, and R. Čapek

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Abstract

Spinal monosynaptic responses evoked by repetitive stimulation undergo homosynaptic depression whose pattern is altered by clonazepam. The dependence of this effect of clonazepam on the GABAergic system was examined in spinal unanaesthetized cats. Topical application of bicuculline to the spinal cord did not change any feature of the biceps-semitendinosus (BST) or triceps surae (TS) early tetanic rundown (ETR) but prevented the action of clonazepam. Semicarbazide (200 mg/kg i.v.) also prevented the benzodiazepine effect but alone had actions of its own. Evidence is presented that clonazepam influenced homosynaptic depression of the BST by lengthening the primary afferent depolarization (PAD). This prolongation of PAD did not last for the entire duration of the train as PAD also underwent depression. Therefore later responses in the train were unaffected by clonazepam. TS ETRs were not similarly affected because activation of TS afferents does not cause significant depolarization of its own afferents.

Introduction

As reported in the companion paper (Davies et al., 1983a), administration of benzodiazepines to spinal cats produced a unique change in the pattern of homosynaptic depression of the stretch monosynaptic response (MSR). The roughly exponential decline to a plateau level seen with repetitive stimulation of the biceps-semitendinosus nerve (BST) was transformed with clonazepam or diazepam into one where the second reflex response was greatly depressed while the first response and the average of the last five responses of the train were generally unchanged. The pattern of depression elicited by stimulation of the triceps surae nerve (TS) was unlike the BST early tetanic rundown (ETR) in that the second response showed less depression while the plateau was unchanged and the first response was increased after clonazepam administration.

In a previous paper by Čapek and Esplin (1977), the question of GABA involvement in homosynaptic depression was addressed because in that study, there was concern that this depressive phenomenon may be due to a GABAergic process. When semicarbazide did not abolish or greatly alleviate homosynaptic depression it was concluded that the reduction of the response was unrelated to the actions of GABA. This information was difficult to reconcile with the fact that benzodiazepines, which are generally thought to act by enhancing GABAergic transmission (Stratten and Barnes, 1971; Polc et al., 1974; Banna et al., 1974), were affecting homosynaptic depression. As there were reported instances of benzodiazepines apparently acting independently of GABAergic transmission (Davies and Polc, 1978; Evans et al., 1977), it was felt that changes in this phenomenon might also be another example. Therefore we re-explored the question of GABA involvement in homosynaptic depression, looking especially for a subtle GABAergic component, and assessed the ability of benzodiazepines to produce their effect on homosynaptic depression under

conditions of impaired GABAergic transmission. A preliminary account of the observations has been reported (Davies et al., 1983b; Esplin et al., 1983).

Methods

The preparation of the cat, data acquisition and manipulation, and statistical tests used, were the same as described in the previous paper (Davies et al., 1983a).

Clonazepam was first dissolved in polyethyleneglycol (10 mg/ml), then the desired amount was added to 5 ml of warm saline and injected immediately. Semicarbazide was dissolved in 5 ml saline. For topical application of bicuculline, a fresh solution was made for each experiment in a manner similar to that described by Benoist et al. (1974). Bicuculline (4 mg/ml) was dissolved in acidified saline, to which 1N NaOH was slowly added to increase the pH to 3.4. A 0.5 x 2.0 cm strip of facial tissue was soaked with this solution and applied to the dorsal surface of the cord under the oil pool and positioned just rostral to the L7 segment.

Results

Two strategies were employed to reduce GABAergic transmission: pretreatment with 200 mg/kg semicarbazide hydrochloride to reduce GABA synthesis and thereby reduce the amount of GABA released, and topical application of a solution of (+)-bicuculline to the spinal cord to block the $GABA_A$ receptor (Hill and Bowery, 1981). Although suicide inhibitors of glutamic acid decarboxylase are known and theoretically would be more appropriate than semicarbazide (Chrystal et al., 1979), their long latency of action and inability to cross the blood brain barrier (Jung, M.J., personal communication) precluded their use.

ETR measurements were made between 90 and 120 min after the administration of 200 mg/kg semicarbazide. Although the GABA depleting action was not yet maximal as shown in a similar preparation by Bell and Anderson (1972), this time interval was chosen because the DRP was significantly depressed but convulsive activity, which would compromise the stability of the measurements, only became a problem after about 3 hours. Semicarbazide reduced the area of the dorsal root potential (DRP) to about 45% of control within 120 min. The dorsal root reflex, when present, was reduced but not abolished. As seen in Fig. 1, semicarbazide caused a slight lessening of depression of the BST second response and plateau, the values reaching statistical significance at 500 ms for the second response and at 100 ms for the plateau. Since the drug seemed to affect the responses at all frequencies, semicarbazide may be affecting a long lasting phasic process with no noticeable decay phase within the 100 to 500 ms range between stimuli. However, the amplitude of the train's first MSR was not significantly changed (Table 1), therefore the general level of excitability in the BST monosynaptic pathway was constant and could not account for an average 10% shift of the second response and plateau. When ETRs were elicited from TS, the most noticeable change after semicarbazide administration was a 15% increase in the first response (Table 1). The second response was not significantly affected (Fig. 1); the large difference at the 100 ms stimulus interval resulted from an abnormally large value included in the calculations. The plateau was significantly raised at 500 ms with the values at the two other intervals being slightly greater than control.

When administered two hours before clonazepam, semicarbazide was able to completely prevent the depression of the second response evoked from the BST nerve caused by the benzodiazepine (Fig. 1). There was also no evidence of reversal of the effects of semicarbazide by clonazepam and even an instance of

enhancement at 200 ms. The slight but significant depressant action of clonazepam on the plateau at 100 ms was also fully reversed by semicarbazide. Clonazepam was unable to reverse the upward shift in the curve caused by semicarbazide. However, clonazepam slightly increased the DRP area depressed by semicarbazide. Because semicarbazide slowly but continually lowers the level of GABA and DRP in the spinal cord (Bell and Anderson, 1972), we assume that the strength of GABAergic transmission was further reduced within the time required to acquire the data. As a consequence, the small change of the measured DRP may in fact be an underestimate of the ability of clonazepam to improve GABAergic transmission. The picture of semicarbazide's antagonism of the effect of clonazepam on homosynaptic depression of BST is however quite clear and is not distorted by the amount of time needed to sample all ETRs.

The interaction of semicarbazide and clonazepam on homosynaptic depression of TS was more complicated than for BST (Fig. 1). Alone, both clonazepam and semicarbazide tended to increase the second response, although this did not reach statistical significance for semicarbazide. Clonazepam did not change the plateau in the absence of semicarbazide, while semicarbazide alone caused a slight upward shift in plateau. When clonazepam was administered after semicarbazide, the plateau remained at the level set by semicarbazide or was even elevated further. The amplitude of the TS first response in the train showed a gradual increase with administration of each drug. Again, the time between comparable ETR measurements might have affected the results. The apparent enhancement of the semicarbazide increase in plateau and first response of TS by clonazepam may, in fact, represent the further deterioration of GABAergic transmission with time.

The existence of a GABAergic component in homosynaptic depression was also tested by the topical application of bicuculline. Intravenous

administration was attempted, but was found to compromise the stability of the preparation to such an extent as to make measurements of ETRs impossible. Theoretically, topical application should limit the area of decreased inhibitory activity to a few spinal segments. Thus the increased variability of the input reaching the monosynaptic pathway of interest from other parts of the spinal cord resulting from a generalized spinal disinhibition, brought about by systemic administration of the convulsant, could be avoided. Typically, the first noticeable effect of bicuculline was a very pronounced convulsive activity within 8 to 10 min, as monitored from the ventral root, with a gradual reduction of the DRP to about 60% within 20 min. Acquisition of ETRs was attempted after this point and only when the convulsive activity was absent and variation between individual trains was not excessive. In the case of wide variation due to seizure activity, the ETR was redone. Bicuculline had no effect on any parameter of BST ETRs (Fig. 2). Bicuculline caused an increase in the amplitude of the TS first response (Table 1). The relative depression of the second response was untouched but that of the plateau was reduced at 10 Hz with a return to control levels at lower frequencies.

Topical application of bicuculline after clonazepam decreased the DRP by the same amount whether or not clonazepam was present (Table 1) and was able to partly reverse the benzodiazepine induced depression of the BST second response (Fig. 3). The lack of full reversal is probably a result of an inability to reach sufficient blockade of GABA receptors with a dose that will allow maintenance of stable recording conditions. In the case of plateau, statistical analysis shows that the slight depression at 10 Hz after clonazepam was reversed by bicuculline. Even though the mean values do not reflect this, they are statistically different because of the large variation that occurred in the presence of bicuculline while with clonazepam only, the variation was small. In the case of TS, the effect of bicuculline on the usual

lessening of the second response depression by clonazepam was somewhat unclear (Fig. 3). Nonparametric statistical analysis showed that after bicuculline the second response at 200 ms was indistinguishable from that during the control period, and therefore had reversed the clonazepam effect. Since, however, bicuculline increased the instability of the preparation, the variance was larger but the means at 200 and 500 ms after bicuculline were above those of clonazepam. Clonazepam alone, did not change TS plateau and did not influence the increase in plateau induced by bicuculline. In this set of experiments, clonazepam did not significantly increase the size of the first response (Table 1). Application of bicuculline showed no trend to reverse this growth and even tended to increase it further, in the same way as when bicuculline was administered alone.

The DRP measured in these experiments were elicited by stimulation of the entire L7 dorsal root and hence many cutaneous as well as muscle afferents were activated. Monitoring of the DRP was originally intended as a gross measurement of the changes in GABAergic transmission and not as a true reflection of the GABAergic influences to which group Ia afferent fibres would be subjected, as it is known that this potential also reflects the depolarization of the terminals of the large afferent cutaneous fibres (Wall, 1958). Nevertheless, it is possible to gain some insight into the changes that occur in the PAD during repetitive activation of this pathway by examining the DRP. As seen in Fig. 4, under control conditions, the first DRP in the train was larger in amplitude and in duration than any subsequent DRP. This difference was most noticeable at 10 Hz as the area of the second DRP was 38% of the first. At 5 and 2 Hz, the area was reduced to 62.5% and 96% respectively. With each stimulation, the DRP became smaller until after about the third or fourth pulse a steady state was reached. After administration of

clonazepam, the first DRP grew in amplitude and duration as seen in the single DRP of Fig. 4. The first DRP in the 10 Hz train appears to last almost until the next stimulus evoked another one, 100 ms later. The second DRP and the remaining DRPs decline in much the same manner as under control situations.

In order to investigate whether clonazepam changes the recovery of the BST pathway after it has been briefly activated by the train, the tenth stimulus of the train was presented at different delays after nine stimuli delivered at 10 Hz. Clonazepam treatment did not change the pattern of recovery seen under control conditions (Fig. 5A). This is in contrast to the ability of clonazepam to depress the second response as measured in the same experiments (Fig 5B).

Discussion

Under the influence of a constant electrical stimulation of a peripheral nerve, the number of motoneurons that fire is influenced by the excitability of the neurons involved in the stretch reflex. As primary afferents furnish only about 1% of the total number of synaptic boutons on the motoneuron (Conradi, 1969), the activity of other terminals can greatly affect the response of the motoneuron. These other boutons come from many sources such as descending tracts, and intersegmental and segmental connections. In the spinal preparation treated with a neuromuscular blocking agent there are no influences from supraspinal regions or from the muscles due to neuromuscular blockade, therefore the level of afferent input into the cord can be assumed to be constant. Two possible sources of variation remain for the first response in the train: a change in the tonic activity of interneurons synapsing on the afferents or motoneurons and a change of the membrane potential of the motoneurons or afferents. Because there is minimal spontaneous afferent traffic from the paralysed muscles, it is unlikely that

activation of reciprocal or recurrent inhibitory pathways plays a major role in influencing this response. For all other responses in the train, other influences may be added, as previous volleys may cause long term changes (>100 ms) in the state of the motoneurons, afferents or the surrounding interneurons.

In this study, it is crucial to ascertain whether semicarbazide and bicuculline are specific blockers of GABAergic transmission in the spinal cord before approaching the question of the dependency of benzodiazepines on a functional GABA system to have their action. If semicarbazide and bicuculline administration created the same conditions for primary afferent terminals, that is, reduced the effect of GABAergic transmission in the same manner, then it could be expected that the observed drug induced changes would be qualitatively similar. This was not the case. While both seemed to reverse the prominent effects of clonazepam on BST, they alone caused unique changes in the pattern of depression. Treatment with semicarbazide and bicuculline appeared to similarly depress the GABA transmission within the L7 segment, as judged by an equivalent reduction of the DRP area and the same changes in general characteristics. However the DRP as recorded here is not a good indicator of the tonic state of polarization of the primary afferents, but reflects the phasic change due to stimulation. It is possible that other GABA mediated processes exist which are not detected by measurement of the DRP, but still influence the stretch reflex.

There is substantial evidence in favour of bicuculline being a GABA receptor antagonist: inhibition of the binding of GABA (Möhler and Okada, 1977; Frere et al., 1982), competitive antagonism of the action of GABA on afferent fibres in the cuneate nucleus (Simmonds, 1982) and on spinal cord and cultured brain neurons (Curtis et al, 1971a,b; Nowak et al., 1982), although

there have been reports of low doses of bicuculline methochloride or bicuculline potentiating the action of GABA (Krnjevic et al., 1977; Godfraind et al., 1970).

The action of bicuculline on primary afferents has received much attention with many contradictory results. Čapek and Esplin (1982) and Simmonds (1978) saw no change in the excitability of the Ia terminals in the spinal cord and cuneate, respectively, when bicuculline was administered. By contrast, Sastry (1979) found that iontophoretic application of bicuculline caused a slight increase in the terminal excitability of TS afferents whereas, in a similar experiment, Curtis and Lodge (1982) showed a decrease and suggested that differences in stimulation conditions were responsible for their opposite findings. On motoneurons, iontophoretic application of bicuculline methochloride resulted in a slow depolarizing change of the membrane resting potential, reaching as much as 20 mV, with an accompanying decrease of input resistance of up to 70% (Krnjevic et al., 1977). From work done on cultured spinal cord neurons, it has been proposed that high concentrations of bicuculline also blocks a K^+ conductance causing an increase in membrane resistance and membrane depolarization (Heyer et al., 1982; Nowak et al., 1982). Bicuculline was shown to prolong calcium dependent action potentials recorded from cultured spinal cord and dorsal root ganglion soma, probably by the same mechanism (Heyer et al., 1982). If this prolongation also occurred in the terminals, an enhancement of transmitter release may be expected. In light of this information, it cannot be assumed that bicuculline affected only the GABA receptor-chloride ionophore complex.

The overall effect of bicuculline on monosynaptic pathway would depend on the relative strength of each change. It is appropriate to look at the changes of the first response of the train to evaluate the importance of each, as it is an indication of the excitability prior to stimulation. The 20%

increase in the first TS MSR after bicuculline application would be in line with the increase of threshold of afferents seen by Curtis and Lodge (1982), depolarization of the motoneuron or increased calcium entry into the terminals. Alternatively, if the slight decrease in terminal excitability seen by Sastry (1979) occurred in our preparation, it would tend to cause a depression of the MSR, and would have to be counteracted by a process which would increase the MSR. Either the increased calcium entry into afferent terminals or depolarization of the motoneurons would increase the probability of motoneurons firing. In the absence of quantitative studies conducted on TS motoneurons and TS primary afferent terminals, it is impossible to distinguish between these possibilities. However, because BST was not similarly affected, it is unlikely that bicuculline is changing a phenomenon common to all pathways such as depolarization of the motoneurons and increased calcium entry.

Semicarbazide has been shown to satisfy many of the requirements of a good depleter of GABA with surprisingly few effects that could not be ascribed to a reduction of GABAergic function (Bell and Anderson, 1972; Banna, 1973). In vitro studies have shown that semicarbazide inhibits glutamic acid decarboxylase without greatly affecting GABA-transaminase (Wood and Abrahams, 1971). In the study of Bell and Anderson (1972), the depression of the DRP and presynaptic inhibition paralleled the reduction of GABA levels although, unlike the DRP, presynaptic inhibition was never eliminated. The monosynaptic response gradually increased but this was thought to be the result of reduced tonic GABA inhibition. This is not in agreement with the work of Sastry (1979) who showed that the threshold for antidromic activation of afferents was only slightly reduced or not changed by semicarbazide. There are no reports suggesting that semicarbazide significantly changes the

function of any other neuronal system although the limited number of studies performed have only dealt with transmission in the spinal cord or cuneate nucleus. Unfortunately, biochemical studies have focused on semicarbazide action on enzymes involved in GABA metabolism. Therefore it is difficult to determine its specificity.

Semicarbazide blocks GABAergic transmission by removing GABA from the synaptic cleft whereas bicuculline binds to the $GABA_A$ receptor. In reducing the amount of GABA released in the synaptic cleft, semicarbazide may stop ongoing activation of the GABA autoreceptor described by Mitchell and Martin (1978) and the presynaptic $GABA_B$ receptor which modulates the release of other neurotransmitters (Bowery et al., 1980). Bicuculline appears to bind to the autoreceptor but does not affect the $GABA_B$ receptor (Hill and Bowery, 1981) and therefore could not be expected to produce similar results as semicarbazide. The lessening of the depression of the BST second and plateau seen with semicarbazide may be a consequence of the lack of activation of $GABA_B$ receptor which may mediate a modulatory mechanism that reduces the excitability of the monosynaptic pathway during repetitive stimulation.

Despite the drawbacks of the agents used to reduce GABA transmission, it is quite clear that for clonazepam to have its action on homosynaptic depression, the GABAergic transmission in the immediate vicinity of the spinal L7 segment must be intact. From the more extensive plot of the BST second response, it appears that clonazepam can cause further deepening of homosynaptic depression for almost 500 ms and a stimulus delivered after longer delays elicits a response which is unaffected by the earlier period of depression. On the other hand, clonazepam did not change the pattern of recovery of the BST monosynaptic pathway which had been previously activated by a train of nine pulses delivered at 10 Hz. This would indicate that the benzodiazepine did not cause cumulative changes in the primary afferent which

would influence its later pattern of functioning, as is also evident in benzodiazepine's inability to affect post-tetanic potentiation (Schlosser, 1971).

The characteristic very low second BST MSR of the ETR train at 10 Hz and the return to control plateau levels after benzodiazepine administration, are most readily explained by a change in the time course of the primary afferent depolarization (PAD). Stimulation of the afferents of different muscles produce differing amounts of PAD (Eccles et al., 1961). Stimulation of the BST or peroneal nerve easily evokes DRP and depolarizes afferents of almost any hindlimb muscle, including their own. In the absence of drug, the PAD of the BST Ia afferents set up by the first stimulus in the train almost disappears within the inter-stimulus interval of 100 ms. Further evidence of this is the inability of bicuculline to influence the second response of BST ETRs. With subsequent pulses in the train, the DRPs diminish due to depression and are less and less able to influence the next MSR. If diazepam or clonazepam are present, the amplitude and duration of the first DRP are greater and upon the arrival of the second volley, a depolarization of the BST primary afferents is still present. As a result, less transmitter is released and the second MSR is drastically reduced. With further stimulations the DRP itself begins to undergo depression and, after a few stimulations, does not last until the arrival of the next volley 100 ms later, and hence does not affect transmitter release. Since PAD in Ia afferents is the result of activation of other Ia fibres (Eccles et al., 1962), the phenomenon would be seen if lower stimulation intensities were used. As Ro15-1788 was able to antagonize the action of clonazepam on both the ETR and DRP, the benzodiazepine effect is probably mediated by the central benzodiazepine receptor and the classic GABA receptor/chloride ionophore.

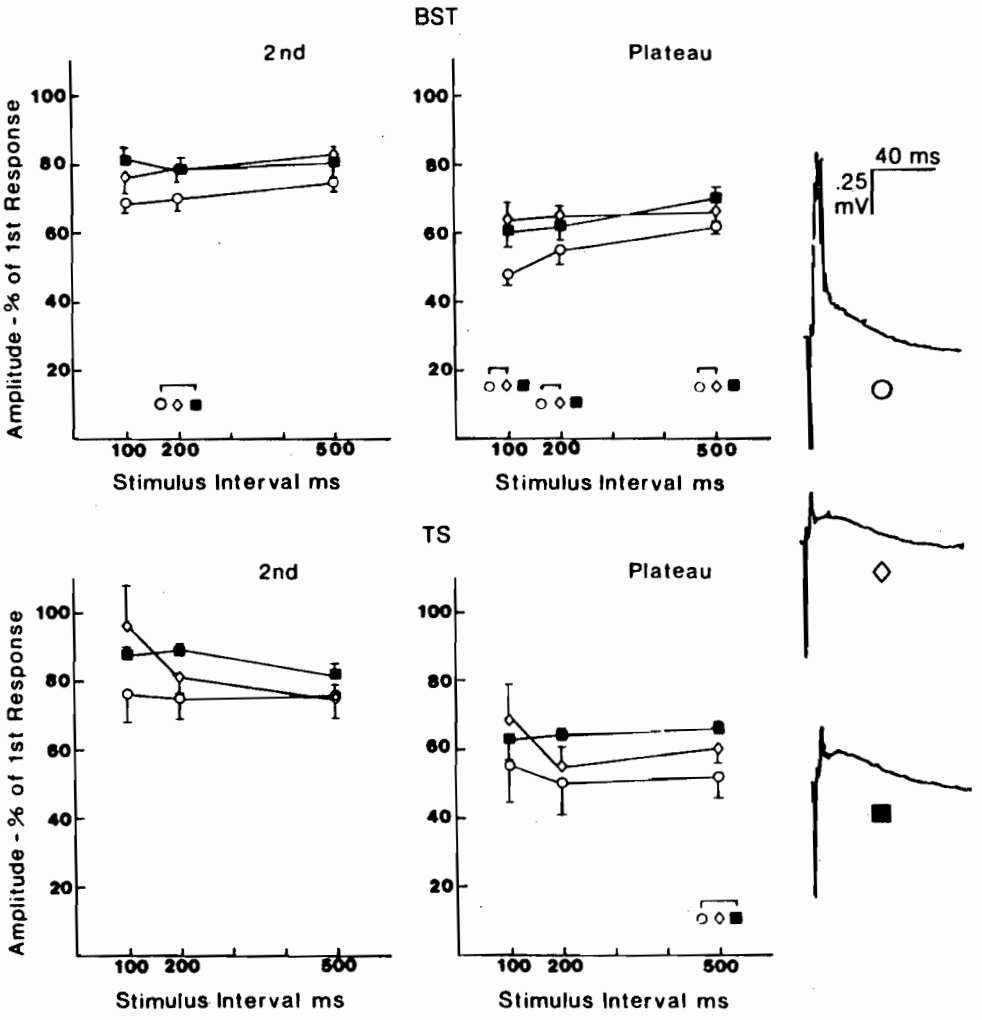
The depression of the DRP with repetitive stimulation was first seen by the group of Eccles (1963). They showed that the maximum depression occurred with stimulation intervals of 50 ms and returned to control levels within 500 ms. Our results are in agreement with these observations and extend them by showing that a plateau level is reached within three or four responses. It is not known why the DRP declines but possibly this could be due to homosynaptic depression within the pathway leading to PAD or desensitization of the GABA receptor on the primary afferent (Krnjevic, 1981). The action of benzodiazepines to lengthen the DRP has also been previously documented (Schmidt et al., 1967).

A similar depressive effect of clonazepam on the second TS MSR in the train as seen for BST does not occur probably because stimulation of the extensors such as TS causes very little PAD in other afferents and in themselves (Eccles et al., 1961). The only report of a significant PAD between afferents of the TS group was based on experiments done in the presence of pentobarbital anaesthesia and using a short burst of pulses to elicit the PAD of more than 100 ms in duration (Decandia et al., 1967). Stimulation by a single pulse produced a very short, small depolarization. Since there is little phasic release of GABA to influence the terminals, no depression at any frequency would be detected.

In summary, the present experiments confirmed the previous observation (Čapek and Esplin, 1977) that homosynaptic depression of the spinal monosynaptic pathway is not contingent on GABAergic transmission. However, the results indicate that the effect of benzodiazepines on the pattern of ETR of monosynaptic responses produced by stimulation of BST nerve is caused by enhancing and lengthening of the GABAergic PAD. The lack of benzodiazepine effects on ETR elicited from the TS nerve is likely due to the paucity of presynaptic influences that the afferents of the extensor muscles exert on

themselves.

Fig. 1 The second response and plateau taken from BST (N=11) and TS (N=6) ETRs under control conditions (○), 90 to 120 minutes after administration of 200 mg/kg semicarbazide (◇), and followed by 0.5 mg/kg clonazepam (■) administered approximately 120 minutes after semicarbazide. DRPs from a typical experiment. In all figures, statistical significance is indicated below the data points, where symbols connected by the brackets are significantly different from each other. All data points are expressed as mean ± the standard error.


BST
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
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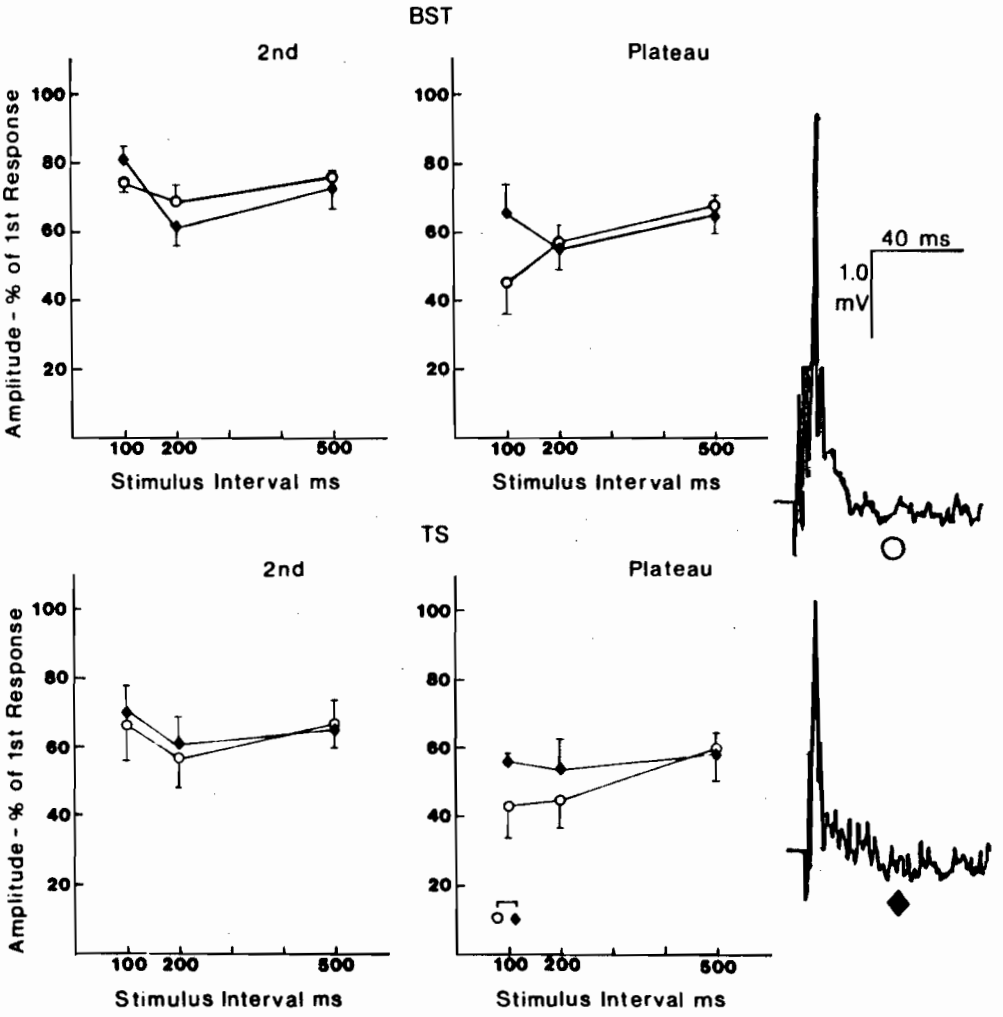
20
100 200 500
Stimulus Interval ms
40 ms
.25
mV
TS
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms

Fig. 2 The second response and plateau taken from BST (N=6) and TS (N=5) ETRs before (○) and after (◆) topical application of a solution of bicuculline to L7 segment of spinal cord. DRPs from a typical experiment.


BST
2nd
Plateau
Amplitude - % of 1st Response
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Stimulus Interval ms
40 ms
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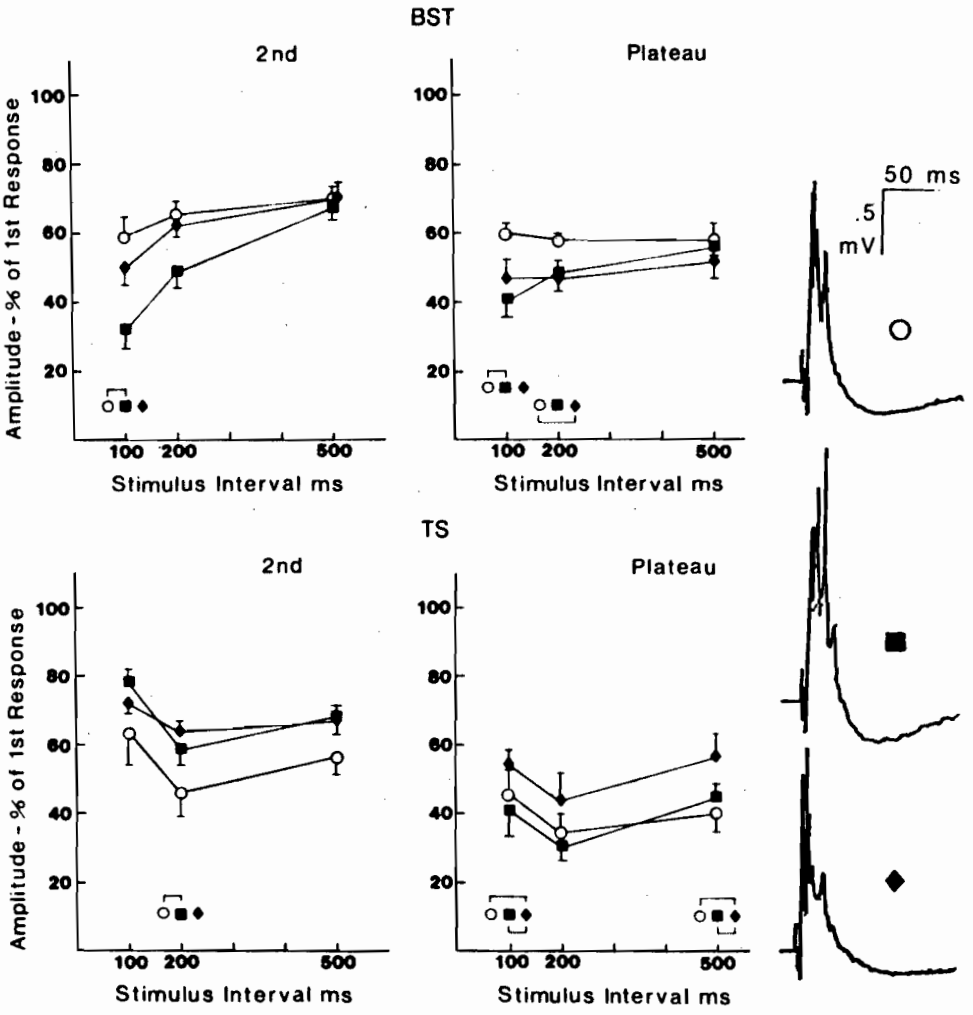
mV
TS
2nd
Plateau
Amplitude - % of 1st Response
100
80
60
40
20
100 200 500
Stimulus Interval ms

Fig. 3 The second response and plateau taken from BST (N=7) and TS (N=7) ETRs under control conditions (○), after administration of 0.5 mg/kg clonazepam (■) and followed by application of bicuculline to the cord (◆). DRPs from a typical experiment.


BST
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Amplitude - % of 1st Response
Stimulus Interval ms

Fig. 4 A single DRP and a train of 5 DRPs at 10, 5, and 2 Hz, before and after 0.5 mg/kg clonazepam. The dots below the traces represent a time interval of 100 ms.

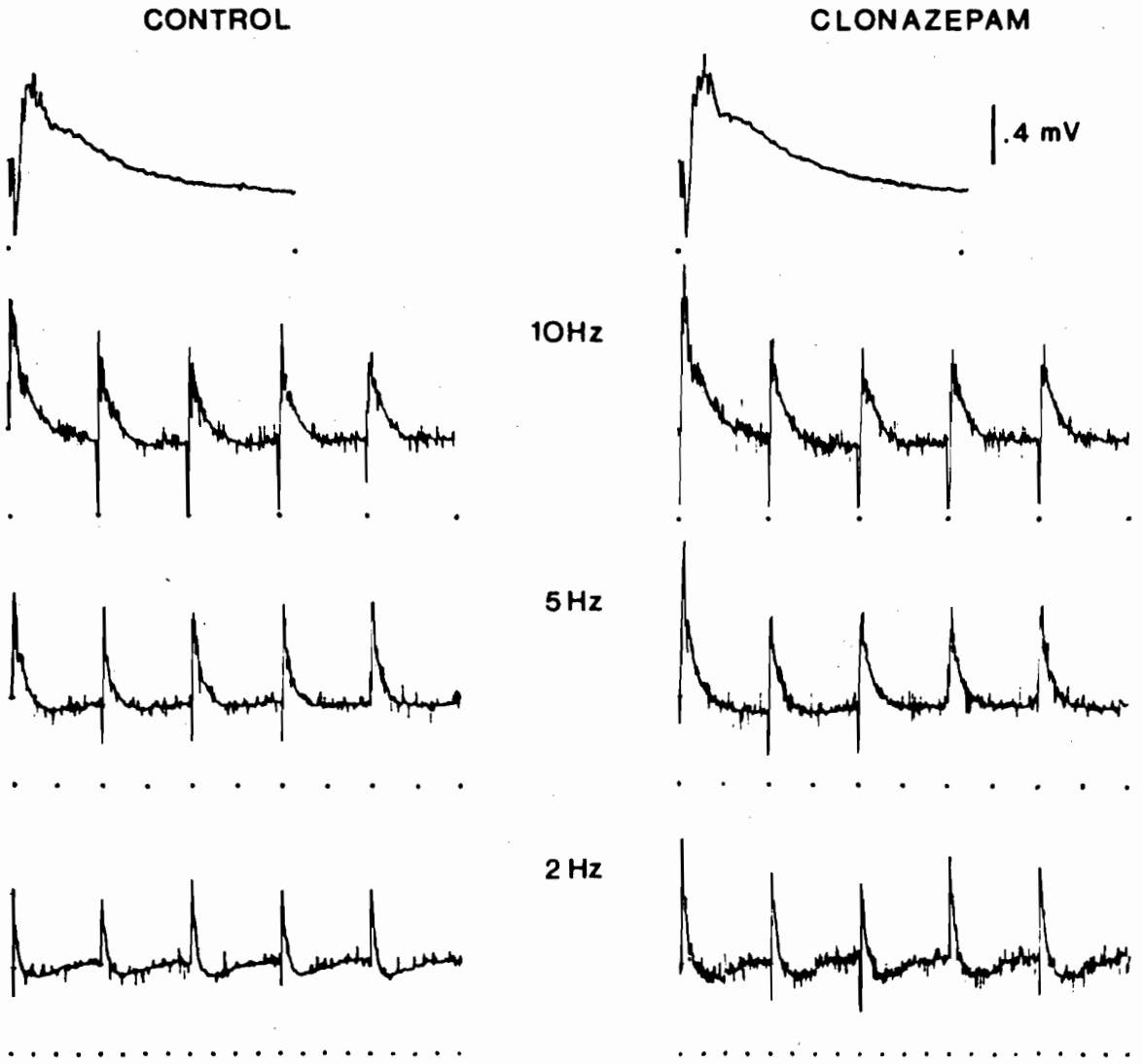
CONTROL
CLONAZEPAM
.4 mV
10Hz
5Hz
2 Hz

Fig. 5 A) The recovery curve of the BST MSR after previous activation by nine stimuli at 10 HZ, before (○) and after (■) 0.5 mg/kg clonazepam. N = 8.

B) An extended time course of synaptic recovery of the second response of the BST ETR, before (○) and after (■) 0.5 mg/kg clonazepam. N = 8.

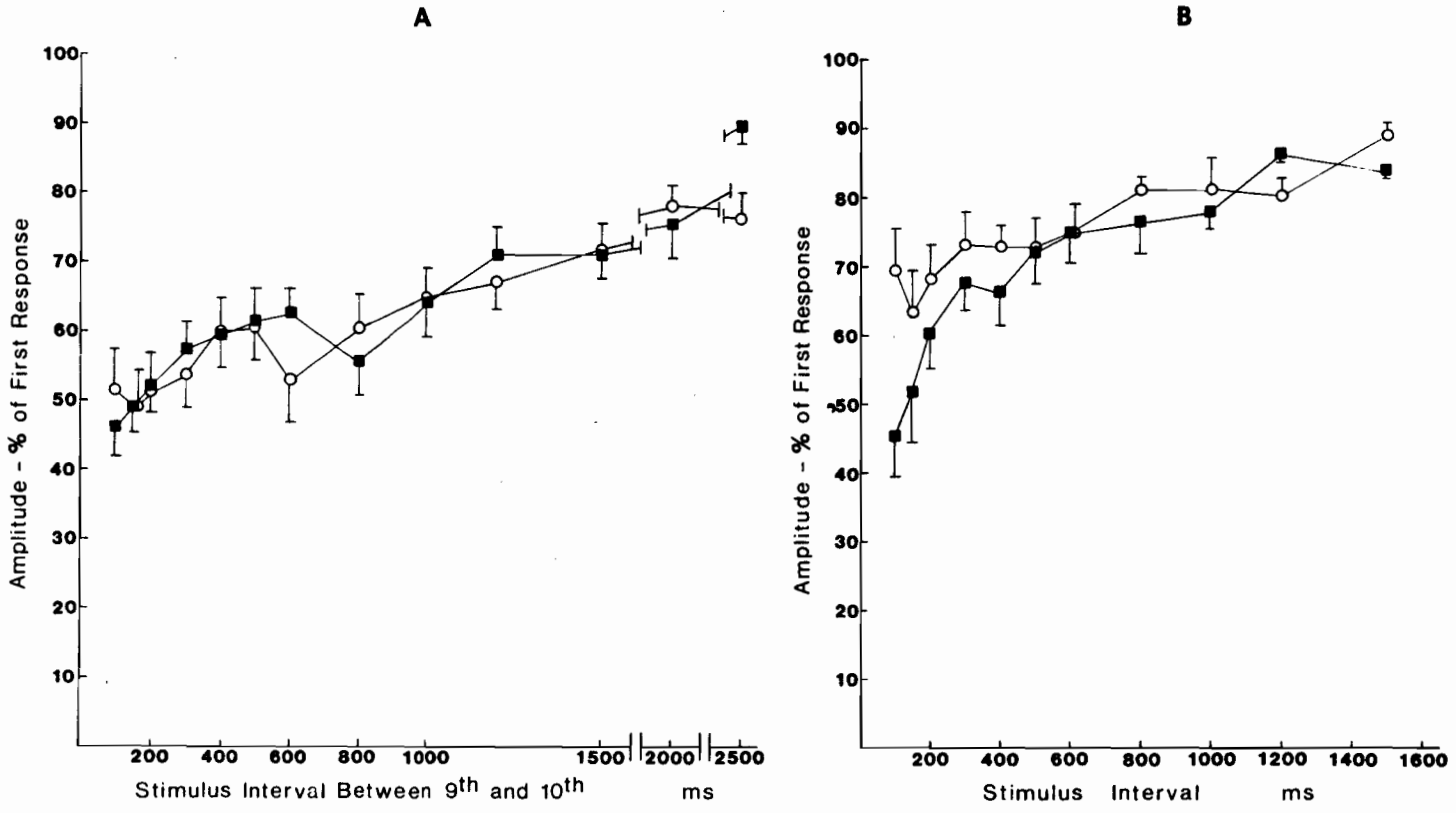
A
B
Amplitude - % of First Response
100
90
80
70
60
50
40
30
20
10
200
400
600
800
1000
1500
2000
2500
Stimulus Interval Between 9th and 10th
ms
1200
1400
1600
Stimulus Interval
ms

Table 1

Summary of the amplitude of the first MSR in the ETR train and the area of the DRP before and after various drug treatments.

(x ± S.E.M.)

Treatment	Amplitude of First Response		DRP Area
	BST (mv)	TS (mv)	percent of control
Control - 10 V	2.1 ± 0.3	1.5 ± 0.4	
Semicarbazide	2.2 ± 0.3	1.7 ± 0.5	46.5 ± 7.4*
Semicarbazide - Clonazepam	2.2 ± 0.3 N=11	1.8 ± 0.4 N=6	51.7 ± 7.4
Control	3.1 ± 0.5	2.4 ± 1.0	
Bicuculline	3.3 ± 0.5 N=6	2.8 ± 1.0* N=5	62.1 ± 12.6*
Control	3.5 ± 1.0	1.7 ± 0.4	
Clonazepam	3.9 ± 1.3	1.8 ± 0.4	123.8 ± 3.7*
Clonazepam - Bicuculline	4.0 ± 1.4 N=7	2.1 ± 0.4 N=7	61.1 ± 15.**

* value is significantly different ($p < 0.05$) than control

** value is significantly different ($p < 0.05$) than control and previous drug value

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CHAPTER 5

DISCUSSION

The original aim of this thesis was to probe how benzodiazepines influence homosynaptic depression. The first question posed was whether this was a GABA dependent phenomenon. It was decided that more than one method for reducing GABA transmission was needed in order to be confident of any conclusions. However, when this project was begun, there were very few options open. The functioning of the GABA receptor could be blocked with either bicuculline or picrotoxin, or GABA synthesis inhibited with a hydrazide, such as semicarbazide. Bicuculline was chosen over picrotoxin because it appeared to interact with the GABA receptor directly and not affect the chloride ionophore (Ticku and Olsen, 1978). The choice of a GABA synthesis blocker was more difficult to make. A literature search indicated that 3-mercaptoproprionic acid might have been a good candidate since it was a competitive inhibitor of GAD and produced short latency convulsions. The first paper describes our experience with this compound and presents our reasoning for using semicarbazide as our GABA depleting agent in a subsequent study. In the second paper, various aspects of the action of benzodiazepines on homosynaptic depression are presented. From this data and that in the third paper it was possible to propose a theory of how clonazepam produces its action on homosynaptic depression.

This study and others (Schmidt et al., 1967) demonstrated that benzodiazepines prolong the DRP. However, it is not clear why an agent which is thought to enhance the action of GABA would not augment the DRP amplitude throughout the whole time course of the PAD instead of preferentially increasing the late phase only. If it is true that benzodiazepines prolong the GABA induced chloride ion flux by influencing the channel opening frequency (Study and Barker, 1981) then the duration of the PAD is still dependent on the presence of GABA in the synaptic cleft although each GABA molecule may be more effective than in the absence of the benzodiazepine. To

get a significant lengthening of the PAD either GABA has to remain around the receptor longer due to a longer release phase or a less efficient reuptake, or there is a threshold effect so a certain amount of chloride efflux is required before there is any measurable DRP. It is difficult to accept that there is a threshold since the efflux of chloride is presumably directly responsible for the depolarization. While it has been shown that chlordiazepoxide causes no change in uptake of GABA (Choi et al., 1977) there is some indication that benzodiazepine can increase the rate of firing of hippocampal basket interneurons (Lee et al., 1979) which presumably release GABA (Ribak et al, 1978). Unfortunately there is no data available on the reaction of spinal interneurons to benzodiazepine administration. There is evidence that GABA release is subject to autoregulation, which is mediated by a novel GABA receptor (Mitchell and Martin, 1978), but this receptor is not coupled to a benzodiazepine receptor (Brennan, 1982). Therefore, benzodiazepines would have to act at a point prior to release to change the amount of GABA released from interneurons.

The benzodiazepine prolongation of PAD is also at odds with the concept of a biphasic PAD presented by Krnjevic and Morris (1975), where GABA is responsible for the early phase, potassium buildup for the later, longer phase. Benzodiazepines would have to prolong the early GABA phase many fold to prolong the entire PAD. This seems unreasonable with the known action of these agents on the single channel kinetics demonstrated by Barker (1981) and would indicate that GABA is present in the synaptic cleft throughout the PAD.

In the second study it was shown that high intensity stimulation lessens the depression seen at 10 Hz on both nerves. If this were due to activity of higher threshold afferents, which would inhibit tonically active interneurons thereby causing primary afferent hyperpolarization (PAH), as suggested by

Rudomin's group (1974), then the neurotransmitter involved must depolarize primary afferents. Mendell (1972) has shown that both PAD and PAH are picrotoxin-sensitive and therefore probably GABAergic. If GABA is the modulated inhibitory transmitter and tonic release does occur, then application of bicuculline or semicarbazide should mimic the state produced by stimulation of high threshold afferents. However their action should also be evident at all frequencies since the drug effect would not be linked to the previous stimulus. As bicuculline has been shown to block the depolarizing action of GABA (Curtis and Lodge, 1982; Sastry, 1979) it is likely that the $GABA_A$ receptor is mediating PAD. However, our results show that bicuculline does not lessen depression of the BST ETR at 10 Hz as predicted, therefore there is probably no tonic GABA release which affects Ia primary afferents by means of the $GABA_A$ receptor. On the other hand, semicarbazide does reduce depression of BST in the appropriate manner. Therefore there maybe a $GABA_B$ component in the depression. There are two other explanations for this phenomenon: semicarbazide affects another transmitter system or the membranes of motoneurons also have bicuculline insensitive GABA receptors on their surface which influence their functioning. Since the depression of TS is not changed by either semicarbazide or bicuculline, GABA is probably not involved in the tonic modulation of homosynaptic depression of this pathway.

After the submission of the MPA paper, evidence was published supporting the finding that the ability of MPA to cause short latency convulsions is probably due to its effect on transmitter release rather than interfering with GABA metabolic processes. MPA enhances the release of excitant amino acids from rat brain slices (Skerritt et al. 1983). While this does not explain our observation of depression of the excitatory transmission in the spinal cord, and is in fact an opposite observation, it further substantiates the inappropriateness of MPA as a tool. Consequently, previous studies (Taberner,

1975; Stone and Savid, 1980) where MPA was thought to be a specific GABA depleting agent should be reinterpreted in light of these new findings.

There is still no ideal agent for the reduction of GABAergic transmission in an in vivo system. Hydrazides such as semicarbazide or thiosemicarbazide have been used successfully (Bell and Anderson, 1972; Polc et al., 1974) but they cannot be entirely trusted because of their very unspecific mode of action. The presently available suicide inhibitors of GAD are of very limited use because they do not cross the blood brain barrier (Jung, personal communication). There is also no published data on their ability to enter the cell although other unpublished data has documented a reduction in cellular GABA levels in chick embryo neuronal cells (from Chrystal et al., 1979).

Despite its very short half-life and potent proconvulsant effect, bicuculline and its methochloride and methiodide derivatives can be successively used to block the $GABA_A$ receptor but not the $GABA_B$ receptor. For this reason it is reasonable to expect that a specific GABA synthesis blocker or an hypothetical agent which would specifically block GABA release would have a different action than bicuculline due to an ability to prevent the activation of both GABA receptors. Whether the observed differences between the action of semicarbazide and bicuculline can be explained in this manner could only be determined with more experimentation using a $GABA_B$ blocking agent in conjunction with bicuculline to see if the action of semicarbazide could be duplicated.

From the observations reported in the last two papers it is evident that the stimulation history of the BST and TS pathways has to be considered since the second response and plateau react differently to some drug manipulations. Because of the complexity of the system, there are probably many factors which influence the size of the MSR. With stimulation, one or a combination of a few

of these factors may change. Therefore it is almost impossible to elucidate all the components responsible for the plateau level. Only with the BST ETR were we able to show that a GABAergic PAD component was one of the changing factors if benzodiazepines were present to prolong its action. However, this conclusion was only arrived at because it was known that anti-GABA agents and Ro15-1788 blocked this action, along with many pieces of information gathered from the literature. Interpreting TS data was more difficult since the first response increased with almost any drug application and all other changes were small.

It is apparent from this study that the single responses evoked by stimulation of the two nerves did not react similarly to drug treatments. This was evident in the MPA study where TS was more profoundly depressed than BST by MPA application and in the homosynaptic depression experiments in which the first TS response was increased by clonazepam, bicuculline and semicarbazide but that of BST remained constant. Since TS MSR grew when GABAergic transmission were presumably enhanced by clonazepam and also with application of antiGABA agents such as semicarbazide and bicuculline, the influence of GABAergic transmission on the TS pathway may be complex. The known differences between TS and BST noted in the introduction are not sufficient to explain these findings. It is widely known by investigators in the field that it is much easier to obtain a stable MSR with stimulation of BST than with TS, unfortunately there is no rigorous explanation for this phenomenon.

While it is clear that something intrinsic to the group Ia afferents causes homosynaptic depression as demonstrated by Lloyd's group (1957), it is also evident from this work that external influences set up by group I or higher threshold afferents can change the basic pattern of depression. Consequently, any drug-induced change cannot be interpreted solely on what is known about homosynaptic depression. It is imperative to catalogue the

reaction of this neuronal system to changes in stimulation conditions in an attempt to determine all possible influences before making statements about a drug's mechanism of action. In our case, such knowledge may have been useful in unravelling the drug action on the behavior of the TS pathway. Some manipulation of stimulation paradigms was done for BST to answer a specific question, that is whether this pathway's recovery pattern was the same after a train of stimuli as after only one stimulus, with and without clonazepam present. However, due to time limitations and no firm grasp as to what was happening in the TS pathway on which to design a discriminating experiment, further work on this nerve was not attempted.

The evidence presented in this thesis indicates that clonazepam indirectly enhances homosynaptic depression by increasing the duration of the primary afferent depolarization caused by the previous stimulation. With further stimulation, the influence of the PAD also undergoes depression so that within a few impulses it is unable to depress transmitter release by the next impulse. The degree to which benzodiazepines change homosynaptic depression of any particular muscle pathway is determined by the amount of primary afferent depolarization produced by the afferents of that same muscle. From this it can be predicted that benzodiazepines will reduce the efficiency of the flexor stretch reflex arcs more than those of the extensors. In conclusion, benzodiazepines do not have a direct effect on homosynaptic depression but increase the strength of the GABAergic presynaptic inhibition.

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CHAPTER 6

SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Clonazepam (0.5 mg/kg) caused a unique change in the pattern of homosynaptic depression of the BST monosynaptic responses. More specifically, clonazepam caused further depression of the second response of the ETR but did not alter the first response or the average of the last five responses.

The ETR elicited by stimulation of TS was altered in a different manner than that of the BST by clonazepam. Clonazepam increased the amplitude of first response and diminished the depression of the second response without changing the plateau. The difference in responses of the two pathways suggests that one or more processes, not common to both pathways, were being modified by clonazepam.

Administration of the central benzodiazepine receptor antagonist, Ro15-1788 did not change the BST or TS ETR, indicating that homosynaptic depression was not modulated at the frequencies tested by an endogenous ligand.

Ro15-1788 antagonized the action of clonazepam on the BST and TS ETR, therefore the central benzodiazepine receptor mediated this action of clonazepam.

Bicuculline directly applied to the cord surface, did not change the BST ETR and only slightly increased the plateau at 10 Hz of TS, indicating that, in this range of frequencies, there is little or no modulation of homosynaptic depression via the $GABA_A$ receptor.

Two hours after the administration of 200 mg/kg semicarbazide, the depression of the second response and plateau of the BST ETR was reduced with no change in the amplitude of the first response. It would appear that homosynaptic depression in the BST pathway is modulated by a $GABA_B$ receptor mediated event or that semicarbazide has an effect in a manner unrelated to the GABAergic system.

The depression of the BST second response normally caused by clonazepam

was prevented by prior administration of semicarbazide and by topical application of bicuculline to the cord. Therefore clonazepam changed the pattern of homosynaptic depression by increasing the efficacy of GABA.

The action of clonazepam on the BST second response was evident up to 500 ms stimulus interval with no effect at longer intervals. When the BST pathway was preconditioned with 9 stimuli presented at 10 Hz, clonazepam did not affect the pattern of synaptic recovery of the tenth response.

It is concluded that clonazepam causes depression of the BST second response by enhancing and lengthening the PAD in BST primary afferents elicited by the first stimulation in the train. In the absence of benzodiazepines the PAD does not last more than 100 ms therefore the BST primary afferent terminals are fully repolarized when the second response is elicited. However, with prolongation of PAD by benzodiazepines, the terminals are not fully repolarized at the instant the second action potential arrives and hence release less transmitter. With further stimulation the PAD also undergoes depression and within 3-5 pulses and, even with a benzodiazepine present, becomes too brief to last the 100 ms between stimuli. As a consequence benzodiazepines are unable to change the plateau level.

A similar depressive effect of clonazepam on TS does not occur because stimulations of extensors, such as TS, causes very little PAD of their own or other afferents.

Compared to 0.5 mg/kg clonazepam, 1.0 mg/kg diazepam caused similar changes in the BST ETR and DRP but had different effects on the TS ETR. Diazepam did not significantly affect the first response or the second response but did reduce the depression of the plateau.

Activation of high threshold afferents will reduce the extent of homosynaptic depression especially that seen with high rates of stimulation.

3-mercaptopropionic acid was shown to be an unsuitable GABA depleting agent in the cat spinal cord because excitatory transmission between the group Ia primary afferents and alpha-motoneurons was affected faster and to a greater extent than the GABAergic dorsal root potential and presynaptic inhibition.