

An Enquiry into the Neurochemical, Neuroanatomical, and
Electrophysiological Basis of Benzodiazepine-Induced Spatial Learning
Deficits in the Rat

by

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Robert Keith McNamara
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M.Sc., University of Victoria, 1990

DEAN

DATE

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We accept this thesis as conforming
to the required standard

Dr. R. W. Skeiton, Supervisor (Department of Psychology)

Dr. M. E. Corcoran, Department Member (Department of Psychology)

Dr. B. Goldwater, Department Member (Department of Psychology)

Dr. D. Paul, Outside Member (Department of Biology)

Dr. A. G. Phillips, External Examiner (Department of Psychology, U.B.C.)

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University of Victoria

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Supervisor: Dr. Ronald W. Skelton

ABSTRACT

Benzodiazepine (BZ) drugs, such as diazepam (Valium®) and chlordiazepoxide (Librium®), are widely prescribed for their sedative/anxiolytic properties but also impair mnemonic processes in both humans and animals. In the Morris water maze, an aversively motivated spatial learning task, BZs impair spatial learning but spare retention/performance. This spatial learning deficit cannot be attributed to sedation, gross sensorimotor impairments, hypothermia, state-dependent learning, or reductions of escape motivation (anxiolysis). The following series of experiments sought to further characterize the neurochemical, neuroanatomical, and electrophysiological substrates of BZ-induced impairments of spatial learning. In Experiment I, the role of endogenous BZs in spatial learning was assessed. The BZ receptor antagonists flumazenil (Ro 15-1788) and CGS 8216, as well as the BZ receptor inverse-agonist β -carboline, enhanced spatial learning in an inverted-U dose-dependent manner, suggesting that endogenously released BZs impede optimal learning. In Experiment II, the role of the BZ ω_1 receptor subtype in spatial learning was assessed. CL 218,872, a selective agonist for the BZ ω_1 receptor subtype, impaired spatial learning in a dose-dependent and flumazenil-reversible manner, thereby implicating the ω_1 receptor subtype in BZ-induced amnesia. Together these results suggest that endogenous BZs activity, like BZ drugs, is detrimental to spatial learning and that specific BZ receptors mediate this impairment.

Several neurochemical systems are important for spatial learning in the MWM and are influenced by BZs. The contributions of two of these neurochemical systems, the opioids and acetylcholine (ACh), to the spatial learning deficit produced by BZs were assessed. In Experiment III, a better understanding of the role of opioid systems in spatial learning was sought. Morphine, a prototypical opioid, impaired spatial learning in a dose-dependent and naloxone-reversible manner. However, morphine also impaired performance and escape to a visible platform and its effects on spatial learning could be attenuated by increasing the escape incentive

(colder water). This impairment pattern suggests that morphine impairs spatial learning by reducing escape motivation. Because both BZs and cold water immersion increase endogenous opioid activity, it seemed possible that the combination of drug- and water-induced opioid release might mediate the spatial learning deficit produced by BZs. In Experiment IV, naloxone, an opioid receptor antagonist, completely blocked the spatial learning deficit produced by morphine but failed, even at a higher dose, to block the spatial learning deficit produced by diazepam. Conversely, flumazenil, a BZ receptor antagonist, completely blocked the spatial learning deficit produced by diazepam but failed to affect the amnesic effects of morphine. Together, these findings strongly suggest that the spatial learning deficit produced by BZs is not due to enhanced opioid activity.

There is also biochemical evidence that BZs interact with ACh systems. In Experiment V, flumazenil attenuated the spatial learning deficit produced by scopolamine, an ACh (muscarinic) antagonist, but physostigmine, an acetylcholinesterase inhibitor, failed to attenuate the spatial learning deficit produced by chlordiazepoxide, even at doses that completely reversed the spatial learning deficit produced by scopolamine. Together these results fail to support the notion that BZs impair spatial learning by reducing ACh activity but suggest that scopolamine impairs spatial learning by enhancing endogenous BZ activity.

Several neuroanatomical regions possess a high density of BZ receptors and are also integral for spatial learning in the MWM. In Experiment VI, infusions of chlordiazepoxide into the medial septum, but not frontal cortex, nucleus basalis magnocellularis, amygdala, hippocampus, or cerebellum, impaired spatial learning but had little effect on anxiety. Conversely, infusions of chlordiazepoxide into the amygdala reduced anxiety but had little effect on spatial learning. These results suggest that the medial septum mediates the amnesic effects of BZs and that the amygdala mediates the anxiolytic effects. In Experiment VII, intraseptal infusions of chlordiazepoxide were additionally found to impair spatial learning in a dose-dependent and flumazenil-reversible manner. However, infusions of flumazenil into the medial septum failed to block the amnesic effects of systemically administered chlordiazepoxide, suggesting that the amnesic

effects of BZs are not mediated by the medial septum exclusively. Tetrahydroaminoacridine, an acetylcholinesterase inhibitor, failed to attenuate the spatial learning deficit produced by intraseptal infusions of chlordiazepoxide, suggesting that the deficit was not due to a disruption of the septohippocampal ACh projection. Together, these results suggest that chlordiazepoxide impairs spatial learning by interacting with the septohippocampal GABAergic projection.

The septohippocampal GABAergic projection regulates the excitability of hippocampal afferents (e.g., perforant path). Experiment VIII assessed the effects of systemically administered BZs on the induction of long-term potentiation (LTP) in the perforant path. CL 218,872, but not chlordiazepoxide or diazepam, significantly suppressed long-term potentiation. However, all drugs impaired spatial learning. These findings suggest that CL 218,872 impairs spatial learning by suppressing LTP but that BZ-induced spatial learning deficits can occur in the absence of perforant path LTP suppression.

Taken together, the above results suggest that endogenous BZ systems, particularly those in the septohippocampal system, are important modulators of mnemonic processes. These findings are discussed in the context of understanding information storage processes and the implications for clinical populations.

Examiners:

Dr. R. W. Skelton, Supervisor (Department of Psychology)

Dr. M. E. Corcoran, Department Member (Department of Psychology)

Dr. B. Goldwater, Department Member (Department of Psychology)

Dr. D. Paul, Outside Member (Department of Biology)

Dr. T. Phillips, External Examiner (Department of Psychology, U.B.C.)

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CHAPTER 1: INTRODUCTION

Benzodiazepine history, use and abuse

Since their synthesis by Hoffmann-La Roche, chlordiazepoxide (Librium®;1955) and its more potent analog diazepam (Valium®;1959), have come to dominate the market for anxiolytic/sedative drugs and are the most widely prescribed drugs in the world (Blackwell, 1973). In North America during 1972, more than 77 million prescriptions for BZs were filled, sixty percent of which were for diazepam (Greenblatt & Shader, 1974). In 1977, 54 million prescriptions were filled for diazepam and 13 million for chlordiazepoxide (Skegg & Perry, 1977). In Canada in 1984, approximately 1 of every 10 Canadians reports using BZs at least once per year, and typically 10 percent of these users continue their use for more than 1 year (Balter, Manheimer & Mellinger, 1984). BZ drug use is higher among adults aged 50 years or over and as the proportion of the aged population in Canada increases, a parallel increase in BZ consumption is expected (Balter et al., 1984). A recent longitudinal study conducted in Canada revealed that BZ consumption between 1978 and 1987 increased by 145 percent and that there is a general trend away from slowly metabolized BZs, such as diazepam and chlordiazepoxide, in favor of the rapidly metabolized BZs, such as lorazepam and triazolam (Busto, Lanctot, Isaac & Adrian, 1989). Because these compounds are so commonly prescribed, their effects on human performance, particularly in the elderly, warrant further characterization.

Although most BZ drugs are prescribed primarily for their anxiolytic and sedative/hypnotic properties (Rickels, 1978), several side effects appear significant: (1) long-term consumption of BZ drugs can lead to dependence (Petursson & Lader, 1981), though BZ-associated deaths are rare (Finkle, McCloskey & Goodman, 1979), (2) BZs are known to produce "paradoxical" rage attacks, increased incidence of hostile-aggressive feelings and BZ drugs are often used, in combination with other sedatives such as alcohol, to commit suicide (Zisook & DeVaul, 1977) and (3) BZ drugs impair cognitive processes, most notably learning and memory (Lister, 1985).

Hence, in addition their efficacy as anxiolytic sedative/hypnotics, BZ drugs also have properties that are clearly undesirable for the clinical population.

Benzodiazepine-induced amnesia: Human studies

BZ drugs were discovered to have amnesic properties soon after their release onto the market in the early 1960s (Feldman, 1963; Knight & Burgess, 1968). The amnesic effects of BZs were first observed in the clinical setting where intravenous diazepam was used as a premedicant (Knight & Burgess, 1968). In these settings, it was discovered that patients administered diazepam prior to surgery could recall little about the presurgical environment or events leading up to surgery (Knight & Burgess, 1968). Initially this effect was considered a positive side-effect of diazepam, since it would "erase" the anxiety-provoking presurgical events from the patient's memory. While these early observations were often poorly controlled and often confounded by the administration of other anesthetics (e.g., Feldman, 1963), numerous, well controlled experiments have subsequently confirmed that BZs impair the storage of information (see Cole, 1986; Curran, 1986; Ghoneim & Mewaldt, 1990; Lister, 1985; Romney & Angus, 1984 for reviews).

Although the amnesic effects of BZs may be considered a positive side-effect in presurgical situations, problems may arise clinically because [1] BZs are so widely prescribed and are typically taken while the patient is engaged in their daily activities, [2] little tolerance develops to the amnesic effects (Ghoneim, Mewaldt, Berie, & Hinrichs, 1981; Griffiths, McLeod, Bigelow, Liebson & Roache, 1984; Petersen & Ghoneim, 1980), [3] subjects may not be subjectively aware of their memory impairment, even after doing poorly on memory tasks (Hinrichs, Mewaldt, Ghoneim, & Berie, 1982), [4] the amnesia can last up to 14 hours after BZ administration (Ghoneim et al., 1981), [5] the use of BZs in a psychiatric context (i.e., to combat phobias) may hinder habituation or the acquisition of appropriate coping skills (Hafner & Marks, 1976), and [6] geriatric populations are primary consumers of BZs despite a heightened susceptibility to their amnesic effects (Cook, Flanagan, & James, 1984; Nikaido, Ellinwood, Heatherly & Gupta, 1990; Pomara, Stanley, Block, Guido, Stanley, Greenblatt, Newton, & Gershon, 1984). Regarding the latter issue, a recent study demonstrated that 25% of nursing home patients

diagnosed with dementia of the Alzheimer's type were receiving BZ medication (Beere, Avorn, Soumerai, Everitt, Sherman & Salem, 1988). Hence, the relative clinical utility of BZ drugs needs to be reevaluated in light of these noted liabilities.

Typically, the influences of BZs on human memory processes are studied through testing the recall and recognition of visually or auditorily presented items, usually pictures or infrequently used English words (Ghoneim & Mewaldt, 1990). Other indexes of memory performance, including serial position (Mewaldt, Hinrichs & Ghoneim, 1983) and Wechsler Memory or Benton Visual Retention scales (Gentil, Gorenstein, Camargo & Singer, 1989), have yielded congruent results. Subjects are typically between the ages of 20 and 35 years and the BZ is administered either orally, intravenously, or intramuscularly in doses ranging from 0.1 - 0.3 mg/kg, doses that are commonly prescribed clinically.

Information storage is considered to be a multiple stage process that can occur either immediately or require several hours before true consolidation occurs. A popular model, both conceptually and intuitively, was proposed by Atkinson and Shiffrin (1968) and involves a two-stages, short-term memory and long-term storage. In this model, information must first be registered in short-term memory from which it is transferred to long-term storage (encoding). The pattern of memory failure observed in humans after BZ administration is striking in its selectivity for impairing the transfer of information from short-term memory to long-term storage while having little effect on short-term memory (immediate recall) or retention/retrieval (Lister, 1985). In fact, BZs may actually produce a significant retrograde facilitation of memory (Brown, Brown & Bowes, 1983; Hinrichs, Ghoneim & Mewaldt, 1984). Increasing the dose of the BZ increases the magnitude and duration of the memory impediment (e.g., Ghoneim, Hinrichs, Mewaldt, 1984) and the time course of the amnesic effect can range from 2 minutes to 14 hours after BZ administration (e.g., Ghoneim, Mewaldt, Berie & Hinrichs, 1981).

In addition to a selectivity of BZ drugs to impede information storage, the type of information also appears to be selectively affected by BZ drugs. According to the theory of Squire (1987), memory can be dichotomized into declarative memory (or explicit memory; Graf & Schacter, 1985), information that is directly accessible to conscious recollection (i.e., facts, names, places etc.), and procedural memory (or implicit memory; Graf & Schacter, 1985), which is memory that is contained within learned skills (e.g., playing darts). BZs impair only the storage of declarative information while procedural memories are spared (Danion, Zimmermann, Willard-Schroeder, Grange & Singer, 1989; Fang, Hinrichs & Ghoneim, 1987). For example, diazepam (0.3 mg/kg) impaired the acquisition of declarative information (free recall of word list) but spared a procedural, word-stem completion task. In this task, subjects are presented with a series of words and later asked to complete three-letter word stems; these word stems typically elicit those words previously presented. The selective impairment of declarative memory and the sparing of procedural memory is also found in patients with organic amnesias such as Korsakoff's disease or patients with hippocampal damage (Squire, 1987). The similarity between the mnemonic deficit of BZ-treated subjects and patients with organic amnesia has led to the suggestion that BZ-induced amnesia may represent a convenient neuropharmacological model which may facilitate the understanding the nature of memory and amnesia (Wolkowitz, Weingartner, Thompson, Pickard, Paul & Hommer, 1987).

The failure of BZs to impede short-term memory or retrieval is taken as evidence that the memory impairment is not secondary to the sedation that is typically experienced during testing (Mewaldt et al., 1983). Additional evidence supports the notion that BZ-induced sedation and amnesia are independent processes. Firstly, there is a differential time course of the amnesic and sedative effects; the amnesic effects persist beyond the sedative effects (Pandit, Heisterkamp & Cohen, 1976; Ghoneim et al., 1981). Secondly, differential tolerance develops to the amnesic and sedative effects of BZs; tolerance develops to the sedative effects of BZ well before it develops to the amnesic effects (Lucki, Rickels & Geller, 1986). Together these findings suggest that the BZ-induced amnesia is not a secondary result of sedation.

In sum, BZ drugs are frequently prescribed for their anxiolytic, sedative, and hypnotic properties and are used by a large segment of the population, most notably the elderly. One side-effect of BZ drug administration is an impairment of the transfer of information from short-term memory to long-term storage. More specifically, information that is declarative cannot be encoded, unlike procedural skills which are readily acquired. The amnesic effects of BZs cannot be attributed to their sedative effects. Despite their side-effects, the large experimental and clinical literature provides an opportunity to identify a congruency between the amnesic effects of BZs in humans and animals.

Benzodiazepine-induced amnesia: Animal studies

BZs have been found to impair performance in animals ranging from monkeys to pigeons on a wide variety of learning and memory tasks (see Cole, 1986; Dantzer, 1977; Thiebot, 1985 for reviews). Animal memory tasks commonly employed to study BZ-induced amnesia include avoidance conditioning, discrimination learning, and spatial learning.

The avoidance conditioning task typically involves placing a rat/mouse into one compartment of a two compartment box (one chamber white, one chamber black) separated by a wall with a door. When the rat enters the opposite chamber (typically the black and preferred side), a brief foot-shock is administered. This is the training session. During the test session, typically given 24 h later, the rat is returned to the white chamber and the latency to reenter the black chamber is the index of acquisition/retention. Good acquisition/retention is manifested as a long latency to enter the black chamber. On avoidance conditioning tasks, pre-training administration of a BZ impairs acquisition of the avoidance response but post-training-trial administration has little effect on acquisition (Broekamp, Le Pichon, & Lloyd, 1984; Essman, 1973; Jensen, Martinez, Vasquez, & McGaugh, 1979; Patel, Ciofalo, & Iorio, 1979; Tohyama, Nabeshima, Ichihara, & Kameyama, 1991). It is difficult to attribute these deficits to amnesia, however, since BZs increase pain thresholds (Houser & Pare, 1973; Wuster, Duka & Herz, 1980) and punished responses (Theibot, 1985). Hence, the analgesic effects of BZs may have reduced the significance of the shock and the motivation to learn

(e.g., Kesner & Calder, 1980). Moreover, the BZ-induced impairments of avoidance conditioning can be overcome by re-administering the drug prior to the test session (state-dependent learning; Patel, Ciofalo & Lorio, 1979). Hence, the avoidance conditioning task does not appear suitable for assessing the amnesic effects of BZs.

A second task used to assess the amnesic effects of BZs is simple discrimination. In these tasks, the animal must respond (press a lever for reward) when a given stimulus (light; S⁺) is presented and withhold responding when a second stimulus is offered (S⁻). The S⁺ and S⁻ can be presented together simultaneously or individually in succession. BZs impair successive discrimination acquisition (Cole & Michaleski, 1986; Fukuda & Iwahara, 1976; Ksir & Slifer, 1982; McNaughton, 1985; Nicholson & Wright, 1974; Thompson, 1974) but spare simultaneous discrimination acquisition in rats (Iwasaki, Ezawa, & Iwahara, 1976) and monkeys (Hasegawa, Ibuka, & Iwahara, 1973; Sahgal & Iversen, 1980). The common deficit observed on discrimination tasks is a failure to withhold responding to the S⁻, an effect attributed to the disinhibitory actions of BZs (Cole, 1982, 1983, 1990; Gray, 1983; Tye, Sahgal, & Iversen, 1977). Hence, BZs impair discrimination acquisition by disinhibiting punished/non-rewarded responses rather than learning and memory per se.

In monkeys, lorazepam impaired spatial delayed response performance which required the monkey to retain the spatial location of a symbol on a computer screen (Rupniak, Samson, Stevenson, & Iversen, 1990). In rats, the effects of BZs on spatial learning has been examined using the appetitively motivated radial arm maze (Olton & Samuelson, 1976). On this task, the rat must retrieve a piece of food from the end of several (usually 8) arms/alleys that project out from a central area. The individual arms can be discriminated only by their location relative to ambient room cues. A reference memory error is recorded if the rat enters an arm that has never been baited and a working memory error is recorded if the rat visits the same arm twice during a given test session. Assessment of BZs on radial maze learning has yielded inconsistent results. For example, chlordiazepoxide impaired the rats' ability to acquire the location of a single baited arm on an 8

arm radial maze (reference memory error; Willner & Birbeck, 1986, see also Shumsky & Lucki, 1991). On a cued version of the radial arm maze (i.e., distinguishable arms), rats treated with chlordiazepoxide made both working and reference memory errors (Hodges & Green, 1986). These authors suggested that the errors committed by chlordiazepoxide-treated rats may be attributed to response perseveration, rather than a specific mnemonic deficit. In a separate study, no deficit was observed when the BZ-treated rat was required to retrieve a food pellet from each of eight arms (Hiraga & Iwasaki, 1984). It should be noted, however, that the latter task could be solved without using spatial cues. For example, the rat merely had to continue to turn left upon exiting each arm to achieve a perfect score. In sum, although BZs impair performance on the radial arm maze, this deficit may be attributed to response perseveration rather than to a true mnemonic impairment.

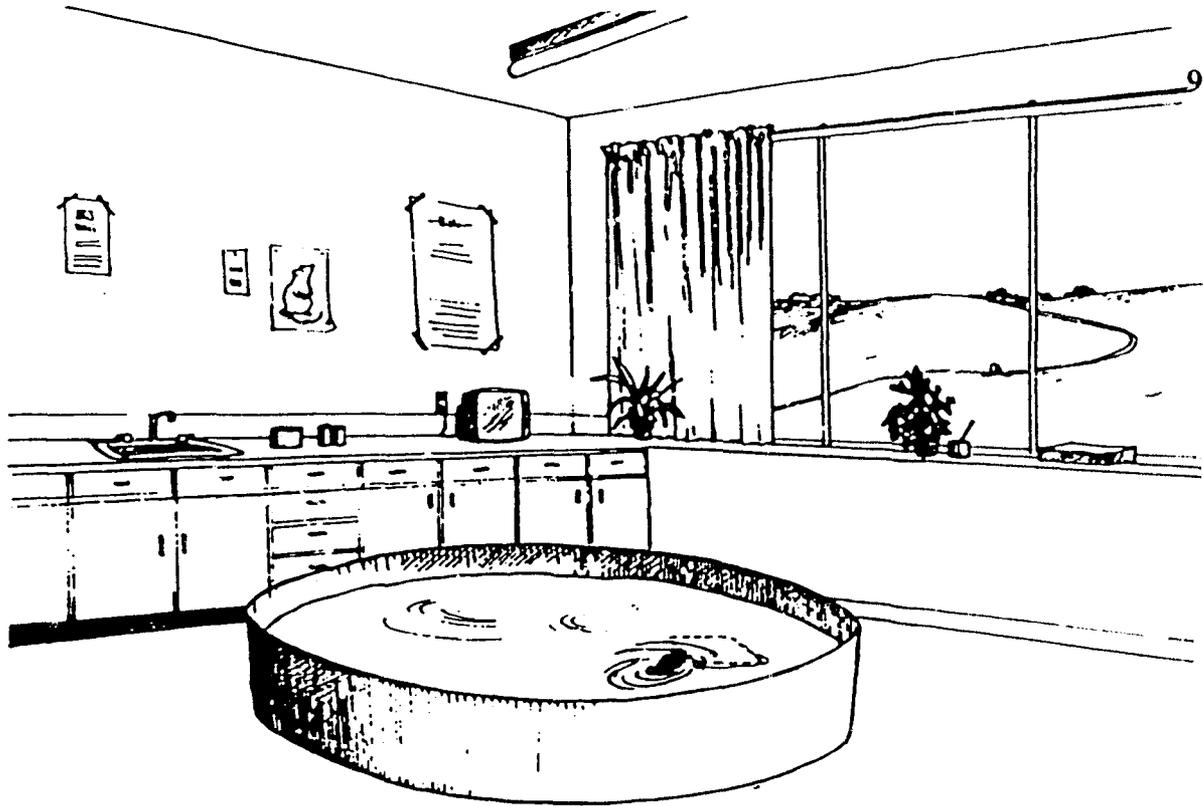
In sum, performance across a variety of memory tasks is impaired by pretraining BZ administration. However, it is difficult to attribute these impairments to a specific disruption of mnemonic processes per se. A task that appears well suited to the assessment of BZs on learning and memory processes is the Morris water maze (Morris, 1981; Morris, 1984).

The Morris water maze

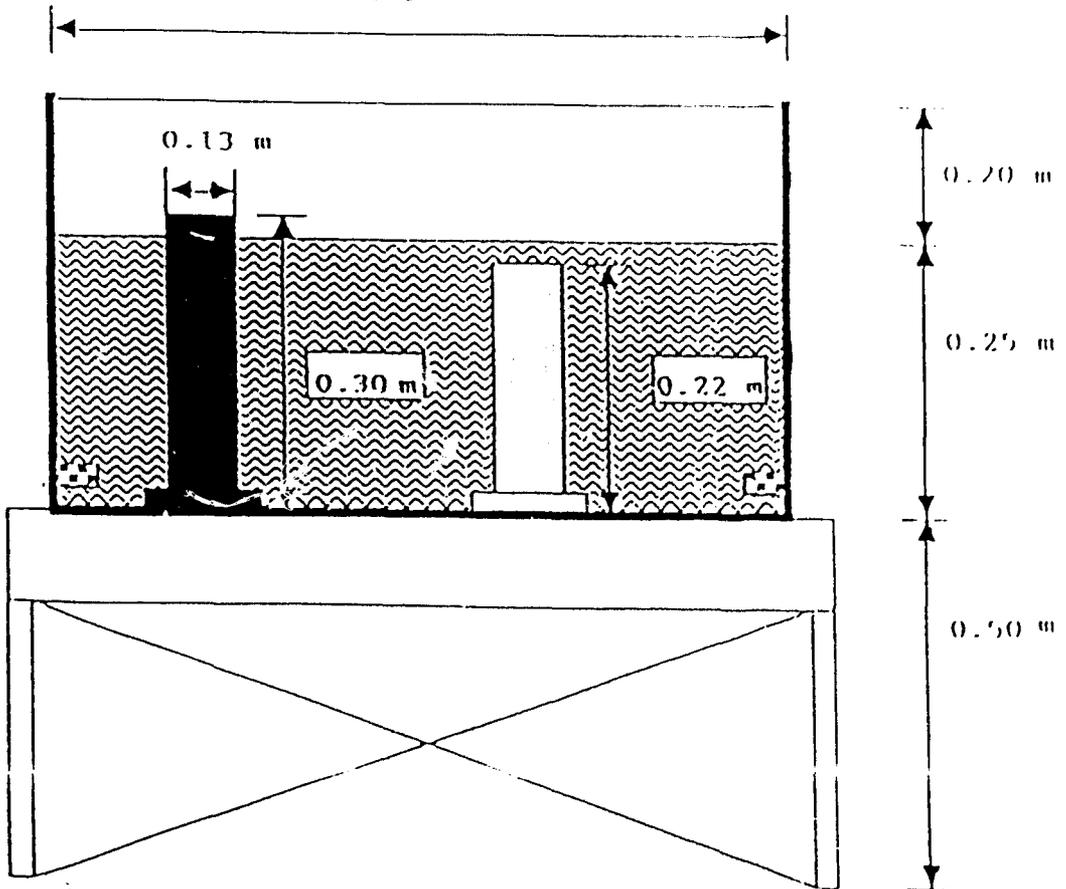
The Morris water maze (MWM; Fig. 1.1) consists of a large circular pool filled with cool water rendered opaque with milk powder. Submerged somewhere within the pool is a platform onto which the rat can climb to emerge from the cool water and escape from the necessity of swimming. In the standard use of the MWM, the rat is placed into the pool at one of several randomly ordered start locations near the wall (e.g., north, south, west, or east "pole") and swims to a submerged platform maintained in a fixed position (e.g., center of the northwest quadrant) throughout training. Though many variations have been used, and many more are possible.

The MWM was originally developed to test rats' abilities to learn, remember, and go to a place in space defined only by its position relative to distal, extramaze cues. Because the rat is generally placed into the pool from

Figure 1.1: Illustration of the Morris water maze. (top illustration adapted from "Plasticity in the Neocortex: Mechanisms Underlying Recovery From Early Brain Damage" by Kolb, B., and Whishaw, I. Q., 1989, Progress in Neurobiology, 32, p. 242. © 1989 by Pergamon Press. Adapted with permission.



1.5 m



a variety of start locations (usually near the wall), and because there are no cues within the pool to guide the rat to the platform, most rats solve this maze by resorting to a spatial (locale) strategy (O'Keefe & Nadel, 1978). Other possible strategies include cue (taxon) strategies in which the rat guides its movements relative to a single cue in the environment, or response (praxis) strategies in which the rat moves according to a specific sequence of movements, though these strategies are less efficient in the MWM and are generally not considered to represent true spatial learning (O'Keefe & Nadel, 1978). In this thesis, "spatial learning" will refer to acquisition of knowledge of a submerged platform location based on extra-maze cues (a locale strategy). The new term "spatial recall" will be used to refer to the demonstration of the knowledge of a submerged platform location acquired prior to anatomical or pharmacological manipulations. "Cue learning" will refer to acquisition of an escape response to a platform clearly identified by an unambiguous, proximal cue (e.g, the platform itself, visible above the surface of the water).

A great strength of the MWM is the availability of procedures for evaluating the strategy being used to locate the platform, and for dissociating pharmacological impairments of memory processes from non-mnemonic performance deficits. First and foremost are probe trials (transfer tests) in which the rat is permitted to swim freely about the pool without any platform present. The use of a place strategy is inferred if the rat spends more time in the quadrant that previously contained the platform, or crosses over the old platform position (annulus) more often than equivalent positions in the other three quadrants. These measures quantify the strength and accuracy of the original learning. Alternative strategies, such as cue (taxon) or response (praxis) strategies, are revealed by swim paths that fail to be biased towards a particular quadrant or platform location and which either describe large circles at the appropriate distance from the pool wall or which seem to be directed towards or away from a single cue (such as the experimenter).

A second valuable procedure is cue learning, which involves the use of a single proximal cue to identify the location of the platform. Usually, the

platform is simply visible above the surface and the rat is required only to learn to swim to it and climb on. This task typically requires only one trial to master, and is capable of revealing deficits in sensory, motor, sensorimotor, or motivational processes. The visible platform can be maintained in one position, or moved from trial to trial in order to prevent the use of a spatial strategy (e.g., Whishaw & Mittleman, 1986). This task may best be viewed as a minimally acceptable control procedure for it may be sensitive only to relatively gross deficits. A more sensitive (though less frequently used) procedure is the spatial recall test. This consists of submerged platform or probe trials given to lesioned or drugged rats that learned the location of the platform earlier, while still intact and undrugged. Spatial recall tests should reveal impairments to almost all of the processes required for proper performance of the task (sensory, motor, motivation, memory retrieval, spatial information processing) except for processes required for learning and for memory formation. A fourth valuable procedure requires the rat to learn to discriminate between two visible platforms, only one of which will support the weight of the rat. The correct platform is distinguished either by its position in space (spatial version) or by its visual appearance (non-spatial version). Deficits of the non-spatial task reveal impairments of simple discrimination learning or sensory/perceptual processes, whereas deficits specific to the spatial version reveal impairments of spatial learning.

In addition to the ability of the MWM to dissociate learning, memory, and performance deficits and its paradigmatic flexibility, several components of this task commend it over other animal memory tasks such as avoidance conditioning or more traditional measures of spatial memory, such as the T-maze or the radial-arm maze. For example: 1) both learning and cognitive performance can be assessed simultaneously over the course of training, 2) swim speed can be used to assess motoric and motivational deficits within each learning trial, 3) intramaze cues such as odour trails are obviated, 4) no pretraining is required and acquisition is quite rapid (5-16 trials to asymptote), allowing for large number of animals/treatments to be assessed in short periods of time, 5) rats can be tested and retested over several days or manipulations, 6) shock administration and food deprivation are not necessary, thereby reducing the stress to the animal, and 7) the level of

motivation [i.e., water temperature (McNamara & Skelton, 1991B)] can be manipulated. Although it is possible that immersion into cool water may cause physiological events which interact with pharmacological manipulations in an undefined manner, the high concordance between the effects of pharmacological manipulations on spatial learning in the aversively motivated MWM and the appetitively motivated radial arm maze [see (Levin, 1988) for a review of radial arm maze pharmacology], suggests that both tasks assess a common substrate of learning and memory and that the cool water produces no substantial alteration in physiological responses to drugs.

Effects of BZs on MWM acquisition

The seminal description of the effects of BZs on spatial learning in the MWM was conducted by McNaughton and Morris (1987). These authors found that a single dose of chlordiazepoxide (5 mg/kg) impaired MWM acquisition, increasing the distance to located the platform over the course of three days training. When the platform was removed from the pool, control rats showed a bias for the quadrant that had contained the platform while rats treated with chlordiazepoxide failed to show such a preference. The latter finding is taken as evidence that the rat did not acquire the spatial location of the platform. In subsequent investigations, it was demonstrated that diazepam impaired spatial learning in a dose-dependent and flumazenil reversible manner (Arolfa & Brioni, 1991; McNamara & Whishaw, 1991). For example, diazepam impaired a learning-set task that required the rat to acquire a new platform location each day over a series of days (McNamara & Whishaw, 1991). This learning-set does not require the retention of the prior days training, in fact forgetting the previous platform location would be advantageous. Hence, diazepam impairs acquisition that requires information to be stored for only a short time (~ 5 min) while sparing retention processes. It was further found in this study that diazepam did not impair cue learning, suggesting that diazepam does not impair simple associative learning processes, escape motivation, or produce gross sensorimotor deficits. These data suggest that BZs impair spatial learning in the MWM by impairing the acquisition of spatial information.

While the above data suggest that BZs selectively impair mnemonic processes, it is still possible that BZs affect other aspects of performance that prevent the expression of what has been acquired. However, we recently demonstrated (McNamara & Skelton, 1991A) that rats that learn the platform location prior to receiving an amnesic dose of diazepam have total saving of the platform location (Fig. 1.2A, B). During the probe trial, rats switched to diazepam post-acquisition show a bias for the correct quadrant to a degree equal with controls (Fig. 1.3A). Representative swim paths taken during the probe trial for each treatment group (Fig. 1.4) shows that the group switched to diazepam (Switch) swim in manner more similar to the diazepam group (wide loops) but are able to concentrate their time in the correct quadrant. Moreover, the switch group showed proficiency despite reductions of core-body temperature (Fig. 1.5). When the location of the submerged platform was moved to a new location, the switch group showed an acquisition impairment (Fig. 1.6, Fig. 1.7), as did rats treated with diazepam from the beginning of training (Diazepam in figures), and rats treated with saline during initial acquisition and given diazepam during reversal training (saline-diazepam: SD in figures). However, rats treated with diazepam during initial acquisition and given saline during reversal (diazepam-saline: DS in figures) training acquired the reversed platform location at control levels.

Diazepam has sedative properties which have two consequences: attentional/perceptual impairments and myorelaxation. In the present study, the place-learning deficit cannot be attributed to the myorelaxation effects of diazepam for three reasons: [1] the Diazepam group swam consistently slower than the Saline group, even as the group reached criterion levels, [2] the Switch group did not show impaired maze performance in spite of a reduction in swim speed (Fig. 1.2C), and [3] the Diazepam group did not swim slower during the probe trials (Figs. 1.4B, 1.7B). These results suggest that the place-learning deficit produced by diazepam cannot be attributed to the myorelaxation. Neither could the deficits have been due to perceptual/attentional factors. In the present study, the Switch group, while receiving diazepam, was still able to navigate to the hidden platform. Because the distance taken to locate the platform did not

Figure 1.2: Effects of diazepam on (A) the distance taken to locate the escape platform, (B) heading errors, and (C) swim speeds over the course of training. Note that the preadministration of diazepam resulted in greater distances taken to locate the platform as well as heading errors. Also when the Switch group was switched from saline to diazepam on day 7, neither the distance nor heading errors increased substantially, despite reductions of swim speed.

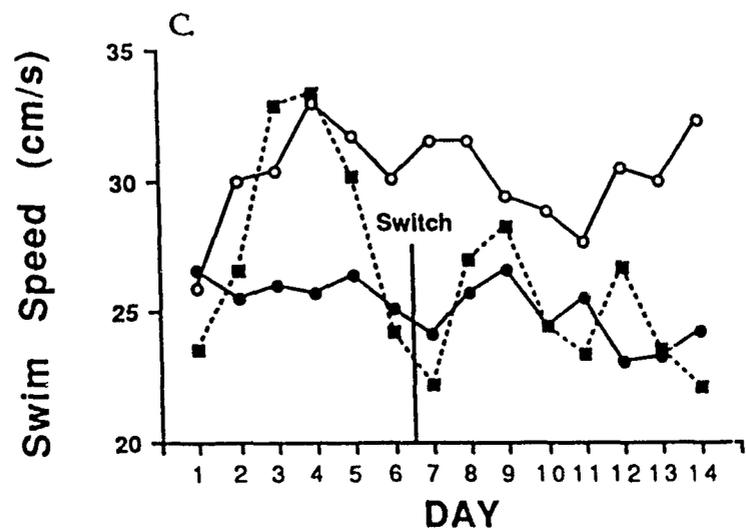
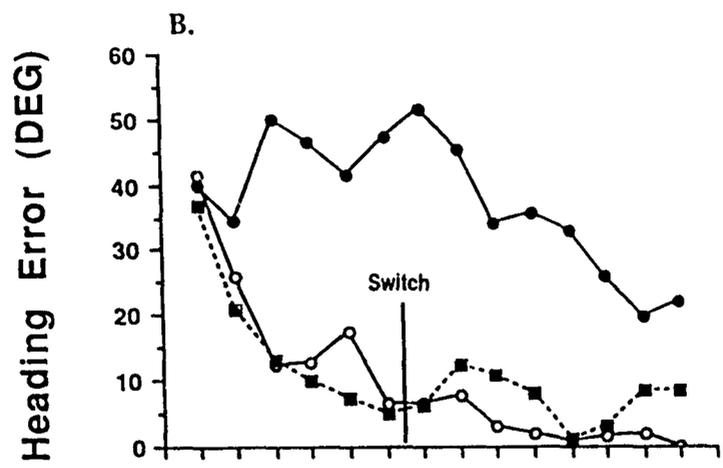
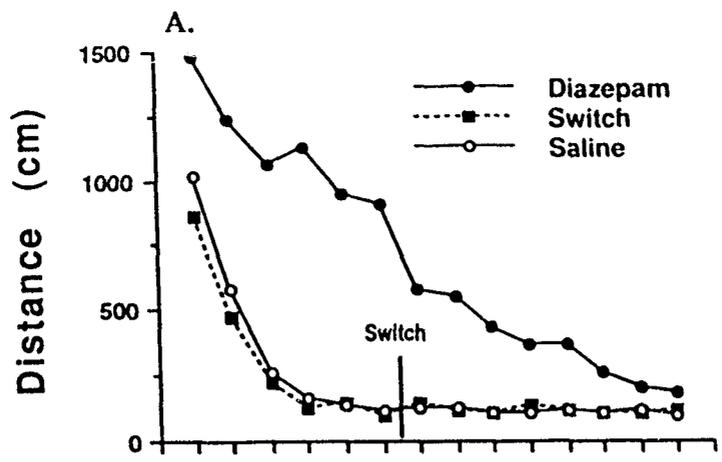


Figure 1.3: Effects of diazepam on (A) the distance spent in the correct quadrant, (B) swim speed, and (C) post-swim T_c (post-swim - pre-swim) during the first probe trial. Note that both the Saline and Switch groups, but not the Diazepam group, demonstrated an 'above chance' bias for the quadrant that previously contained the escape platform. Also, none of the group's swim speeds differed during the probe trial. Finally, only the Saline group's post-swim T_c was reduced. Data expressed as mean \pm S.E.M.. * $p < 0.01$ compared to chance level (25%) in (A) and to Saline T_c in (C).

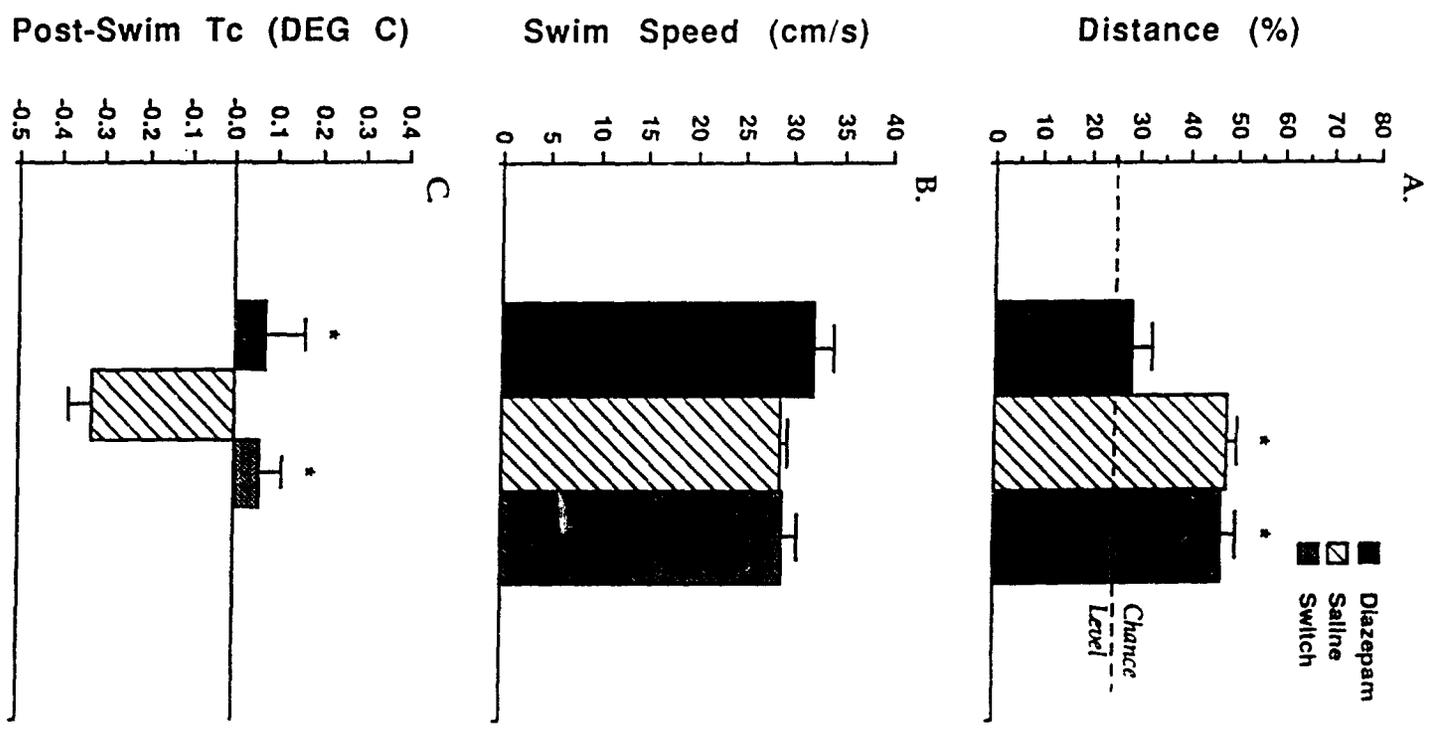
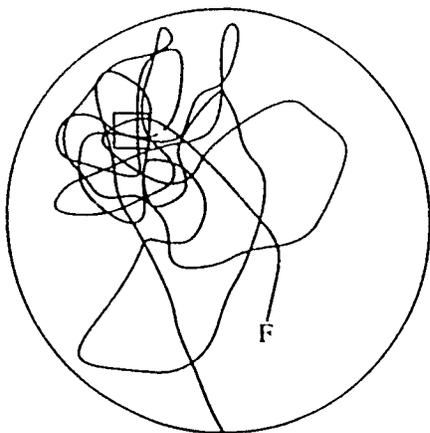
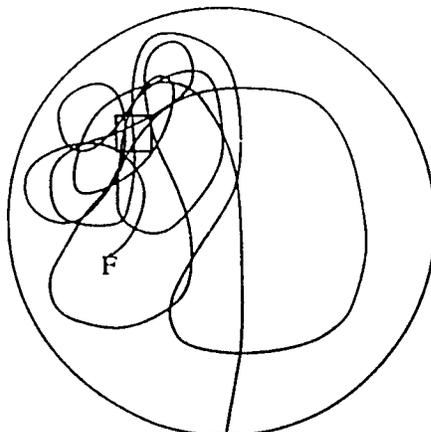


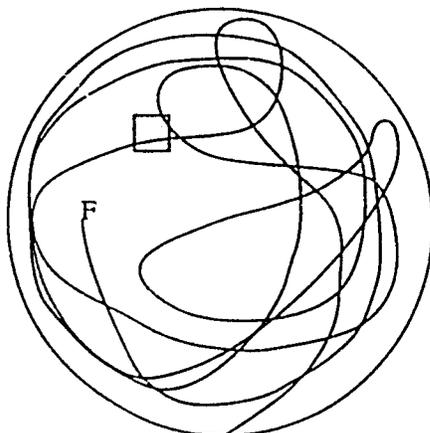
Figure 1.4: Representative swim paths during the first probe trial. Note that the Switch group rat, despite having an elongated and circuitous swim path, still spent the majority of time in the correct quadrant. The 'F' denotes where the rat was removed from the pool after the trial was finished.



Saline

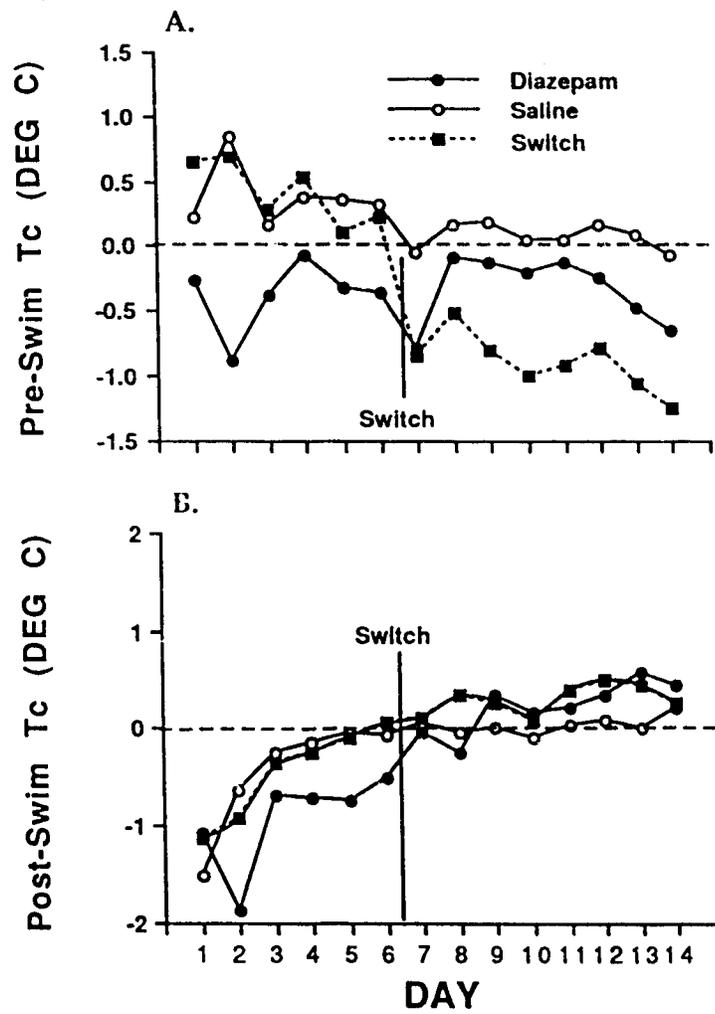


Switch



Diazepam

Figure 1.5: Effects of diazepam on (A) pre-swim T_c (pre-swim - pre-drug) and (B) post-swim T_c (post-swim - pre-swim) during initial acquisition. Note: [1] the consistently lower pre-swim T_c over the course of testing, [2] when the Switch group is switched to diazepam on day 7, the pre-swim T_c is reduced and [3] all three groups show increases in post-swim T_c over the course of testing (B).



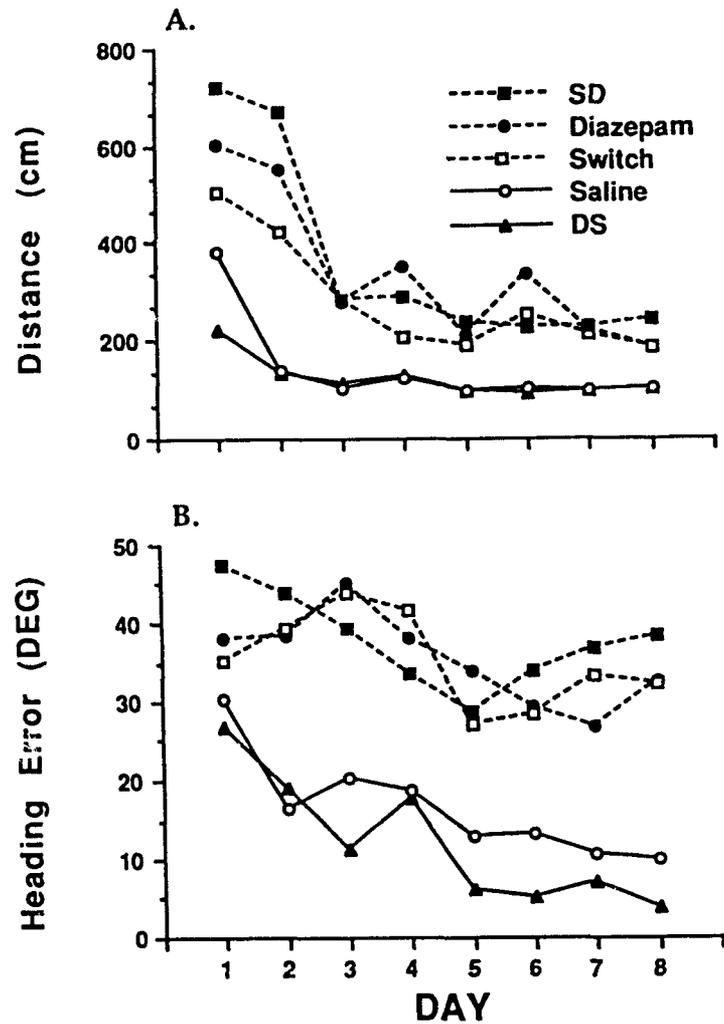
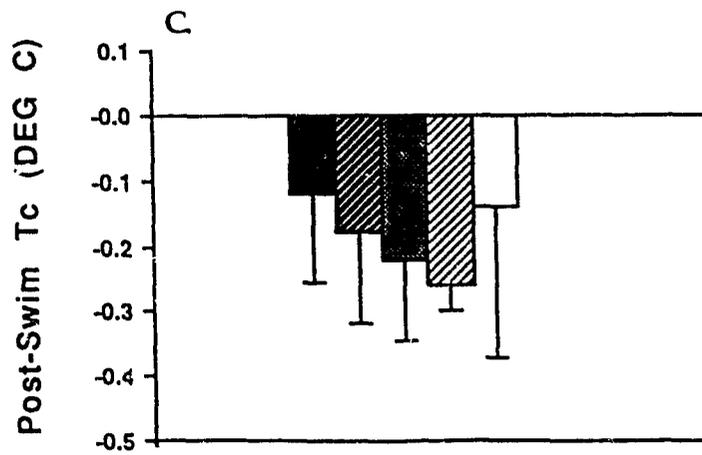
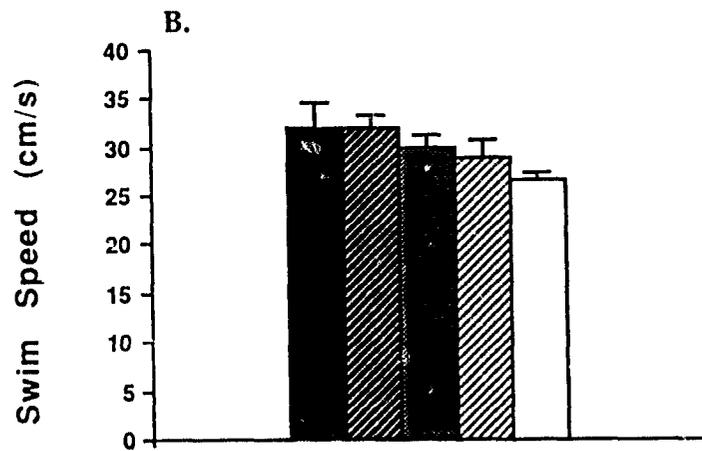
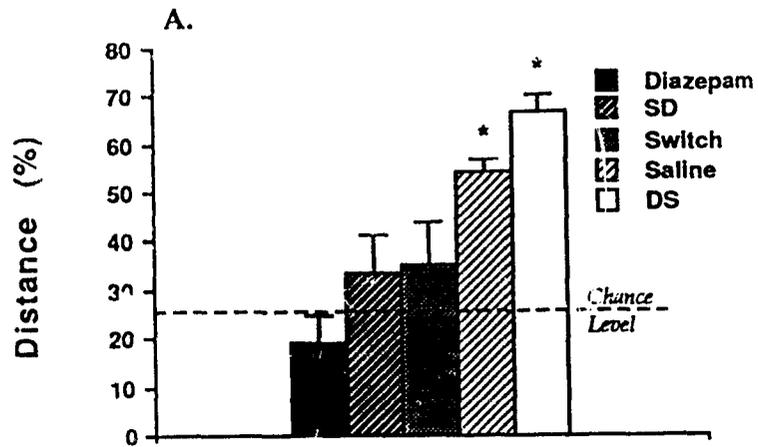


Figure 1.6: An illustration of (A) the distance taken to locate the submerged platform and (B) heading errors during reversal acquisition. Note that those groups receiving diazepam (saline → diazepam-SD, Diazepam, Switch) have greater distances and heading errors.

Figure 1.7: The percentage distance in the correct quadrant (A), swim speed (B), and post-swim T_c (pre-swim - post-swim; C) during the final probe trial. Note that the saline treated groups (Saline and DS), but not the groups receiving diazepam (Diazepam, SD, Switch), demonstrate a preference for the correct quadrant, despite comparable swim speeds and post-swim T_c s. * $p < 0.01$ compared to chance level (25%).



increase when diazepam was administered, it is likely that the Switch group continued to use the same efficient strategy. Further, the Diazepam group was not impaired when required to navigate to single, visible platform. These findings suggest that the diazepam-treated rat can perceive and use distal spatial cues to locate the platform.

The BZ-induced acquisition impairment in the MWM may be due to hypothermia. Studies have demonstrated that diazepam can induce hypothermia (present results, Zarrindast & Dibayan, 1989), and hypothermia alone can produce anterograde amnesia (Richardson et al., 1983). Hence, the combination of both drug-induced and water-induced hypothermia might be sufficient to impair spatial learning. However, three results argue against this interpretation: [1] the pre-swim hypothermia remained consistently low throughout acquisition (even though the animals acquired the platform location (Fig. 1.3A), [2] the body temperature in the Diazepam group did not decrease during swimming (post-swim T_c) more than that of controls (Fig. 1.3B), and [3] the Diazepam group was impaired but not hypothermic on all three probe trials (Figs. 1.4C, 1.7C). Further, the average change in the body temperature for the groups receiving diazepam was -0.35°C (± 0.1) prior to swimming and -0.21°C ($\pm 0.2^{\circ}\text{C}$) after swimming. These drops in body temperature are not as severe as those previously found to induce amnesia, which typically exceed 5°C above or below normothermia (Richardson et al., 1983). Together, these results suggest that hypothermia did not produce the observed anterograde amnesia.

Previously, the amnesic effects of BZs have been attributed to state-dependent learning (Patel et al., 1979), but such was not the case here. Here, rats administered diazepam during both acquisition and retrieval (Diazepam group) were impaired whereas rats trained under saline and switched to diazepam (Switch group) were not. Furthermore, the group which displayed the most proactive interference (as indicated by performance on first day of reversal training) in the reversal phase was the one that had been trained under saline and then reversed to diazepam. These results are opposite to what would have been predicted by the state-dependent learning hypothesis (Overton, 1974).

Although the diazepam groups eventually learned to swim to the submerged platform in both the initial acquisition and reversal phases, it was clear that these rats never acquired its spatial location. This was demonstrated in the probe trials, in which the diazepam-treated rats swam randomly about the pool, failing to concentrate their search in the correct quadrant. Indeed, it appeared that diazepam produced a total anterograde amnesia and the rats adopted alternative strategies to locate the platform. For example, this may have included a 'taxon' strategy, such as swimming towards or away from a single cue, or a 'praxis' strategy, such as swimming in a particular pattern (e.g., sequence of loops). Support for the latter strategy comes from the finding that diazepam-treated rats typically swam in a circular pattern until eventually bumping into the platform. This 'praxis' strategy can be seen in the illustrative swim paths drawn from the first probe trial (Fig. 1.5). These findings suggest that diazepam produced a severe and persisting anterograde amnesia which necessitated the adoption of a response-based search strategy.

In summary, BZs impair spatial learning, but not spatial recall, in the MWM. This impairment does not appear to be the result of BZ-induced impairments of motorical efficiency, perception/attention, retention/retrieval, or motivation (anxiolysis). Further, the deficit cannot be accounted for by hypothermia or state-dependent learning. Finally, BZ-treated rats appear to adopt non-mnemonic search strategies to compensate for their impairment. In conclusion, the MWM appears well suited for assessing the amnesic actions of BZs.

Neurochemistry of Benzodiazepines

In order to understand how BZs impair spatial learning, it is first important to understand the neurochemical mechanisms by which BZs exert their effects. There is now little doubt that BZs exert their actions by enhancing the actions of the inhibitory neurotransmitter γ -aminobutyric acid (GABA). Hence, the following section will review what is currently known about GABA as well as the interactions between GABA and BZs.

GABAergic cell morphology and localization

GABA is a four carbon amino-acid which functions as a major inhibitory neurotransmitter in the mammalian CNS. Only trace amounts of GABA are present in peripheral nerve tissue (Cooper, Bloom, & Roth, 1986). Glutamic acid decarboxylase (GAD, an anabolic enzyme of GABA) immunohistochemistry has revealed that GABA is distributed throughout the rat CNS, though there are regional variations (Mugnaini & Oertel, 1985). High density (GAD-positive cells representing >90% of total neurons) regions include the septum, amygdala (central nucleus), nucleus accumbens, corpus callosum, some regions of the hippocampus and dentate gyrus (see below), suprachiasmatic nucleus, and certain laminae of the cerebellum. Low density (GAD-positive cells representing <15% of total neurons) regions include bed nucleus of the anterior commissure, most of the thalamus, mammillary bodies, locus coeruleus, and the reticular formation. Within the hippocampus proper (CA1-CA4), GABAergic neurons and axonal varicosities are differentially distributed, with the greatest density of GAD-positive cells and terminals located in the stratum lacunosum-moleculare and stratum radiatum; moderate densities are located in the pyramidal and oriens layers (Babb et al., 1988; Berger et al., 1977; Camrani et al., 1986; Mugnaini & Oertel, 1985; Penny et al., 1981). In the dentate gyrus, the greatest density of GAD-positive cells and terminals are located in the hilus (60% of hilar neurons are GAD-positive) and molecular layer, with a lower density in the stratum granulosum (cell body; Mugnaini & Oertel, 1985).

GABAergic neurons are both local circuit neurons (interneurons) and projection neurons. GAD-positive cells are non-pyramidal interneurons (Storm-Mathisen, 1972), projection neurons (Seress & Ribak, 1983) as well as neurons that have both local and distal innervations (Schwerdtfeger & Buhl, 1986). The morphology of the GABAergic interneurons is more heterogenous than once thought. In the hippocampus proper, pyramidal basket cells, inverted basket cells, and horizontal basket cells have been identified in stratum oriens; stellate cells and pyramidal-like aspiny cells have been identified in the stratum radiatum (CA1 and CA3; Knowles & Schwartzkroin, 1981; Lacaille et al., 1987; Schwerdtfeger & Buhl, 1986; Seress & Ribak, 1985). In the dentate gyrus, four different GABAergic cell

morphologies have been identified: pyramidal basket cell, fusiform basket cell, horizontal basket cell, inverted fusiform cell (Seress & Riback, 1983). Despite their morphological variability, these interneurons all have short, locally arborizing axons that form a dense plexus around the somata and 'appendages' of principle cells (granule in the dentate gyrus and pyramidal cells in hippocampus proper).

Three populations of GABAergic projection neurons have been identified that are afferent to the hippocampus. The first is a small band of fibers that originate in the entorhinal cortex (layers II and III), travel via the perforant path (medial/lateral not specified) and terminate in the dentate gyrus (Germroth et al., 1989). The second band of fibers originate in the medial septum and terminate in the CA1, CA3 and dentate gyrus (Chronister & DeFrance, 1979; Kohler et al., 1984). The third major band of projection neurons, arising largely from the hilar region, innervates the contralateral hippocampus (CA1, CA3, dentate gyrus; Seress & Riback, 1983; Seroogy et al., 1983).

The main action of GABAergic interneurons is to mediate presynaptic and postsynaptic inhibition, recurrent and/or lateral inhibition, and feed-forward inhibition. In most brain regions, postsynaptic inhibition predominates and is mediated by axodendritic and axosomatic synapses onto principle cells (Alfer & Nicoll, 1982A; Ben-Ari et al., 1981; Frotscher & Zimmer, 1983; Frotscher, 1989; Muller & Misgeld, 1990; Schwatzkroin, 1986; Seress & Riback, 1983; Soriano & Frotscher, 1989; see Buzsaki, 1984 for a review) and local circuit neurons (Bilkey & Goddard, 1985; Gulyas et al., 1991; Krnjevic et al., 1988). An example of GABA-mediated recurrent inhibition can be demonstrated in the dentate gyrus when paired pulses are applied to the perforant path. The first pulse fires granule cells, which trigger recurrent inhibition and depress the response to a second pulse if it is elicited in temporal proximity (<100 mses; paired-pulse depression). Paired-pulse depression is augmented by GABAergic agonists (muscimol) and blocked by GABAergic antagonists (bicuculline; Albertson & Joy, 1987; Tuff et al., 1983). GABAergic projection neurons mediate inhibition at distal sites. For example, the GABAergic component of the septohippocampal projection

innervates inhibitory interneurons (Freund & Antal, 1988), producing 'disinhibition' when activated (e.g., Krnjevic et al., 1988).

GABA release

Neurochemical and pharmacological studies have revealed that GABA release is activated by several different trigger mechanisms. For example, GABA release is activated by excitatory amino acid (EAA) neurotransmitter glutamate (Tonnum, 1984). [³H]GABA release from hippocampal neurons in primary cell culture can be stimulated by glutamate as well as by *N*-methyl-D-aspartate (NMDA) and kainate (Drejer et al., 1987; Harris & Miller, 1989). [³H]GABA release induced by glutamate, NMDA and kainate is blocked by the NMDA receptor antagonists CPP and D-AP5 (Drejer et al., 1987; Harris & Miller, 1989). EAA-induced [³H]GABA release can be achieved by either a Ca²⁺-dependent or Ca²⁺-independent mechanism. For example, NMDA-induced [³H]GABA release is inhibited by the removal of external Ca²⁺ while the [³H]GABA release evoked by glutamate and kainate are unaffected by the removal of external Ca²⁺ (Harris & Miller, 1989). Moreover, the depolarization of neurons (*in vitro*) by high external K⁺ levels elicits the release of [³H]-GABA from pre-synaptic terminals by a Ca²⁺-dependent mechanism (Fan et al., 1982; Pin et al., 1988; Srinivasan et al., 1969). Ca²⁺-independent [³H]GABA release has been suggested to occur by the depolarization-induced reversal of electrogenic, Na⁺-coupled GABA uptake (Nelson & Blaustein, 1982).

The synaptic actions of GABA are terminated by reuptake into terminals and glia cells (Krogsgaard-Larsen, 1980). Newly taken up GABA is stored in at least two different pools, one of which includes newly synthesized GABA (Abe & Matsuda, 1983).

GABA receptors

GABA released from presynaptic terminals binds to stereospecific, high affinity pre- and postsynaptic recognition sites (Enna et al., 1977; Harrison et al., 1988). Combined evidence from pharmacological and physiological studies support the existence of at least two major subclasses of GABA receptors, termed GABA_A and GABA_B. While both receptor subtypes are

receptive to GABA and both mediate neuronal inhibition, the pharmacology, physiology, and anatomical distribution of these two receptors is quite distinct. However, as there is no evidence that BZs interact with GABA_B receptors, they will not be discussed here.

GABA_A receptors. Initial characterization of the GABA_A receptor showed that it consisted of two glycosylated polypeptide subunits, designated α and β (see Stephenson, 1988 and Villar et al., 1991 for reviews). Subsequently, molecular cloning of GABA_A receptor cDNAs has led to the identification of several closely related $\alpha_{1,2,3}$ and β_{1-3} subunit variants as well as two additional subclasses, γ_2 and δ (Benke et al., 1991; Olsen & Tobin, 1990; Pritchett et al., 1989; Schofield et al., 1987). Each subunit has a transmembrane domain (oligomer), one or more of which contributes to the wall of the Cl⁻ ionophore. Each oligomer contains at least one copy of an α and β subunit and may combine with other oligomers (e.g., $\alpha\delta$; Olsen & Tobin, 1990). Channel sensitivity to GABA depends on both the combination of subunits (e.g., $\alpha_4\beta\gamma > \beta\gamma\alpha_1$ or α_2), the molecular weight of individual subunits (58-kilodalton [kDa] $\beta > 56$ kDa β) as well as the concentration of GABA at the receptor (Yasui et al., 1985). In general, however, the recognition site for GABA, as well as picrotoxin (Sigel et al., 1989), requires the presence of a β subunit (Schofield et al., 1987).

The relative distribution of the subunits differs anatomically, as determined by Northern blot analysis and in situ hybridization to locate subunit mRNAs (Levitan et al., 1988). For example, the highest density of α_1 mRNA is in the cerebellum, α_2 mRNA in the hippocampus and α_3 mRNA in cortex (Levitan et al., 1988). Within the hippocampus, α_2 mRNA predominates in the dentate gyrus and CA3 region whereas α_1 is evenly distributed; α_3 is present only at low levels (Wisden et al., 1988). Distribution of α and β mRNAs show considerable overlap, although there is not a 100 percent correspondence (Sequier et al., 1988). The distribution of γ mRNAs is similar to that of α_1 mRNAs while δ mRNAs are more abundant in the cerebellum and almost nil in the hippocampus (Shivers et al., 1989). Hence, subunit distribution is heterogeneous.

Physiologically, the GABA_A receptor is directly coupled to the Cl⁻ ionophore, a transmembrane ion-conducting channel. Activation of the GABA_A receptor opens the Cl⁻ ionophore, increasing the membrane conductance to Cl⁻ and, to a lesser extent, other anions (e.g., Br⁻, F⁻). Depending on the number of ionophores and the concentration of Cl⁻ anions on either side of the membrane, GABA_A receptor activation may hyperpolarize (Cl⁻ influx) or depolarize (Cl⁻ efflux) the neuron (Bormann, 1988; Farrant et al., 1990; Segal & Barker, 1984). Indeed, the neuronal response to GABA_A receptor activation also depends on the location of the receptor. For example, the iontophoretic application of GABA to the soma of hippocampal pyramidal neurons elicits hyperpolarization while the application of GABA to basilar or apical dendrites elicits depolarization (Thalmann et al., 1981). However, under normal conditions, the net effect will be hyperpolarization with the depolarization being masked by the more powerful somatic input (Nicoll & Dutar, 1989).

In sum, the GABA_A receptor is a hetero-oligomeric protein complex that is coupled to the Cl⁻ ionophore. Activation of the GABA_A receptor increases membrane permeability to Cl⁻, hyperpolarizing the cell. If the neuron that is hyperpolarized is excitatory, net inhibition ensues. If the neuron is itself inhibitory, then net excitation ensues.

Allosteric modulation of the GABA_A receptor

BZs exert their effects by enhancing the efficacy of GABA at the GABA_A receptor, an oligomeric protein complex which possesses several distinct high affinity receptors. Modulators of the GABA_A receptor do not act directly on the GABA receptor but, rather, influence the efficacy of the receptor to open the Cl⁻ channel in an allosteric manner (Costa, Alho, Favaron & Manev, 1989). GABA_A receptors are positively modulated by a number of different drugs, including BZs, barbiturates and ethanol, and negatively modulated by inverse-agonist drugs such as β -carbolines. For example, BZ agonists enhance the affinity of GABA to its receptor, thereby increasing the frequency that GABA opens the Cl⁻ ionophore (Costa, Rodbard, & Pert, 1979; Marangos & Martino, 1981; Unnerstall, Kuhar, Niehoff, & Palacios, 1981; Vicini, Mienville, & Costa, 1986) and β -carbolines

reduce the efficacy of GABA at the GABA_A receptor (Jensen, Petersen, Honore, & Drejer, 1986; Ngur, Rosenberg, & Chiu, 1990; Vicini, Mienville, & Costa, 1987). BZ receptor antagonists, such as flumazenil (formerly Ro 15-1788) or CGS 8216, block the receptor and neither enhance nor reduce GABA_A receptor affinity at the level of the synapse (Haefely, 1990; Hunkeler, Mohler, Pieri, Polc, Bonetti, Cummin, Schaffner & Haefely, 1981). However, flumazenil, at high concentrations, has been shown to enhance GABAergic responses to a moderate degree (e.g., Skerritt & Macdonald, 1983).

Summary of experiments

The following series of experiments were designed to further delineate the neurochemical, neuroanatomical and electrophysiological basis of BZ-induced deficits of spatial learning in the MWM. In Experiment I, the effects of BZ receptor antagonists and inverse-agonists on spatial learning were assessed. It is speculated that if endogenous BZ ligands do impede mnemonic processes, then blockade of BZ receptors should enhance spatial learning. BZ receptors are heterogenous and differentially distributed throughout the CNS. To assess the contribution of the BZ receptor ω_1 subtype to spatial learning, the effects of CL 218, 872, a selective ligand for the ω_1 receptor, on spatial learning was assessed in Experiment II. Several different neurotransmitter systems have an active role in spatial learning and an interaction between BZs and these systems may be responsible for the spatial learning impairment produced by BZs. In Experiment III, the importance of opioid systems on spatial learning was assessed and, in Experiment IV, an interaction between BZs, opioid systems, and spatial learning was assessed pharmacologically. In Experiment V, an interaction between BZs, cholinergic systems and spatial learning was assessed. Which region of the brain mediating the amnesic actions of BZs has yet to be revealed. In Experiment VI, neuroanatomical mapping revealed that out of the several sites that possess a high density of BZ receptors, infusions of chlordiazepoxide only into the medial septum impaired spatial learning. Experiment VII sought to determine if BZ receptors in the medial septum mediate the amnesic effects of chlordiazepoxide infusions and if the deficit is related to the septohippocampal cholinergic system. Finally, Experiment VIII assessed the effects of amnesic doses of chlordiazepoxide, CL 218,872 and diazepam on the

induction of long-term potentiation, a neural model of long-term information storage in the mammalian CNS.

CHAPTER 2: ROLE OF BZs IN SPATIAL LEARNING

Experiment I. Endozepines and memory

Experiment I will assess the effects of BZ receptor antagonists on spatial learning. BZ receptor antagonists, by definition, do not activate the receptor site to which it binds; therefore, any behavioral effects resulting from BZ receptor blockade can be attributed to the inactivity of an endogenous ligand. The term "endozepines" has been adopted to refer to *endogenous benzodiazepines* (Rothstein, Garland, Puia, Guidotti & Costa, 1991). Since the discovery of high affinity binding sites for BZs in the CNS (see below), a search for an endogenous ligand has ensued (see Haefely, 1988 for review). To date, a number of endogenous substances that bind with high affinity to the BZ receptor have been identified (see Haefely, 1988 for review). These include β -carbolines (Pena, Medina, Novas, Paladini, & De Robertis, 1986), a number of polypeptides (Guidotti, Forchetti, Corda, Konkel, Bennett, & Costa, 1983) as well as diazepam and its primary metabolite N-desmethyldiazepam (De blas & Sangameswaran, 1986; Rothstein, Garland, Puia, Guidotti & Costa, 1990) and endozepine-2, a newly discovered endogenous BZ agonist (Rothstein, Garland, Puia, Guidotti & Costa, 1991). Although purines and purine nucleosides have also been proposed to be endozepines, their low affinity for the BZ receptor (approximately 200,000 times less than that of diazepam; Mohler, 1981) make them unlikely candidate ligands for the BZ receptor.

The β -carbolines are negative allosteric modulators of the GABA_A receptor (i.e., they decrease the efficacy of the GABA receptor by interacting with the BZ receptor) and have, therefore, been termed inverse-agonists (as above; Braestrup, Nielson, Honore, Jensen, & Petersen, 1983; Paterson & Roberts, 1983). The endogenous β -carbolines, ethyl- β -carboline-3-carboxylate and *n*-butyl β -carboline-3-carboxylate, have been detected in human urine (Braestrup, Nielson, & Olsen, 1980; Nutt & Cowan, 1983) and bovine brain tissue and are potent displacers of [³H]flunitrazepam from BZ receptors (Pena et al., 1986). The behavioral effects of β -carbolines are opposite to those of BZ agonists. For example, β -carbolines possess anxiogenic (Corda, Blaker, Mendelsen, Guidotti, & Costa, 1983; File, Lister, & Nutt, 1982), proconvulsant

(Oakley & Jones, 1980; Novas, Wolfman, Medina, & De Robertis, 1988), and memory enhancing properties (Izquierdo, Pereira, & Medina, 1990; Venault, Chapouthier, Prado de Carvalho, Simiand, Morre, Dodd, & Rossier, 1986). Hence, endogenous β -carbolines may have an important role in reducing the actions of GABA.

A second candidate endozepine is an 11 kDa polypeptide termed 'diazepam binding inhibitor' (DBI) due to its ability to inhibit [3 H]diazepam binding (Guidotti et al., 1983). DBI has 104 amino acid residues and contains two identical octadecapeptide chains (Gray, Glaister, Seeburg, Guidotti, & Costa, 1986). In most respects, the actions of DBI resemble those of β -carboline inverse-agonists. That is, DBI is a negative allosteric modulator of the GABA_A receptor. Electrophysiological studies have shown that DBI reduces GABA-induced current in spinal neurons by 42%, an effect that is antagonized by the BZ receptor antagonist flumazenil (Bormann, Ferrero, Guidotti & Costa, 1985). In the latter study, β -carboline reduced the GABA-induced current by 52%. Moreover, DBI preferentially displaces [3 H] β -carboline over [3 H]diazepam (Costa, Corda, Guidotti, 1983) and is present in high concentrations in cerebellar cortex (Alho et al., 1985), suggesting that DBI, like β -carboline, has a preferential affinity for the ω_1 BZ receptor. DBI does not inhibit the binding of Ro 5-4864, a selective agonist of the peripheral type (ω_3) BZ receptor (DeBlas & Sangameswaran, 1986). Moreover, DBI, like β -carbolines, has anxiogenic actions (Guidotti et al., 1983) and is elevated in brain tissue after acute stress exposure (Ferrarese et al., 1991). Interestingly, chronic, but not acute, treatment with BZs is found to increase DBI content in several brain regions and may be responsible the desensitization of GABA_A receptor found in BZ tolerant rats (Miyata et al., 1987). Hence, DBI has inverse-agonist properties that resemble those of the β -carbolines.

DBI has been detected in rat brain tissue (Alho et al., 1985; De Blas & Sangameswaran, 1986), human urine (Nielsen et al., 1979), CSF, and brain tissue (Barbaccia et al., 1986; Ferrero et al., 1986). DBI-like immunoreactivity is highly concentrated in the hypothalamus, cerebellum and, to a lesser extent, in the hippocampus in neuronal populations known to be GAD-immunoreactive (i.e., basket cells in dentate gyrus, Alho et al., 1985). In some

cultured neurons, DBI is co-localized and co-released with GABA (Ferrarese et al., 1987a, b). DBI-like immunoreactivity of CSF is higher in human males than in females until 50 years of age, at which point sex differences are not significant (Barbaccia et al., 1989, 1990). Moreover, DBI-like immunoreactivity of CSF is found to be higher in depressed patients, regardless of gender (Barbaccia et al., 1989). No significant differences in DBI-like immunoreactivity was found in the CSF of schizophrenics or patients with dementia of the Alzheimer's type (Barbaccia et al., 1986). Patients with hepatic encephalopathy, a complex syndrome of altered mental status associated with chronic and acute liver disease, have significantly higher CSF concentrations of DBI relative to patients with liver disease but without encephalopathy and non-hepatic encephalopathy (Rothstein et al., 1989; Rothstein & Olasmaa, 1990). Hence, DBI is found endogenously and may be related to certain pathologies.

DBI coexists with at least three active 'DBI-processing products': octadecaneuropeptide (ODN), eicosapentaneuropeptide (EPN) and triakontatetraneuropeptide (TTN; Ferrarese et al., 1987; Guidotti et al., 1989), suggesting that DBI functions as a precursor for these peptide fragments. These DBI products have proconflict (anxiogenic) actions that are more potent than DBI itself. The proconflict action of ODN is blocked by flumazenil, but not PK 11195 (a peripheral BZ receptor antagonist), while the proconflict action of TTN is more potently blocked by PK 11195 than flumazenil (Guidotti et al., 1989). These latter findings suggest that ODN has a selective affinity for central BZ receptors and TTN has a selective affinity for the peripheral receptor (Guidotti et al., 1988). However, both central and peripheral BZ receptors are found in the CNS, implicating both ODN and TTN in mnemonic processes.

Recent studies have shown that diazepam and its primary active metabolite N-desmethyldiazepam could be detected in the mammalian brain (De Blas & Sangameswaran, 1986; Rothstein et al., 1990; Sangameswaran & De Blas, 1985; Wildmann et al., 1987), adrenal tissue (Wildmann et al., 1987), mothers' milk (Klotz, 1990), plasma (Wildmann et al., 1986), several plant sources including certain grains, and standard rat chow (Wildmann et al.,

1987). The quantity of diazepam and N-desmethyldiazepam found in adrenal tissue ranges from 6 - 10 ng/g and in mammalian brain tissue ranges from 2 - 5 ng/g. These quantities are comparable to brain concentrations of 3 - 30 ng/g found after the oral administration of therapeutic doses of diazepam (Haefely, Kyburz, Gerecke, & Mohler, 1985). Therefore, the quantities of diazepam and N-desmethyldiazepam present endogenously are in a biologically active range. The quantity of diazepam and N-desmethyldiazepam found in plant sources range from 0.1 - 0.5 ng/g (Wildmann et al., 1987). Thus, it is possible that the detected diazepam and N-desmethyldiazepam may have accumulated from dietary sources. However, the possibility that diazepam-like compounds are synthesized endogenously cannot be ruled out at present.

More recently, an endogenous substance, termed endozepine-2, has been identified that has several characteristics that are suggestive of an endozepine (Rothstein et al., 1991). For example, endozepine-2 binds to central BZ receptors with high affinity and potentiates GABA-mediated Cl⁻ currents, an effect that is blocked by flumazenil (Rothstein et al., 1991). Endozepine-2 is released by potassium depolarization and detected in rat brain using *in vivo* microdialysis. The chemical structure of endozepine-2 is currently under investigation.

Hence, endogenous BZ ligands are of two types, BZ receptor agonists and BZ receptor inverse-agonists. However, behavioral data suggests that it is the BZ agonists that are principle modulators of the BZ receptor. For example, flumazenil, a BZ-receptor antagonist which therefore blocks the actions of both BZ agonists and inverse-agonists, has anxiogenic, not anxiolytic, actions on some tests of experimental anxiety (File et al., 1982; Lee & Rodgers, 1991) but not others (Crawley, Skolnick & Paul, 1984; Prado de Carvalho et al., 1983; Treit, 1987) and mnemonic enhancing properties (Brioni, Arolfo, Jerusalinsky, Medina & Izquierdo, 1991; Izquierdo et al., 1990; Lal, Kumar, & Forster, 1988; Raffalli-Sebille & Chapouthier, 1991). However, flumazenil lacks the proconvulsant properties of the β -carbolines (e.g., Marescaux, Micheletti, Vergnes, Depaulis, Rumbach and Warter, 1984). The behavioral properties of CGS 8216, a pyrazoloquinoline BZ receptor

antagonist, more closely resemble the β -carboline in that it possesses proconvulsant (File, 1983), anxiogenic (File & Lister, 1983) and mnemonic enhancing properties (Kumar, Forster & Lal, 1988). Thus, unlike their actions at the GABA_A receptor, at the behavioral level BZ receptor antagonists act like inverse-agonists, suggesting that they are blocking an endogenously released BZ receptor agonist.

It is important to note that BZ receptor antagonists, as well as inverse-agonists, increase both anxiety and cognitive ability while BZ receptor agonists reduce both anxiety and cognition. Hence, anxiety and cognitive processes appear to be positively related. However, there have been few studies that have clearly dissociated the anxiolytic and amnesic effects of BZs. One approach to this problem would be to assess several doses of an anxiogenic drug on tests of learning and anxiety. Thus, it would be predicted that only those doses that enhance learning in the MWM would also increase exploratory anxiety. Experiment I sought to determine the effects of several doses of the BZ receptor antagonists flumazenil and CGS 8216 and the BZ receptor inverse-agonist methyl β -carboline-3-carboxylate (β -CCM) on spatial learning and on thigmotaxia in an open field. These effects were contrasted with an amnesic dose of the BZ receptor agonist diazepam. The training protocol in the MWM was modified such that 2 trials, rather than the usual 4 trials, were given each day in order to slow acquisition rates and increase the possibility of detecting drug-enhancement effects (e.g., Mandel, Gage, & Thal, 1989).

METHODS

Animals

Eighty-eight hooded, male rats of the Long-Evans strain served as subjects. They were housed in pairs in shoebox cages and maintained on a 12:12 h light-dark cycle. Testing was conducted during the light phase of the cycle. The rats weighed approximately 400 g at the beginning of the experiment and food and water were available ad lib.

Apparatus and procedure

Water maze. The MWM consisted of a circular pool (diameter: 150 cm, height: 45 cm), with a featureless white inner surface. The pool was filled to a height of 25 cm with 22°C ($\pm 1^\circ\text{C}$) water, in which 1500 ml of powdered skim milk was dissolved. The hidden escape platform was a clear Plexiglas stand (13 X 13 cm) submerged 3 cm below the water surface so that it was invisible at water level. The visible platform was a black stand (13 X 13 cm) that protruded 5 cm above the surface of the water.

During initial acquisition, the submerged escape platform was located in the center of the northwest quadrant. All groups were given only two trials each day for five consecutive days. For each trial, the rat was placed in the water facing the pool wall at one of two quasi-randomly determined starting locations with the only restriction that daily sessions have one trial start from a location close to the platform and one start from further away (e.g., S-W, N-S, E-N, etc.). During each trial, the rat's swim path length and escape latency were recorded with a video-tracking system (Chromotrack; SD Instruments). Swim speed was calculated as the distance divided by the latency (cm/s) obtained for each trial. Once the rat located the platform, it was permitted to remain on it for 15 s. If the rat did not locate the platform within 60 s, it was placed on the platform for 15 s. After each trial, the rat was returned to a holding cage positioned 90 cm under a 250 W brooding lamp (for warmth) and allowed to remain there for the intertrial interval (approximately 4 min).

On the day following the final day of acquisition, a drug-free probe trial was given to assess the strength and accuracy of initial learning as well as to determine the strategy adopted by the rat to locate the platform during training. Rats were required to swim in the pool without an escape platform for 60 s. All rats were released from the southern pole and the time spent in each quadrant was recorded and analysed with the video-tracking system.

Open-field. Potential anxiogenic drug effects were assessed by using the empty pool as a circular open field (diameter: 150 cm, height: 45 cm). The pool/open field was kept in the same location to reduce novelty-induced

anxiety. The field was illuminated to 100 ft-c by 8 100 Watt bulbs and constant background noise was maintained with a stereo system. The inner surface was white and featureless except for a line drawn on the floor 6 cm from the wall around the periphery. This line divided the floor of the field into a central area (85% of field) and a peripheral area (15% of field). Rats were placed one at a time into the center of the open field and allowed to explore freely for 5 min. During this time the rat's path in both the peripheral region (thigmotaxia; Treit and Fundytus, 1989), and central regions of the field were measured by the video-tracking system. Anxiety was assessed as thigmotaxia, the proportion of time spent in the peripheral region of the open field. Total number of boli excreted were counted to provide an additional measure of anxiety.

Drugs and group assignment

Subjects were randomly divided into the following eight treatment groups: (1) vehicle (1 ml/kg; 0.9% NaCl with a drop of Tween 80 (1 drop/10 ml) n=10), (2) FLU (10, 20, and 30 mg/kg; n=6/dose; Hoffmann-La Roche Inc.), (3) CGS 8216 (10, 20, and 30 mg/kg; n=6/dose; Ciba-Geigy Inc.), (4) β -CCM (0.3, 0.6, and 1.0 mg/kg; n=6/dose; Sigma Chemical Co.), (5) diazepam (3 mg/kg; n=6; Hoffmann-La Roche Inc.), (6) diazepam (3 mg/kg) + β -CCM (0.3 mg/kg; n=6), (7) diazepam (3 mg/kg) + FLU (10 mg/kg; n=6), and (8) FLU (10 mg/kg) + β -CCM (0.3 mg/kg; n=6). All drugs were suspended in physiological saline with Tween 80 (1 drop/10 ml) and injected IP in the rat's home cage 15 minutes prior to behavioral tests.

Data analysis

Group differences in percentage distances in the peripheral region of the open-field, number of boli, probe test data, and swim speeds were assessed using Dunnett's test. Escape latencies and swim path lengths were assessed using an analysis of variance (ANOVA) procedure with repeated measures. Post hoc comparisons were assessed using Dunnett's t-test. In every case the acceptable level for statistical significance was $p < 0.05$.

RESULTS

Water maze acquisition

Rats treated with the drug vehicle showed a gradual improvement in performance over training but failed to reach asymptotic levels of proficiency by the end of testing. This pattern is consistent with previous observations that a 2 trial/day protocol produces slower acquisition, allowing for potential enhancement effects to be revealed (e.g., Mandel, Gage and Thal, 1989). Relative to the drug vehicle, the BZ receptor antagonists flumazenil and CGS 8216 and the inverse-agonist β -CCM all enhanced spatial learning in a dose-dependent manner (Fig. 2.1 and Fig. 2.2). Rats treated with the two low doses of flumazenil (10 and 20 mg/kg) took shorter distances to locate the platform, though the high dose of flumazenil (30 mg/kg) had little effect relative to controls (Fig. 2.1A and 2.2A). An overall ANOVA on the escape distances of groups treated with flumazenil revealed a significant group difference, $F(3,56)=4.84$, $p<0.005$, day difference, $F(4,224)=25.2$, $p<0.001$, but not a significant interaction between groups and day, $F(12,224)<1.0$, N.S. Post hoc tests revealed that rats treated with the low (10 mg/kg) and intermediate (20 mg/kg) doses of flumazenil, but not the high (30 mg/kg) dose, had significantly shorter distances relative vehicle-treated controls ($p<0.01$). Rats treated with all three doses of CGS 8216 had shorter distances to locate the escape platform, with the intermediate dose of CGS 8216 (20 mg/kg) producing the most drastic reduction of distances (Figs 2.1B and 2.2B). An overall ANOVA on the distances of groups treated with CGS 8216 revealed a significant group difference, $F(3,56)=4.41$, $p<0.001$, day difference, $F(4,224)=30.6$, $p<0.001$, but not a significant interaction between groups and day, $F(12,224)<1.0$, N.S. Post hoc tests revealed that rats treated with both the low (10 mg/kg; $p<0.05$) and intermediate (20 mg/kg; $p<0.01$) doses of CGS 8216, but not the high dose (30 mg/kg), had significantly shorter distances relative to vehicle-treated rats. Rats treated with the two low doses (0.3 and 0.6 mg/kg) of β -CCM showed distance reductions whereas rats treated with the high dose (1.0 mg/kg) of β -CCM performed at control levels (Fig. 2.1C and 2.2C). An overall ANOVA on the distances of groups treated with β -CCM revealed a significant group difference, $F(3,56)=3.61$, $p<0.01$, day difference, $F(4,224)=22.9$, $p<0.001$, but not a significant interaction between groups and

Figure 2.1: Effects of (A) flumazenil (FLU), (B) CGS 8216 (CGS) and (C) β -CCM on the distance taken to locate the submerged escape platform over the course of training. FLU = flumazenil, CGS = CGS 8216, β -CCM = β -carboline, Numbers = drug dose in mg/kg. Note that rats treated with the medium doses of flumazenil and CGS 8216 and the low dose of β -carboline acquire the location of the platform at a faster rate than controls.

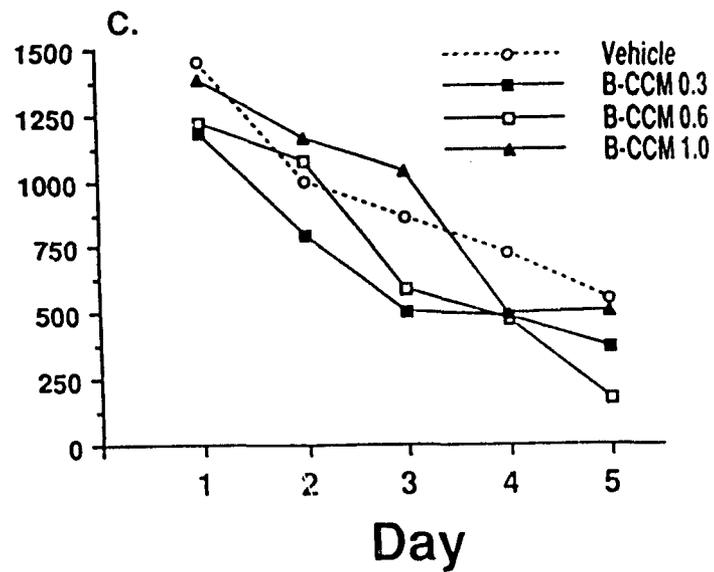
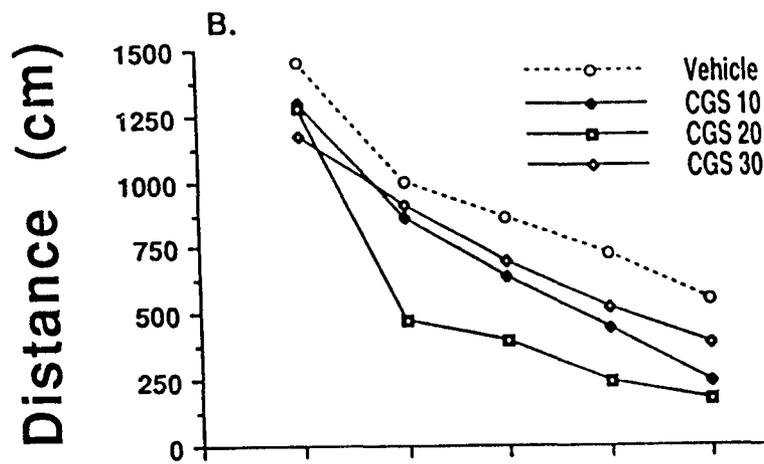
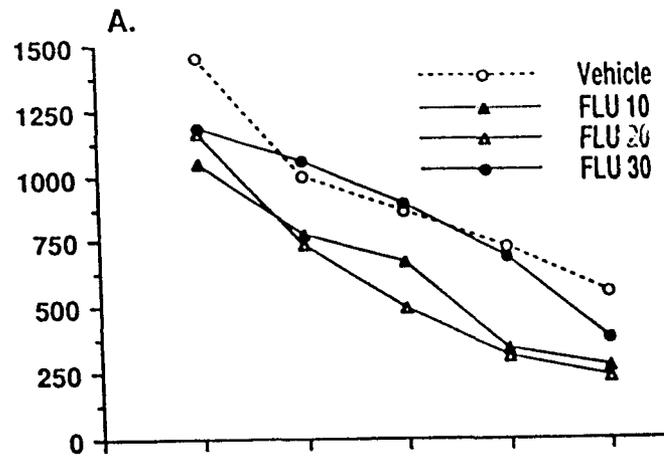
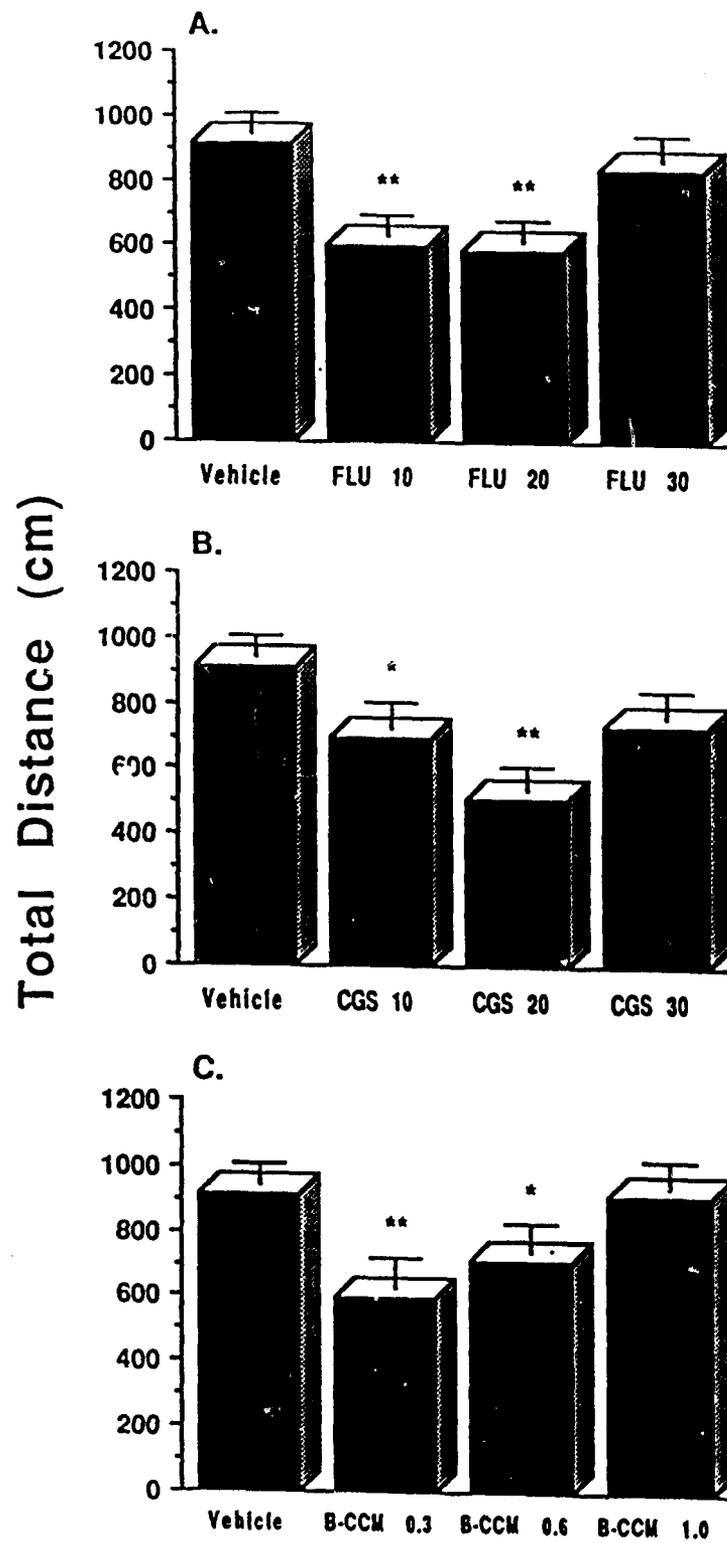


Figure 2.2: Effects of (A) flumazenil (FLU), (B) CGS 8216 (CGS) and (C) β -CCM on the distance taken to locate the submerged escape platform averaged across days. Note the U-shaped dose-dependent reductions of total distance in each treatment group. * p <0.05 and ** p <0.01 compared to vehicle. (error bars represent S.E.M.



day, $F(12,224)=1.08$, N.S. Post hoc tests revealed that only those rats treated with the low (0.3 mg/kg; $p<0.01$) and intermediate (0.6 mg/kg; $p<0.05$) dose of β -CCM had significantly shorter distances relative to controls. Latency data (not shown) followed the same pattern of deficits and statistical significances.

The pattern of facilitated acquisition revealed during training was largely confirmed during the drug-free probe trial (Fig. 2.3). Rats treated with the drug vehicle failed to demonstrate a bias for the goal quadrant, suggesting that the spatial location of the platform had not been acquired in so few trials (5 X 2). In contrast, rats treated with any dose of flumazenil (Fig. 2.3A) demonstrated a clear bias for the goal quadrant, with those given the two lowest doses ($p<0.01$) showing the greatest biases and those given the highest dose showing only a moderate quadrant bias ($p<0.05$). Rats treated with CGS 8216 demonstrated a quadrant bias in a similar inverted-U dose-response curve, with the low (10 mg/kg) and high (30 mg/kg) doses resulting in a modest quadrant bias ($p<0.05$) and the intermediate dose (20 mg/kg) resulting in a more robust quadrant bias ($p<0.01$; Fig. 2.3B). Rats treated with β -CCM, despite showing enhanced acquisition at the two lower doses, displayed surprisingly weak quadrant biases; only those treated with the lowest dose (0.3 mg/kg) showed a significant quadrant bias ($p<0.05$; Fig. 2.3C).

The effects of different drugs and doses on swim speed are illustrated in Figure 2.4. Analysis of the swim speeds over the course of training failed to reveal any significant day effect and the data are therefore collapsed across days for clarity. Rats treated with the vehicle swam at an overall average swim speed of 31 ± 0.8 cm/s, and the means of all other groups but one were not significantly different, ranging from 29.5 ± 0.8 - 33.5 ± 0.85 cm/s. Only the lowest dose of flumazenil (10 mg/kg) produced a significant swim speed reduction (28 ± 0.7 cm/s; $p<0.05$).

Flumazenil antagonized both the impairment produced by diazepam and the enhancement produced by β -CCM, whereas β -CCM exacerbated the deficit produced by diazepam (Fig. 2.5). Rats treated with diazepam alone (3 mg/kg) showed a small but significant increase in swim path lengths relative to vehicle controls ($p<0.05$), an effect that was completely antagonized by the

Figure 2.3: Effects of (A) flumazenil (FLU), (B) CGS 8216 (CGS) and (C) β -CCM on the distance traveled in the correct quadrant during the 60-sec probe trial. Note that rats treated with flumazenil or CGS 8216 during training spend a greater percentage of their time in the correct quadrant relative to controls. Note also that rats treated with *b*-carboline during acquisition do not show a strong bias for the correct quadrant. * $p < 0.05$ and ** $p < 0.01$ compared to chance levels (25%; dotted line).

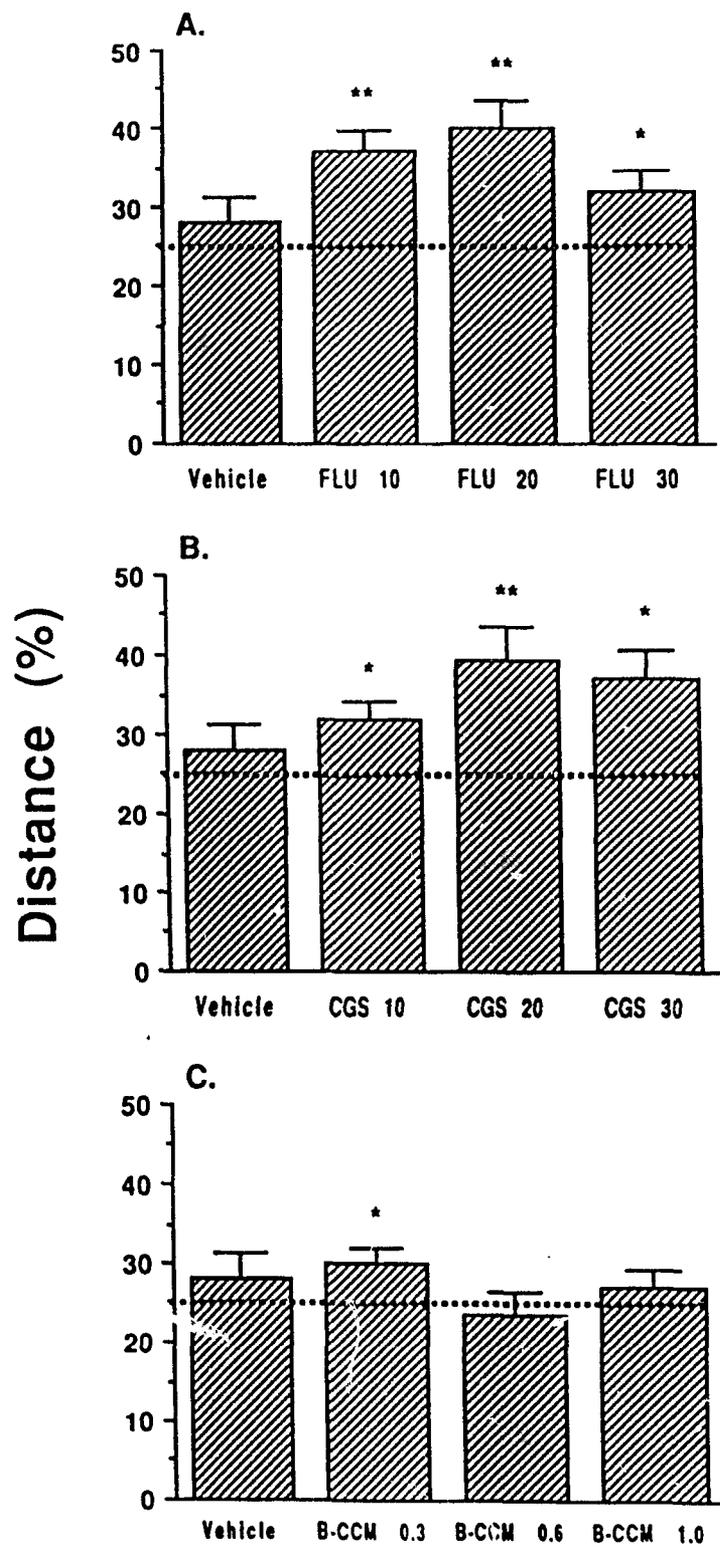


Figure 2.4: Effects of (A) flumazenil (FLU), (B) CGS 8216 (CGS) and (C) β -CCM on swim speed averaged over the five days of training. Note that the doses of flumazenil and CGS 8216 that enhanced acquisition did not affect swim speed. * $p < 0.05$ compared to vehicle controls.

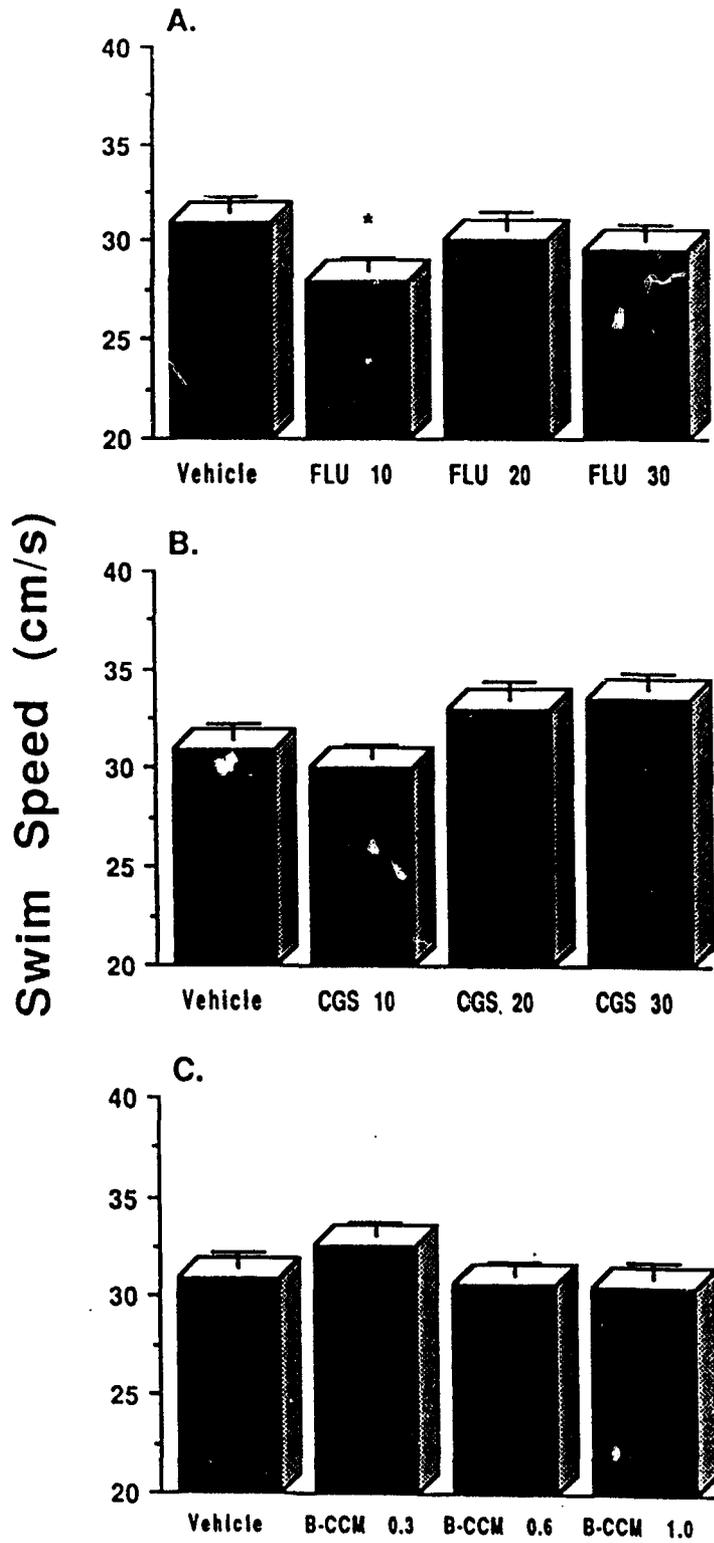
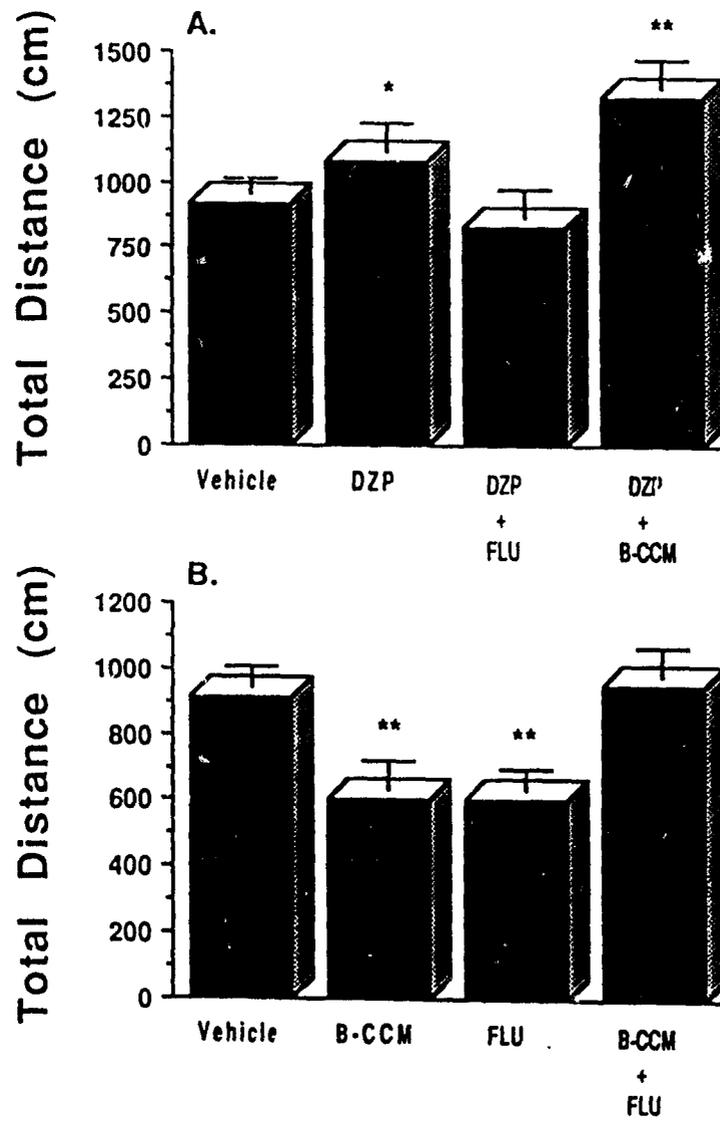


Figure 2.5: Effects of (A) diazepam (3 mg/kg) on the distance taken to locate the submerged escape platform averaged across days either alone or in combination with FLU (10 mg/kg) and β -CCM (0.3 mg/kg). (B) Effects of FLU (10 mg/kg) and β -CCM (0.3 mg/kg) on the distance taken to locate the submerged escape platform averaged across days either alone or in combination. Note that FLU, but not β -CCM, blocks the effects of diazepam in (A) and β -CCM in (B). * p <0.05, ** p <0.01 compared to vehicle-treated controls. (DZP = diazepam).



low dose of flumazenil (10 mg/kg; Fig. 2.5A; $p < 0.05$ relative to rats treated with diazepam alone). Flumazenil (10 mg/kg) also antagonized the enhancement produced by β -CCM (0.3 mg/kg; Fig. 2.5B; $p < 0.01$ compared to either flumazenil or β -CCM alone). The low dose of β -CCM failed to antagonize, and actually appeared to exacerbate, the deficit produced by diazepam ($p < 0.01$ relative to controls; Fig. 2.5A). Given the difficult training protocol, and the lack of a quadrant bias in vehicle-treated rats, it is not surprising that none of the groups given diazepam demonstrated a quadrant bias during the drug-free probe trial (data not shown).

Open-field behavior (thigmotaxia)

The effects of each drug treatment on open-field behavior are summarized in Table I. Several treatment groups showed a reduction in the total distance travelled in the open field. Most notably were the CGS 8216 groups where all three doses of CGS 8216 significantly reduced the total distance travelled relative to controls ($p < 0.05$). Additionally, the moderate doses of flumazenil and β -CCM also reduced total distances relative to controls ($p < 0.05$).

Assessment of regional preferences in the open field revealed anxiogenic drug effects. Rats treated with the vehicle spent a good proportion (42%) of their total distance in the central region of the field. In a separate investigation (see Fig. 2.7B in Experiment II) we found that naive control rats typically spend only $\approx 15\%$ of their total distance in the central region. Apparently, the greater familiarity with the open field environment resulting from water maze training reduced exploratory anxiety. In contrast, rats treated with the moderate dose of flumazenil (20 mg/kg), the low (10 mg/kg) and high (30 mg/kg) doses of CGS 8216, the intermediate (0.6 mg/kg) and high doses (1.0 mg/kg) of β -CCM all demonstrated a significant preference for the peripheral region (thigmotaxia; $p < 0.05$). The peripheral preference (anxiety) produced by the moderate dose of β -CCM (0.6 mg/kg) was antagonized by flumazenil (10 mg/kg). Diazepam had little effect on region preference relative to controls. Interestingly, the coadministration of a non-anxiogenic dose of flumazenil (10 mg/kg) and diazepam resulted in a significant preference for the peripheral region ($p < 0.05$). The anxiogenic

TABLE I

Effect of flumazenil (FLU), CGS 8216, β -CCM and diazepam on total distance traveled, proportion of distance traveled in peripheral region and number of boli excreted during 5 min. open field test.

Drug Treatment	Total Distance Travelled	Distance in Periphery (%)	Bolus Count
Vehicle	8336 \pm 172	58 \pm 2.7	20 \pm 0.46
FLU 10	8021 \pm 381	59 \pm 5.9	18 \pm 3.9
FLU 20	7561 \pm 167**	70 \pm 2.6*	35 \pm 1.3*
FLU 30	8131 \pm 478	66 \pm 7.4	18 \pm 0.5
CGS 8216 10	7531 \pm 133**	71 \pm 6.7**	37 \pm 0.9*
CGS 8216 20	7131 \pm 565*	57 \pm 8.2	42 \pm 0.7**
CGS 8216 30	7169 \pm 140**	71 \pm 4.0**	38 \pm 1.2*
β -CCM 0.3	8295 \pm 327	62 \pm 4.9	52 \pm 0.6**
β -CCM 0.6	7248 \pm 372*	67 \pm 5.1*	48 \pm 0.5**
β -CCM 1.0	8069 \pm 404	66 \pm 4.0*	43 \pm 1.1*
β -CCM 0.6+FLU 10	8098 \pm 277	63 \pm 5.7	30 \pm 5.5
DZP	8086 \pm 548	61 \pm 4.6	60 \pm 1.1**
DZP + FLU 10	8211 \pm 416	68 \pm 2.3*	54 \pm 1.1**
DZP + β -CCM 0.6	8277 \pm 267	71 \pm 2.6**	41 \pm 1.2*

* $P < 0.05$ compared to vehicle-treated controls

** $P < 0.01$ compared to vehicle-treated controls

effects of β -CCM (0.6 mg/kg) were unaltered when diazepam was given concomitantly. The fact that the moderate dose of CGS 8216 had little effect on thigmotaxia but reduced total distance suggests that the thigmotaxia was not the result of secondary sedative effects.

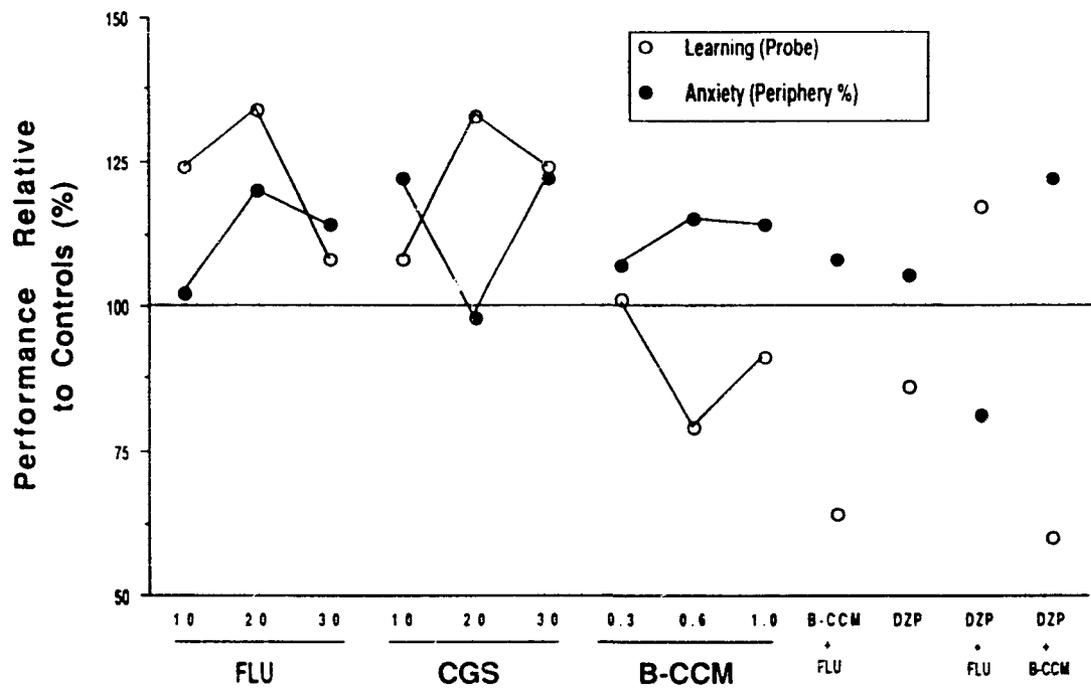
All of the drugs tested increased the number of boli emitted while in the open field. The number of boli emitted increased with the moderate dose of flumazenil (20 mg/kg), each dose of CGS 8216 and β -CCM as well as with diazepam, diazepam + flumazenil (10 mg/kg) and diazepam + β -CCM (0.6 mg/kg). The low dose of flumazenil (10 mg/kg) antagonized the increased bolus emission produced by the moderate dose of β -CCM. The fact that diazepam, an anxiolytic, increased boli emission suggests that this index of anxiety is of little predictive value.

Overall, the mnemonic enhancing effects of each drug treatment showed a poor relationship with their anxiogenic effects (Fig. 2.6). In Figure 2.6, it can be seen that with each drug and dose, anxiogenesis (percentage distance spent in the periphery; thigmotaxia) and mnemonic enhancement (percentage time spent in the target quadrant during the probe trial) show little directional congruency. For example, and most notable, the dose-response curve of CGS 8216 for anxiety is U-shaped whereas the curve for learning is an inverted U-shape. A similar, although less dramatic, lack of congruence between anxiety and learning can be seen with each drug and dose.

DISCUSSION

The present investigation demonstrates that the BZ receptor antagonists flumazenil and CGS 8216 and the inverse-agonist β -CCM enhance spatial learning in the MWM. Each of these compounds, in a dose-dependent manner, reduced the distance taken to locate the submerged escape platform and increased the bias for the correct quadrant during the probe trial. Conversely, diazepam impaired spatial learning, increasing the distance taken to reach the escape platform. The moderate dose of flumazenil antagonized the enhancement produced by the low dose of β -

Figure 2.6: A composite illustration comparing the percentage time spent in the target quadrant during the probe trial (Learning—probe) and percentage distance spent in the peripheral region of the open field (Anxiety—periphery %) for each drug and dose (numbers). Values expressed as percentage of control levels (dotted line). Note the lack of congruence between the learning and anxiety measures.



CCM and the impairment produced by diazepam. However, the low dose of β -CCM failed to block the impairment produced by diazepam. None of the drug treatments, either alone or in combination, altered swim speed except the low dose of flumazenil, suggesting that alterations in motivation or sensorimotor ability were not responsible for the observed changes in spatial learning. Together, these findings confirm earlier reports that diazepam impairs spatial learning in the MWM in a flumazenil-reversible manner (McNamara and Whishaw, 1991) and that flumazenil can enhance retention (Brioni et al., 1991). The present findings extend earlier reports by showing that flumazenil, CGS 8216, and β -CCM also enhance spatial learning.

The present study also confirms previous reports that flumazenil (Lee and Rodgers, 1991), CGS 8216 (File and Lister, 1983), and β -CCM (Cutler and Aitken, 1991) have anxiogenic properties. In the open field test given in a familiar environment, vehicle-treated controls spent close to half of their total distance in the central region of the field. This contrasts sharply with the thigmotaxic tendencies of naive rats who spent the majority of their total distance in the peripheral region (> 85%; thigmotaxia) and suggests that control rats were not anxious in this environment. Flumazenil, CGS 8216, and β -CCM all increased the time spent in the peripheral region and decreased the total distance travelled, indicating that all three of these compounds increased anxiety. This increase in anxiety cannot be attributed to a lack of familiarity with the spatial environment since rats treated with flumazenil, CGS 8216, and β -CCM showed better spatial learning than controls.

Several observations dissociate the mnemonic enhancing effects of flumazenil, CGS 8216, and β -CCM from their anxiogenic properties. First, the low dose of flumazenil enhanced learning but had no effect on anxiety. Second, the moderate dose of CGS 8216 produced the greatest enhancement of learning, but the least anxiety. Third, the high dose of β -CCM did not enhance learning but did increase anxiety. Finally, β -CCM in combination with either diazepam or flumazenil impaired learning while increasing anxiety. Thus, it appears unlikely that the processes mediating the anxiogenic effects of flumazenil, CGS 8216 and β -CCM in the open field are

responsible for enhancing spatial learning, agreeing with other findings in which the anxiogenic compound Ro5-4864, a ligand at the peripheral BZ receptor, failed to enhance avoidance conditioning (Holmes and Drugan, 1991). However, it is possible that other experimental indices of anxiety (social interaction test; File et al., 1982) would correlate with the mnemonic enhancing effects of these drugs. Indeed, the effective anxiogenic dose of flumazenil appears to vary with the type of anxiety test employed (see File and Pellow, 1986).

In addition to their anxiogenic actions, CGS 8216 (File, 1983) and β -CCM (e.g., Cowan et al., 1981) also have proconvulsant effects at higher doses. It is, therefore, possible that the enhancement of neuronal excitability that subserves seizure susceptibility also has a beneficial effect on mnemonic processes. However, this account seems unlikely since limbic kindling, a proconvulsant consequence of repetitive brain stimulation (Goddard, 1967) has been found to both impair (Leung, Boon, Kaibara and Innis, 1990) or have no effect on spatial learning (McNamara, Kirkby, de Pape and Corcoran, 1992) and seizures severely impair spatial learning (McNamara et al., 1992). Moreover, the low dose of flumazenil (10 mg/kg) used in the present study were found to actually retard the development of amygdaloid kindling (Robertson and Riives, 1983). Thus, enhanced neuronal excitability of the type subserving long-term seizure susceptibility does not appear to be responsible for the enhanced learning observed in the present study.

The finding that diazepam impairs spatial learning in a flumazenil reversible manner and that flumazenil, CGS 8216 and β -CCM enhance spatial learning suggests that endogenous BZ agonists activated during water maze training prevent optimal learning. Several endogenous substances with a high affinity for the BZ receptor have been identified in mammalian tissues including inverse-agonists (Guidotti, Forchetti, Corda, Konkel, Bennett and Costa, 1983; Pena, Medina, Novas, Paladini, De Robertis, 1986) as well as agonists, such as diazepam (Wildmann, Mohler, Vetter, Ranalder, Schmidt and Maurer, 1987) and endozepine-2 (Rothstein, Garland, Puia, Guidotti and Costa, 1991). It is unlikely that an endogenous β -CCM-like substance is activated during training since the dose of flumazenil which

enhanced spatial learning when administered alone blocked the enhancement effect of β -CCM. Rather, the present results suggest that during the stress and anxiety of maze training, an endogenous BZ agonist, such as endozepine-2, is released, resulting in sub-optimal learning. Surprising, then, is the finding that β -CCM worsened rather than attenuated the amnesic effects of diazepam, although it is possible that a higher dose of β -CCM might block the amnesic effects (e.g., Tensen and Hirsch, 1980). Nonetheless, these data tend to support the existence of an endogenous BZ agonist that, like its exogenous analog, is detrimental to mnemonic processing.

Flumazenil, CGS 8216, and β -CCM may have facilitated spatial learning by enhancing acetylcholine activity by disinhibiting cholinergic neurons. Cholinergic blockade impairs spatial learning in the MWM (Sutherland, Whishaw and Regehr, 1982) and cholinergic-enhancing drugs such as physostigmine have been found to enhance mnemonic processes across a variety of tasks (see Hagen and Morris, 1988 for a review). The finding that the amnesic effects of scopolamine can be attenuated by flumazenil and β -CCM inverse-agonists (McNamara and Skelton, 1992A; Jensen, Stephens, Sarter, & Petersen, 1987) suggests that the mnemonic enhancing effects of flumazenil, CGS 8216 and β -CCM may be due to an enhancement of ACh activity (NOTE: ACh activity is determined using either sodium-dependent, high affinity choline uptake (HACU) or choline-acetyltransferase (ChAT) measures which implicate presynaptic ACh terminals and not postsynaptic ACh receptors).

In sum, the present experiment found that the pre-training administration of BZ receptor antagonists flumazenil or CGS 8216 and the BZ receptor inverse-agonist β -CCM dose-dependently enhanced, while diazepam impaired, spatial learning in the MWM. The low dose of flumazenil blocked both the enhancing effect of β -CCM and the impairing effect of diazepam. In the same rats, flumazenil, CGS 8216 and β -CCM increased exploratory anxiety in a dose-dependent manner. However, the dose-response relationship between the observed mnemonic facilitation and the anxiogenesis did not concur. Taken together, these results support the

view that BZ receptor activation, either endogenously or exogenously, is detrimental to optimal learning and blocking such activity can benefit mnemonic processes, possibly by reducing endogenous BZ activity or by enhancing ACh activity.

Overall, the results of Experiment I provide further evidence that BZs are involved in mnemonic processes. Because β -CCM has a selective affinity for the BZ receptor ω_1 subtype (Braestrup & Nielson, 1981) and enhanced spatial learning, it seems possible that selective activation of the ω_1 subtype would impair spatial learning. Experiment II sought to assess the effects of CL 218,872, a selective ω_1 subtype agonist, on spatial learning.

Experiment II: Benzodiazepine receptor specificity

BZ receptors are complex structures and therefore deserve some discussion. The finding that [3 H]diazepam binds with high affinity to peripheral tissues (i.e., heart, kidney, adrenal gland) as well as tissue in the CNS led to the differentiation of BZ receptors into peripheral BZ receptors and central BZ receptors, despite the finding that peripheral BZ receptors are also located in the CNS (see Drugan & Holmes, 1991 for review). Unlike the central BZ receptor, though, the peripheral BZ receptor is not coupled to the GABA/ Cl^- receptor-ionophore in either the CNS or PNS.

In 1977, high affinity binding sites for BZs were discovered in mammalian brain tissue (Mohler & Okada, 1988; Squires & Braestrup, 1977). Since this initial discovery, several reports have confirmed the existence of BZ receptors in the mammalian brain *in vivo* (Chang & Snyder, 1978) and have demonstrated that BZ agonists, such as diazepam, bind stereospecifically to these receptors (Braestrup & Squires, 1981). The degree of receptor occupation by diazepam correlates well with its anticonvulsant (Duka et al., 1979) and anxiolytic actions (Lippa et al., 1978). Using autoradiography, BZ receptors have also been identified in fish, amphibians and reptiles (Hebebrand et al., 1987), non-human primates (Meinecke et al., 1989), pre-mortum and post-mortum human brain tissue (Manchon et al., 1985; Iyo et al., 1991). There is similarity between the distribution of BZ binding sites in rats and humans (Seighart et al., 1985; Young & Kuhar, 1979).

The quantity and distribution of BZ receptors in rats can vary as a function of strain, sex and stress exposure (Shephard et al., 1982). For example, [³H]flunitrazepam binding was shown to be significantly higher (relative to males) in female (Roman rat strain) neocortex and cerebellum and lower in female striatum and hippocampus. Male rats and mice selected for their high 'emotionality' or 'reactivity' show lower [³H]diazepam binding in the hippocampus, hypothalamus, and midbrain (Robertson et al., 1978; Robertson, 1979, see also Gallagher et al., 1987). Moreover, exposure to chronic stress (cold water 18°C swim; 2 min daily for 7 consecutive days) reversibly decreases [³H]diazepam binding in cerebral cortex, hippocampus, hypothalamus, midbrain, and striatum (Medina, Novas, & De Robertis, 1983; Weizman et al., 1989; see Drugan & Holmes for review). Exposure to acute stress (cold-water swim; once for 3 min in 6°C water or surgery) elevates [³H]flunitrazepam binding in the cerebral cortex (Okun et al., 1988; Soubrie et al., 1980). Hence, BZ receptors are regulated by both genetics and environmental factors.

Central BZ receptors have been dissociated into two subtypes (Braestrup & Nielson, 1980; Guidotti et al., 1990; Martin et al., 1983; Squires, 1983). The two BZ receptor subtypes were pharmacologically dissociated with the non-BZ triazolopyridazine CL 218,872 (3-methyl-6-[3-(trifluoromethyl) phenyl]-1,2,4,-triazolo [4,3- β] pyridazine), which was found to have a different regional distribution relative to classic BZs (Klepnar et al., 1979; Squires et al., 1979; Young et al., 1981). This led to the naming of BZ receptors as ω_1 , those receptors binding CL 218,872, and ω_2 , those receptors not binding CL 218,872 (classic BZs such as diazepam bind with high affinity to both subtypes). These BZ receptor subtypes are differentially distributed throughout rodent, monkey and human CNS (Benavides et al., 1988; Dennis et al., 1988; Gee & Yamamura, 1982; Niddam et al., 1987; Young et al., 1981). Regional displacement studies have revealed that ω_1 sites predominate in sensorimotor cortical regions and in the extrapyramidal motor system (globus pallidus, substantia nigra and cerebellum). In contrast, the ω_2 site predominates in limbic areas including the hippocampus proper, dentate gyrus, cingulate and parahippocampal gyrus, amygdala and nucleus

accumbens (Dennis et al., 1988; Niehoff & Whitehouse, 1983; Supavilia & Karobath, 1980).

Additional evidence for the dissociation of BZ receptors comes from the finding that the BZ receptor subtypes mature at different rates. For example, the ω_1 site represents only a very small relative proportion of BZ receptors on postnatal day 1, but increases in number after postnatal day 6 while the ω_2 receptor is present in high quantity from postnatal day 1 (Bacon et al., 1991; Lippa et al., 1981). Whether or not this ontogenetic lag has a functional consequence is unknown.

Elucidating the molecular biology of the ω_1 site and the ω_2 site has provided further differentiation. The ω_1 site is a 50 kDa α -subunit-like protein whereas the ω_2 site is a 54 kDa β -subunit-like protein (Sato & Neale, 1989; Sieghart et al., 1987). CL 218,872 and the β -carboline methyl β -carboline-3-carboxylate, a ω_1 receptor agonist and inverse-agonist respectively, selectively bind to receptors containing the α_1 subunit ($\alpha_1\beta_1\gamma_2 > \alpha_2\beta_1\gamma_2$ or $\alpha_3\beta_1\gamma_2$) whereas classic BZs have similar affinities for the $\alpha_{1,2,3}$ subunit variants in combination with a β_1 and γ_2 subunit (i.e., $\alpha_1\beta_1\gamma_2 = \alpha_2\beta_1\gamma_2 = \alpha_3\beta_1\gamma_2$; Olsen & Tobin, 1990; Pritchett et al., 1989).

From a behavioral perspective, the finding that CL 218,872 could reduce experimental anxiety at doses below that required to cause sedation offered the possibility that BZ-induced sedation and anxiolysis could be pharmacologically dissociated (Lippa et al. 1979). However, attempts to replicate this finding have been unsuccessful, with CL 218,872 being found to produce sedation at doses comparable to those required to produce anxiolysis (File et al. 1985; McElroy et al. 1985; Oakley et al. 1984). The effects of CL 218,872 on learning and memory have not been assessed in either humans or animals. The relative distribution of BZ receptor subtypes suggest that selective activation of the ω_1 receptor subtype may be less deleterious to mnemonic processes than would be the co-activation of both ω_1 and ω_2 receptor subtypes. For example, the hippocampus, a forebrain structure that is integral for accurate spatial learning (O'Keefe and Nadel, 1978), possesses a lower relative proportion (50:50) of the ω_1 receptor subtype than does the

cerebellum (75:25; Braestrup and Nielson 1980; Oakley et al. 1984; Young et al. 1981), a structure that does not appear to be important for spatial learning (Thompson 1983). By this reasoning, the ω_2 receptor subtype alone may mediate the amnesic effects of BZs and CL 218,872 should have little effect on spatial learning. Alternatively, if the amnesic effects of BZs are mediated by the ω_1 receptor subtype, then CL 218,872 should produce a deficit in spatial learning.

Experiment II assessed the sedative, anxiolytic and amnesic actions of CL 218,872 and diazepam. Sedation was indexed by spontaneous locomotion in a running wheel, amnesia by spatial learning in the MWM, and anxiety by thigmotaxia in an open field. Thigmotaxia (wall-hugging) manifests as a preference for peripheral areas of an open field and is a robust and reliable defensive behavior that is selectively blocked by anxiolytic agents (Treit and Fundytus 1989). The effects of flumazenil, a BZ receptor antagonist with a similar affinity for both ω_1 and ω_2 subtypes (e.g., Benavides et al. 1988), were assessed on the actions of CL 218,872 and diazepam.

METHODS

Animals

Fifty hooded, male rats of the Long-Evans strain served as subjects. They were housed in pairs in shoebox cages and maintained on a 12:12 h light-dark cycle. Testing was conducted during the light phase of the cycle. The rats weighed approximately 400 g at the beginning of the experiment and food and water were continuously available.

Apparatus and procedure

Running wheel. Spontaneous locomotion was investigated in a freely rotating running wheel (diameter: 40 cm). The floor of the wheel was steel grating and the interior of the wheel was dark. Revolutions were counted mechanically. Each daily session consisted of a 10-min trial in the running wheel, given at the same time each day. Rats were given two days to habituate and establish baseline performance in the running wheel. Following habituation, rats were given three days of testing with their appropriate drug treatments.

Open-field. Anxiety was assessed in a circular open field described in Experiment I.

Water maze. Amnesia was assessed in the MWM described in Experiment I. However, all groups were given four trials each day rather than two.

Drugs and group assignment

At the beginning of the experiment, rats were divided into nine treatment groups. The first four groups (n=20, 5/group) were treated with CL 218,872 (1, 5, 10, or 20 mg/kg; Lederle). The remaining groups received either diazepam (3 mg/kg; Hoffmann-La Roche Inc.; n=5), flumazenil (10 mg/kg; Hoffmann-La Roche Inc.; n=5), diazepam (3 mg/kg) + flumazenil (10 mg/kg; n=5), CL 218,872 (20 mg/kg) + flumazenil (10 mg/kg; n=5), or saline (2 ml/kg; 0.9% NaCl; n=10). CL 218,872, and flumazenil were suspended in 0.9% NaCl with a drop of Tween 80. Diazepam was suspended in the commercial vehicle consisting of propylene glycol, ethanol and saline (40:10:50). Pilot experiments have found that equivalent volumes of the commercial diazepam vehicle do not have any significant effect on spatial learning or open field behavior. All drugs were prepared in concentrations of 2 ml/kg and given IP in the rats' home cage. Diazepam and CL 218,872 were given 30 min before testing and flumazenil was given 15 min before testing.

Data analysis

Group differences in running wheel activity (revolutions per 10 min), percentage distances in the central area of the open-field, probe test data and swim speeds were assessed using individual *t*-tests. Escape latencies and swim path lengths were assessed using an analysis of variance (ANOVA) procedure with repeated measures. Post hoc comparisons were assessed using Tukey's (HSD) method. In every case the acceptable level for statistical significance was $p < 0.05$.

RESULTS

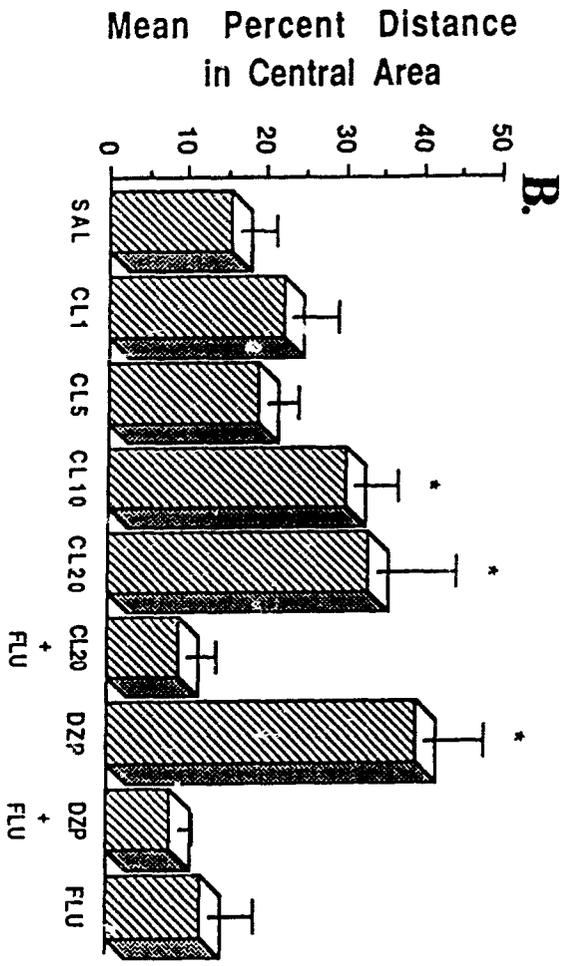
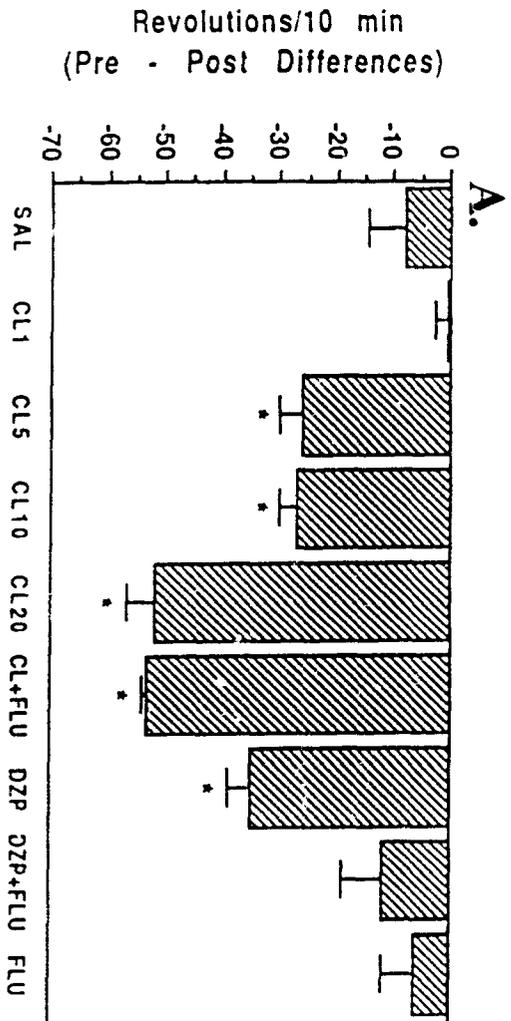
Spontaneous locomotion

None of the treatment groups differed from controls in running wheel activity during the predrug baseline phase, with an overall average of 63.5 ± 4.2 revolutions per 10 min. CL 218,872 reduced activity in a dose-dependent manner (Fig. 2.7A): 1 mg/kg dose had no effect whereas the three higher doses produced significant reductions ($p < 0.01$), with the largest dose of CL 218,872 (20 mg/kg) producing the greatest reduction. Diazepam also significantly reduced activity relative to controls ($p < 0.01$) to a degree equivalent to the moderate dose of CL 218,872. Flumazenil reversed the activity reduction produced by diazepam ($p < 0.05$), but had no effect on the reduction produced by the highest dose of CL 218,872 (20 mg/kg). Flumazenil had little effect on spontaneous activity when administered alone ($p > 0.05$).

Open-field behavior

None of the treatment groups differed from controls in the total amount of distance travelled in the five minute period in the open field ($p > 0.05$). Rats treated with saline spent a small proportion of their total distance inside the center of the open-field, spending over 85% of their exploration in the peripheral area (thigmotaxis; Fig. 2.7B). Rats treated with the two higher doses of CL 218,872 (10 & 20 mg/kg) spent a significantly greater proportion of their total distance in the center of the field relative to controls ($p < 0.01$) but did not show as great a reduction in thigmotaxis as rats treated with diazepam, though the latter difference was not statistically significant. Rats treated with the lower doses of CL 218,872 (1 & 5 mg/kg) were not significantly different from controls. The coadministration of flumazenil antagonized the antithigmotaxic effects of both CL 218,872 and diazepam ($p < 0.01$ —compared to each drug alone) while having a slight, but not statistically significant, anxiogenic effect when administered alone. It was incidentally observed that rats treated with CL 218,872 displayed muscle rigidity similar to controls, but quite unlike diazepam-treated rats which were typically quite flaccid when handled.

Figure 2.7: (A) The differences between pre-drug and post-drug activity levels in the running wheel. Note that the four higher doses of CL 218,872 reduced spontaneous activity and that flumazenil did not antagonize the largest dose of CL 218,872. (B) Effects of diazepam, CL 218,872, and flumazenil on the percent distance spent in the center of the open field. Note that CL 218,872, like diazepam, increased the percent distance spent in the center of the field and flumazenil antagonized the antithigmotaxic effects of both diazepam and CL 218,872. * $p < 0.01$ compared to saline-treated controls. (SAL=saline; CL=CL 218,872; FLU=flumazenil; DZP=diazepam; Numbers represent dose in mg/kg).



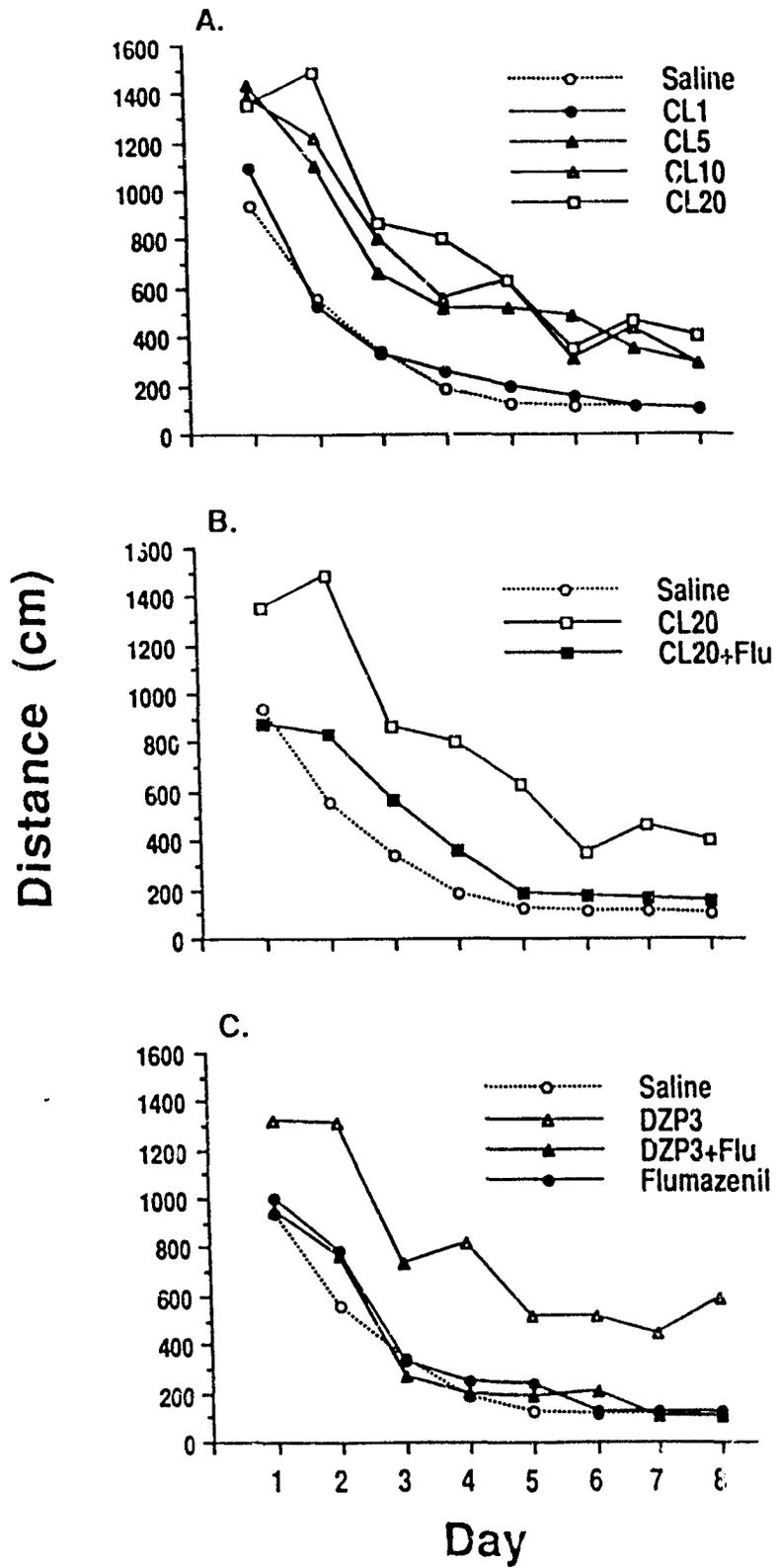
Water maze

The distance required by each of the treatment groups to locate the submerged platform over the 8 days of testing is shown in Figure 2.8A, B, and C. Rats treated with saline rapidly acquired the platform position, reaching asymptotic levels by the fourth day of testing. Rats treated with CL 218,872 demonstrated impaired acquisition at all but the lowest dose (Fig. 2.8A), to a degree comparable to diazepam-treated rats (Fig. 2.8C). Rats treated with diazepam showed a severe impairment of place learning, showing slower acquisition and poorer asymptotic levels relative to controls (Fig. 2.8C). The impairments produced by diazepam and the largest dose of CL 218,872 (20 mg/kg) were reversed by flumazenil. An overall ANOVA on swim path length revealed a significant group difference, $F(8,211)=27.1$, $p<0.001$, day difference $F(7,1477)=166.1$, $p<0.001$, and a significant interaction between groups and day, $F(56,1477)=1.6$, $p<0.01$. Post hoc comparisons revealed that rats treated with diazepam and the three largest doses of CL 218,872 (5, 10, 20 mg/kg) had longer swim paths relative to controls ($p<0.01$). Latency data revealed a similar pattern of deficits and statistical significances.

Results from the probe trial confirmed the pattern of impairments revealed during acquisition (Fig. 2.9). Rats treated with diazepam or the three highest doses of CL 218,872 (5, 10, or 20 mg/kg) failed to show a bias for the correct quadrant, unlike rats treated with saline or the lowest dose of CL 218,872 (1 mg/kg) who showed a robust bias for the correct quadrant ($p<0.01$ compared to chance=25%). Rats treated with flumazenil, alone or in combination with diazepam or CL 218,872, demonstrated a bias for the correct quadrant ($p<0.01$). None of the treatment groups were impaired when required to escape onto the single visible platform (data not shown).

The effects of CL 218,872, diazepam, and flumazenil on swim speed are shown in Figure 2.10. Diazepam and every dose of CL 218,872 significantly reduced swim speed relative to controls ($p<0.01$). Flumazenil attenuated the reductions in swim speed produced by either diazepam or CL 218,872 (20 mg/kg) to levels greater than each drug alone but less than controls and not significantly different from either ($p>0.05$). Flumazenil had little effect on swim speed when administered alone.

Figure 2.8: Effects of diazepam, CL 218,872, and flumazenil on the distance taken to locate the submerged escape platform. Note that the place learning deficit produced by CL 218,872 is comparable to that produced by diazepam and is reversed by flumazenil.



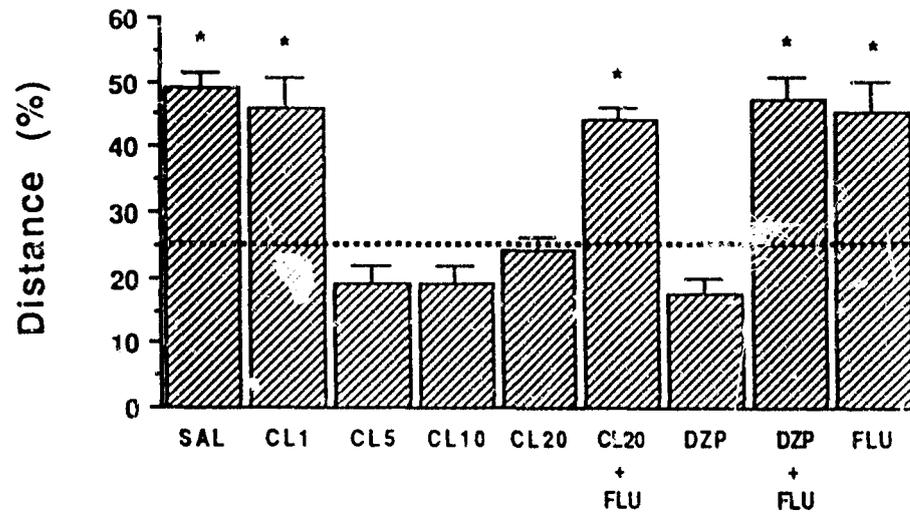


Figure 2.9: Effects of diazepam, CL 218,872, and flumazenil on the distance spent in the correct quadrant during the drug-free 60 s probe trial. * $p < 0.01$ compared to chance level (25% represented by a dotted line).

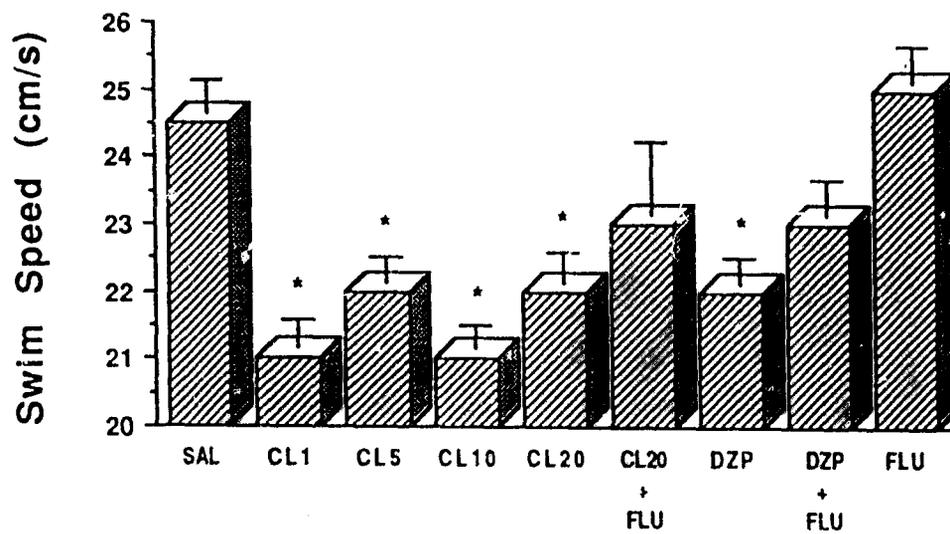


Figure 2.10: Effects of diazepam, CL 218,872 and flumazenil on swim speed averaged over the 8 d of training. * $p < 0.01$ compared to saline-treated controls.

DISCUSSION

The present results reveal that both CL 218,872 and diazepam produce comparable sedation, anxiolysis, and amnesia. CL 218,872 produced sedation and anxiolysis in a dose-dependent manner, suppressing spontaneous activity in the running wheel and thigmotaxis in the open field. CL 218,872 also impaired spatial learning; the three highest doses of CL 218,872 increased swim path lengths to the submerged platform, worsened asymptotic performance, and prevented the development of a quadrant bias during the probe trial. Although CL 218,872 also reduced swim speeds, it did not impair cue learning on the visible platform task, suggesting that the disruption of spatial learning was not due to sensorimotor or motivational deficits. Finally, the spatial learning deficit produced by both diazepam and CL 218,872 was blocked by flumazenil, suggesting BZ receptor mediation.

It was suggested that the sedative and anxiolytic properties of BZs could be dissociated with CL 218,872 (Lippa et al. 1979). Lippa et al. (1979) found that CL 218,872 produced anxiolysis at doses below those required to produce sedation. Subsequently however, CL 218,872 was found to produce significant sedation at doses below those required to produce anxiolysis (File et al. 1985; McElroy et al. 1985; Oakley et al. 1984). In the present study, CL 218,872 reduced activity in the running wheel (sedation) and impaired spatial learning in the water maze (amnesia) at doses lower than that required to reduce thigmotaxis (anxiety). These findings suggest that the thresholds for sedation and amnesia are lower than the threshold for anxiolysis, agreeing with the notion that CL 218,872 does not selectively reduce anxiety in the absence of sedation or, as revealed in the present study, anterograde amnesia.

Interestingly, flumazenil antagonized the anxiolytic and amnesic actions of both diazepam and CL 218,872, but only antagonized the sedative actions of diazepam, and not CL 218,872. This latter finding is congruent with File et al. (1985) who found that larger doses of flumazenil were required to completely antagonize the sedation produced by CL 218,872. Why the sedative effects of CL 218,872 are more resistant to flumazenil is unclear.

However, flumazenil did attenuate the swim speed reduction produced by both CL 218,872 and diazepam, suggesting that flumazenil's effectiveness against the sedative effects of CL 218,872 may be task specific. Nevertheless, these results confirm that the anxiolytic, sedative and amnesic actions of CL 218,872 and diazepam are mediated by endogenous BZ receptors.

The spatial learning impairment produced by CL 218,872 was comparable to that produced by diazepam (present results; McNamara & Skelton, 1991; McNamara and Whishaw 1990), a BZ receptor agonist which has approximately equal affinity for both ω_1 and ω_2 receptor subtypes (e.g., Sieghart and Schuster, 1984). This result suggests that the selective activation of the ω_1 receptor subtype with CL 218,872 is sufficient to impair spatial learning and, therefore, the ω_1 receptor subtype alone may mediate the amnesic actions of classic BZs. This notion is supported by the findings in Experiment I, where β -CCM, an inverse-agonist with a preferential affinity for the ω_1 receptor subtype (Braestrup and Nielsen, 1981), enhanced spatial learning. Future experiments should investigate the effects of a selective ω_2 receptor ligand on sedation, anxiety and mnemonic processes.

The results of the present study suggest that the behavioral actions of BZs (sedation, anxiety, amnesia) cannot be dissociated using a ligand selective for the ω_1 receptor subtype. Indeed, these results support the argument that the two BZ receptor subtypes may both contribute to the same functions (see Martin et al. 1983). However, this conclusion may be premature given recent findings that zolpidem, a novel non-BZ hypnotic, has been reported to have both a preferential affinity for the ω_1 receptor subtype (Benavides et al. 1988) and preferential ability to induce hypnosis (Depoortere et al. 1986).

Together, the results of Experiment I and II, as well as the previous findings that BZs impair spatial learning in the MWM, suggest that endogenous BZ systems are important modulators of mnemonic processes. That is, activation of endogenous BZ receptors, either by drugs or the activation of endozepines, impedes spatial learning while blockade of BZ receptors enhances spatial learning. Moreover, these actions may be

selectively mediated by the ω_1 receptor subtype. However, BZ systems do not exist in isolation but rather interact with other neurotransmitter systems. As mentioned, BZs enhance the actions of GABA, an inhibitory neurotransmitter. The principle role of GABA is to inhibit the activities of both proximal and distal cells, cells which may use neurotransmitters other than GABA. Hence, while BZ systems are clearly implicated in mnemonic processes, it is necessary to expand the picture to account for the several other neurotransmitters that are affected by BZ receptor activation. The following chapter explores first those neurotransmitter systems that are implicated in spatial learning in the MWM and, second, possible interactions between these systems and BZs.

CHAPTER 3: Neurochemical Interactions

Water maze neuropharmacology/neurochemistry

To date, several neurotransmitter systems, other than GABA, have been identified as playing an important role in spatial learning in the MWM including acetylcholine (ACh), glutamate, somatostatin and the opioids. Further, other neurotransmitter systems do not appear to be required for spatial learning per se and include the catecholamines and serotonin. Because BZs and GABA have been found to interact with acetylcholine (Sarter et al., 1990), glutamate (Baba, Okumura, Mizouo, & Iwata, 1983), somatostatin (Stryker, Conlin, & Reichlin, 1986), opioids (Wuster, Duka, & Herz, 1980A), corticosterone (Lahti & Barsuhn, 1974), catecholamines (e.g., Yang, Lou, & Zhou, 1988), and serotonin (Mennini, Gobbi, & Romandini, 1986; Thiebot, 1986), the contribution of each of these neurochemicals to spatial learning in the MWM will be reviewed.

Acetylcholine

Renewed interest in the role of ACh systems in mnemonic processes has been generated by the discovery of cholinergic system deterioration in patients with geriatric memory dysfunction or Alzheimer's disease [see (Bartus, Dean, Beer, & Lippa, 1982) for a review]. Animal studies have tended to confirm that cholinergic systems play an important role in learning and memory processes (Hagen & Morris, 1987). In the MWM, the role of cholinergic systems has been assessed by measuring spatial learning deficits produced by: (1) lesions of cholinergic cell groups in either nucleus basalis magnocellularis (NBM) or medial septum (MS), (2) age-related decreases in ACh activity, (3) pharmacological blockade of ACh receptors. The effects of each of these manipulations on spatial learning in the MWM are presented separately below.

Lesions of the NBM or MS selectively reduce choline acetyltransferase (ChAT) levels, an index of ACh activity, in the forebrain and hippocampus, respectively (Hartgraves, Mensah, & Kelly, 1982; Miyamoto, Kato, Narumi, & Nagaoka, 1987). However, such lesions have been found to produce contradictory effects on spatial learning in the MWM. Some reports have

found that lesions of the NBM impair spatial learning (Carl, Dolka, Gardner, Hann, Passebet, Fitzgerald, & Leiman, 1985; Dokla & Thal, 1988; Dunnett, Toniolo, Fine, Ryan, Bjorklund, & Iversen, 1985; Mandel, Gage, & Thal, 1989; Mandel & Thal, 1988; Riekkinen, Sirvio, & Riekkinen, 1990; Wishaw, O'Connor, & Dunnett, 1985) while others have failed to find a persistent impairment (Hagan, Salamone, Simpson, Iversen, & Morris, 1988; Mayo, Kharouby, Loal, & Simon, 1988; Mundy, Barone, & Tilson, 1990; Mundy & Tilson, 1988; Riekkinen, Sirvio, & Riekkinen, 1990). Similarly, MS lesions have been found to produce either a persistent deficit (Hagan, Salamone, Simpson, Iversen, & Morris, 1988; Kelsey & Landry, 1988; Miyamoto, Kato, Narumi, & Nagaoka, 1987), a transient disruption (Sutherland & Rodriguez, 1989) or no effect (Decker, Radek, Majchrzak, & Anderson, 1992). Transection of the fimbria-fornix, the main pathway connecting the hippocampus to subcortical structures which also carries the cholinergic pathway from the MS to the hippocampus (Dudar, 1975), has been found to impair spatial learning (Nilsson, Shapiro, Gage, Olton, & Bjorklund, 1987; Segal, Greenberger, & Pearl, 1989; Sutherland & Rodriguez, 1989). Interestingly, the two studies that assessed both MS and fimbria-fornix lesions on MWM performance found that fimbria-fornix lesions impaired spatial learning far more than did MS lesions (Segal, Greenberger, & Pearl, 1989; Sutherland & Rodriguez, 1989). Overall, these results suggest that ACh plays an necessary, though possibly not sufficient role in spatial learning, and that the deficit resulting from fimbria-fornix lesions is due to more than just loss of cholinergic input.

While the degree of ACh reduction in frontal cortices resulting from NBM lesions has been found to be correlated with the severity of the spatial learning deficit (Mandel, Gage, & Thal, 1989), there are reasons to doubt whether ACh depletion is the sole cause of the spatial learning deficit. Colchicine, which has been reported to be selectively toxic to cholinergic neurons in the medial septum (Emerich & Walsh, 1990) infused either directly into the MS or intracerebroventricularly (ICV) both produce similar reductions of hippocampal ChAT levels, but only the ICV infusions produce a spatial learning deficit (Barone, Nanry, Mundy, McGinity, & Tilson, 1991). Moreover, different neurotoxins (e.g., ibotenic acid vs. quisqualic acid)

infused into the NBM region produce comparable reductions of cortical ChAT activity but differing degrees of spatial learning deficits (Connor, Langlais, & Thal, 1991; Dunnett, Everitt, & Robbins, 1991; Dunnett, Whishaw, Jones, & Bunch, 1987). Thus, although the NBM and MS have been shown to be important for spatial learning in the MWM, it is unlikely that lesion-induced deficits are due entirely to ACh reductions. Indeed, there is some evidence that this deficit may be related to the degree of non-specific subcortical cell loss (Dellu, Mayo, Cherkaoui, Le Moal, & Simon, 1991; Riekkinen, Sirvio, & Riekkinen, 1990).

If NBM and MS lesions impair spatial learning by reducing cortical and hippocampal ACh activity respectively, then enhancing the activity of remaining ACh should attenuate this impairment. This approach has been taken using acetylcholinesterase inhibitors, ACh agonists, nerve growth factor (NGF), and the transplantation of ACh-rich fetal tissue into depleted areas. Spatial learning deficits produced by NBM lesions are attenuated by the ACh agonist oxotremorine (Miyamoto, Narumi, Nagaoka, & Coyle, 1989) as well as by the cholinesterase inhibitors physostigmine (Dokla & Thal, 1988; Mandel, Chen, Connor, & Thal, 1989; Miyamoto, Narumi, Nagaoka, & Coyle, 1989; Mandel & Thal, 1988) and tetrahydroaminoacridine (THA; Kwo-On-Yeun, Mendel, Chen, & Thal, 1990). The spatial learning deficit produced by NBM lesions is attenuated by the neuronotrophic factor NGF (Mandel, Gage, & Thal, 1989), which promotes the survival and maintenance of basal forebrain cholinergic neurons and stimulates ACh activity (e.g., Hefti, Dravid, & Hartikka, 1984). The spatial learning deficit produced by MS lesions is attenuated by nicotine (Decker, Majchrzak, & Anderson, 1992) and by THA (Riekkinen, Aaltonen, Sirvio, & Riekkinen, 1991; Riekkinen, Sirvio & Riekkinen, 1990) but not physostigmine (Dokla & Boitano, 1991). Finally, the spatial learning deficit produced by fimbria-fornix transections is attenuated by intrahippocampal transplantation of fetal MS cholinergic neurons (Nilsson, Shapiro, Gage, Olton, & Bjorklund, 1987; Segal, Greenberger, & Pearl, 1989; Tarricone, Keim, Simon, & Low, 1991) whereas deficits from NBM lesions are attenuated by cortically-placed cholinergic-rich embryonic ventral forebrain cells (Dunnett, Toniolo, Ryan, Bjorklund, & Iversen, 1985). In sum, the spatial learning deficit produced by NBM and MS

lesions can be attenuated by enhancing remaining ACh activity, suggesting that lesion-induced reductions of ACh are at least partly responsible for the observed deficits.

Although rats show a general trend toward poorer spatial learning with age, some aged rats (>18 months old) perform as well as young rats (8 - 12 months old) while others show severe spatial learning deficits (Barnes, 1988; Gallagher & Pelleymounter, 1988; Means & Kennard, 1991; Rapp, Rosenberg, & Gallagher, 1987). Aged "impaired" rats have been found to have a significant reduction of ChAT in the basal forebrain, striatum, and frontal and sensorimotor cortices, but not in the hippocampus (Fischer, Chen, Gage, & Bjorklund, 1992; Fischer, Nilsson, & Bjorklund, 1992; Callagher, Burwell, Kodosi, McKinney, Southerland, Vella-Rountree, & Lewis, 1990; Hellweg, Fischer, Hock, Gage, Bjorklund, & Thoenen, 1990). Aged "impaired" rats also show a reduction of NGF receptor immunoreactivity in basal forebrain neurons which may contribute to the noted reductions of ChAT activity (Koh, Chang, Collier, & Loy, 1989; Koy & Loy, 1988). Consistent, then, is the observation that age-related spatial learning deficits are attenuated by transplantation of cholinergic septal grafts into the hippocampus (Gage & Bjorklund, 1986; 1984), NGF treatment (Fischer, Bjorklund, Chen, & Gage, 1992; Fischer, Victorin, Bjorklund, Williams, Varon, & Gage, 1987) and THA (Riekkinen, Aaltonen, Sirvio, & Riekkinen, 1991), suggesting that age-related declines in ACh activity are at least partially responsible for the ensuing spatial learning deficit. However, it might be noted that aged "impaired" rats also show elevated hippocampal dynorphin levels (Jiang, Owyang, Hong, & Gallagher, 1989) which are correlated with a reduction of ^3H -CPP binding to NMDA receptors (see below) in the hippocampus (Pelleymounter, Beatty, & Gallagher, 1990; Zhang, Mundy, Thai, Hudson, Gallagher, Tilson, & Hong, 1991), suggesting that a unitary account of age-related spatial learning deficits may be overly simplistic.

Pharmacological blockade of cholinergic (muscarinic) receptors has been found to impair spatial learning in the MWM. Pretraining blockade of central muscarinic receptors by atropine sulphate dose dependently impairs spatial learning when either the reference memory or learning-set procedure

is employed (Whishaw, 1985; 1985; Whishaw, O'Connor, & Dunnett, 1985). Blockade of peripheral cholinergic receptors with atropine methylnitrate does not impair spatial learning (Sutherland, Whishaw, & Regehr, 1982), suggesting that deficits are not due to peripheral side-effects [e.g., pupillary dilation (Leopold & Comroe, 1948)]. Central muscarinic blockade with atropine sulphate does not appear to impair spatial recall [but see (Hagan, Tweedie, & Morris, 1986)], cue learning, or the use of praxis strategies (Whishaw, 1985; Whishaw, 1989; Whishaw & Tomie, 1987), suggesting that the spatial learning impairment is not due to sensorimotor, motivational, perceptual or even navigational deficits. Administration of atropine sulphate after each daily training session does not impair spatial learning (Hagan, Tweedie, & Morris, 1986), suggesting that cholinergic systems are not required for the "consolidation" process.

The effects of atropine have also been assessed using spatial and non-spatial simultaneous discrimination tasks in the MWM. Atropine sulphate (10 & 50 mg/kg) impairs both discrimination problems, leading to the suggestion that cholinergic blockade produces a learning deficit that is not specific to spatial learning (Hagan, Tweedie, & Morris, 1986). However, in a follow-up experiment, it was found that atropine produces only moderate impairments if the discriminative cues in the non-spatial version are made more salient and contiguous (Whishaw & Petrie, 1988). Inferior performance was attributed to a failure of strategy selection rather than learning and memory processes (Whishaw & Petrie, 1988). An alternative account may be that cholinergic blockade disrupts attentional processes and that the proficiency of spatial learning may depend on the salience of ambient cues (Muir, Robbins, & Everitt, 1991). Of course, this account applies equally well to all manipulations which reduce ACh function.

Scopolamine, a muscarinic antagonist that is approximately five times more potent than atropine, also impairs spatial learning in a dose-dependent manner (Buresova, Bolhuis, & Bures; McMamara & Skelton, 1992; McNaughton & Morris, 1987) with a 0.1 mg/kg dose selectively impairing spatial learning (Buresova, Bolhuis, & Bures, 1986; Hunter, Roberts & Tutty, 1986) whereas a 1.0 mg/kg dose impaired spatial recall as well. When

administered ICV, scopolamine produces a severe, and dose-dependent, spatial learning deficit (Hagan, Jansen & Broekkamp, 1987).

Muscarinic receptors have been subdivided into M₁ and M₂ receptors based on ligand binding and biochemical evidence [e.g., (Hamer & Giachetti, 1982)]. Selective antagonism of the M₁ muscarinic receptor with pirenzepine impaired spatial learning when infused ICV at doses three times lower than that required by ICV scopolamine (Hagan, Jansen, & Broekkamp, 1987), suggesting that the M₁ muscarinic receptor may be more directly related to mnemonic processes.

Central nicotinic receptors also appear to be important for spatial learning. For example, mecamylamine (central nicotinic antagonist) impaired spatial learning while hexamethonium (peripheral nicotinic antagonist) had no effect (Riekkinen, Riekkinen, Sirvo, & Riekkinen, 1992). Systemically administered (-)nicotine (0.1 or 0.3 mg/kg) also has little effect on the acquisition of a spatial discrimination version of the MWM (Decker, Majchrzak, & Anderson, 1992).

Depletion of ACh with hemicholinium-3 (HC-3) also impairs spatial learning (Hagan, Jansen, & Broekkamp, 1989) at doses that had been previously shown to reduce central ACh levels by 80 - 90% (Freeman, Marci, Choi, & Jenden, 1979). This impairment was reversed by the administration of acetylcholinesterase inhibitors physostigmine and THA as well as by cholinomimetics such as arecoline, aceclidine and oxotremorine (Hagan, Jansen, & Broekkamp, 1989).

As mentioned earlier, enhancement of cholinergic activity by reducing enzymatic breakdown with the acetylcholinesterase inhibitor physostigmine attenuates spatial learning deficits produced by lesions of cholinergic nuclei [e.g., (Mandel & Thal, 1988)], choline uptake blockade (Hagan, Jansen, & Broekkamp, 1989), and receptor blockade (McNamara & Skelton, 1992). However, when administered alone, physostigmine impairs place, but not cue, learning at higher doses [0.50 but not 0.05, 0.10 or 0.25 mg/kg (McNamara & Skelton, 1992)]. This deficit may be due to: (1) overstimulation

of post-synaptic cells, (2) overstimulation of opposing systems, or (3) motorical side-effects, mediated centrally (e.g., striatum) or peripherally (e.g., muscles). The enhancement of cholinergic activity via ACh receptor agonists does not impair spatial learning. For example, spatial learning is not affected by either oxotremorine [0.25 - 2 mg/kg/day s.c. (Miyamoto, Narumi, Nagaoka, & Coyle, 1989; Wade & Maier, 1985)] or nicotine [0.1 & 0.3 mg/kg (Decker, Majchrzak, & Anderson, 1992)].

In sum, several different lines of evidence suggest that the functional integrity of central cholinergic systems (muscarinic and nicotinic) is important for spatial learning in the MWM. Reductions of ACh activity resulting from lesions, age, or pharmacological blockade impairs spatial learning. Conversely, restoration of ACh activity attenuates the deficit resulting from ACh hypofunction, implicating ACh as the neurotransmitter responsible. However, there is also evidence that other factors in addition to ACh reduction contribute to these spatial learning deficits. Moreover, it is possible that ACh hypofunction may impair spatial learning by impairing attentional rather than mnemonic processes.

Glutamate

The role of the excitatory amino acid transmitter glutamate has been investigated with antagonists of the *N*-methyl-D-aspartate (NMDA) receptor. NMDA receptors have gained notoriety because of their suspected role in long-term potentiation (LTP), a cellular phenomenon speculated to be involved in the encoding of new information [e.g., (Teyler & Discenna, 1984)]. Several studies have suggested that learning and memory deficits produced by NMDA receptor antagonists may be related to the blockade of LTP [e.g., (Morris, Anderson, Lynch, & Baudry, 1986)], though this interpretation remains controversial (Keith & Rudy, 1990). The present review of the literature will examine only the contribution of NMDA receptors to spatial learning and will not evaluate the validity of the relationship between NMDA receptor blockade, LTP, and spatial learning.

Several studies have demonstrated that NMDA blockade impairs spatial learning in the MWM. D-2-amino-5-phosphonopentanoic acid (AP5)

is a competitive NMDA receptor antagonist that does not cross the blood-brain barrier and must therefore be infused intracranially. AP5, but not the inactive stereo isomer D,L-AP5, dose-dependently impaired spatial learning when infused either ICV (Butcher, Davis, & Morris, 1990; Davis, Butcher, & Morris, 1992; Morris, Anderson, Lynch, & Baudry, 1986) or intrahippocampally (Morris, Halliwell, & Bowerly, 1989). The ICV infusion of AP5 impaired spatial learning even when the inter-trial interval was reduced from 4 hrs to 30 seconds (Morris, Anderson, Lynch, & Baudry, 1986) to make the task easier. AP5-treated rats were also impaired when the platform was moved to the diagonally opposite quadrant, but were not impaired on a non-spatial simultaneous discrimination task, suggesting that AP5 did not disrupt sensorimotor and/or motivational processes (Morris et al., 1986). Visual discrimination learning was not impaired when AP5 was infused close to visual cortices (Butcher, Hamberger, & Morris, 1991), suggesting that NMDA receptors in this region were not required for accurate discrimination learning and that AP5-induced spatial learning deficits were not the result of drug-induced visual deficits. Subsequent studies have shown that AP5 does not impair spatial recall (Butcher, Hendry, & Morris, 1989; Heale & Harley, 1990), suggesting that AP5 does not impair spatial learning by impairing perceptual or other nonassociative processes. Another competitive NMDA receptor antagonist, 3-[2-carboxypiperazine-4-yl] propyl-1-phosphonic acid (CPP), also impaired spatial learning in mice (Upchurch & Wehner, 1990), although the one dose (5 mg/kg) tested also impaired cue learning and produced non-specific motor effects.

The effects of noncompetitive antagonists of the NMDA receptor have also been assessed on spatial learning in the MWM. Some studies have found that systemically administered 5-methyl-10, 11-dihydro-5H-dibenzo-[a,d] cyclohepten-5, 10-imine maleate (MK-801) impairs spatial learning (Mondadori, Weiskrantz, Buerki, Petschke, & Fagg, 1989; Robinson, Crooks, Shinkerman, & Gallagher, 1989; Wishaw & Auer, 1989) whereas other studies have found such treatment to be without effect (Halliwell & Morris, 1987; Shapiro & Bohbot, 1990). In those studies finding an effect with MK-801, doses ranged from 0.01 - 1.0 mg/kg and only a narrow dose range was found to selectively impair place but not cue learning. For example, a dose of

0.05 mg/kg impairs place but not cue learning while a dose of 0.08 mg/kg impairs both place and cue learning (Robinson, Crooks, Shinkman, & Gallagher, 1989). Doses above 0.25 mg/kg produce somnolence and impair the rats' ability to swim (Whishaw & Auer, 1989), but even very large doses (i.e., 1 mg/kg) of MK-801 fail to impair acquisition when administered after each daily training session (Mohler & Okada, 1977). A 0.07 mg/kg dose which impaired spatial learning did not impair spatial recall (Heale & Harley, 1990). Another noncompetitive NMDA receptor antagonist, ketamine, dose dependently impairs place but not cue learning (Alessandri, Battig, & Welzl, 1989), though larger doses also impair spatial recall (Wesierska, Macias-Gonzalez, & Bures, 1990).

In sum, both competitive and noncompetitive antagonists of the NMDA receptor impair spatial learning when administered prior to training, but not when administered after each trial block or after spatial learning is complete (spatial recall). Although high doses of NMDA receptor antagonists severely impair motor proficiency, spatial learning deficits can be observed in the absence of sensorimotor, motivational or perceptual deficits. Thus, NMDA receptors appear to play an integral role in spatial learning. The effect of NMDA agonists on spatial learning have not yet been investigated in the MWM. The effects of glutamate agonists and antagonists acting at the kainate/AMPA receptor also have not been tested.

Somatostatin

Somatostatin (SOM) is a putative neurotransmitter found in various cortical regions including the hippocampus (Storm-Mathisen & Ottersen, 1984). SOM has been implicated in mnemonic processes by the finding that SOM-like immunoreactivity is reduced in the cortex and hippocampus in patients with Alzheimer's disease (Davies, Katzman, & Terry, 1980). The observation that fifty percent reductions of SOM-like immunoreactivity in cortex and hippocampus via the ICV infusion of cystamine (100 mg/kg) impairs spatial learning (Fitzgerald & Dokla, 1989) provides evidence for a role for SOM in spatial learning. However, since the effects of SOM depletion on spatial recall and cue learning have not yet been assessed, it is not clear whether such depletions disrupt mnemonic processes exclusively.

Opioids

Several endogenous peptides are known to influence cognitive processes. Perhaps the most studied of these peptides are the opioids. Opioidergic activity has been found to impede learning and memory processes on other memory tasks (Gallagher, 1984; McGaugh, 1989). The MWM requires the rat to be repeatedly submerged into cold water (18 - 26°C), and similar stress procedures have been shown to elicit the release of endogenous opioid peptides, particularly in limbic structures (Barta & Yashpal, 1981). Interesting, then, is the finding that naloxone (3 mg/kg) produces a slight enhancement of spatial learning when given before, but not after training using a difficult 2 trials/day procedure (Decker, 1989). This finding suggests that the endogenous opioid activity elicited by cold water stress can be detrimental to spatial learning. However, the same dose of naloxone has been shown to impair, not enhance, spatial learning when a 4 trials/day procedure is used (McDaniel, Mundy, & Tilson, 1990). In the latter study, naloxone-treated rats performed poorly during training and did not show a preference for the correct quadrant during the probe trial. Dynorphin, an endogenous opioid peptide, is elevated in the hippocampus of aged rats with, but not without, spatial learning deficits (Jiang, Owyang, Hong, & Gallagher, 1989). Further, spatial learning is impaired when dynorphin is infused into the dorsal, but not ventral hippocampus, posttrial (McDaniel, Mundy, & Tilson, 1990). This impairment is antagonized by systemic naloxone, suggesting the impairment is mediated via endogenous opioid receptors. In sum, endogenous opioid activity appears to be detrimental to spatial learning, but does not appear to occur under normal circumstances.

Corticosteroids

Upon exposure to novel and/or stressful situations, corticosteroids are released from the adrenal gland (Lefur, Guilloux, Mitrani, & Uzan, 1979). Removal of circulating corticosteroids via adrenalectomy, impairs spatial learning, but not cue learning, in the MWM (Oitzl & Kloet, 1992). Further, pretraining administration of the mineralcorticoid antagonist spironolactone also impaired spatial learning in the MWM (Oitzl & Kloet,

1992). Hence, the rise in circulating corticosteroids elicited by novel learning situations is an important component of accurate spatial learning.

Catecholamines

Noradrenaline does not appear to have an important role in spatial learning in the MWM. For example, 6-hydroxydopamine (6-OHDA) lesions of the dorsal noradrenergic bundle, which arises from the locus coeruleus to innervate cortex and hippocampus (Moore & Bloom, 1979), do not impair spatial learning in 22 - 26°C water (Valjakka, Riekkinen, Sirvio, Nieminen, Airaksinen, Miettinen, & Riekkinen, 1990) but may either facilitate (Selden, Cole, Everitt, & Robbins, 1990) or have no effect in colder water (11 - 12°C; Valjakka, Riekkinen, Sirvio, Nieminen, Airaksinen, Miettinen, & Riekkinen, 1990). Lesions of the ventral noradrenergic bundle do not impair spatial learning in either warm or cold water (Selden, Cole, Everitt, & Robbins, 1990). Similarly, selective depletion of forebrain noradrenaline with 6-OHDA does not impair spatial learning in either adult rats (Hagan, Alpert, Morris, & Iversen, 1983), neonates (Whishaw, Sutherland, Kolb, & Becker, 1986) or adult rats treated with 6-OHDA as neonates (Sutherland, Kolb, Becker, & Whishaw, 1981).

Pharmacological manipulations of noradrenergic systems have tended to confirm the lesion data. For example, propranolol (10 mg/kg), a β -adrenergic antagonist, does not impair spatial learning (Decker, Gill, & McGaugh, 1990). Further, guanfacine (4 ml/kg), an α_2 agonist, failed to affect spatial learning as indexed by distance taken to reach the platform (Sirvio, Riekkinen, Vajanto, Koivisto, & Riekkinen, 1991). The α_2 agonist dexmedetomidine slowed spatial learning at lower doses (0.3 and 0.9 μ g/kg) but was without effect at higher doses (3.0 and 9.0 μ g/kg; Sirvio, Riekkinen, Ekonsalo, Lammintausta, & Riekkinen, 1992). However, in the latter study, a probe test was not given to rats treated with the lower doses to determine if spatial learning had occurred by the end of training; moreover, no tests for sensorimotor impairment were administered. Taken together, the pharmacological manipulations tend to support the lesion data to suggest that noradrenergic systems do not have a major role in spatial learning.

However, better controlled studies are warranted to further clarify the effects of other noradrenergic drugs on MWM acquisition.

Like noradrenaline, dopamine systems do not appear to be required for spatial learning per se. Neostriatal and cortical dopamine depletion (>80%) via bilateral 6-OHDA lesions of the nigrostriatal or mesocorticolimbic dopamine systems does not impair spatial learning (Hagan, Alpert, Morris, & Iversen, 1983). Although dopamine-depleted rats were slower to learn the platform location, they showed a normal preference for the correct quadrant during the probe trial (Hagan, Alpert, Morris, & Iversen, 1983). More extensive depletions (>95%) impaired spatial learning, spatial recall and cue learning (Whishaw & Dunnett, 1985). In the latter study, a probe trial was not administered, so it remains possible that a spatial bias did develop in dopamine-depleted rats. Selective dopamine depletions in the caudate-putamen produced a small spatial learning deficit, although a probe trial was not used and the authors noted that treated rats could not swim in a straight line (Selden, Cole, Everitt, & Robbins, 1990). Further, dopaminergic agonists (methamphetamine & apomorphine) and antagonists (haloperidol & α -flupenthixol) produce a dose-dependent (Whishaw & Dunnett, 1985) and trial-dependent (Whishaw, Mittleman, & Evenden, 1989) impairment of spatial recall and cue performance. Together, these findings suggest that alterations of dopamine activity (depletion, blockade or activation) impede spatial learning by producing sensory, motor, sensorimotor or motivational impairments rather than by disrupting mnemonic processes.

In sum, noradrenaline depletion, blockade or activation does not impair spatial learning in the MWM. Dopamine depletion impairs spatial learning, but also impairs cue learning and spatial recall. In addition, dopamine agonists and antagonists impair spatial recall and cue learning, both indicative of sensorimotor impairment. Therefore, neither central catecholamine system appears to be required for spatial learning, though dopamine may be required for proficient performance.

Serotonin

Serotonin (5-HT) does not appear to be important for spatial learning in the MWM. Reducing 5-HT synthesis by inhibiting tryptophan hydroxylase with *p*-chlorophenylalanine (PCPA) does not impair spatial learning (Richter-Levin & Segal, 1989; Riekkinen, Riekkinen, Sirvio, & Riekkinen, 1992). Further, >70% reductions of cortical and hippocampal 5-HT with ICV infusions of 5,7-dihydroxytryptamine (5,7-DHT) also does not impair spatial learning (Nilsson, Strecker, Daszuta, & Bjorklund, 1988; Riekkinen, Sirvio, & Riekkinen, 1990). In contrast, buspirone, a 5-HT_{1A} partial agonist/antagonist, was found to dose-dependently impair spatial learning (McNaughton & Moris, in press) but not spatial recall (Rowan, Cullen, & Moulton, 1990). However, the single dose of buspirone (2 mg/kg) tested in the latter study did not produce the postural alterations common to 5-HT activation (Skorinick, Weismann, & Youdim, 1985), suggesting that the place deficit may not be mediated by 5-HT systems. Overall, then, 5-HT by itself does not appear to be critical for spatial learning. However, while selective 5-HT depletions do not impair spatial learning, 5-HT depletions do potentiate spatial learning deficits produced by septal (Richter-Levin & Segal, 1989) and NBM lesions (Riekkinen, Sirvio, & Riekkinen, 1990). The latter finding suggests an interaction of 5-HT and ACh systems in spatial learning.

Summary

A great deal has been learned about the neurochemical basis of spatial learning in the rodent using the MWM. Blockade of ACh and NMDA receptors impair spatial learning at doses that do not impair cue learning or spatial recall, suggesting that these transmitter systems play a critical role in the formation of spatial memories. In addition, preliminary analysis of somatostatin and corticosteroids reveals that they too may be important. Conversely, activation of opioid systems impairs spatial learning, suggesting that this transmitter system is detrimental to spatial memory formation. Noradrenaline, dopamine or serotonin systems do not appear to be required for spatial memory formation, though dopamine may be required for normal performance.

It is unlikely that a single transmitter system mediates the mnemonic processes required for spatial learning in the MWM, and as the above review reveals, several transmitter systems are critical. Some investigations have demonstrated that interactions among these systems may also be important. For example, ACh appears to interact with both 5-HT (Nilsson, Strecker, Daszuta, & Bjorklund, 1988; Richter-Levin & Segal, 1989; Riekkinen, Sirvio, & Riekkinen, 1990), and noradrenaline (Decker, Gill, & McGaugh, 1990; Riekkinen, Sirvio, Valjakka, 1990).

In each case except the opioids, BZs decrease the level of the neurotransmitter/neuromodulator. That is, BZs decrease acetylcholine (Sarter et al., 1990), glutamate (Baba, Okumura, Mizuo, & Iwata, 1983), somatostatin (Stryker, Conlin, & Reichlin, 1986), corticosterone (Lahti & Barsuhn, 1974), catecholamines (e.g., Yang, Lou, & Zhou, 1988), and serotonin (Mennini, Gobbi, & Romandini, 1986; Thiebot, 1986). Conversely, BZs increase opioid activity (Wuster, Duka, & Herz, 1980A). However, given that catecholamine and serotonergic systems do not appear to be important for spatial learning by themselves, it is unlikely that BZ-induced reductions of these systems are responsible for the ensuing spatial learning deficit. Hence, BZ-induced reductions of acetylcholine, glutamate, somatostatin and corticosteroids may be responsible for the observed impairment. As well, BZ-induced increases in opioidergic activity may also be responsible for the spatial learning deficit. In the following sections, investigations were conducted to assess the interactions between BZs, acetylcholine and spatial learning as well as BZs, opioids and spatial learning. First, however, the interaction between opioid systems and spatial learning was better characterized.

Experiment III: Opioidergic systems and memory

The role of endogenous opioid systems in learning and memory processes has been the focus of increasing experimental interest, and a consistent pattern has emerged (for reviews, see Gallagher, 1984, 1985). Briefly, opioid agonists impair and opioid antagonists facilitate acquisition when administered either prior to or immediately following training trials. This pattern has been observed across a variety of animal memory tasks,

including heart rate conditioning (Gallagher, Kapp, McNall, & Pascoe, 1981), avoidance conditioning (Izquierdo & Dias, 1981; Martinez & Rigter, 1980; Messing et al., 1979), and aversively motivated simultaneous discrimination (Castellano, 1975). The role of opioid systems in spatial memory is less clear. Rats administered naloxone after being trained on a radial maze show faster reacquisition when the maze is moved into a new environment (Gallagher, King, & Young, 1983), yet neither naloxone nor morphine administered prior to, or immediately following, training trials affected performance in the radial maze was maintained in a constant environment (Beatty, 1983). The rats used in the latter study were highly overtrained, which may have reduced the sensitivity of the task to potential drug effects. More recently, it was demonstrated that naloxone administered prior to, but not immediately after, training marginally facilitated spatial learning in the MWM (Decker, Introini-Collison, & McGaugh, 1989) and spatial working memory in the radial maze (Canli, Cook, & Miczek, 1990). Conversely, posttraining infusion of dynorphin, an endogenous opioid peptide, in the dorsal hippocampus *impaired* acquisition in the MWM (McDaniel, Mundy, & Tilson, 1990). The latter two findings, which are consistent with the mnemonic pattern of impairment/facilitation found with other tasks, suggest that opioid activity is detrimental to spatial learning. However, McDaniel et al. (1990) also found that naloxone (3mg/kg) alone paradoxically produced a small but significant impairment of spatial learning. Thus, the effects of pretraining administration of either an opioid agonist or an antagonist on spatial learning in the MWM require further delineation.

Experiment III sought to characterize the effects of pretraining morphine on spatial learning, place retention, and cue learning in the MWM. Place retention and cue learning procedures were incorporated to determine whether morphine produces motivation or sensorimotor deficits that may account for its effects on spatial learning. The effect of posttraining morphine on spatial learning was also assessed. Because tolerance develops to the effects of morphine (Madden, Akil, Patrick, & Barchas, 1977; Rosow, Miller, Poulsen-Burke, & Cochin, 1982b; Shippenberg, Emmett-Oglesby, Ayesta, & Herz, 1988), Experiment IIIa also assessed whether tolerance develops to spatial learning deficits after chronic morphine treatment.

Experiment IIIa also examined the effects of morphine and naloxone on body temperature, since: (1) alterations of approximately 3°C in either direction of normothermia can produce both anterograde (Ahlers & Riccio, 1987) and retrograde amnesia (Rauch, Welch, & Gallego, 1989), (2) the MWM requires the rat to escape from a pool of cool (18°-26°C) water (Morris, 1981, 1984), and (3) opioid agonists and antagonists, such as morphine (Rosow, Miller, Pelkan, & Cochin, 1980) and naloxone (Rosow et al., 1982a) influence thermoregulatory mechanisms. Experiment IIIb assessed whether increased incentive to escape (cold water) could reverse place and cue deficits produced by morphine.

METHODS

Animals

The subjects were 55 naive hooded male rats of the Long-Evans strain, housed in pairs in shoebox cages and maintained on a 12:12-h light:dark cycle. The rats weighed approximately 400 g at the beginning of the experiment, and food and water were available ad lib

Apparatus and procedure

Amnesia was assessed in the MWM described in Experiment I except that all groups were given four trials each day for 14 consecutive days.

Drugs and Group Assignment

At the beginning of the experiment, rats were divided into nine treatment groups. The first three groups ($n = 15$, 5/group) were given pretraining injections of morphine sulfate (5, 10, or 20 mg/kg). the fourth group ($n = 5$) was treated with naloxone hydrochloride (2 mg/kg; Sigma), the fifth group ($n = 5$) was co-administered morphine (20 mg/kg) and naloxone (2 mg/kg), the sixth group ($n = 10$) received saline (0.9% NaCl), the seventh group ($n = 5$) received morphine (20 mg/kg) immediately after each daily training session, and the eighth group ($n = 5$) received saline posttraining. The ninth, or *switch*, group ($n = 5$) received saline for the first 7 days of acquisition and was switched to morphine (20 mg/kg) for the remaining 7 days of training. All injections were 1 ml/kg given i.p. in the animals' home

cages. Pretraining morphine and naloxone were injected 25 and 15 min prior to testing, respectively.

Procedures

Colonic temperature. Core body temperature was assessed colonically with an Atkins Technical Inc. Thermal probe. The probe was inserted 6 cm into the rat's rectum for 30 sec. Colonic temperature was sampled three times daily: (1) after rats had been removed from their home cages to the holding cage (predrug normothermia), (2) 15 min after injection (postdrug), and (3) after rats had swum in a pool filled with 22°C water for 60 sec.

Tolerance test. Upon completion of the acquisition, probe trials and visible-platform task, the submerged platform was moved to the center of the diagonally opposite (NW - SE) quadrant of the pool. The saline group was divided into two ($n = 5$) groups. Half of the saline group continued to receive saline while the other half was administered morphine (10 mg/kg) and served as the *acute* group. The group that received morphine (10 mg/kg) throughout training continued to receive morphine during reversal training and served as the *chronic* group. These three groups were trained to the new platform locations for 6 days (4 trials/day) and a probe trial was given on Day 7.

Data Analysis

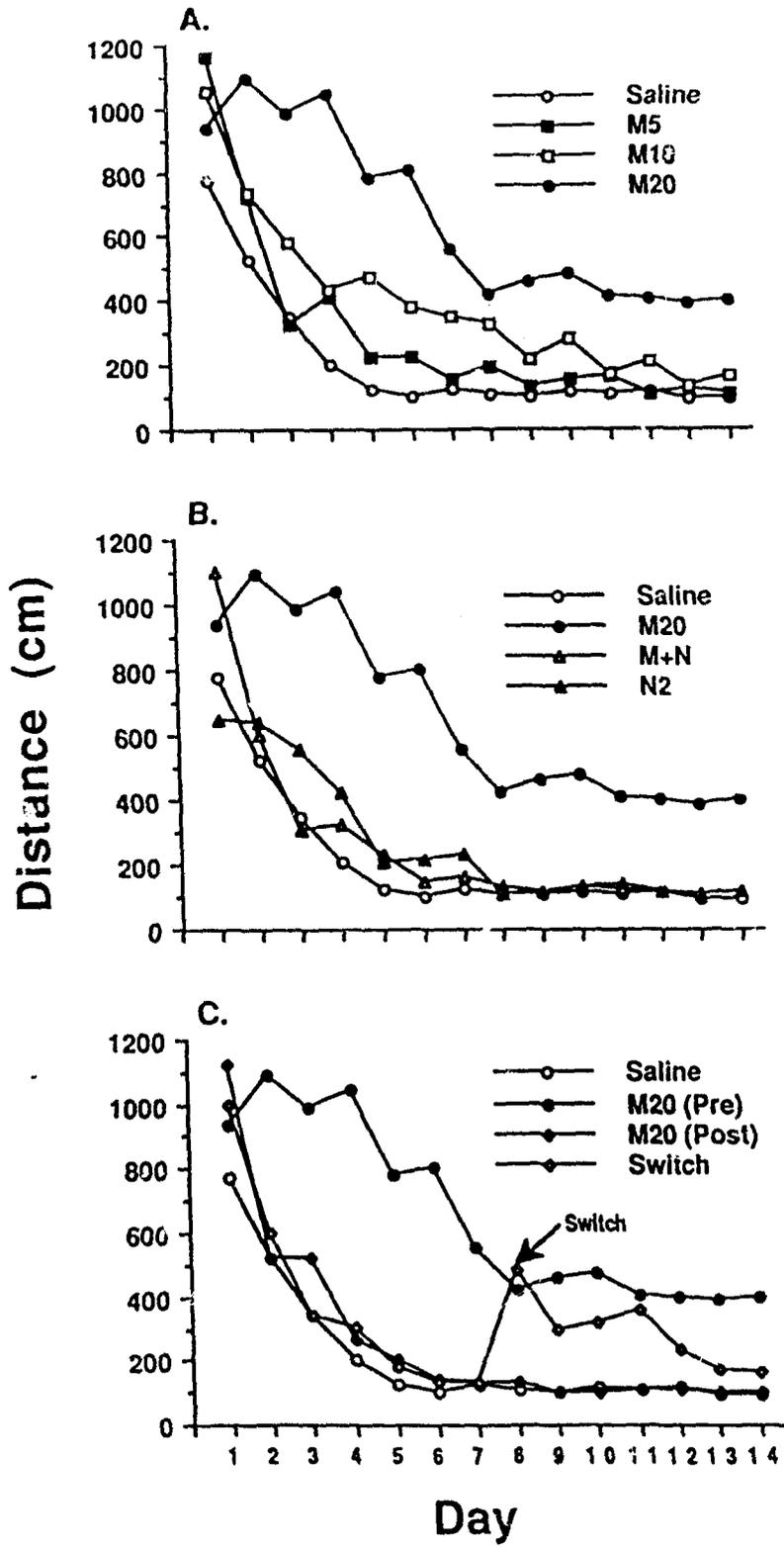
The differences in escape latency, swim path length, and heading error (degrees deviation from a straight line between the start position and the platform over the first 12 cm of the swim path) were assessed with a repeated measures analysis of variance (ANOVA). Post hoc comparisons were assessed with the use of Tukey's (HSD) method and individual t tests. In every case, the minimum acceptable level for statistical significance was $p < .05$.

RESULTS

Maze Testing

Pretraining morphine produced a dose-dependent increase in swim path lengths (Fig. 3.1A). The 10- and 20-mg/kg doses, but not the 5-mg/kg dose, increased the distance required to locate the submerged platform relative to

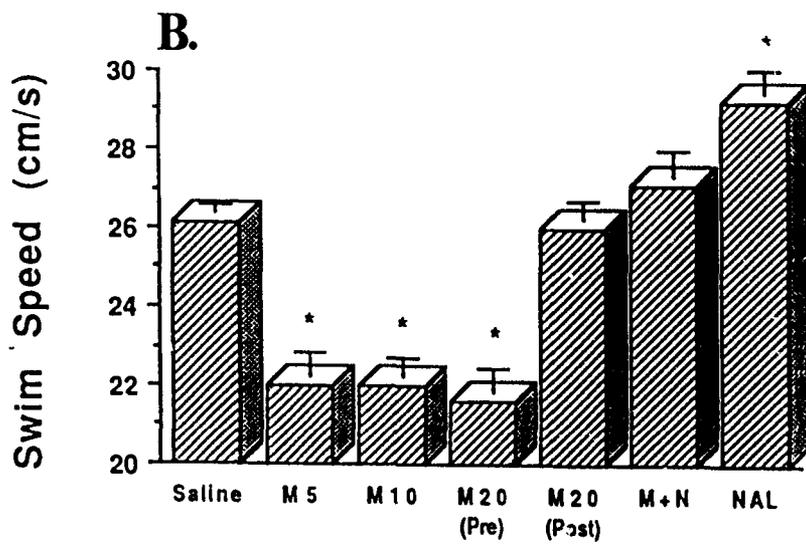
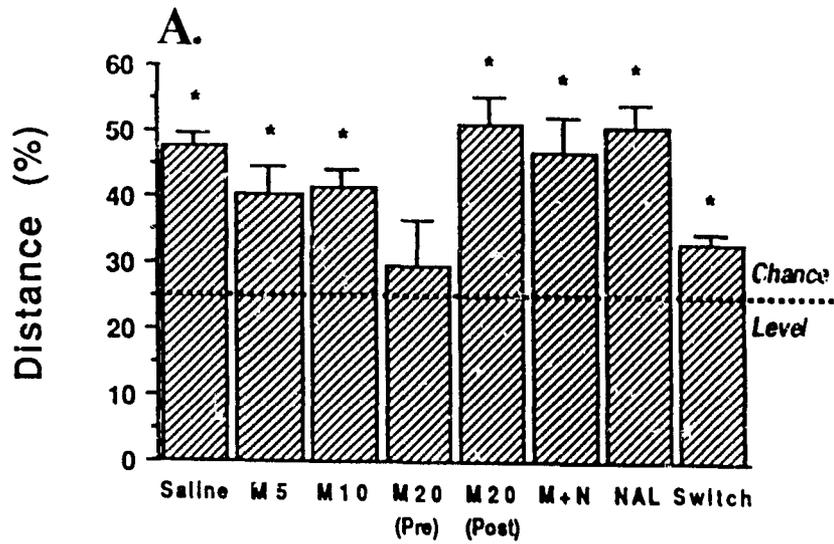
Figure 3.1: Effects of (A) three doses of morphine, (B) naloxone and naloxone + morphine, and (C) posttraining and postacquisition morphine on the distance taken to locate the submerged escape platform. Note: (1) the gradual improvement of morphine-treated rats, (2) the reversal of the morphine impairment by naloxone, (3) the lack of effect of posttraining morphine, and (4) the impairment of performance when rats are switched to morphine after having acquired the platform location under saline. (M + morphine; N = naloxone; numbers indicate dose in mg/kg of body weight).



control performance. Naloxone blocked the increase in swim paths produced by the highest dose of morphine but had little effect on swim path lengths when given alone (Fig. 3.1B). Posttraining morphine (20 mg/kg) had no effect on swim path lengths (Fig. 3.1C). When rats were switched from saline to pretraining morphine (20 mg/kg) on the 8th day of training, swim path lengths increased to levels comparable to those of rats receiving morphine from the outset of training (Fig. 3.1C). An overall ANOVA on swim path length revealed a significant group difference, $F(3,96) = 26.96$, $p < 0.001$, and day difference, $F(13,1248) = 57.13$, $p < 0.001$, and a significant interaction between groups and day, $F(39,1248) = 3.15$, $p < 0.001$. Escape latencies (data not shown) showed a similar pattern. An ANOVA of heading error (data not show) also revealed a significant group difference, $F(3,96) = 40.18$, $p < 0.001$, and day difference, $F(13,1248) = 18.89$, $p < 0.001$, and an interaction of group and day, $F(39,1248) = 1.53$, $p < 0.05$. Post hoc analysis revealed that the 10- and 20-mg/kg doses of morphine, but not the 5-mg/kg dose, increased swim path lengths, escape latencies, and heading errors relative to the performance of controls ($p < .01$). The escape latencies and swim path lengths of the 20 mg/kg groups were greater than those of the 10-mg/kg group ($p < .01$). Naloxone significantly antagonized morphine ($p < .01$), but had no effect when administered alone ($p < .05$). The naloxone + morphine group showed no significant differences in spatial learning performance (distance, latency, and heading error) relative to the control group or the naloxone group ($p < .05$). The naloxone group did not differ from controls on any measure ($p < .05$). Rats administered posttrial morphine did not differ from controls on any measure. The switch group did not differ from controls on any measure during the first 7 days of training but had significantly longer swim paths than controls, once switched to morphine ($p < .01$). During administration of pretraining morphine, the swim path lengths of the switch group did not differ significantly from those of the rats treated with morphine (20mg/kg) from the outset.

The effects of morphine and naloxone on acquisition were confirmed in the posttraining probe trial. The distance traveled in the correct quadrant during the probe trial is show in Figure 3.2A. Only the rats that were

Figure 3.2: (A) Effect of pretraining and posttraining morphine (M), postacquisition morphine (switch), naloxone (NAL), and naloxone plus morphine (M + N) on the distance traveled in the correct quadrant during the 60-sec probe trial. (B) Effect of pretraining and posttraining morphine, naloxone, and naloxone plus morphine on swim speed averaged over the 14 days of testing. Note the swim speed reduction produced by the three doses of morphine and the swim speed increase produced by naloxone. * $p < 0.01$ relative to chance level (25%; dotted line) in A and relative to saline performance in B. (Error bars represent *S.E.M.*)



administered 20-mg/kg morphine prior to daily training sessions failed to show a significant preference for the correct quadrant. Rats given 5 or 10 mg/kg morphine, posttrial morphine (20 mg/kg), morphine + naloxone, or naloxone and rats switched to morphine demonstrated an above chance (25%) preference for the correct quadrant ($p < .01$). Although the switch group did show a preference for the correct quadrant, this preference was significantly lower than that shown by controls ($p < .01$). Swim speeds were reduced by all three doses of morphine ($p < .01$; see Figure 3.2B); naloxone reversed this effect and also increased swim speeds when given alone ($p < .05$). Swim speed was not reduced when morphine was administered posttraining. When rats were switched to morphine, there was a significant drop in swim speed relative to preswitch and control swim (Preswitch, 25 ± 0.6 cm/sec vs. postswitch, 23 ± 0.6 cm/sec ; $p < .05$). It was observed incidentally that morphine-pretreated rats, but not naloxone- or saline-treated rats, would occasionally jump off the platform after having climbed on. Further, morphine-pretreated rats displayed muscular rigidity when handled. Both of these effects were blocked by naloxone.

Unexpectedly, the 10- and 20- mg/kg doses of morphine impaired performance on the cue task relative to the performance of control animals ($p < .01$; see Figure 3.3). Naloxone antagonized the deficit produced by the 20-mg/kg dose of morphine but had no effect when administered alone. Rats switched to morphine were also impaired when required to escape to the visible platform ($p < .05$), although not to the same extent as rats given morphine from the outset.

Tolerance Test

The swim path distances of rats receiving morphine chronically or acutely are illustrated in Figure 3.4A. When the platform was reversed to the center of the opposite quadrant, rats treated chronically with morphine (10 mg/kg) showed faster acquisition relative to acutely treated rats, but they were still impaired relative to controls. There was an overall group effect, $F(3,76)=40.19$, $p < 0.001$, and post hoc analysis revealed that the chronic morphine group had shorter swim paths than did the acute morphine group ($p < .05$), although both groups had longer swim paths than did saline-treated

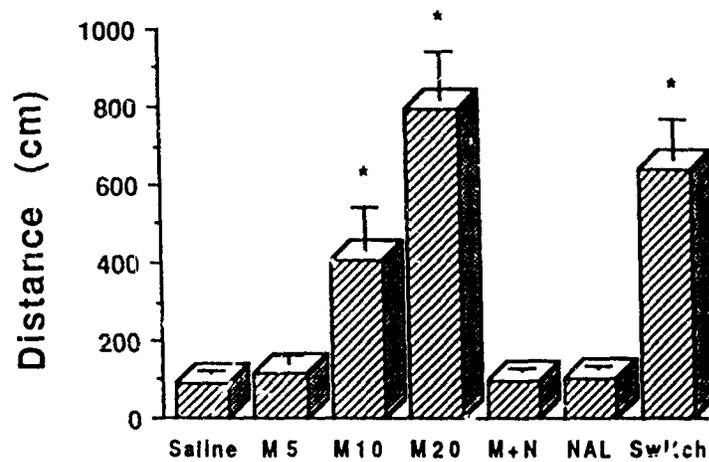
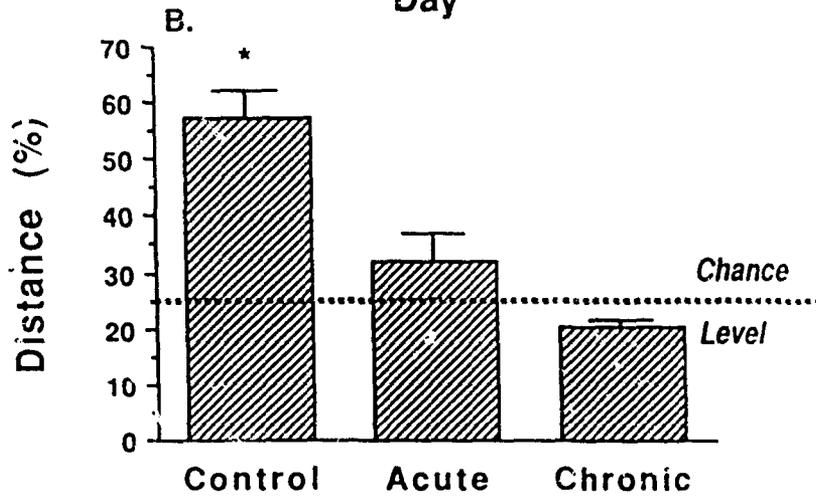
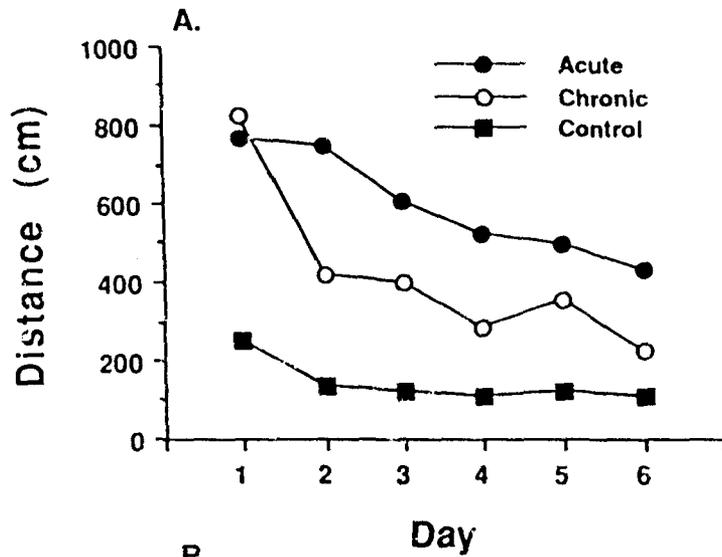


Figure 3.3: The distance, averaged over all trials, taken by each treatment group to swim to a single visible platform. M = morphine. NAL = naloxone. M + N = naloxone plus morphine. numbers = drug dose in mg/kg. Note the dose-dependent impairment caused by morphine. * $p < .05$ relative to saline performance.

Figure 3.4: A comparison between rats treated chronically or acutely with morphine on (A) the distance to locate the submerged platform, and (B) the percent distance traveled in the correct quadrant during the probe trial. Note that rats treated chronically with morphine fail to show a preference for the correct quadrant despite improvements in acquisition. * $p < 0.01$ relative to chance level (dotted line) in panel B.



controls ($p < .01$). During the probe trial (Figure 3.4B), only the control group showed a preference for the quadrant that had contained the submerged escape platform ($p < .01$), suggesting that the chronic morphine rats were still impaired despite evidence of tolerance.

Colonic Temperature

Both the 10- and 20-mg/kg doses of morphine increased colonic temperature during both the postdrug and postswim phases, and this effect was antagonized by naloxone (Table II). Naloxone had no effect on colonic temperature when given alone. An overall ANOVA revealed a significant difference between groups, $F(3,16)=4.11$, $p < 0.05$, and between sampling periods, $F(1,16) = 7.5$, $p < 0.01$, but no significant interaction between groups and sampling periods, $F(3,16)=0.32$, N.S. Post hoc comparisons revealed that the 10- and 20-mg/kg morphine groups demonstrated statistically significant hyperthermia during both the postdrug and postswim phases relative to saline-treated controls. The 10- and 20-mg/kg morphine groups also demonstrated hyperthermia during the postswim phase, relative to saline-treated controls ($p < .05$), which showed a reduction of colonic temperature. The naloxone and naloxone + morphine groups did not differ from saline-treated controls during any sampling phase. The naloxone + morphine group did not differ significantly from the naloxone group. It should be noted that the greatest (average) deviation from normothermia was only 0.7°C.

DISCUSSION

In the present experiment pretraining morphine impaired spatial learning in dose-dependent manner. Medium and high doses of morphine increased the swim path lengths to the hidden platform and increased the number of days required to reach asymptotic performance. The highest dose of morphine (20 mg/kg) prevented attainment of control-level performance, increasing path lengths threefold over control levels on the last 7 days. This dose of morphine also impaired performance on the probe trial; morphine-pretreated rats failed to show a preference for the correct quadrant. The medium dose of morphine did not prevent spatial learning; by the end of the

TABLE II

Effects of three different doses of morphine, naloxone, and naloxone + morphine on core body temperature

<u>Group</u>	<u>Predrug</u>	<u>Postdrug</u>	<u>Postswim</u>
Vehicle	38.0 ± 0.08	38.1 ± 0.30	37.3 ± 0.50
M5	38.0 ± 0.02	38.2 ± 0.30	37.5 ± 0.40
M10	38.0 ± 0.03	38.4 ± 0.19*	38.1 ± 0.19*
M20	38.1 ± 0.08	38.7 ± 0.08**	38.1 ± 0.08**
NAL	38.1 ± 0.09	37.9 ± 0.08	37.4 ± 0.04
NAL + M20	38.1 ± 0.04	38.1 ± 0.07	37.4 ± 0.60

Data expressed as mean ± S.E.M.

* $P < 0.05$ compared to controls

** $P < 0.01$ compared to controls

Note—The 20 mg/kg dose induces significant increases in core body temperature during both the post-drug and post-swim phases and is reversed to control levels by naloxone.

acquisition period, swim distance and quadrant preferences were comparable to control levels. Pretraining morphine (20 mg/kg) also impaired performance (retention). Rats given morphine (20 mg/kg) after acquiring the platform location (switch) demonstrated a deficit comparable to that for rats treated with morphine from the outset of training. Post-training morphine (20 mg/kg) did not impair spatial learning.

Partial tolerance developed to morphine over the 15 days of acquisition training, but could not fully account for the control-level performance of the rats given the moderate dose of morphine. When tested with a new platform location, rats treated with the medium dose of morphine continued to be impaired, though they were not as impaired as comparably trained rats newly exposed to morphine. However, during the probe trial, neither group of morphine-treated rats demonstrated a preference for the correct quadrant, suggesting that both groups had not acquired the spatial location of the platform. Therefore, it does not appear that complete tolerance developed to the spatial learning deficits produced by morphine.

Morphine effects were not limited to spatial learning. Morphine also reduced swim speeds and increased the likelihood of jumping off the platform once the platform had been attained. Surprisingly, both the moderate and high doses of morphine impaired performance of the visible-platform task. It should be noted that rats treated with either the moderate or high doses of morphine were capable of climbing onto the visible platform, despite their seeming lack of desire to do so. Finally morphine impaired the performance of a previously acquired spatial location. Together, these results suggest that morphine impairs one or several performance variables that are required for spatial learning, rather than spatial learning per se.

Naloxone antagonized all the effects of morphine, while having negligible effects when administered alone, suggesting that the effects of morphine were mediated by opioid receptors.

The failure of morphine to affect spatial learning when administered after each trial contrasts with the impairment of acquisition found in several

studies when an opioid agonist was administered posttraining, which has often been interpreted as an interference with "consolidation" processes (see Gallagher, 1984). Our finding suggests that posttraining consolidation processes required for accurate acquisition on this task are either not disrupted by increased opioid activity or are completed prior to morphine administration. This is also supported by the finding that naloxone has little effect on spatial learning when administered posttraining (Decker et al., 1989), although the finding that posttrial infusion of dynorphin into the dorsal hippocampus impairs spatial learning would argue against it. Reconciliation of this discrepancy will require further experimentation.

Morphine has many physiological actions, and it is important to consider which ones were most critical to the deficits observed, and whether these deficits represent a disruption of learning and memory or a disruption of sensory, motor, thermoregulatory, or motivational processes. Two critical findings in this regard are the impairment of visible-platform task and spatial recall in the switch group. The retention tests and the visible-platform task were employed to assess the possible contribution of non-mnemonic factors such as sensorimotor or motivational processes. The deficits of cue learning and spatial recall observed in the present experiment suggest that morphine was not disrupting spatial learning specifically, but rather was impairing some other process necessary for learning in general. For example, morphine induces both hyperthermia (Tulunay, 1980) and muscular rigidity (Walter & Kuschinsky, 1989), two effects that could potentially impair spatial learning. Although morphine did produce moderate hyperthermia in the present experiment, the magnitude of this hyperthermia (0.7°C) has been demonstrated to be insufficient to impair MWM performance (Rauch et al., 1989). Morphine also produced muscular rigidity, particularly at the highest dose. Despite this, however, morphine-treated rats were capable of climbing onto and maintaining themselves on the escape platforms, and they showed no obvious disruption of swimming. Furthermore, unlike morphine's effect on spatial learning, morphine's effect on swim speed was not dose dependent; the highest dose of morphine reduced swim speed to the same extent as the 5-mg/kg dose did. In addition, swim distances would not be expected to be affected by changes in swim

speed. Therefore, although morphine did produce hyperthermia and muscular rigidity in the present experiment, these effects were not sufficient to account for the observed acquisition deficit.

A third non-mnemonic effect of morphine that could have interfered with learning is an impairment of visual perception (Grilly, Genovese, & Nowak, 1980; West, Hernandez, & Appel, 1982). An essential feature of the submerged-platform version of the MWM is the utilization of ambient room cues to accurately navigate to a hidden escape platform, although other less efficient response-based strategies can be adopted (Sutherland & Dyck, 1984). In the present experiment, morphine-treated rats were impaired even when a single local and highly visible cue (i.e., the visible platform) was available. A similar impairment was obtained in a previous study in which 10- and 30-mg/kg, but not 5-mg/kg, morphine impaired light/dark discrimination in a Y water maze (Castellano, 1975). Nevertheless, it is difficult to argue that rats in the present experiment were unable to locate the visible platform, because morphine-treated rats did on occasion contact the platform only to push off and continue swimming. Visual impairments were further ruled out by the observation that rats treated with a moderate dose of morphine (10 mg/kg) eventually became as proficient as controls at locating the platform and at concentrating their search in the correct quadrant during the probe trial. It might be noted that in an earlier study, rats treated with atropine methylnitrate, a peripherally acting muscarinic antagonist that causes pupillary dilation (Leopold & Comroe, 1984), did not show spatial learning deficits in the MWM (Sutherland, Whishaw, & Regehr, 1982). Therefore, it seems unlikely that visual/perceptual deficits alone could account for the observed learning deficit.

A final non-mnemonic effect of morphine that may have produced the observed learning impairments is analgesia/antinociception (Lloyd, Appel, & McGowan, 1978; Martin, 1984; Rochat, Cervo, Romandini, & Samanin, 1982). In the present experiment, morphine could have attenuated the aversiveness of the cold water, thereby reducing the rat's incentive to escape. Three observations from the present experiment indicate that morphine-treated rats did not find the cold water as aversive as controls did:

Morphine-treated rats swam slower, took longer to initiate swimming when placed into the water and were more likely to jump off the platform than were controls. All of these effects resemble the consequences of reducing the incentive value of water by making it warmer (Wever, 1932). In Experiment IIIb, this interpretation of the morphine-induced deficits was investigated.

EXPERIMENT IIIb

In Experiment IIIA, morphine-treated rats demonstrated several behaviors that suggested that the perceived aversiveness of the cold water was diminished. Indeed, the behavior of morphine-treated rats resembled that displayed by rats trained to escape from warmer water. For example, Wever (1932) noted that at warmer water temperatures, rats "did not head for the goal immediately, but spent some time in casual exploration, or for a few moments remained motionless at the entrance... and then finally at a relatively slow and leisurely gait swam to the landing platform" (p. 222). Therefore, it seems possible that the poorer performance of morphine-treated rats was due not to interruptions of memory processes, but rather to a decrease in motivation due to attenuation of the aversiveness of the 22°C water. The present experiment assessed whether increased incentive to escape (cold water) could reverse the place and cue deficits produced by morphine.

METHOD

Animals and Apparatus

Eighteen naive hooded male rats of the Long-Evans strain served as subjects. The rats were maintained as described in the previous experiment, and the same MWM was used.

Drugs and Group Assignment

The rats were divided into one of three treatment groups receiving morphine sulfate ($n = 6$; 10 mg/kg), naloxone hydrochloride ($n = 6$; 2 mg/kg), or saline ($n = 6$, 1 ml/kg). All drugs were dissolved in saline (0.9% NaCl) and administered i.p. in the rat's home cage 20 min prior to

behavioral testing. All rats in each group were tested in both conditions (cold water and warm water).

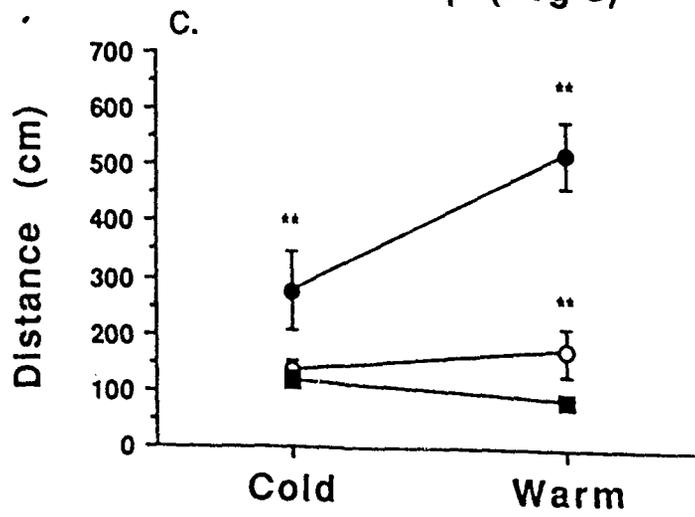
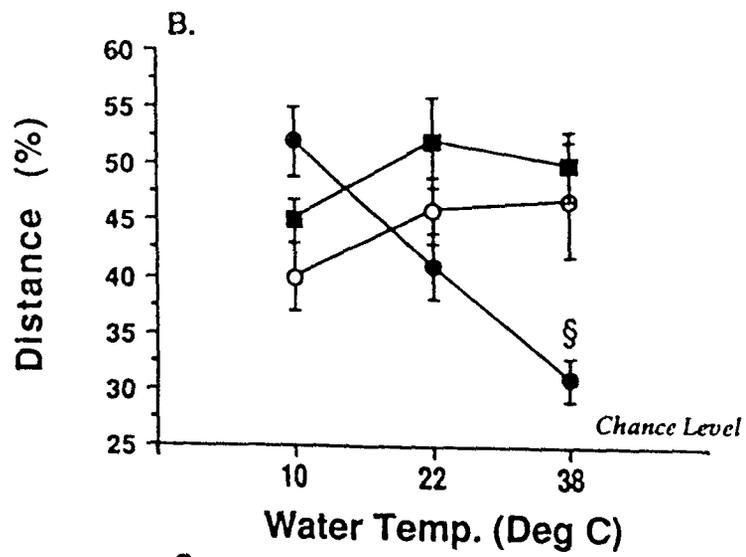
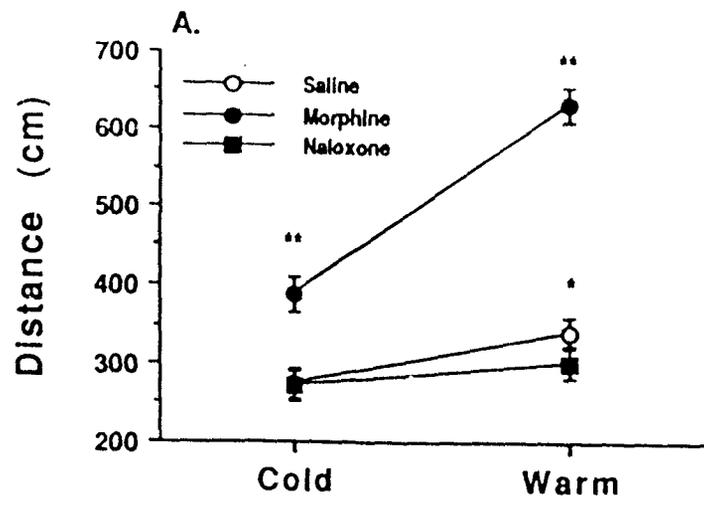
Procedure

The maze training was a modification of the "moving platform" task described by Whishaw (1985); the rats were required to learn a *new* platform location on each test day (eight trials/day). The procedure differed from Whishaw's (1985), in that each trial was separated by an interval of approximately 4 min. Water temperature alternated between $38^{\circ} \pm 1^{\circ}\text{C}$ and $10^{\circ} \pm 1^{\circ}\text{C}$ on successive days. Each rat was tested six times in both water temperatures (one temperature/day), with a new platform position each time (total of 12 new platform positions). In addition to latency and distance measures, the incidence of jumping off the platform was also recorded. On the 6th and final day of each incentive condition, the platform was removed after the eighth trial and each rat was given a 60-sec probe trial. During the final 2 days of testing, colonic temperatures were sampled at six phases during testing to determine whether hyper- or hypothermia resulted from the drug and/or water temperature. When the moving platform task was completed, rats were tested with the visible platform (as described in Experiment IIIa) under both incentive conditions.

RESULTS

Water temperature affected spatial learning in both saline- and morphine-treated rats. Morphine-treated rats demonstrated a marked impairment relative to saline- or naloxone-treated rats ($p < .01$) in warm water. Morphine-treated rats performed nearly as well as controls in cold water, although they did differ from controls significantly ($p < .05$; see Figure 3.5A). Saline-treated rats were better able to find the submerged platform in cold water, with distances significantly shorter than in the warm-water condition ($p < .05$). Naloxone-treated rats were significantly faster than controls in warm water ($p < .05$) and did not show a significant improvement between cold- and warm-water conditions.

Figure 3.5: Effects of morphine and naloxone on (A) the distance taken to locate the submerged platform in either cold ($10^{\circ}\pm 1^{\circ}\text{C}$) or warm ($38^{\circ}\pm 1^{\circ}\text{C}$) water (averaged over all trials under each condition) and (B) the distance traveled in the correct quadrant during cold- and warm-water probe trials as well as performance on the Experiment IIIA probe trial ($22^{\circ}\pm 1^{\circ}\text{C}$). (C) The distance taken to navigate to the visible platform (averaged over all trials in each condition). * $p<0.05$ compared to naloxone performance as well as cold water performance. ** $p<0.01$ compared with control levels, § $p<0.05$ compared with chance level (25%) in panel B; all other means are significantly greater than chance levels (25%).



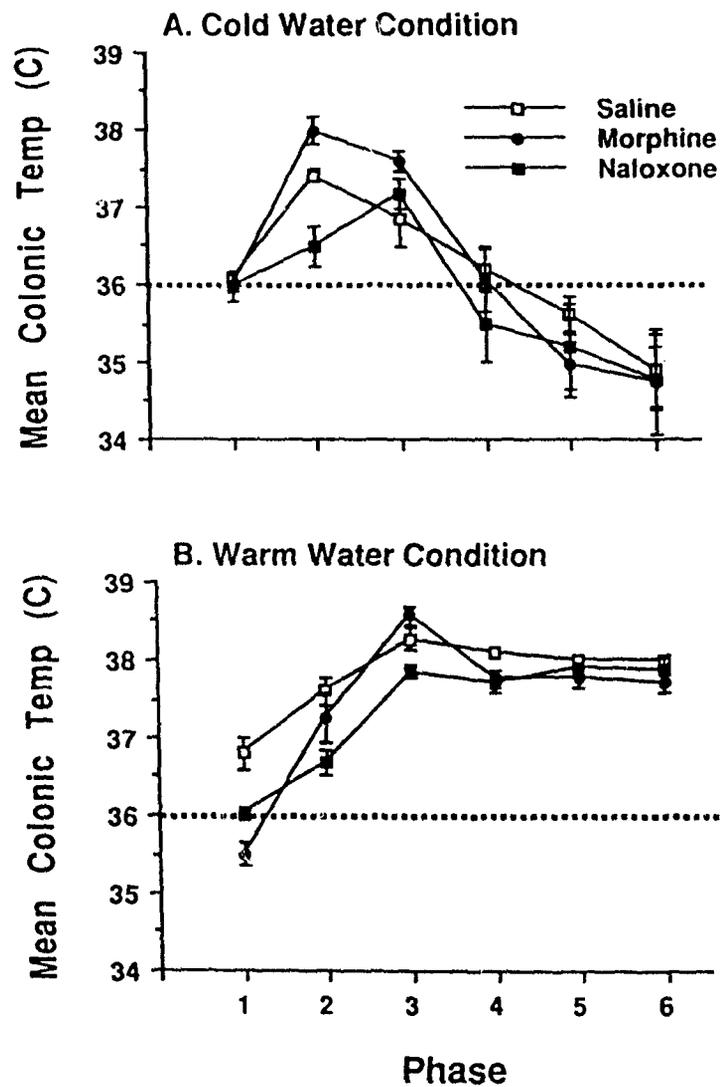
None of the rats from any treatment group jumped off the platform when the water was cold. However, when the water was warm, the morphine-treated rats frequently jumped off the platform (probability = 0.42 ± 0.06), unlike the controls, who jumped off infrequently (0.03 ± 0.03), and the naloxone-treated rats, who did not jump off at all.

In the probe trials, morphine-treated rats did not show a preference for the correct quadrant when the water was warm, yet they showed a clear preference ($p < .01$) when the water was cold (Figure 3.5B). Saline- and naloxone-treated rats showed a clear preference for the correct quadrant under both temperatures, with naloxone-treated rats tending to show a greater preference relative to controls ($p < .05$). The probe data from Experiments IIIa and IIIb are plotted together in Figure 3.5B, to show that water temperature had a systematic effect only on morphine-treated rats. For these rats, the diligence of the search in the correct quadrant was directly related to the temperature of the water.

Morphine-treated rats showed exactly the same pattern of impairments in the visible-platform task as they had during the submerged-platform task (Figure 3.5C). During the cold-water condition, morphine-treated rats showed only a slight impairment relative to saline- and naloxone-treated rats ($p < .01$), but they showed a large impairment during the warm-water condition relative to both controls and cold-water performance ($p < .01$). Saline-treated rats showed similar performance during both cold- and warm water conditions. Naloxone-treated rats actually performed better in warm water than in cold, although this effect failed to reach statistical significance, and they were significantly better than controls in warm water ($p < .05$). Swim speeds were lower for all groups in the warm-water condition ($p < .01$) in both the visible- and the submerged-platform tasks (data not shown).

Morphine produced significant hyperthermia between Phase 1 and Phase 2 in both cold- and warm-water conditions ($p < .01$), producing a 2°C increase in core body temperature in the warm-water condition (Figure 3.6). Saline and naloxone also increased core body temperature, although these

Figure 3.6: Effects of morphine and naloxone on core body temperature sampled before testing (Phase 1), 20 min after drug administration (Phase 2), and at four points (after every second trial) during testing in the water maze in either (A) cold or (B) warm water. (Dotted line represents mean for three treatment groups on Phase 1.



increases failed to reach statistical significance relative to baseline levels. Overall, colonic temperatures declined in cold water and rose in warm water. The mean colonic temperature for each group under each water temperature did not increase or decrease more than 2°C. An overall ANOVA failed to reveal significant differences between groups ($p > .05$), though there were significant ($p < .05$) changes between sampling periods.

DISCUSSION

The main finding of Experiment IIIb is that the learning deficit produced by morphine in warm water can be largely overcome by lowering the water temperature used to motivate escape. In the warm-water condition, morphine-treated rats demonstrated an acquisition impairment relative to their cold-water performance, were more likely to jump off the platform than controls, and failed to show a preference for the correct quadrant during the probe trial. In the cold-water condition, morphine-treated rats demonstrated only a marginal deficit relative to controls during acquisition, showed a greater preference than controls for the correct quadrant during the probe trial, and did not jump off the platform. Further, saline-treated rats performed better in cold water, suggesting that the cold water was sufficient to increase escape motivation. The latter result is consistent with the finding of Selden, Cole, Everitt, and Robbins (1990) that rats trained in cold water (12°C) showed a stronger spatial bias for the correct quadrant during the probe trial than did rats trained in warm water (26°C). Interestingly, in the present study, naloxone-treated rats performed optimally in both warm and cold water, suggesting the rather paradoxical conclusion that the warm water elicited endogenous opioid activity that was detrimental to performance. If present, this endogenous opioid activity may have exacerbated the morphine deficit. It is possible, then, that cold water does not elicit the same degree of endogenous opioid activity as warm water does, thereby attenuating the morphine deficit. However, this conclusion contrasts with previous reports that cold water elicits endogenous opioid activity (Barta & Yashpal, 1981). An alternative interpretation is that the cold water may have elicited the release of hormones (e.g., epinephrine) that facilitated the performance of morphine-treated rats. The spatial learning

deficits produced by morphine in the warm-water condition are unlikely to be due to drug-induced alteration of body temperature, since the magnitude of this change was below that found to affect mnemonic processes (Ahlers & Riccio, 1987). These data, along with the data from Experiment IIIb, suggest that morphine-treated rats can learn the location of a place, or swim to a single visual cue, when the incentive to escape is increased to overcome the antinociceptive effects of the drug.

GENERAL DISCUSSION

Experiment IIIa demonstrated that morphine impairs spatial learning, spatial recall, and cue learning, lengthening swim paths and increasing heading errors. The place and cue learning deficits were mediated via endogenous opioid receptors since they were reversed by naloxone. Rats treated with a moderate dose of morphine (10 mg/kg) showed a gradual improvement in performance over training and developed a preference for the correct quadrant. However, this improvement was not completely due to the development of tolerance, since the same rats were impaired at learning the reversed platform position. Experiment IIIb demonstrated that morphine-treated rats can acquire spatial relationships with an efficiency comparable to that of controls if the incentive value of escape is increased. Morphine-treated rats showed large deficits in warm water but minimal deficits in cold water. These findings suggest that in the MWM, morphine does not impair spatial learning *per se*, but rather impairs the motivation to learn. This effect is consistent with morphine's characteristic analgesic/antinociception actions (Martin, 1984).

The finding that the preadministration of an opioid agonist impairs acquisition contrasts with the findings of Beatty (1983), who found that the preadministration of comparable doses of morphine did not impair spatial working memory on an appetitively motivated eight-arm radial maze. Perhaps the difference between the present findings and those of Beatty is related to the difference between appetitively and aversively motivated tasks. Interestingly, both naloxone and morphine suppress food intake in the rat (e.g., Frenk & Rogers, 1979), suggesting that naloxone- and morphine-

treated rats would also lose their incentive to acquire the radial maze. Surprising, then, is the finding that naloxone enhances spatial working memory in this task (Canli et al., 1990).

Naloxone has been shown to enhance learning and memory processes across a wide variety of tasks (Gallagher, 1984), suggesting that endogenous opioid activity can disrupt optimal learning. Recently, Decker et al. (1989) demonstrated that the pretraining administration of naloxone enhanced acquisition in the MWM. However, in the present study, naloxone had little or no effect on acquisition. This discrepancy may be accounted for by the different number of trials given each day. For example, the two-trial-per-day procedure employed by Decker et al. would be more difficult for control rats, taking longer to reach asymptotic levels, thereby allowing for an enhancement to be revealed. Furthermore, the use of different rat strains and/or drug doses may also account for the discrepancy. However, the 3-mg/kg dose of naloxone that Decker et al. found to enhance acquisition has also been shown to impair acquisition in the MWM using the same procedures as those of the present study (McDaniel et al., 1990). Thus, the role of endogenous opioid systems in spatial learning requires further clarification.

In sum, it was demonstrated that morphine impaired both place and cue learning via opioid receptors, in a dose-dependent manner. This impairment could not be attributed to drug- or environmentally induced alterations in body temperature, or to motor or perceptual deficits. However, the results of Experiment IIIb suggested that morphine impaired place and cue learning by reducing the incentive to escape, rather than by interfering with memory processes per se. These results suggest that morphine's antinociceptive/analgesic action reduced the aversive nature of the water, thereby reducing the motivation to escape from it. Finally, naloxone had little effect either on place or cue learning. These results suggest that endogenous opioid systems have a negligible role in spatial learning, and they emphasize that one should be cautious when interpreting experiments in which the preadministration of morphine seems to disrupt aversively motivated learning.

Experiment IV: BZs interaction with opioids

The results of Experiment III suggest that opioidergic activation is detrimental to spatial learning in the MWM. There is good reason to suspect that the spatial learning impairment produced by BZs in the MWM may be mediated by opioidergic systems. For example, biochemical studies have found that opioidergic agonists alter the pharmacodynamic properties of BZ receptors in hippocampus and cortex (Lopez et al. 1990; Miller et al. 1987; Smith et al. 1984) and GABA content in several brain regions (Moroni et al. 1978; Kuriyama and Yoneda 1978). Secondly, acute BZ administration decreases met⁵-enkephalin release in the striatum (Harsing et al. 1982; Wuster et al. 1980a,b) and increases met⁵-enkephalin release in the hypothalamus (Duka et al. 1979, 1980). Thirdly, behavioral analysis has shown that acute BZ administration increases pain thresholds (Chapman and Feather 1973; Houser and Pare 1973; Wuster et al. 1980a). Finally, naloxone, a selective opioid antagonist, blocks several of the behavioral effects of BZs, including hyperactivity (Sansone and Vetulani 1988), sedation (Billingsley and Kubena 1978), hyperphagia and hyperdipsia (Cooper 1983; Stapleton et al. 1979), anxiolysis (Duka et al. 1981, 1982; Soubrie et al. 1980) and some learning deficits (Tripp et al. 1987). Hence, the spatial learning deficit produced by BZs may be caused by BZ-induced opioid release. If this is the case, it would be expected that naloxone would block the spatial learning deficit produced by diazepam. Experiment IV examined the effects of naloxone, an opioid antagonist, and flumazenil, a BZ antagonist, on the spatial memory deficits produced by either the BZ agonist diazepam or the opioid agonist morphine.

METHODS

Animals

Sixty-five hooded, male Long-Evans rats served as subjects. They were housed in pairs and maintained on a 12:12 h light-dark cycle. All testing was conducted during the light phase of the cycle. Rats weighed 350-500 g at the beginning of testing. Food and water were continuously available.

Apparatus and procedure

The MWM is the same as that described in Experiment I except that rats were given four trials each day for eight consecutive days.

Drugs and group assignment

Diazepam (Roche) was dissolved in a vehicle of 40% propylene glycol, 10% ethanol, and 50% distilled water at a concentration of 0.6 mg/ml. Flumazenil (Roche) was suspended in saline with Tween 80 (1 drop/10 ml) at a concentration of 2 mg/ml. Morphine sulphate (5 mg/ml) and naloxone hydrochloride (2 or 10 mg/ml) were dissolved in saline (0.9% NaCl). All drugs were injected IP in the rat's home cage. Diazepam and morphine were given 30 min prior to testing and naloxone and flumazenil were given 15 min prior to testing.

Rats were randomly divided into one of eleven treatment groups: vehicle (1 ml/kg; saline, n=5; diazepam vehicle, n=5), diazepam (3 mg/kg; n=10), flumazenil (10 mg/kg; n=5), morphine sulfate (15 mg/kg; n=5), naloxone hydrochloride (2 mg/kg; n=5, or 10 mg/kg; n=5), flumazenil (10 mg/kg) + diazepam (3 mg/kg; n=5), flumazenil (10 mg/kg) + morphine (15 mg/kg; n=5), naloxone (2 mg/kg; n=5, or 10 mg/kg; n=5) + diazepam (3 mg/kg), naloxone (2 mg/kg) + morphine (15 mg/kg; n=5).

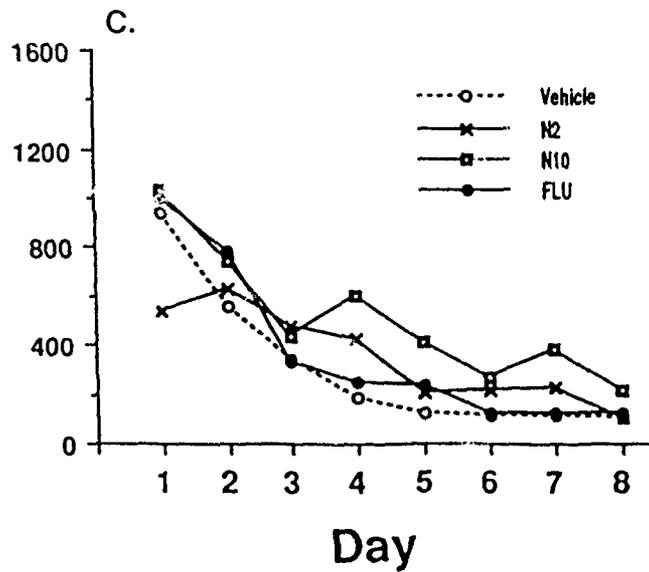
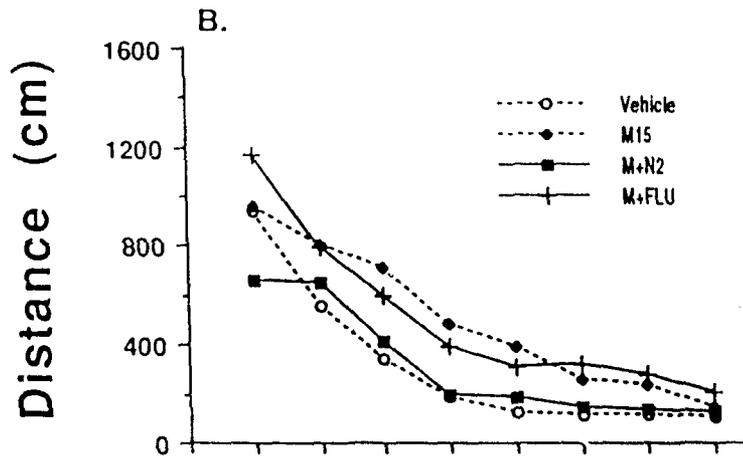
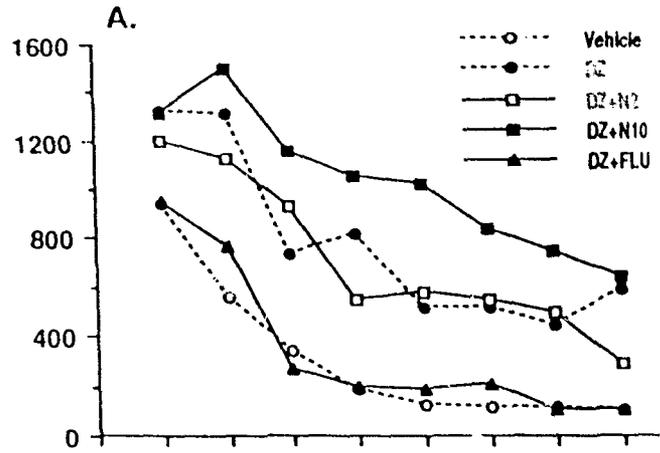
Data analysis

Escape latencies and swim path lengths were assessed using an analysis of variance (ANOVA) procedure with repeated measures. Post hoc comparisons were assessed using Dunnett's test. In every case the acceptable level for statistical significance was $P < 0.05$.

RESULTS

The distance required by each treatment group to locate the submerged platform over the eight days of training is shown in Figure 3.7A, B, C. Rats treated with drug vehicles rapidly acquired the platform position, reaching asymptotic levels by the fifth day of testing. Rats treated with diazepam took longer paths to locate the platform, had poorer asymptotic levels relative to

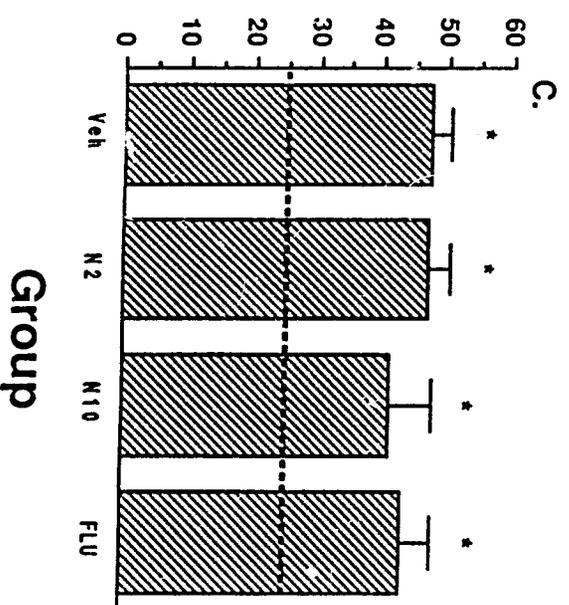
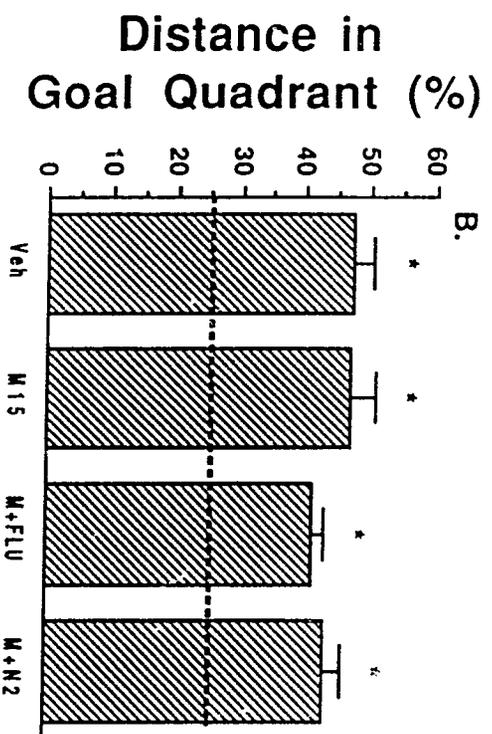
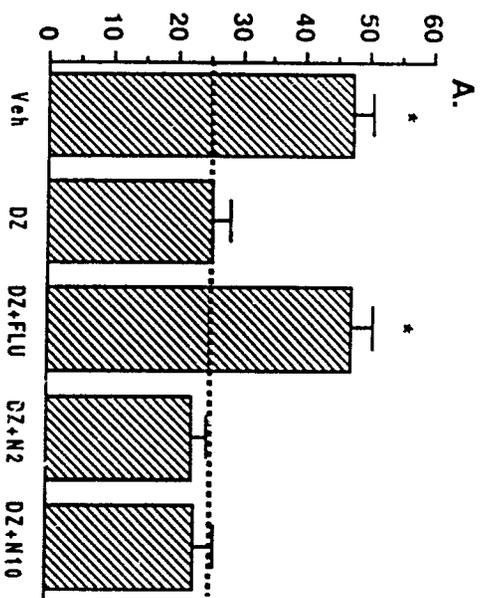
Figure 3.7: Effects of diazepam, morphine, naloxone and flumazenil on the distance taken to locate the submerged escape platform over the eight days of training. Note that flumazenil, but neither dose of naloxone, blocks the deficit produced by diazepam. Note also that the low dose of naloxone, but not flumazenil, blocks the deficit produced by morphine. (DZ=diazepam; N=naloxone; FLU=flumazenil; M=morphine; Numbers=drug dose in mg/kg).



controls and tended to swim in broad circuitous loops until meeting with the platform. The concurrent administration of flumazenil completely reversed the deficit produced by diazepam. In contrast, naloxone (2 mg/kg) failed to influence the deficit produced by diazepam and the higher dose of naloxone (10 mg/kg) actually exacerbated this deficit. Rats treated with morphine took longer paths to locate the platform and tended to swim in large loops during initial training but swam directly to the platform during the final days of training. The deficit produced by morphine was reversed by the low dose of naloxone (2 mg/kg), but not flumazenil. When administered alone, neither flumazenil nor the low dose of naloxone had any effect on swim path lengths, but the higher dose of naloxone increased swim path lengths. An overall ANOVA on swim path lengths revealed a significant group difference, $F(10,249)=28.25$, $p<0.0001$, day difference, $F(7,1743)=88.34$, $p<0.0001$, and a significant interaction between groups and day, $F(70,1743)=1.67$, $p<0.001$. Post hoc comparisons revealed that rats treated with diazepam, diazepam+naloxone (2 or 10 mg/kg), morphine, morphine+flumazenil and naloxone (10 mg/kg) had significantly longer swim path lengths compared to controls ($p<0.05$). Further, rats treated with diazepam+flumazenil and morphine+naloxone had significantly shorter swim paths compared to rats treated with diazepam or morphine, respectively ($p<0.05$). Latency data (not shown) revealed a similar pattern of deficits and statistical significances. It was observed incidentally that morphine-treated rats would occasionally take longer to initiate swimming when placed in the pool and would occasionally jump off the platform once mounted. This effect was not observed in rats treated with morphine+naloxone or diazepam.

During the probe trial (Fig. 3.8), rats treated with the drug vehicle showed a robust preference for the quadrant that contained the platform during training ($p<0.01$, compared to chance). In contrast, rats treated with diazepam failed to demonstrate a quadrant preference, ($p>0.05$). This effect was antagonized by flumazenil ($p<0.05$), but not by either dose of naloxone (Fig. 3.8A). Unlike rats treated with diazepam, rats treated with morphine showed a preference for the correct quadrant, as did rats treated with

Figure 3.8: Effects of diazepam, morphine, naloxone and flumazenil on the percentage distance spent in the correct (goal) quadrant during the 60 s probe trial. Note the failure of naloxone to attenuate the deficit produced by diazepam. * $p < 0.01$ compared to chance level (25%) represented by dotted line.



morphine+naloxone, morphine+flumazenil ($p < 0.01$; Fig. 3.8B), flumazenil or either dose of naloxone alone (Fig. 3.8C).

The effects of each drug treatment on swim speed over the course of training are shown in Figure 3.9. Both diazepam and morphine significantly decreased swim speed ($p < 0.01$). Flumazenil antagonized the swim speed reduction produced by diazepam ($p < 0.01$), but not morphine. Naloxone (2 mg/kg) antagonized the swim speed reduction produced by morphine ($p < 0.01$), but not diazepam at either dose. Both naloxone (2 or 10 mg/kg) and flumazenil produced small, but non-significant, increases in swim speeds when administered alone.

The effects of each drug treatment on visible platform acquisition are shown in Figure 3.10. Morphine significantly increased the swim path distance taken to reach the visible platform ($p < 0.01$). Naloxone, but not flumazenil, antagonized this impairment ($p < 0.05$). None of the other drug treatments significantly altered the distance to reach the visible platform. Latency data (not shown) revealed a similar pattern of deficits and statistical significances.

DISCUSSION

The above results replicate previous findings that diazepam impairs spatial learning in the MWM, increasing swim path lengths and preventing the formation of a quadrant preference as revealed by the probe trial (Arolofa and Brioni 1991; McNamara and Skelton 1991a). Although diazepam reduced swim speeds over the course of training, it did not impair cue learning, suggesting that it did not impair motivational or sensorimotor processes. Morphine also slowed acquisition and reduced swim speeds over the course of training, replicating the findings of Experiment III, but unlike diazepam, it impaired cue learning and did not impair the formation of a quadrant bias during the probe trial. Furthermore, morphine increased the latency to initiate swimming and the frequency with which rats would jump off the platform once mounted. We have previously found that increasing the escape incentive (colder water) attenuates the spatial learning deficit

Figure 3.9: Effects of diazepam, morphine, naloxone and flumazenil on swim speed over the 8 days of training. Note: 1) rats treated with diazepam have slower swim speeds, an effect reversed by flumazenil but not naloxone, 2) rats treated with morphine have slower swim speeds, an effect reversed by the low dose of naloxone but not flumazenil.

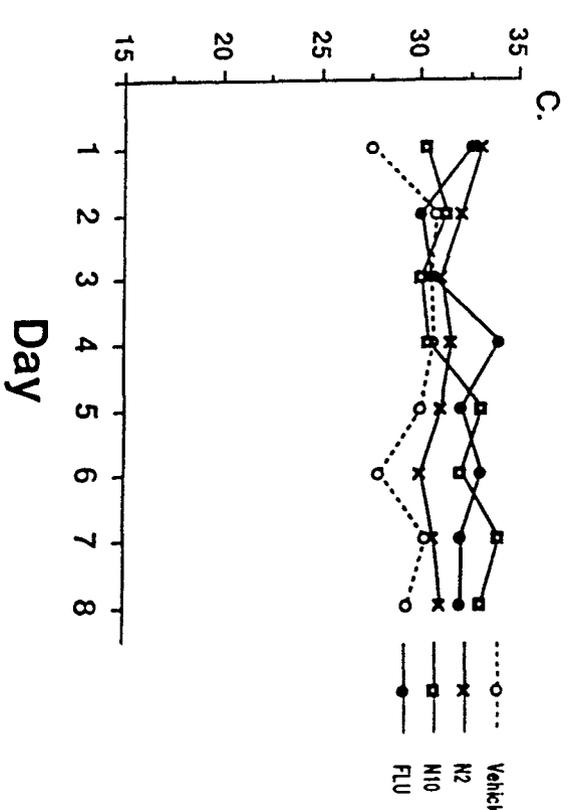
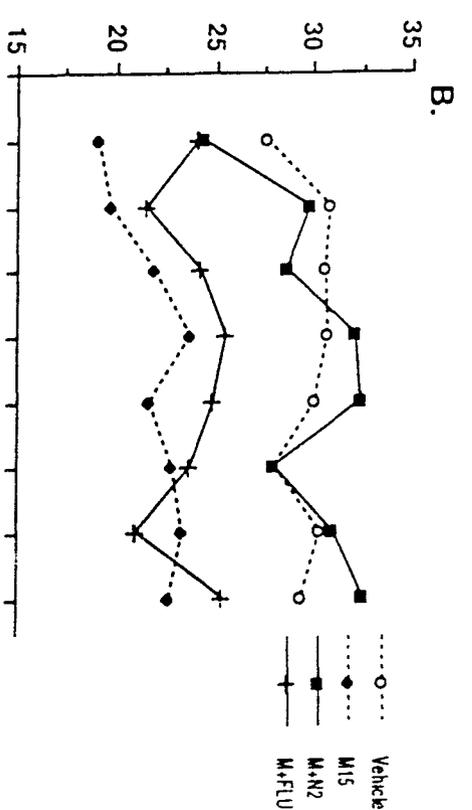
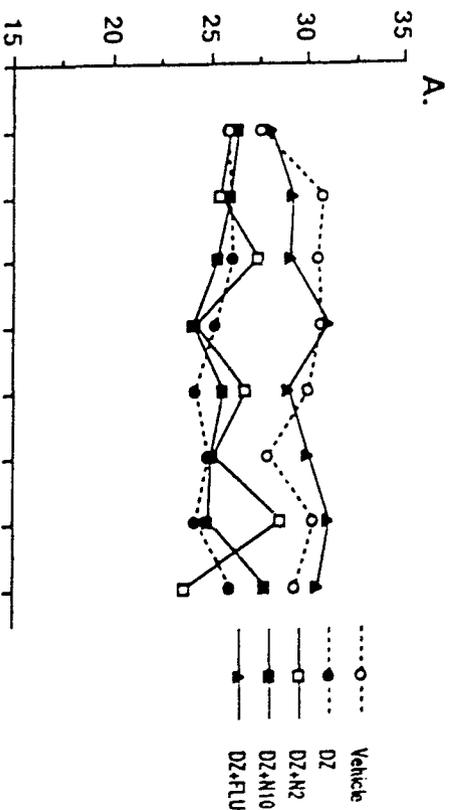
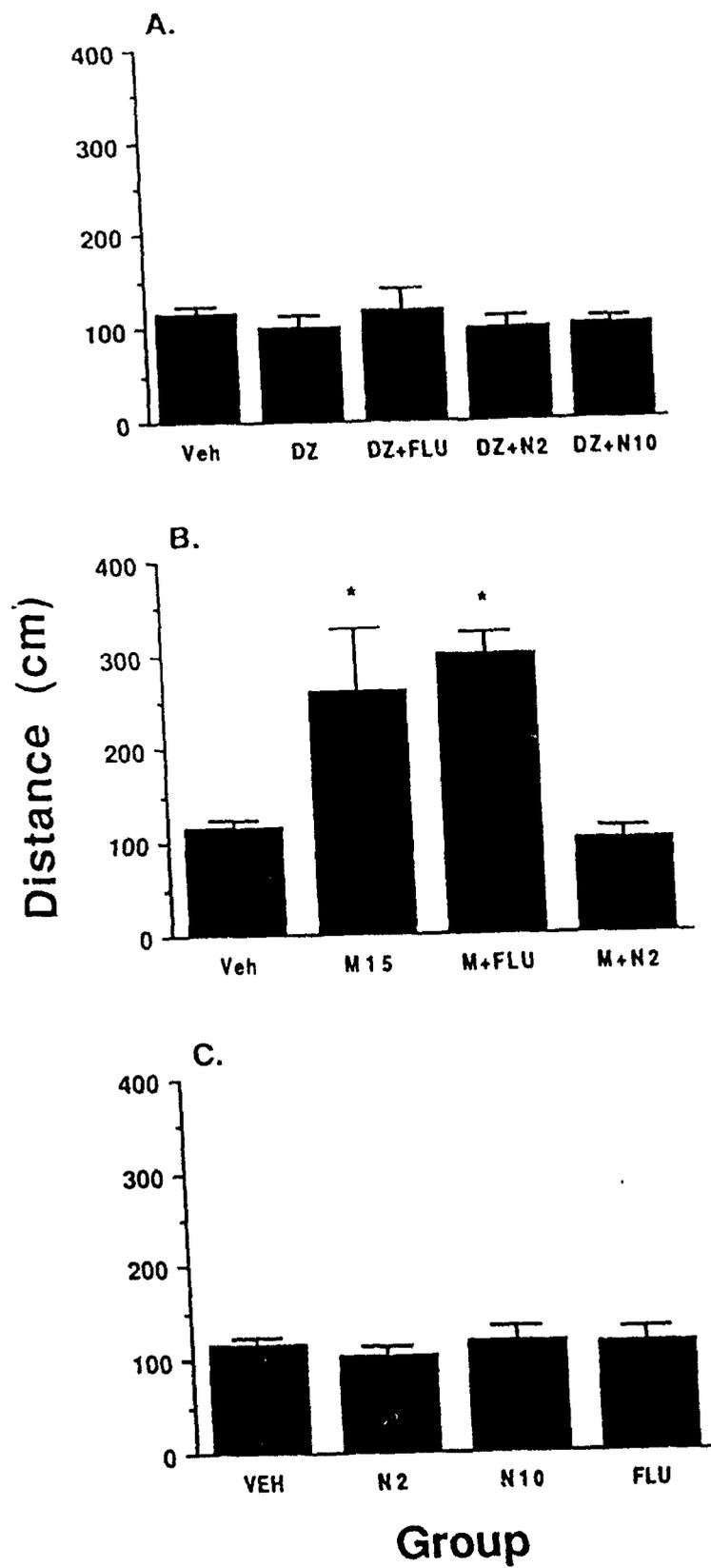


Figure 3.10: Effects of diazepam, morphine, naloxone and flumazenil on the distance taken to reach the visible platform averaged over trials. Note that only the morphine and morphine+flumazenil treatments impair performance. * $p < 0.01$ compared to vehicle-treated controls.



produced by morphine (Experiment III), supporting the contention that the spatial learning deficit produced by morphine in the present study was due to reduced escape incentive rather than mnemonic or motorical deficits. Moreover, in the present study morphine reduced swim speed over the course of training although acquisition performance approached control levels by the end of training. This latter finding provides strong evidence that the motor slowing effects of morphine were not responsible for the poor acquisition performance observed during initial training. In sum, although diazepam and morphine slowed acquisition and reduced swim speeds in a similar manner, the nature of the learning deficits was quite different and suggests mediation by different mechanisms.

The present results indicate that the spatial learning deficit produced by diazepam was not mediated by opioidergic systems. Naloxone, at a dose sufficient to completely block impairments produced by morphine, failed to antagonize the spatial learning deficit produced by diazepam. Indeed, a larger dose of naloxone exacerbated (not reversed) the deficit produced by diazepam. Flumazenil, a selective BZ receptor antagonist, at a dose sufficient to block the acquisition deficit produced by diazepam, failed to block the place and cue deficits produced by morphine. Taken together, these results suggest that the spatial learning impairment produced by diazepam is not mediated via endogenous opioid receptors, but rather, via endogenous BZ receptors. Conversely, the impairments produced by morphine are mediated via endogenous opioid receptors, not BZ receptors (e.g., Lopez et al. 1990). Furthermore, the different behavioral and pharmacological profiles of diazepam and morphine suggest that these deficits are mediated by parallel systems.

Naloxone has a high affinity for the μ opioid receptor and, to a lesser degree, for the δ and κ receptors (Chang et al. 1980). It is possible that diazepam elicited opioid activity at a specific sub-population of opioid receptors that are not sensitive to naloxone but detrimental to spatial learning when activated. However, this account seems unlikely since the behavioral effects of morphine, which binds to μ , δ , and κ receptors (Paterson et al. 1983), were completely blocked by naloxone. Naloxone has

also been found to interact directly with GABA receptors, acting as an antagonist at very high doses (i.e., 100 mg/kg; Dingledine et al. 1978). In the present study, the lowest dose of naloxone (2 mg/kg) failed to antagonize the effects of diazepam while completely blocking the effects of morphine. Indeed, the larger dose of naloxone (10 mg/kg) actually exacerbated the deleterious effects of diazepam on spatial learning. Together, these results provide strong evidence that the doses of naloxone used in the present study were selective for opioid receptors and free of antagonist actions at the GABA receptor.

The present finding that the amnesic effects of diazepam are not opioid mediated agrees with previous findings in which naloxone was ineffective at preventing acquisition deficits (errors of commission) produced by a BZ in a successive discrimination task (Tripp & McNaughton, 1987) and a signalled punishment task (Tripp & McNaughton, 1991). The finding contrasts with the observation that naloxone blocked chlordiazepoxide-induced acquisition deficits of differential reinforcement of low rates of response task (Tripp et al. 1987). It is difficult to specify which of many possible behavioral differences between these tasks can account for the differences and this will require further investigation.

The finding that naloxone, at a dose sufficient to block the deficits produced by morphine (i.e., 2 mg/kg), had little effect on spatial learning suggests that the endogenous opioid systems mediating morphine's effects have a negligible role in spatial learning processes. Since opioid activity is detrimental to spatial learning (Experiment III; Decker et al. 1989), it would be expected that naloxone would facilitate performance. However, naloxone-induced facilitation was not found in the present study, agreeing with our previous findings (Experiment III) but running contrary to other findings (Canli et al. 1990; Decker et al. 1989). In the present study, rats treated with the low dose of naloxone (2 mg/kg) performed better on the first day of training relative to controls, although subsequent performance was poorer relative to controls (see Fig. 3.7C). Thus, it seems unlikely that a floor effect concealed an enhancement effect of naloxone. Although the high dose of naloxone slowed acquisition, this group did show a preference for the correct quadrant

during the probe trial, suggesting that spatial learning did occur. Hence, the precise nature of naloxone's effects on mnemonic processes will require further investigation.

Like naloxone, flumazenil has been shown to enhance learning in other tasks at doses comparable to that used in the present study (Lal et al. 1988; Raffalli-Sebille and Chapouthier, 1991). In the present study, flumazenil failed to enhance learning, with flumazenil-treated rats performing at control levels (see Fig. 3.7C). However, Experiment I revealed that a more difficult 2 trials/day procedure is required to reveal the facilitatory actions of flumazenil.

In sum, the present investigation sought to clarify the neurochemical basis of BZ-induced impairments of spatial learning. Diazepam produced a specific deficit in spatial learning whereas morphine impaired acquisition in a manner indicative of a motivational deficit rather than a mnemonic deficit. Naloxone (2 mg/kg) blocked the effects of morphine, suggesting its effects were mediated via opioid receptors, but failed to affect spatial learning when administered alone. Flumazenil blocked the effects of diazepam, indicative of BZ-receptor mediation, but failed to influence spatial learning when administered alone. The dissimilarity between the deficit produced by morphine and diazepam, as well as the ineffectiveness of naloxone against the diazepam deficit, suggests that an interaction between BZs and opioid systems is not responsible for the observed spatial learning deficit. Taken together, these results provide definitive evidence that diazepam-induced impairments of spatial learning are not mediated by endogenous opioid systems. A second neurotransmitter system that may mediate the spatial learning deficits produced by BZs is ACh. The following section will explore the relationship between BZs, ACh, and spatial learning.

Experiment V: BZs interaction with cholinergic systems

A considerable body of research has implicated acetylcholine (ACh) as an important neurotransmitter in learning and memory processes. As reviewed above, cholinergic hypofunction, brought about by receptor blockers, lesions of ACh systems or aging, is detrimental to spatial learning

in the MWM. Hence, the MWM is a task that is sensitive to the mnemonic deficits associated with ACh hypofunction.

There is neurochemical and behavioral evidence that BZ/GABA_A agonists inhibit ACh systems (see Sarter, Bruno & Dudchenko, 1990 for a review). Neurochemical studies have shown that: 1) the systemic administration of BZ/GABA_A agonists reduces ACh activity in the hippocampus and forebrain region, as determined by high-affinity choline uptake (Miller & Richter, 1985; Richter, Gormley, Holtman & Simon, 1982) and ACh turnover rates (Wood & Richard, 1982; Zsilla, Cheney & Costa, 1976), 2) infusions of BZ/GABA_A agonists into the medial septum reduce ACh activity in the hippocampus (Blaker, Peruzzi & Costa, 1984; Costa, Panula, Thompson & Cheney, 1983; Wood, 1986), while the GABA_A antagonist bicuculline increases it (Zucker, Calkins, Zabawska, Lai & Horita, 1987), whereas infusions of BZ/GABA_A agonists into the nucleus basalis region reduce forebrain ACh turnover (Casamenti, Deffenu, Abbamondi & Pepeu, 1986; Wenk, 1984; Wood, 1986; Wood, McQuade, & Vasavan Nair, 1984), and 3) BZ agonists decrease muscarinic binding affinity and capacity (Miyamoto, Kato, Narumi & Nagaoka, 1987). Behavioral studies have shown that: 1) ACh blockers and BZ/GABA_A agonists produce a similar pattern of memory impairment (Curran, Schifano & Lader, 1991), 2) infusions of BZ/GABA_A agonists into either the medial septum (Brioni, Decker, Gamboa, Izquierdo & McGaugh, 1990; Chrobak, Stackman & Walsh, 1989; Givens & Olton, 1990; Stackman & Walsh, 1992) or nucleus basalis (Dudchenko & Sarter, 1991; Majchrzak, Brailowski & Will, 1990) impair acquisition in rats, and 3) BZ antagonists attenuate scopolamine-induced acquisition deficits on passive avoidance (Jensen, Stephens, Sarter & Petersen, 1987; Lal, Kumar & Forster, 1988) and spontaneous alternation tasks (Sarter, Bodewitz & Stephens, 1988). Together, these results suggest that BZ/GABA_A agonists inhibit the release of ACh which in turn results in an impairment of mnemonic processes (Sarter et al., 1990).

There is also neurochemical evidence that ACh activity inhibits GABA release. For example, ACh disinhibits neurons in the dorsolateral septal nucleus (Hasuo, Gallagher & Shinnick-Callagher, 1988) and dorsal

hippocampus in a manner comparable to GABA_A antagonists (Krnjevic, Reiffenstein & Ropert, 1981). This disinhibitory action of ACh (Hasuo et al., 1988; Rovira, Ben-Ari, Cherubini, Krnjevic & Ropert, 1983) is blocked by pirenzepine, a selective muscarinic M₁ receptor blocker, atropine and scopolamine, non-specific M₁ and M₂ receptor blockers. Since ACh does not affect the postsynaptic inhibitory actions of GABA (Ben-Ari, Krnjevic, Reinhardt & Ropert, 1981), it appears that ACh inhibits the release of GABA from presynaptic terminals. Together, these results suggest that ACh reduces GABA release via muscarinic receptors located on the presynaptic terminals of inhibitory interneurons.

The present experiment sought to better characterize the interaction between BZ/GABA_A and ACh systems in spatial learning in the MWM. In Experiment Va, the effects of flumazenil, a selective BZ receptor antagonist, on chlordiazepoxide- and scopolamine-induced impairments of spatial learning were assessed. In Experiment Vb, the effects of physostigmine, an acetylcholinesterase inhibitor, on chlordiazepoxide- and scopolamine-induced impairments of spatial learning were assessed.

EXPERIMENT Va

METHODS

Animals

Forty-six, male Long-Evans rats (Charles-River, Quebec, Canada) served as subjects. They were housed in pairs and maintained on a 12:12 hr light-dark cycle. All testing was conducted during the light phase of the cycle. Rats weighed 350-450 g at the beginning of testing. Food and water were available ad libitum.

Apparatus and procedure

The MWM and procedure used was the same as that used in Experiment I except that rats were given four trials each day for six consecutive days.

Drugs and group assignment

Rats were divided into one of the following eight treatment groups. The first group received chlordiazepoxide hydrochloride+saline (n=5; 5 mg/kg;

dissolved in 0.9% NaCl; Hoffmann La Roche Inc.). The second group received scopolamine hydrobromide+saline (n=5; 1 mg/kg; dissolved in 0.9% NaCl; Sigma Chemical Co.). The third and fourth groups received one of two doses of flumazenil (n=5/group; 15 or 30 mg/kg; suspended in 0.9% NaCl with a drop of Tween 80; Hoffmann La Roche Inc.). The fifth group received chlordiazepoxide hydrochloride (5 mg/kg) +15 mg/kg flumazenil (n=5). The sixth and seventh groups received scopolamine hydrobromide (1 mg/kg)+15 mg/kg flumazenil (n=8) or 30 mg/kg flumazenil (n=8). The eighth group received saline (n=5; 1 mg/ml; 0.9% NaCl) and served as controls. All injections were administered in a volume of 1 ml/kg and administered in the rat's home cage. Chlordiazepoxide, scopolamine and saline were administered 30 min prior to testing and flumazenil was administered 15 min prior to testing.

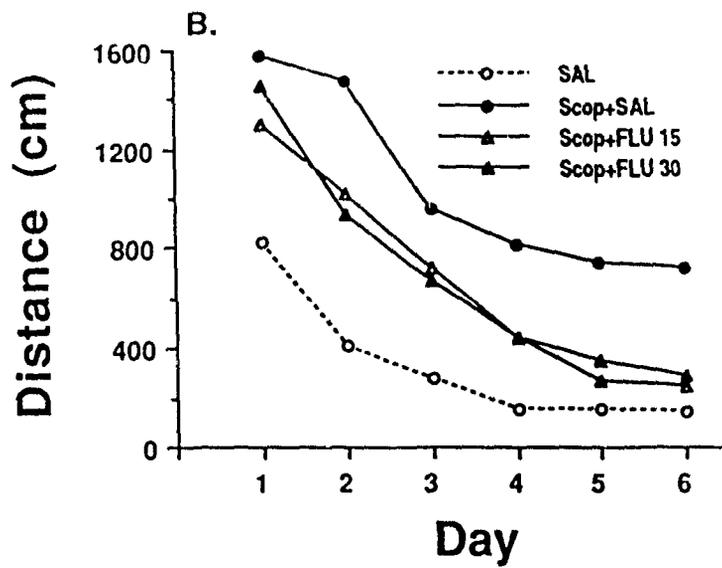
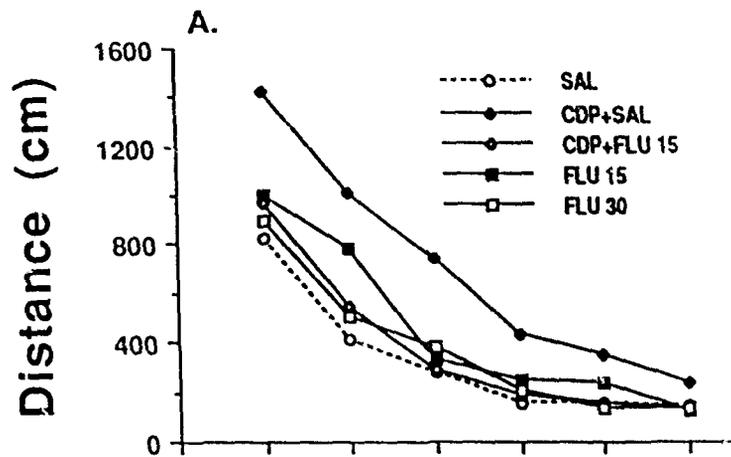
Data analysis

Escape latencies and swim path lengths were assessed using an analysis of variance (ANOVA) procedure with repeated measures. Post hoc comparisons were assessed using Tukey's (HSD) method. In every case the acceptable level for statistical significance was $p < 0.05$.

RESULTS

Chlordiazepoxide increased the distances required by rats to locate the submerged platform. The low dose of flumazenil (15 mg/kg) reversed this deficit while having little effect when administered alone (Fig. 3.11A). The high dose of flumazenil (30 mg/kg) also had no effect when administered alone (Fig. 3.11A). Scopolamine drastically increased the distance taken to reach the submerged platform (Fig. 3.11B). Both doses of flumazenil attenuated, but did not reverse, the distance increase produced by scopolamine (Fig. 3.11B). An overall ANOVA on the swim path lengths of groups treated with chlordiazepoxide and flumazenil or flumazenil alone revealed a significant group difference, $F(4,95)=9.17$, $p < 0.001$, day difference, $F(5,475)=84.3$, $p < 0.001$, but not a significant interaction between groups and day, $F(20,475)=1.30$, N.S. Post hoc tests revealed that rats treated with chlordiazepoxide had significantly longer swim paths relative to both

Figure 3.11: Effects of (A) chlordiazepoxide and flumazenil and (B) scopolamine and flumazenil on the distance taken to locate the submerged platform over the six days of training. Note that the low dose of flumazenil reversed the place learning deficit produced by chlordiazepoxide while both doses of flumazenil attenuated the place learning deficit produced by scopolamine. (SAL=saline; CDP=chlordiazepoxide; FLU=flumazenil; Scop=scopolamine; numbers=drug dose in mg/kg).



controls ($p < 0.01$) and rats treated with chlordiazepoxide+flumazenil ($p < 0.01$). The swim path lengths of controls and rats treated with chlordiazepoxide+flumazenil did not differ significantly ($p > 0.05$). An overall ANOVA on the swim path lengths of groups treated with scopolamine and flumazenil revealed a significant group difference, $F(3,96)=33.38$, $p < 0.001$, day difference, $F(5,480)=61.4$, $p < 0.001$, but not a significant interaction between groups and day, $F(15,480)=1.27$, N.S. Post hoc tests revealed that rats treated with scopolamine+flumazenil (15 mg/kg) or scopolamine+flumazenil (30 mg/kg) had significantly shorter swim paths relative to rats treated with scopolamine alone ($p < 0.01$) but still had significantly longer swim paths relative to controls ($p < 0.01$). Latency data (not shown) showed the same pattern of impairments and statistical significances.

Data from the drug free probe trial confirmed the pattern of impairments found during training. Rats treated with saline demonstrated a significant bias for the quadrant that had contained the platform during training ($p < 0.01$, relative to chance (25%); Fig. 3.12) revealing their accurate knowledge of the platform's location and their use of a spatial strategy. Rats treated with chlordiazepoxide failed to demonstrate a quadrant bias, revealing their failure to accurately learn the platform's location in space, while rats treated with chlordiazepoxide+flumazenil, or either dose of flumazenil alone, demonstrated a significant bias for the correct quadrant. Rats treated with scopolamine alone failed to demonstrate a bias for the correct quadrant while rats treated with scopolamine and either dose of flumazenil did show a bias for the correct quadrant ($p < 0.01$). The quadrant bias demonstrated by rats treated with scopolamine plus the high dose, but not the low dose, of flumazenil (30 mg/kg) was significantly less than the bias demonstrated by controls ($p < 0.05$).

Swim speed, averaged over the six days of training, was affected only by scopolamine (Fig. 3.13). Rats treated chlordiazepoxide, chlordiazepoxide + flumazenil, or either dose of flumazenil swam at approximately the same speed as controls (28 ± 1.7 cm/s; Fig. 3.13A). Surprisingly, rats treated with scopolamine swam significantly faster than controls ($p < 0.01$; Fig. 3.13B), as did rats treated with scopolamine and the low dose of flumazenil (15 mg/kg;

Figure 3.12: Effects of (A) chlordiazepoxide and flumazenil and (B) scopolamine and flumazenil on the percentage distance spent in the correct quadrant during the 60 s probe trial. Note that rats treated with scopolamine plus either dose of flumazenil demonstrate a bias for the quadrant that had contained the platform. * $p < 0.01$ compared to chance level (25%) represented by dotted line.

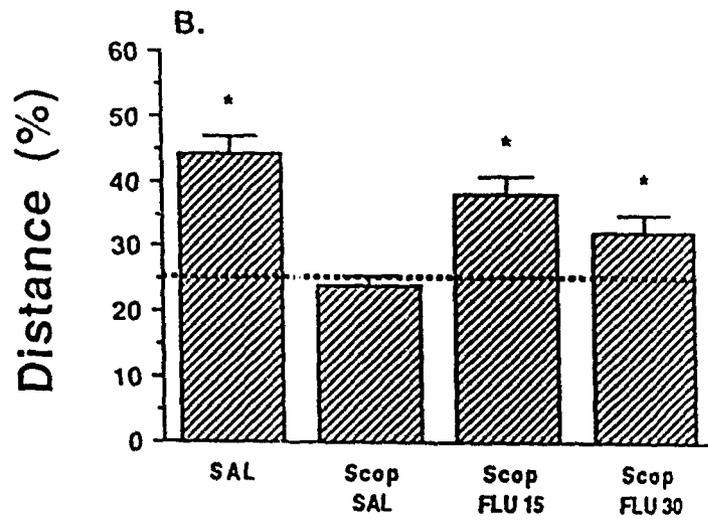
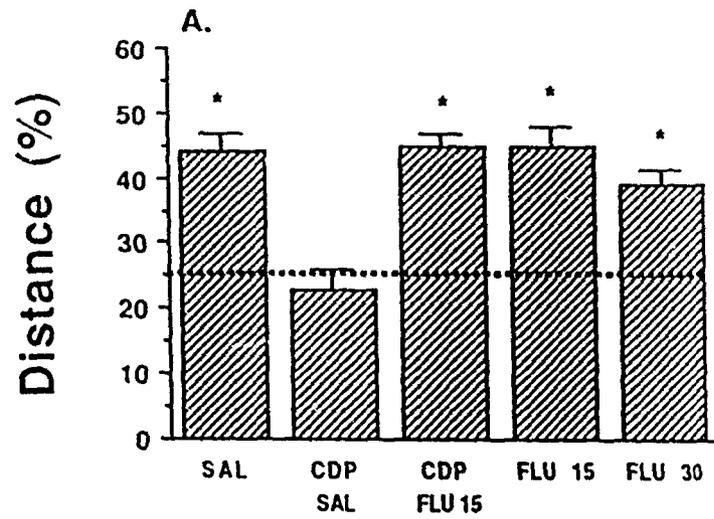
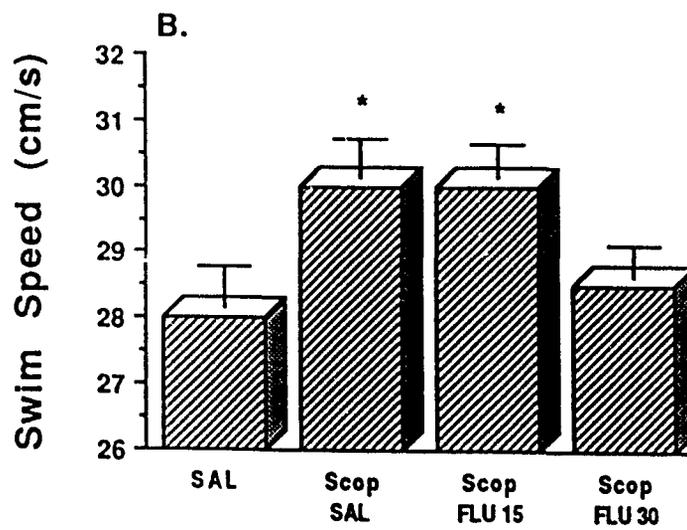
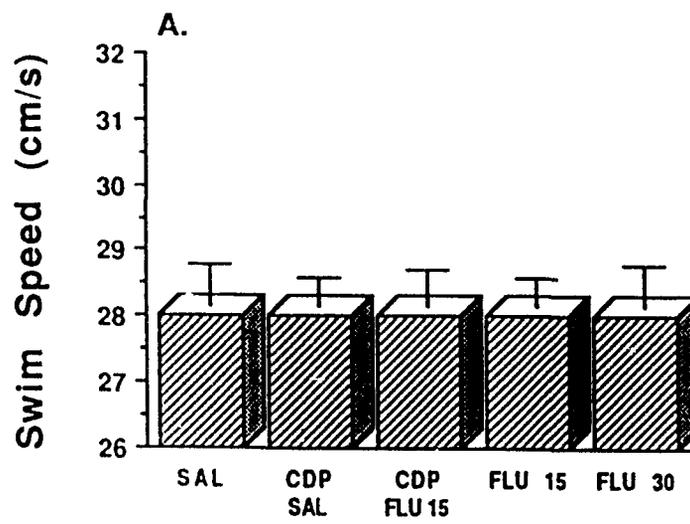


Figure 3.13: Effects of (A) chlordiazepoxide and flumazenil and (B) scopolamine and flumazenil on swim speed averaged over the six days of training. Note that the increased swim speed produced by scopolamine is reversed by the high dose of flumazenil. * $p < 0.01$ compared to saline-treated controls.



$p < 0.01$). The highest dose of flumazenil (30 mg/kg) reversed the increased swim speed produced by scopolamine.

None of the drug treatments resulted in a significant impairment of visible platform training (data not shown). Rats in each treatment rapidly learned to escape to the visible platform.

DISCUSSION

The results of Experiment Va replicate previous findings that both chlordiazepoxide and scopolamine produce a severe impairment of spatial learning in the MWM (McNaughton & Morris, 1987), as evidenced by longer distances taken to locate the submerged platform during training and a failure to show a quadrant bias during the probe trial. A novel finding of the present experiment was that flumazenil (10 mg/kg) completely reversed the impairment produced by chlordiazepoxide, suggesting that this deficit was mediated through endogenous BZ receptors. When administered alone, neither dose of flumazenil affected spatial learning, suggesting that optimal control performance concealed an enhancement effect. The most important finding of Experiment Va was that both doses of flumazenil attenuated the scopolamine-induced deficit, shortening the distance required to find the submerged platform and increasing the quadrant bias during the probe trial. The degree of attenuation produced by flumazenil was not dose-dependent, suggesting that the lowest dose of flumazenil produced a maximal attenuation of the spatial learning deficit produced by scopolamine. Since only a portion of the scopolamine-induced deficit, albeit a significant one, was attenuated by both doses of flumazenil, it would appear that only a portion of the deficit was mediated by BZ/GABA_A receptors.

The finding that flumazenil attenuated the spatial learning deficit produced by scopolamine replicates findings in other learning tasks (Jensen et al., 1987; Lal et al., 1988; Sarter et al., 1988) and suggests that BZs and ACh systems interact in a manner that is consequential to spatial learning. The nature of this interaction, however, is unknown. One possibility is that flumazenil increases arousal/vigilance (by preventing endogenous BZ

activity), thereby overcoming the sedative effects of scopolamine (e.g., Mewaldt & Ghoneim, 1979). This interpretation seems unlikely, however, since rats treated with scopolamine alone swam faster than controls, suggesting that rat treated with scopolamine were in a highly aroused state. Further, the increased swim speed produced by scopolamine was antagonized (not enhanced) by the high dose of flumazenil. A second possible interpretation is that scopolamine blocked the inhibitory actions of ACh on BZ/GABA_A systems, thereby increasing BZ/GABA mediated inhibition (Krnjevic, Reiffenstein, & Ropert, 1981). The resulting increase of endogenous BZ/GABA activity could be blocked by flumazenil (e.g., Izquierdo, Da Cunha, Huang, Walz, Wolfman, & Medina, 1990). A third interpretation is that flumazenil blocked the inhibitory actions of BZ/GABA_A receptors on cholinergic neurons, thereby disinhibiting ACh release which then competed with scopolamine for receptor sites (Sarter et al., 1990). The results of Experiment Va are consistent the latter two interpretations.

EXPERIMENT Vb

The results of Experiment Va confirm that BZs and ACh systems interact in some manner to impair spatial learning. However, the nature of this interaction remains uncertain. In Experiment Va, it was reasoned that if chlordiazepoxide and scopolamine both impair spatial learning by reducing ACh activity, then prolonging ACh activity, by reducing its catabolism with physostigmine, should attenuate both impairments. Indeed, cholinesterase inhibitors attenuate spatial learning deficits produced by ACh depletion (Hagen, Jansen, & Broekkamp, 1989) and by lesions of the nucleus basalis (Dolka & Thal, 1988; Mandel & Thal, 1988; Murray & Fibiger, 1985; Tilson, McLamb, Shaw, Rogers, Pediaditakis, & Cook, 1988) or medial septum (Riekkinen, Sirvo, & Riekkinen, 1990). Alternatively, if ACh is inhibiting the presynaptic release of GABA, but not its postsynaptic actions, physostigmine would fail to block the place deficit produced by chlordiazepoxide but would attenuate the deficit produced by scopolamine. In the present experiment, rats were treated with amnesic doses of either

chlordiazepoxide or scopolamine, alone or in combination with one of four doses of physostigmine, and trained in the MWM.

METHODS

Animals, apparatus and procedure

Ninety, naive, hooded, male rats of the Long-Evans strain served as subjects. Rats weighed between 350-450 g at the beginning of the experiment and food and water were available ad libitum. Rats were maintained as described in Experiment I. The same MWM and procedures used in Experiment Va were used in Experiment Vb.

Drugs and group assignment

Prior to experimentation, rats were divided into one of fifteen treatment groups. Five groups of rats were administered chlordiazepoxide hydrochloride (5 mg/kg; dissolved in 0.9% NaCl; Hoffmann-La Roche Inc.) concomitantly with either saline (n=10; 0.9% NaCl) or one of four doses of physostigmine hemisulfate (n=5/group; 0.05, 0.10, 0.25 or 0.50 mg/kg; dissolved in saline; Sigma Chemical Co.). An additional five groups of rats were administered scopolamine hydrobromide (1 mg/kg; dissolved in saline; Sigma) concomitantly with saline (n=10) or one of four doses of physostigmine hemisulfate (n=5/group; 0.05, 0.10, 0.25 or 0.50 mg/kg). Another four groups of rats were administered one of four doses of physostigmine hemisulfate (n=5/group; 0.05, 0.10, 0.25 & 0.50 mg/kg). The control group was administered saline (n=10; 0.9% NaCl). All injections were administered in a volume of 1 ml/kg and administered in the rat's home cage. Chlordiazepoxide, scopolamine and saline were administered 30 min prior to testing and physostigmine was administered 15 min prior to testing.

RESULTS

The effects of each drug treatment on the distance taken to reach the submerged platform are illustrated in Figures 3.14 and 3.15. Saline-treated rats rapidly acquired the location of the submerged platform, reaching asymptotic levels by the fourth day of testing. As in Experiment Va, both chlordiazepoxide- and scopolamine-treated rats demonstrated severe spatial

Figure 3.14: Effects of (A) chlordiazepoxide and physostigmine, (B) scopolamine and physostigmine and (C) physostigmine alone on the distance taken to locate the submerged platform over the six days of training. Note that physostigmine dose-dependently attenuates the deficit produced by scopolamine but not chlordiazepoxide. (Ph=physostigmine)

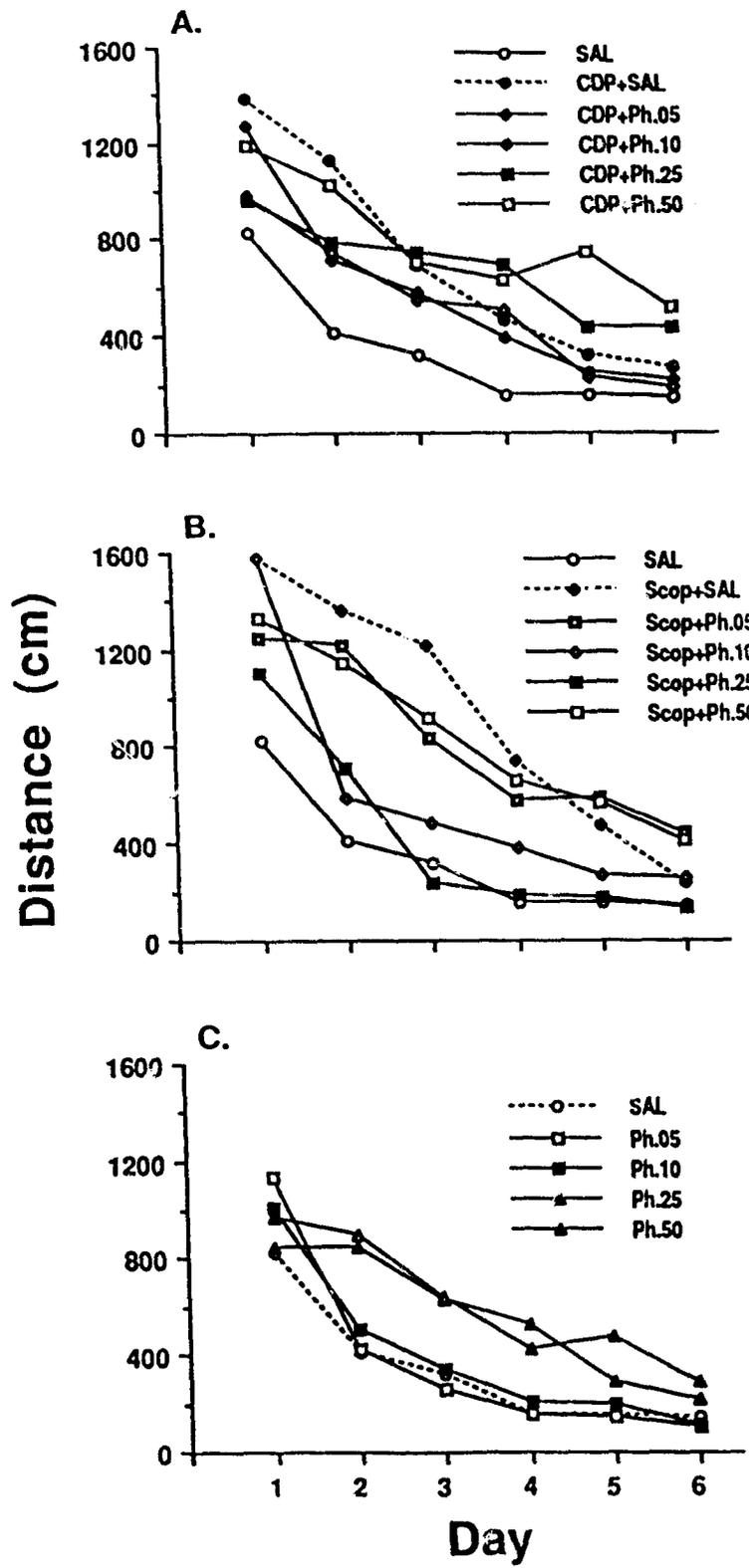
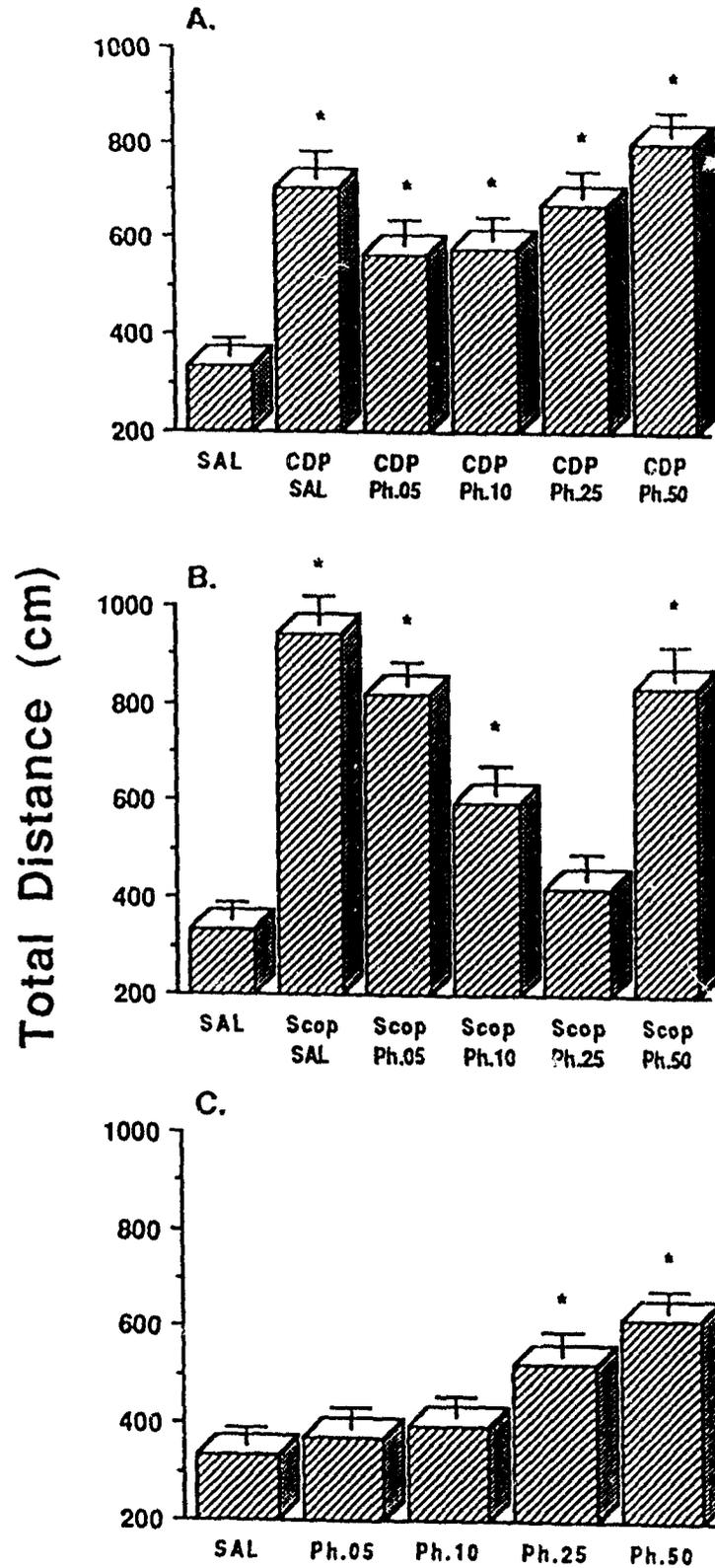


Figure 3.15: Effects of (A) chlordiazepoxide and physostigmine, (B) scopolamine and or (C) physostigmine alone on the distance taken to locate the submerged platform averaged over the six days of training. Note: 1) the U-shape dose-response curve of distances taken by rats treated with scopolamine plus physostigmine and 2) the failure of physostigmine to significantly attenuate the place learning deficit produced by chlordiazepoxide. * $p < 0.01$ compared to control (SAL) levels.



learning deficits, as evidenced by longer distances to locate the platform. Physostigmine failed to attenuate the spatial learning deficit produced by chlordiazepoxide at any dose (Fig. 3.14A & 3.15A). An overall ANOVA on the swim path lengths of groups treated with chlordiazepoxide and physostigmine revealed a significant group difference, $F(5,154)=12.98$, $p<0.001$, day difference, $F(5,770)=74.61$, $p<0.001$, and a significant interaction between groups and day, $F(25,770)=2.13$, $p<0.001$. Post hoc tests revealed that all rats treated with chlordiazepoxide, including those treated concomitantly with physostigmine, had significantly longer swim paths relative to saline-treated controls ($p<0.01$). Further, physostigmine failed, at any dose, to reduce swim path lengths relative to rats treated with chlordiazepoxide alone ($p>0.05$).

Physostigmine attenuated the spatial learning deficit produced by scopolamine in a U-shaped manner (see Fig. 3.15B). The smallest doses of physostigmine (0.05 & 0.10 mg/kg) attenuated the scopolamine deficit while the 0.25 mg/kg dose of physostigmine completely reversed the deficit. The highest dose of physostigmine (0.50 mg/kg) failed to attenuate the scopolamine-induced deficit. An overall ANOVA on the swim path lengths of groups treated with scopolamine and physostigmine revealed a significant group differences, $F(5,154)=24.02$, $p<0.001$, day difference, $F(5,570)=67.75$, $p<0.001$, and a significant interaction between groups and day, $F(25,570)=3.51$, $p<0.001$. Post hoc tests revealed that rats treated with scopolamine or scopolamine+physostigmine (0.05, 0.10 and 0.50 mg/kg) had significantly longer swim paths relative to saline-treated controls ($p<0.01$). Rats treated with scopolamine plus the second highest dose of physostigmine (0.25 mg/kg) had swim distances comparable to controls ($p>0.05$). Rats treated with scopolamine plus the two medium doses of physostigmine (0.10 & 0.25 mg/kg) had significantly shorter swim path lengths relative to rats treated with scopolamine+saline ($p<0.01$). Latency data (not shown) followed the same pattern of deficits and statistical significances.

The two largest doses of physostigmine (0.25 & 0.50 mg/kg) increased swim path lengths while the two smallest doses (0.05 & 0.10 mg/kg) had little effect (Fig. 3.15C). An overall ANOVA on the swim path lengths of groups

treated with physostigmine revealed a significant group difference, $F(4,95)=10.08$, $p<0.001$, day difference, $F(5,475)=61.30$, $p<0.001$, and a significant interaction between groups and day, $F(20, 475)=2.23$, $p<0.001$. Post hoc tests revealed that rats treated the two largest doses of physostigmine had significantly longer swim paths relative to controls ($p<0.01$).

The pattern of impairments observed during the drug free probe trial tended to confirm the pattern of impairments observed during training (Fig. 3.16). Rats treated with saline demonstrated a robust bias for the correct quadrant (44%; $p<0.01$ compared to chance (25%) levels; Fig. 3.16), indicating that they had acquired the spatial location of the platform. Conversely, rats treated with chlordiazepoxide or chlordiazepoxide+physostigmine (all doses) failed to show a quadrant bias (Fig. 3.16A), indicating that they had not acquired the spatial location of the platform. Rats treated with scopolamine+saline similarly failed to show a quadrant bias (Fig. 3.16B). Rats treated with scopolamine and the three highest doses of physostigmine showed a quadrant bias ($p<0.01$), while rats treated with scopolamine and lowest dose of physostigmine (0.05 mg/kg) failed to show a quadrant bias ($p>0.05$). Rats treated with the three lower doses of physostigmine showed a quadrant bias ($p<0.01$), while rats treated with the highest dose of physostigmine (0.50 mg/kg) did not show a quadrant bias (Fig. 3.16C).

Some of the drug treatments also affected motor and/or motivational performance as indexed by swim speed (Fig. 3.17). Rats treated with saline swam at an average speed of 28 ± 1 cm/s over the course of training. Rats treated with chlordiazepoxide swam at approximately the same speed as controls, though rats treated with chlordiazepoxide and the two highest doses of physostigmine swam significantly slower than controls ($p<0.01$). Rats treated with scopolamine swam significantly faster than controls ($p<0.01$) while rats treated with scopolamine and the two highest doses of physostigmine swam at the same speed as controls. Rats treated with the two highest doses of physostigmine (0.25 and 0.50 mg/kg) swam significantly slower than controls ($p<0.01$).

Figure 3.16: Effects of (A) chlordiazepoxide and physostigmine, (B) scopolamine and physostigmine or (C) physostigmine alone on the percentage distance spent in the correct quadrant during the 60 s probe trial. Note the failure of rats treated with chlordiazepoxide to prefer the correct quadrant. Note also the attenuation of the scopolamine deficit by physostigmine. * $p < 0.01$ compared to chance level (25%) represented by dotted line.

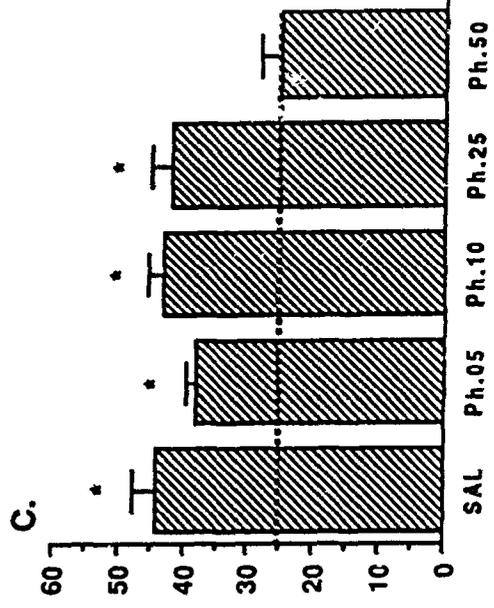
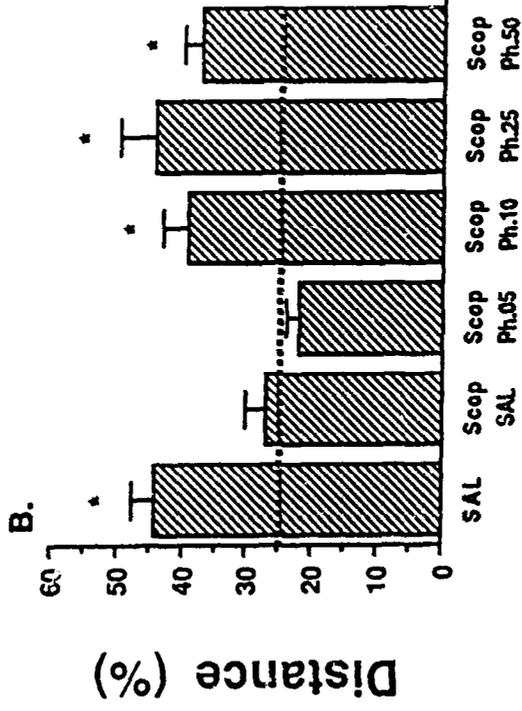
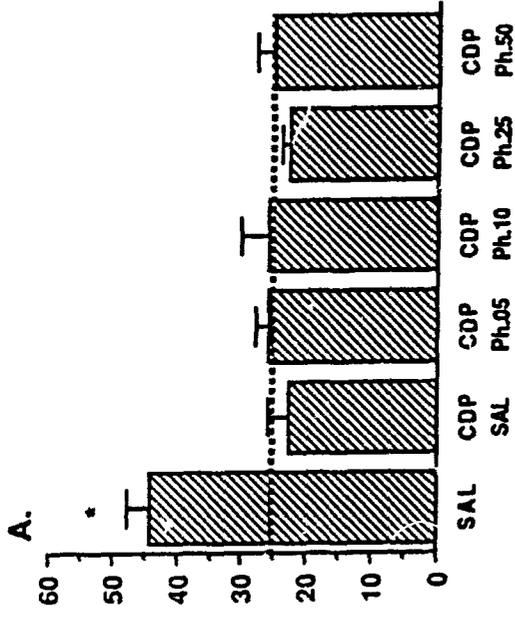
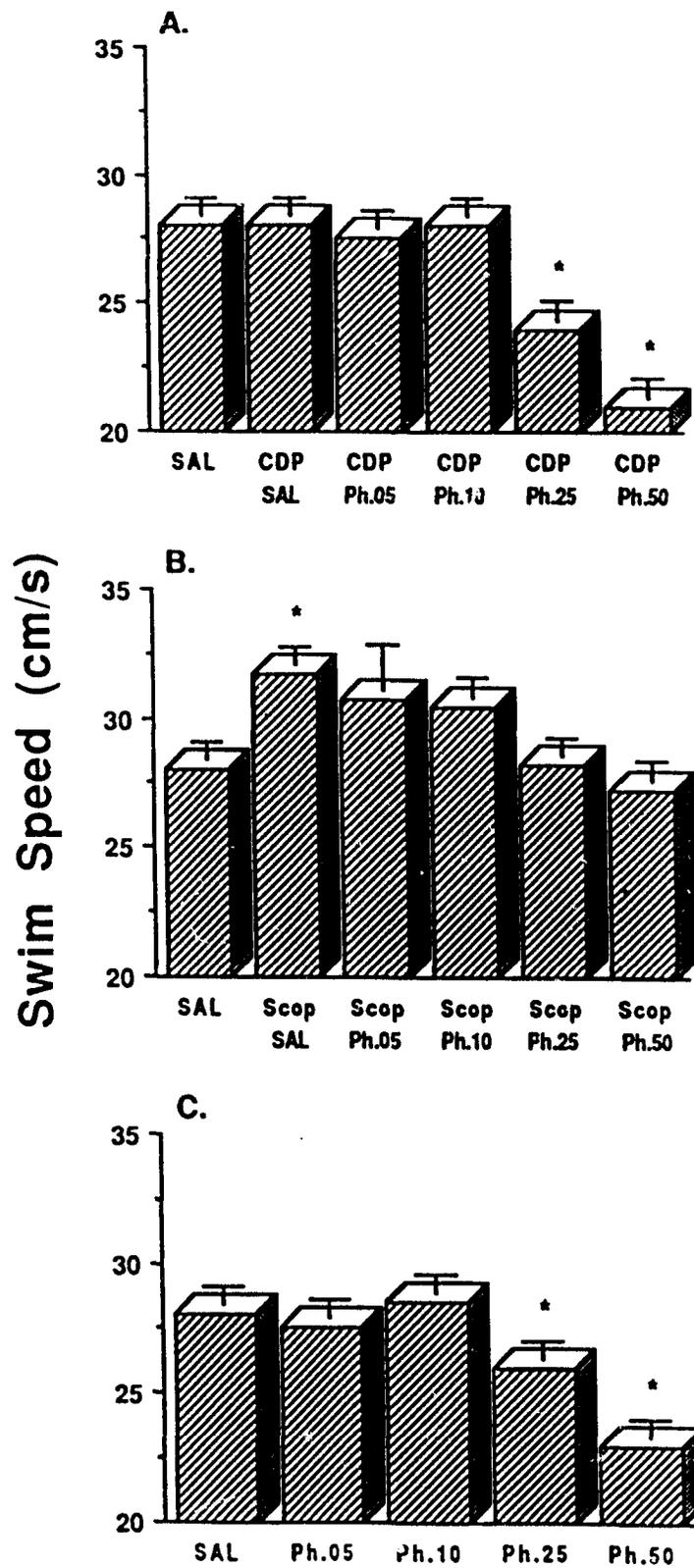


Figure 3.17: Effects of (A) chlordiazepoxide and physostigmine, (B) scopolamine and physostigmine and (C) physostigmine alone on swim speed averaged over the six days of training. Note that physostigmine dose-dependently reduced swim speed. * $p < 0.01$ compared to saline-treated controls.



Unlike the deficits seen during training with the submerged platform, none of the drug treatments produced a significant impairment of visible platform training (data not shown). Each treatment group rapidly learned to navigate to the visible platform, swimming directly to it on each trial.

DISCUSSION

The results of Experiment Vb demonstrate once again that chlordiazepoxide- and scopolamine-treated rats have severe spatial learning impairments. Although these rats eventually became proficient at locating the submerged escape platform, as evidenced by the gradual decrease in swim path lengths over training, it is unlikely that such a reduction reflected acquisition of the spatial location of the platform because both scopolamine- and chlordiazepoxide-treated rats failed to show a preference for the correct quadrant during the subsequent probe trial. Rather, scopolamine- and chlordiazepoxide-treated rats appeared to adopt an efficient response strategy, such as swimming towards or away from a particular cue or circling the pool a particular distance from the wall. Indeed, the control level performance on the visible platform task in Experiments I and II suggests that scopolamine- and chlordiazepoxide-treated rats are capable of accurately navigating to a single cue. Furthermore, good performance on the visible platform task and normal swim speeds suggests that scopolamine- and chlordiazepoxide-treated rats were motivated to escape from the water and did not suffer from sensorimotor impairments. Indeed, chlordiazepoxide-treated rats swam as fast as controls and scopolamine-treated rats actually swam faster than controls, suggesting that neither drug interfered with motorical proficiency or escape motivation.

Physostigmine also produced a dose-dependent impairment of spatial learning. Swim speeds of rats treated with the two highest doses of physostigmine were significantly reduced, suggesting that impairments of motorical proficiency may have contributed to the spatial learning deficit. Additionally or alternatively, it is possible that the higher doses of physostigmine impaired spatial learning by increasing synaptic ACh levels to the extent that presynaptic autoreceptors reduced subsequent ACh release

(Kilbinger, 1984). The latter actions would be expected to have the same consequences as scopolamine. A third alternative is that the high doses of physostigmine impaired spatial learning by overstimulating postsynaptic ACh receptors. Whatever the mechanism, the failure to observe a facilitatory effect with the low doses of physostigmine contrasts with previous reports (see Hagen & Morris, 1988, for review), while the impairment produced by the large dose is consistent with these previous reports.

Physostigmine, in a U-shaped manner, attenuated the spatial learning deficit produced by scopolamine. Rats treated with scopolamine and the highest dose of physostigmine were impaired during initial training, but, surprisingly, demonstrated a quadrant bias during the probe trial, suggesting that these rats had adopted a spatial strategy. The slowed acquisition may have resulted from a non-specific performance impairment produced by the high dose of physostigmine. Together, these findings are consistent with previous reports that mnemonic deficits produced by scopolamine in human subjects can be attenuated by physostigmine (Ghoneim & Mewaldt, 1977; Mewaldt & Ghoneim, 1979; Preston, Brazell, Ward, Broks, Traub, & Stahl, 1988) and confirm that spatial learning deficits produced by ACh blockade can be overcome by prolonging ACh activity.

Physostigmine failed, at every dose, to attenuate the spatial learning deficits produced by chlordiazepoxide, a finding that is consistent with previous human studies in which memory deficits produced by BZs were not attenuated by physostigmine (Mewaldt & Ghoneim, 1979; Preston, Ward, Lines, Poppleton, Haigh, & Traub, 1989). Rats treated with chlordiazepoxide and any of the doses of physostigmine demonstrated severe spatial learning deficits, as revealed by longer swim distances and a failure to demonstrate a quadrant bias. Given that spatial learning deficits produced by ACh blockade (present results), depletion (Hagen & Jansen, 1989), nucleus basalis lesions (Dolka & Thal, 1989; Mandel & Thal, 1988; Murray & Fibiger, 1985; Tilson, McLamb, Shaw, Rogers, Pediaditakis, & Cook, 1988) and septal lesions (Riekkinen, Sirvo, & Riekkinen, 1990) are attenuated by inhibiting cholinesterase, the failure of physostigmine to attenuate the spatial learning

deficits produced by chlordiazepoxide suggests that the impairment is not mediated by GABA-induced reductions of ACh.

GENERAL DISCUSSION

The results of Experiment Va and Experiment Vb demonstrated that blockade of ACh receptors with scopolamine and activation of BZ receptors with chlordiazepoxide impairs spatial learning, but not cue learning, in the MWM. Experiment Va demonstrated that flumazenil, a BZ receptor blocker, could reverse the spatial learning deficit produced by chlordiazepoxide and could attenuate the deficit produced by scopolamine without affecting place or cue learning when given alone. Experiment Vb showed that physostigmine, in a dose-dependent manner, could attenuate the spatial learning deficit produced by scopolamine, but not chlordiazepoxide, and could impair spatial learning at high doses. Together, these results suggest that both BZ and ACh systems are important modulators of rodent spatial memory and that a specific interaction between the two systems is partially responsible for the observed spatial learning deficits.

Recently, Sarter et al. (1990) proposed that the cognitive decline associated with ACh hypofunction may be partly due to (or exacerbated by) tonic inhibition of surviving cholinergic cells by endogenous activity at the BZ/GABA_A receptors on cholinergic neurons. Sarter et al. (1990) further propose that the disinhibition of these cholinergic neurons with the BZ antagonist/inverse agonist ZK 93 426 could attenuate the cognitive deficit. BZ/GABA_A receptor agonists are known to reduce hippocampal and forebrain ACh activity (Miller & Richter, 1985; Richter, Gormley, Holtman, & Simon, 1982; Wood & Richard, 1982; Zsilla, Cheney, & Costa, 1976) and also impair spatial learning (present findings; McNamara & Skelton, 1991; McNaughton & Morris, 1987), but it is not clear that the ACh reduction causes the spatial learning deficit. According to the proposal of Sarter et al. (1990), prolonging ACh activity with physostigmine should have compensated for chlordiazepoxide-induced ACh hypofunction and resulting spatial learning deficit, just as prolonging ACh activity compensates for memory deficits associated with ACh hypofunction produced by global

(Hagen, Jansen, & Broekkamp, 1989) or selective hippocampal (Riekkinen, Sirvo, & Riekkinen, 1990) or forebrain (Dolka & Thal, 1988; Mandel & Thal, 1988; Murray & Fibiger, 1985; Tilson, McLamb, Shaw, Rogers, Padiaditakis, & Cook, 1988) ACh depletion. However, physostigmine failed to attenuate the chlordiazepoxide-induced deficit, thereby contradicting the notion that the spatial learning deficit was due to chlordiazepoxide-induced reductions of ACh activity. Indeed, a recent study employing *in vivo* microdialysis revealed that chlordiazepoxide, at doses equal to and above that used in the present study, had little effect on ACh release in frontoparietal cortices (Moore, Bernston, Sarter, & Bruno, 1991).

The present results also fail to support the notion that the spatial learning deficits produced by ACh antagonists (scopolamine) are due to the disinhibition of GABA release from the presynaptic terminal (Ben-Ari, Krnjevic, Reinhardt, & Ropert, 1981; Krnjevic, Reiffenstein, & Ropert, 1981). For example, by this model physostigmine would be expected to attenuate the deficits produced by CDP, since the reinstatement of ACh-mediated inhibition of GABA release would negate the GABA-enhancing effects of CDP at the postsynaptic receptor. Further, flumazenil, a BZ receptor antagonist, would not be expected to block, postsynaptically, scopolamine-induced increase of GABA release. Hence, further experimentation will be required to reveal the nature of the interaction between ACh and BZ systems as it pertains to mnemonic processes.

In sum, the present investigation replicated previous observations that both scopolamine and chlordiazepoxide produce a severe impairment of spatial learning, but not cue learning, in the MWM (McNamara & Skelton, 1991a; McNaughton & Morris, 1987). It extended these results by showing that spatial learning deficits produced by chlordiazepoxide are reversed by flumazenil but not physostigmine and the spatial learning deficit produced by scopolamine is reversed by physostigmine and flumazenil. Together these results contradict the notion that the BZ-induced impairment of spatial learning is mediated by cholinergic systems and that scopolamine impairs spatial learning by enhancing GABA release.

CHAPTER 4: Neuroanatomy

The identification of which brain regions mediate the behavioral effects of BZs has received little experimental attention. In order to begin to understand how BZs produce their effects on memory and anxiety, it is first necessary to determine which neuroanatomical site(s) mediate these actions. One approach is to infuse micro-quantities of BZs into suspect sites. Deciding which region to infuse BZs should have the following two prerequisites: 1) the site should have BZ receptors, and 2) the site should subservise mnemonic or anxiolytic functions, as determined by lesioning, for example. Several neuroanatomical sites possess a high density of central BZ receptors (Young & Kuhar, 1979; 1980) and have been implicated in learning and memory and/or anxiolytic processes. For example, the frontal cortex has a high density of BZ receptors (Young & Kuhar, 1980) and aspiration lesions of this region impair spatial learning in the MWM (Kolb, Sutherland & Whishaw, 1983). Thus, it may be expected that the amnesic effects of BZs are mediated via BZ receptors in the frontal cortex. Several additional suspect sites will now be discussed separately.

The medial septum (MS) possesses a high density of BZ receptors (Young & Kuhar, 1980) and lesions of the medial septum impair spatial learning in the MWM (Hagen, Salamone, Simpson, Iversen & Morris, 1988; Kelsey & Landry, 1988). Moreover, MS lesions also reduce indices of anxiety (Gray, 1983). Further, infusion of BZs into the septal region produces both anxiolysis (Grishkat, 1991) and spatial learning deficits (Stackman & Walsh, 1992). Hence it is predicted from these data that intra-septal infusions of CDP will reduce anxiety and impair spatial learning.

Another possible site mediating amnesic actions of BZs is the nucleus basalis magnocellularis (NBM). The NBM is a cholinergic cell group in the basal forebrain that innervates the frontal cortex (Woolf, 1991). Lesions of the NBM impair spatial learning across a variety of tasks (see Hagen & Morris, 1986 for review), including the MWM (Whishaw, O'Connor & Dunnett, 1985). Although the effects of intra-NBM infusions of BZs have not been assessed, intra-NBM infusions of the GABA agonist muscimol (Dudchenko

& Sarter, 1991) or GABA (Majchrzak, Brailowsky & Will, 1990) impair spatial learning. While the effects of NBM lesions on anxiety are uncertain, it is expected that intra-NBM infusions of CDP will impair spatial acquisition.

The hippocampal formation is also suspected of mediating the anxiolytic and amnesic actions of BZs. The hippocampal formation possesses a high density of BZ receptors ($\sim 50\% \omega_1 \sim 50\% \omega_2$; Braestrup & Nielson, 1980), particularly in the dentate gyrus and CA1 regions (Young & Kuhar, 1980). Additionally, the hippocampus is well known for its role in learning and memory processes (Squire, 1987). For example, lesions of the hippocampal formation produce a profound impairment of spatial learning across a variety of tasks (O'Keefe & Nadel, 1978), including the MWM (Morris, Garrud, Rawlins & O'Keefe, 1982; Sutherland, Whishaw & Kolb, 1983). Furthermore, the hippocampus and its afferents have been proposed to be involved in anxiety-related behavioral inhibition on the basis of the similarities between the behavioral effects of hippocampal lesions and BZ drugs (Gray, 1983). Hence, from these data it is expected that activation of hippocampal BZ receptors will both impair spatial acquisition as well as reduce anxiety.

The amygdaloid complex is also suspected of mediating the anxiolytic and mnemonic actions of BZs. For example, the amygdala has long been known to mediate the acquisition of fear conditioning (Davis, Hitchcock & Rosen, 1987) and lesions of the central nucleus of the amygdala have been shown to produce anticonflict effects (Shibata, Kataoka, Yamashita & Ueki, 1986; Yadin, Thomas, Strickland & Grishkat, 1991), an effect indicative of anxiolysis. Secondly, the amygdala possesses a high density of BZ receptors, mostly in the basolateral nuclear complex and predominantly ω_2 (Dennis et al., 1988; Niehoff & Kuhar, 1983; Niehoff & Whitehouse, 1983; Young & Kuhar, 1980). Finally, infusions of BZs into the amygdaloid region reduce experimental indices of anxiety (Nagy, Zambo & Decsi, 1979; Shibata, Kataoka, Gomita & Ueki, 1982). Hence, current evidence suggests that the BZ receptors in the amygdala mediate the anxiolytic effects of BZs. Moreover, recent evidence suggests that the amygdala may mediate the amnesic effects of BZs; lesions of the amygdaloid region were found to block the amnesic

effects of diazepam on avoidance conditioning (Dickinson-Anson, Tomaz, & McGaugh, 1991, 1992). In the MWM, however, amygdala lesions have little effect on spatial learning (Sutherland & McDonald, 1990). Hence, it is predicted that activation of BZ receptors in the amygdala will reduce anxiety but have little effect on spatial learning.

The cerebellum may also mediate the anxiolytic and/or amnesic actions of BZs. The cerebellum possesses a disproportionately high quantity of BZ ω_1 receptors relative to BZ ω_2 receptors (75:25; Braestrup & Nielson, 1980; Young, Niehoff, Kuhar, Beer & Lippa, 1981). The functional significance of this BZ receptor distribution is presently unknown. Selective activation of the ω_1 receptor with CL 218,872 impairs spatial learning, suggesting that these receptors can mediate amnesia (McNamara & Skelton, 1992). However, although cerebellar lesions have been found to impair certain types of learning, including avoidance conditioning (Guillaumin, Dahhaoui & Caston, 1991) and classical conditioning (McCormick & Thompson, 1984), they have little effect on spatial learning (Thompson, 1983). Hence, it is unclear what effect the coactivation of both ω_1 and ω_2 receptors in the cerebellum will have on spatial learning. The cerebellum may also be involved in the anxiolytic effects of BZs as cerebellar vermal lesions reduce experimental indices of anxiety (Supple, Leaton & Fanselow, 1987). Hence, the cerebellum may mediate both the anxiolytic and amnesic actions of BZs.

Experiment VI: Intracranial infusions of BZs

Experiment VI sought to investigate the effects of intracranial infusions of CDP on behavioral assays sensitive to the anxiolytic and amnesic effects of systemically administered CDP. If the differential distribution of BZ receptors in the brain is correlated with the differential behavioral effects observed after systemically administered CDP, then it should be possible to fractionate these behaviors by selective infusions. On the basis of the data presented above, the following sites were targeted for CDP infusions: frontal cortex, medial septum, nucleus basalis magnocellularis (NBM), hippocampus, amygdala and cerebellum. Firstly, however, the effects of systemically administered CDP on spatial learning and open field activity were assessed to determine that the behavioral

procedures used in the present study are indeed sensitive to the amnesic and anxiolytic actions of CDP.

METHODS

Animals

Eighty-five naive hooded male rats of the Long-Evans strain served as subjects. Rats were housed in pairs in shoebox cages and maintained on a 12:12-h light:dark cycle. The rats weighed approximately 400 g at the beginning of the experiment, and food and water were available ad lib.

Apparatus and procedure

Open-field. The empty pool served as the open field as described in Experiment I.

Morris water maze. The pool used was the same as that described in Experiment I except that it was filled with 26°C ($\pm 1^\circ\text{C}$) water, instead of 22°C water, for added warmth over the course of training (see below). The basic training procedure is the same as that outlined in Experiment I with the following amendments. Rats were run individually and over the course of one day such that spatial learning could be assessed after acute infusions (i.e., to avoid gliotic plugging and lesioning associated with chronic infusions). A total of 20 trials were given with the submerged platform in the center of northwest quadrant. Rats were started from each of the four poles sequenced randomly. Once the rat located the platform, it was permitted to remain on it for 15 sec. The rat was placed on the platform for 15 sec if it did not locate the platform within 60 sec. After each trial, the rat was returned to a holding cage positioned 90 cm under a 250-W brooding lamp (for warmth) and allowed to remain there for the intertrial interval (45 s). Hence trials were given once every minute. On the 21st trial a probe trial was given and rats were required to swim in the pool without the escape platform for 30 sec. All rats were released from the southern pole, and the distance traveled in each quadrant was recorded. Following this single trial, four visible platform trials were given. Rats were required to navigate to a visible platform located in a different quadrant on each trial for four trials, and swim path

lengths were recorded. Swim path lengths (distance) and escape latencies were recorded with an overhead video tracking system.

Surgery

Rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and given scopolamine methylnitrate (1 mg/kg, i.p.). Stainless-steel guide cannulae (23 gauge, 13.5 mm long) were implanted stereotaxically at the following coordinates relative to bregma (anterior-posterior) and skull (dorsal-ventral): Bilateral frontal cortex (AP +3.0, ML 5.0, DV ~1); Medial septum (AP +0.5, ML, 0.0, DV 5.5); bilateral NBM (AP -1.4, ML 2.5, DV 5.5); bilateral amygdala (AP -2.8, ML 5.0, DV 8.0); bilateral hippocampus (AP -3.8, ML 2.0, DV -2.6); bilateral cerebellum (AP -11.3, ML 3.0, DV -4.0). All anterior-posterior coordinates are relative to bregma and dorsal-ventral relative to skull. Cannulae were affixed to the skull using four screws and dental acrylic. An obturator (fashioned insect pin, 00) was inserted into the guide cannula to prevent occlusion. Animals were allowed one week to recover from surgery prior to behavioral testing.

Drug and injection procedures

Intracranial injections. The obturator was removed and the injection needle was inserted. The injection needle was connected to polyethylene tubing which was in turn attached to a 10 μ l microsyringe. The syringe was driven by a syringe minipump (Razel Scientific Instruments Inc.) which delivered a 1 μ l volume over a 3 min period. Injections were either artificial cerebrospinal fluid (CSF in mM: NaCl 147, KCl 2.9, MgCl₂ 1.6, CaCl₂-2H₂O 1.7, NaHCO₃ 35.9, and dextrose 2.2) or chlordiazepoxide hydrochloride (CDP, Hoffmann-La Roche Inc., 60 nmol concentration dissolved in CSF). The injection needle was retained in the guide cannula for 1 min post injection to facilitate passive drug diffusion. After the injection needle was removed, the obturator was reinserted and 5 min elapsed prior to behavioral testing.

Systemic injections. Rats received either saline (0.9% NaCl; 1 ml/kg) or chlordiazepoxide hydrochloride (5 mg/kg, 1 ml/kg) 30 min prior to testing. All injections were administered IP in the animal's home cage.

Histological analysis

After the completion of behavioral training, cannulae placements were verified histologically. Rats were injected with 1 μ l of black dye, to permit estimates of drug diffusion, and anesthetized with an overdose of sodium pentobarbital. Rats were then perfused transcardially with saline (0.9%) and the brains were stored in a 10% formaldehyde solution for 2 weeks. Brains were cut coronally in 80 μ m thick slices and mounted on slides for microscopic analysis. The position of the injection needle tips were documented.

Data analysis

An analysis of variance with repeated measures (ANOVA) was used to assess distances, escape latencies and swim speeds (cm/s). Probe trial performance and open field measures were assessed using Dunnett's t-test.

RESULTS

Open-field

Systemic injections. Rats receiving systemic saline demonstrated the characteristic thigmotaxic response when placed in the open field. Namely, control rats would circle the periphery of the field, rarely entering the central region. Conversely, rats treated with CDP not only circled the periphery of the field but would also enter the central region, occasionally stopping and rearing while in the center. Consequently, CDP-treated rats spent a greater relative duration in the central region relative to controls ($p < 0.01$; Table III). CDP-treated rats did travel a shorter total distance relative to controls, though this difference was not statistically significant.

Intracranial injections. Intracranial infusions of CDP, regardless of the site, failed to affect the total distance travelled in the open field (Table III). However, there was some variability between the different injection sites, which ranged from 4958 ± 300 cm (amygdala-CSF) to 6508 ± 407 cm (hippocampus; CSF). CDP increased the relative distance spent in the central region of the field only when it was infused into the amygdala ($p < 0.01$); infusions of CDP into every other site failed to affect thigmotaxia, relative to

TABLE III

Effects of systemic and intracranial infusions of chlordiazepoxide on total distance traveled, proportion of distance traveled in the central region, and number of boli excreted during 5 minute open field test.

Brain Site + Treatment	N	Total Distance	Percent Inside	Bolus Count
<u>Systemic injection</u>				
Saline (1 ml/kg)	6	6732 ± 453	17.2 ± 3.8	3.6 ± 1.1
CDP (5 mg/kg)	6	6243 ± 495	27.4 ± 3.2*	3.0 ± 1.1
<u>Intracranial injection</u>				
Frontal Cortex				
CSF	6	5338 ± 560	24.5 ± 5.5	1.7 ± 0.9
CDP	6	6003 ± 353	20.0 ± 5.0	1.7 ± 0.3
Nucleus Basalis				
CSF	6	5450 ± 362	18.0 ± 1.9	1.2 ± 0.5
CDP	7	5294 ± 359	23.1 ± 2.8	1.1 ± 0.5
Medial septum				
CSF	6	5909 ± 785	14.6 ± 2.5	2.5 ± 1.2
CDP	6	6321 ± 292	11.6 ± 1.2	1.3 ± 0.6
Hippocampus				
CSF	6	6508 ± 407	13.2 ± 2.8	1.7 ± 0.6
CDP	7	6217 ± 293	13.8 ± 2.9	0.4 ± 0.4*
Amygdala				
CSF	6	4958 ± 300	8.6 ± 1.1	0.4 ± 0.4
CDP	6	5366 ± 639	21.0 ± 2.2**	1.7 ± 0.7*
Cerebellum				
CSF	6	5883 ± 264	18.1 ± 2.6	0.8 ± 0.5
CDP	6	5498 ± 164	15.7 ± 2.5	2.0 ± 0.6*

*P<0.05 compared to CSF-treated controls

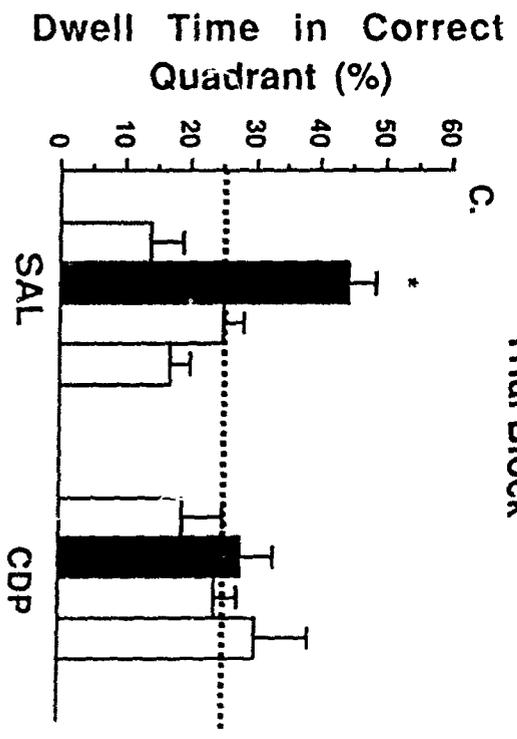
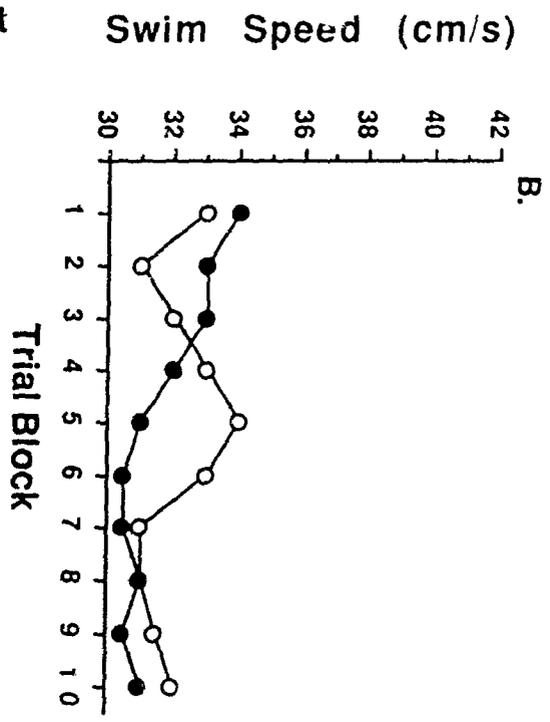
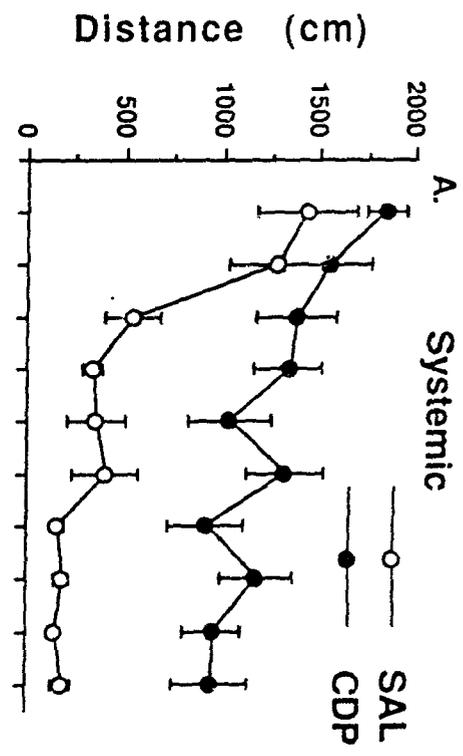
**P<0.01 compared to CSF-treated controls

CSF injections ($p > 0.05$). Once again there was some variability in the distance spent in the central region between each site ranging from 8.6 ± 1.1 (amygdala-CSF) to 24.5 ± 5.5 (frontal cortex-CSF). Further, the distance spent in the central region by rats receiving the CSF infusion differed across infusion sites as well as from systemic controls. The latter discrepancy could have been due to: 1) surgery having some long-lasting influence on the rats anxiety level, 2) infusions of CSF producing an effect of their own (anxiogenesis), and/or 3) anxiety being a trait which naturally varies across different rat litters. It should be noted that for each infusion site, CSF and CDP rats were drawn from the same rat pool and tested on the same day. Because of this between-site variability, comparisons were made only within each site.

Water maze

Systemic injections. Rats treated with saline learned the location of the platform quickly (~ 8 trials) and continued to swim directly to the platform over the remaining trials (Fig. 4.1A), a rate half that required when 4 trial blocks are separated by 24 h intervals (see Fig. 3.7A). Rats treated with systemic CDP showed a severe disruption of spatial learning. An ANOVA on distances revealed a significant group difference, $F(1,22)=27.7$, $p < 0.001$, trial-block difference, $F(9,198)=14.1$, $p < 0.001$, but not a significant group by trial-block interaction, $F(9,198)=1.0$, N.S. Analysis of escape latency data (not shown) revealed a similar pattern of deficits and statistical significances. Over the course of training, both the saline and the CDP groups swam at approximately the same rate (Fig. 4.1B). An ANOVA revealed a significant trial-block difference, $F(9,198)=4.7$, $p < 0.001$, but not a group difference, $F(1,22)=0.5$, N.S., or a group by trial-block interaction, $F(9,198)=1.3$, N.S. During the 30s probe trial (Fig. 4.1C), rats in the CDP group failed to demonstrate a bias for any of the four quadrants, unlike the saline group, which showed a robust bias for the quadrant that had contained the platform ($p < 0.01$ relative to chance). The mean distance travelled to reach the visible platform (data not shown) by the saline (157 ± 21) and CDP groups (148 ± 14) were not significantly different.

Figure 4.1: Effects of systemically administered chlordiazepoxide (CDP) or saline on (A) the distance taken to locate the submerged platform on the twenty trials (shown as ten trial blocks of two trials)/day procedure, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note the profound deficit produced by chlordiazepoxide in the absence of swim speed reductions. * $p < 0.01$ compared to chance level (25%-dotted line; four bars in (C) represent dwell times in each of the four pool quadrants in the following order from left to right: NE - NW - SW - SE quadrants).



Intracranial injections. The infusion rate and volume chosen worked well for all sites except the hippocampus. Initial injections of 1 μ l CSF into the hippocampus produced "wet-dog shakes" (WDS), a behavioral manifestation of ictal activity, and impaired spatial learning. Subsequently, it was found that a smaller volume (0.5 μ l) of CSF, as well as CDP, could be infused into the hippocampus without causing WDS or an impairment of spatial learning. Statistical analysis revealed that injections of CSF into each site did not impair spatial learning relative to rats treated systemically with saline. The infusion sites will be discussed beginning with the most anterior site (frontal cortex) and ending with the most posterior site (cerebellum).

The positions of the injection needle tips in the frontal cortex were similar for the CSF and CDP groups (Figure 4.2). In the CDP group, one rat was found to have placements more medial, locating them in the cingulate cortex; performance of this rat did not differ from those in which placements were restricted to frontal cortex. Assessment of dye diffusion revealed that the 1 μ l infusion spread throughout the frontal cortex region, with some spread to the cingulate cortex. The effects of intra-cortical infusions of CSF and CDP on spatial learning are illustrated in Figure 4.3. Rats receiving infusions of either CSF or CDP into the frontal cortex region acquired the platform location quite rapidly and continued to show good asymptotic performance over the remaining trials (Fig. 4.3A). An ANOVA on distance data revealed a significant difference over trials, $F(9,198)=38.2$, $p<0.001$, but not a significant group difference, $F(1,22)=1.7$, N.S., or group by trial-block interaction, $F(9,198)=1.2$, N.S. For both groups, swim speeds were constant over the course of training and both groups swam at the same rate (Fig. 4.3B). An ANOVA on swim speed data revealed a significant group by trial-block interaction, $F(9,198)=2.1$, $p<0.05$, but not a group difference, $F(1,22)=1.4$, N.S., or trial-block difference, $F(9,198)=0.9$, N.S. During the probe trial (Fig. 4.3C), both the CSF and CDP groups showed a robust bias for the correct quadrant ($p<0.01$). On the visible platform task, swim distances taken by the CSF and CDP groups did not differ significantly (CSF: 179 ± 23 cm vs. CDP: 178 ± 19 cm; $p>0.05$).

Figure 4.2: Illustration of bilateral injection needle tip placements in the frontal cortex (Fr). Note the similarity of injection sites of the two groups. Closed circles = CSF infusions, closed squares = chlordiazepoxide (CDP; 60 nmol) infusions. (Cg = cingulate cortex; CSF = artificial cerebrospinal fluid; bar = 1 mm).

Frontal Cortex

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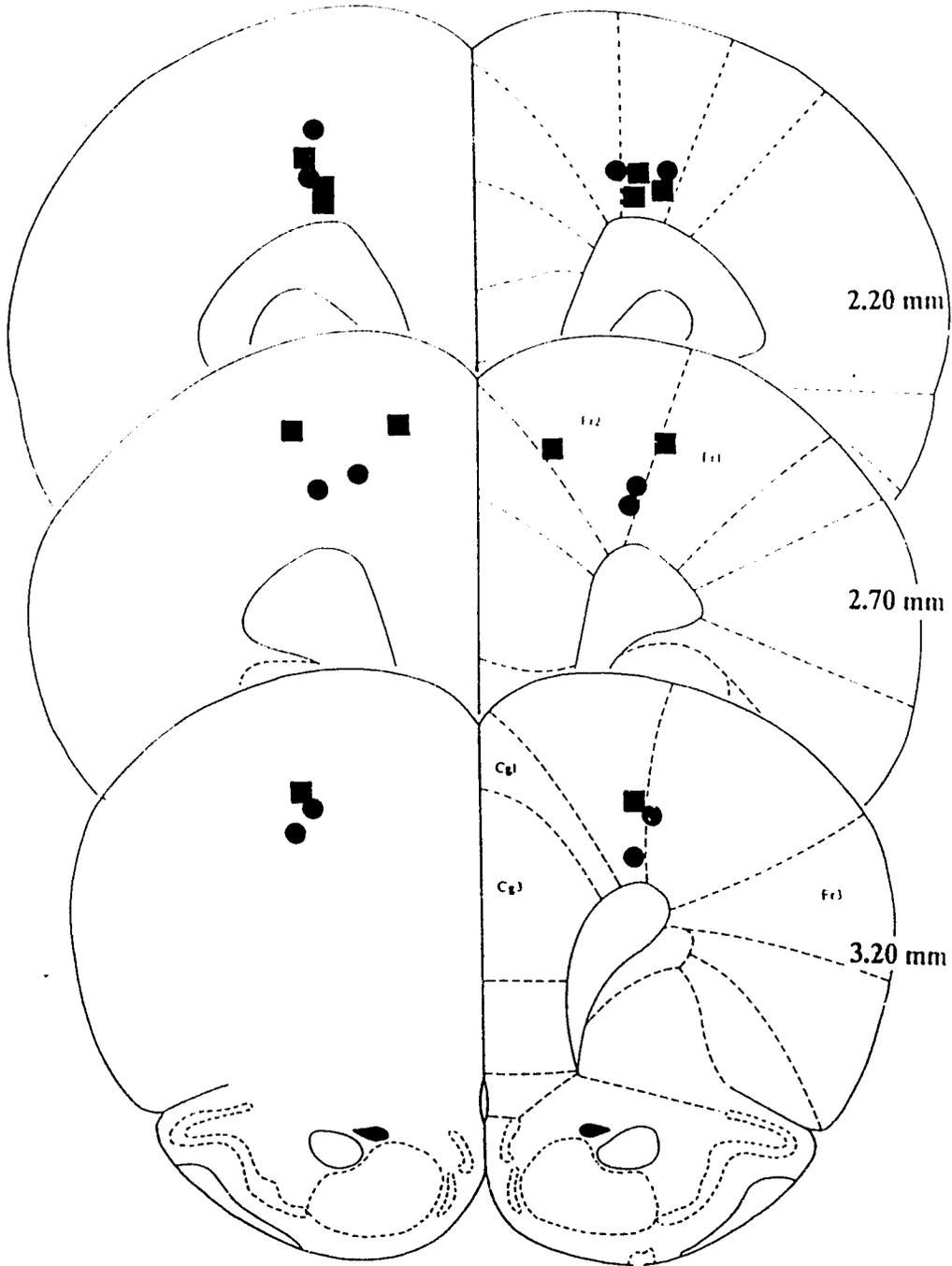
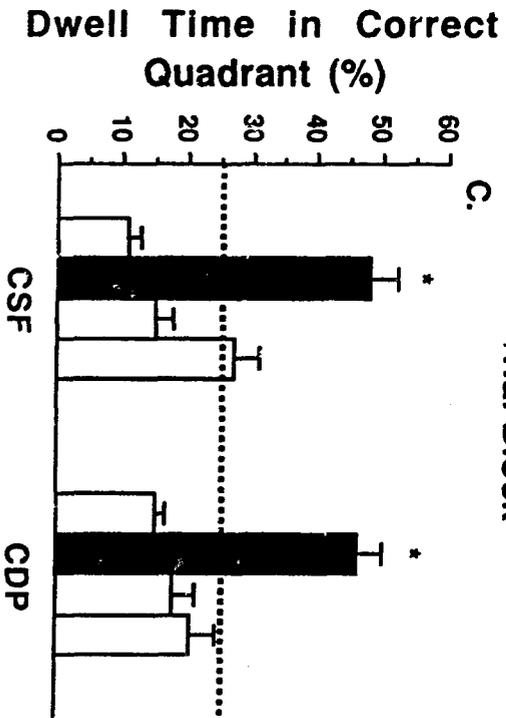
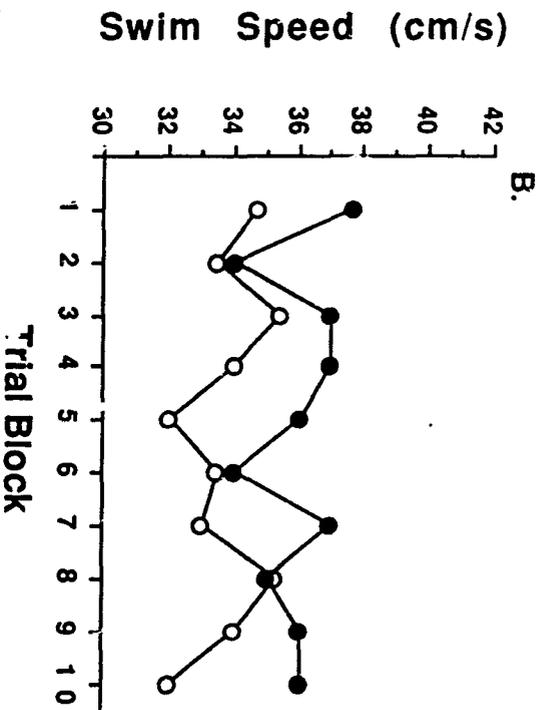
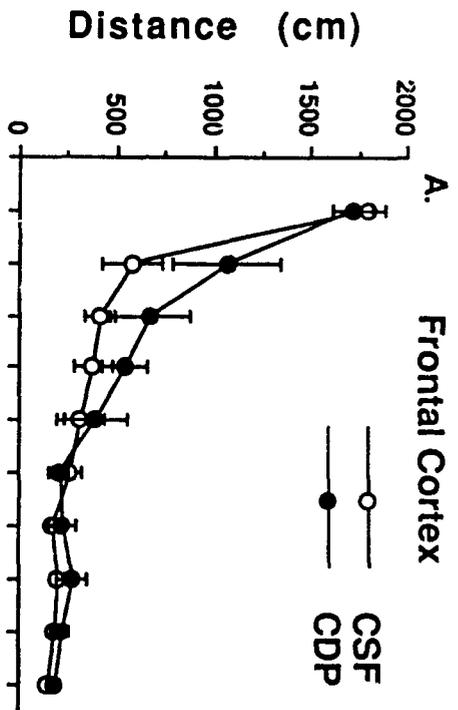


Figure 4.3: Effects of intra-cortical infusions of chlordiazepoxide (CDP; 60 nmol) or CSF on (A) the distance taken to locate the submerged platform, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note that both CSF- and CDP-treated rats acquire the platform location quickly and show a bias for the target quadrant during the probe trial. * $p < 0.01$ compared to chance level (25%-dotted line).



The positions of the injection needle tips in the NBM were similar in the CDP and CSF groups (Figure 4.4). Some placements from both groups were more dorsal, terminating in the reticular thalamic nucleus. Analysis of dye diffusion revealed that even the more dorsally placed cannulas were sufficiently close to deliver drug to the NBM region. There was some spread into the globus pallidus region and into the substantia inominata, just ventral to the NBM. Dye was also detected in the lateral ventricles at sites quite distant from the needle tip placements. The effects of infusions of CSF or CDP on spatial learning are illustrated in Figure 4.5. The CSF and CDP groups acquired the platform location at the same fast rate (Fig. 4.5A). An ANOVA on distance data revealed a significant trial-block difference, $F(9,216)=30.8$, $p<0.001$, but not a significant group difference, $F(1,24)=0.1$, N.S., or group by trial-block interaction. Swim speeds of the CDP group tended to be slower toward the beginning of training but eventually returned to CSF levels by the end of training (Fig. 4.5B). An ANOVA on swim speed data failed to reveal a significant group difference, $F(1,24)=2.3$, N.S., trial-block difference, $F(9,216)=1.4$, N.S. or group by trial-block interaction, $F(9,216)=1.6$, N.S. During the probe trial (Fig. 4.5C), both the CSF and the CDP groups demonstrated a significant bias for the correct quadrant ($p<0.01$). On the visible platform task, the swim distances taken by the CSF and CDP groups did not differ significantly (CSF: 168 ± 22 cm vs. CDP: 204 ± 26 cm; $p>0.05$).

The positions of the injection needle tips in the medial septum were similarly concentrated in the medial septum (Figure 4.6). Assessment of dye diffusion revealed that the infusions reached the medial septum, vertical limb of the diagonal band, and some aspects of the lateral septum. The effects of intra-septal infusions on spatial learning are illustrated in Figure 4.7. Relative to the CSF group, the CDP group showed a severe spatial learning impairment (Fig. 4.7A). An ANOVA on distance data revealed a significant group difference, $F(1,22)=11.8$, $p<0.001$, trial-block difference, $F(9,198)=15.5$, $p<0.001$, but not a significant group by trial-block interaction, $F(9,198)=0.8$, N.S. The CDP group also swam at a slower rate than the CSF group (Fig. 4.7B). An ANOVA on swim speed data revealed a significant group difference, $F(1,22)=10.5$, $p<0.01$, but not a significant trial-block difference, $F(9,198)=1.2$, N.S. or group by trial-block interaction, $F(9,198)=1.8$, N. S. During the probe

Figure 4.4: Illustration of injection of needle tip placements in the nucleus basalis magnocellularis. Closed circles = CSF infusions, closed squares = CDP infusions. (bar = 1 mm)

Nucleus Basalis Magnocellularis

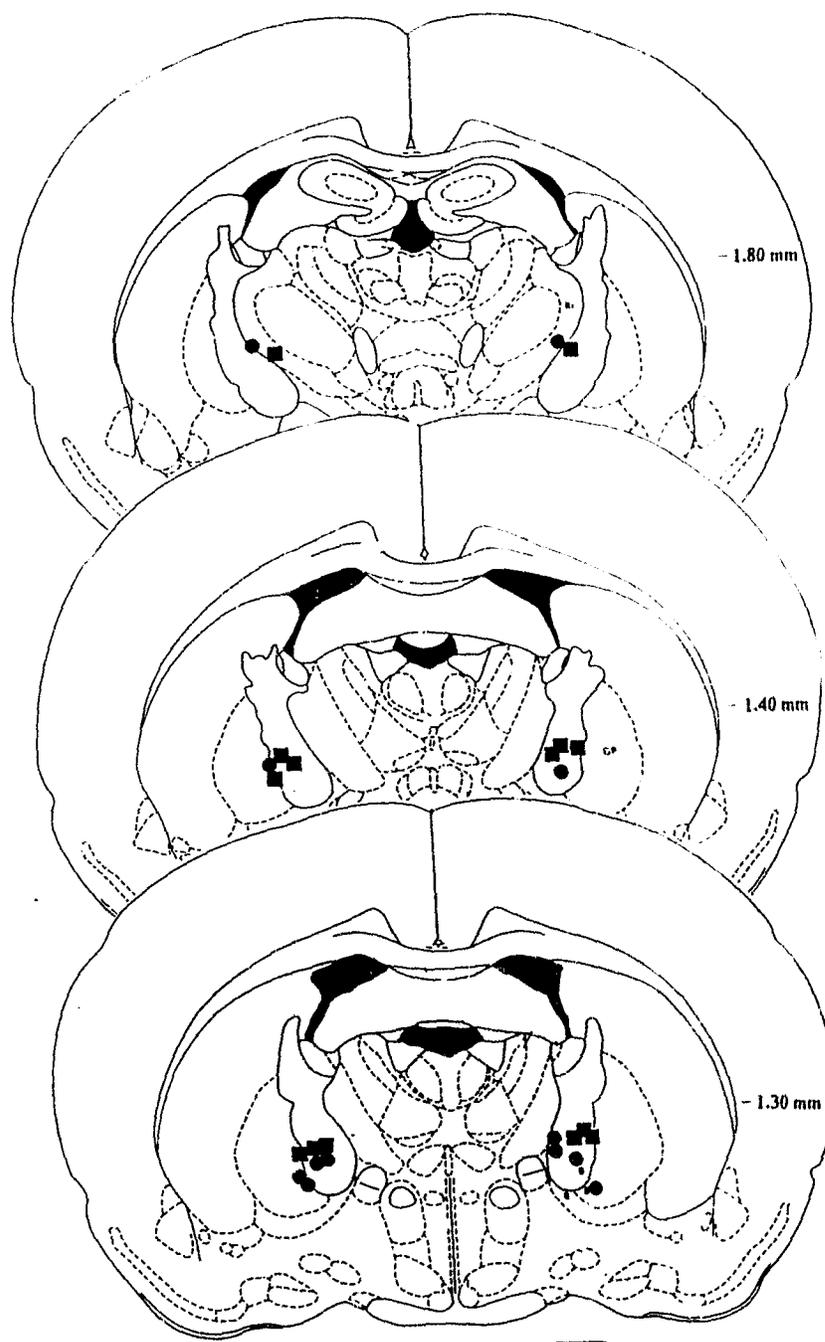


Figure 4.5: Effects of chlordiazepoxide (60 nmol; CDP) or CSF infusions into the NBM on (A) the distance taken to locate the submerged platform, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note: 1) that both CSF- and CDP-treated rats acquire the platform location quickly and show a bias for the target quadrant during the probe trial, and 2) that the chlordiazepoxide group had slower swim speeds during the initial phase of training. * $p < 0.01$ compared to chance level (25%-dotted line).

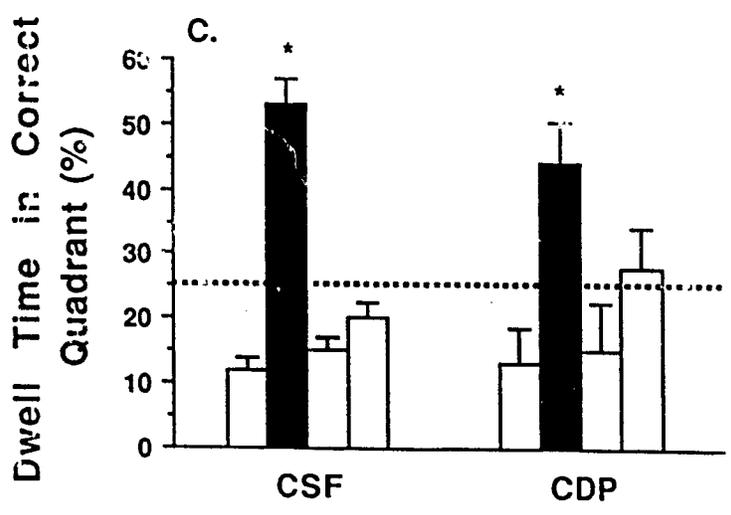
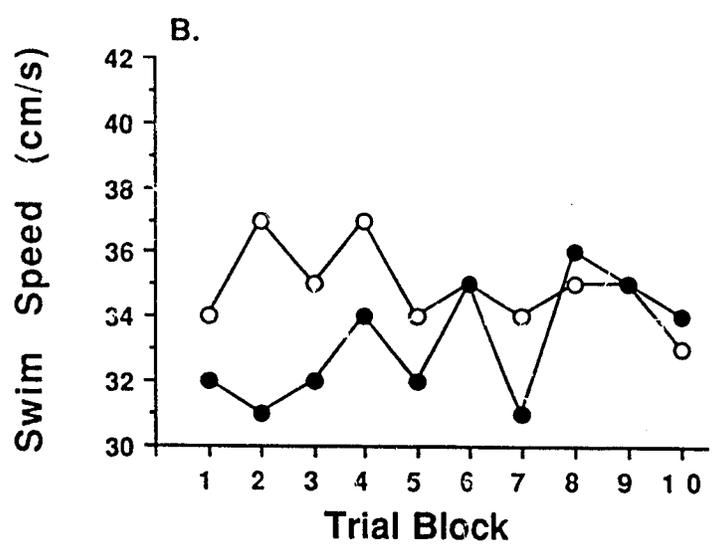
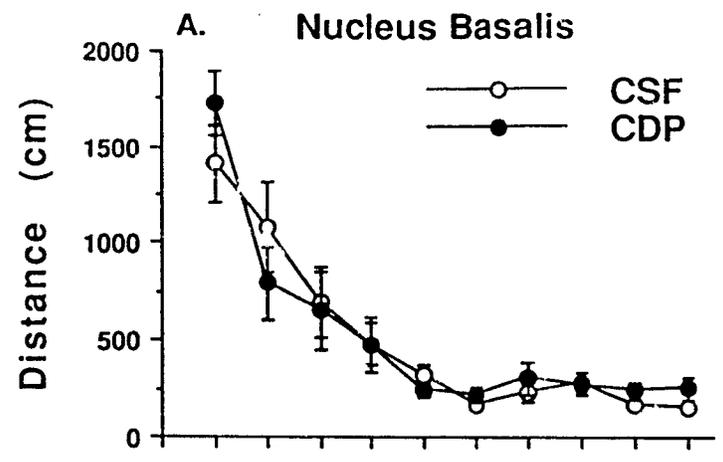


Figure 4.6: Illustration of injection needle tip placements in the medial septum. Note the similar placements in both groups. Closed circles = CSF infusions, closed squares = chlordiazepoxide infusions. (LV = lateral ventricles; Ld, LSt = lateral septum; VDB = ventral band of the diagonal band of Broca; bar = 1 mm).

Medial Septum

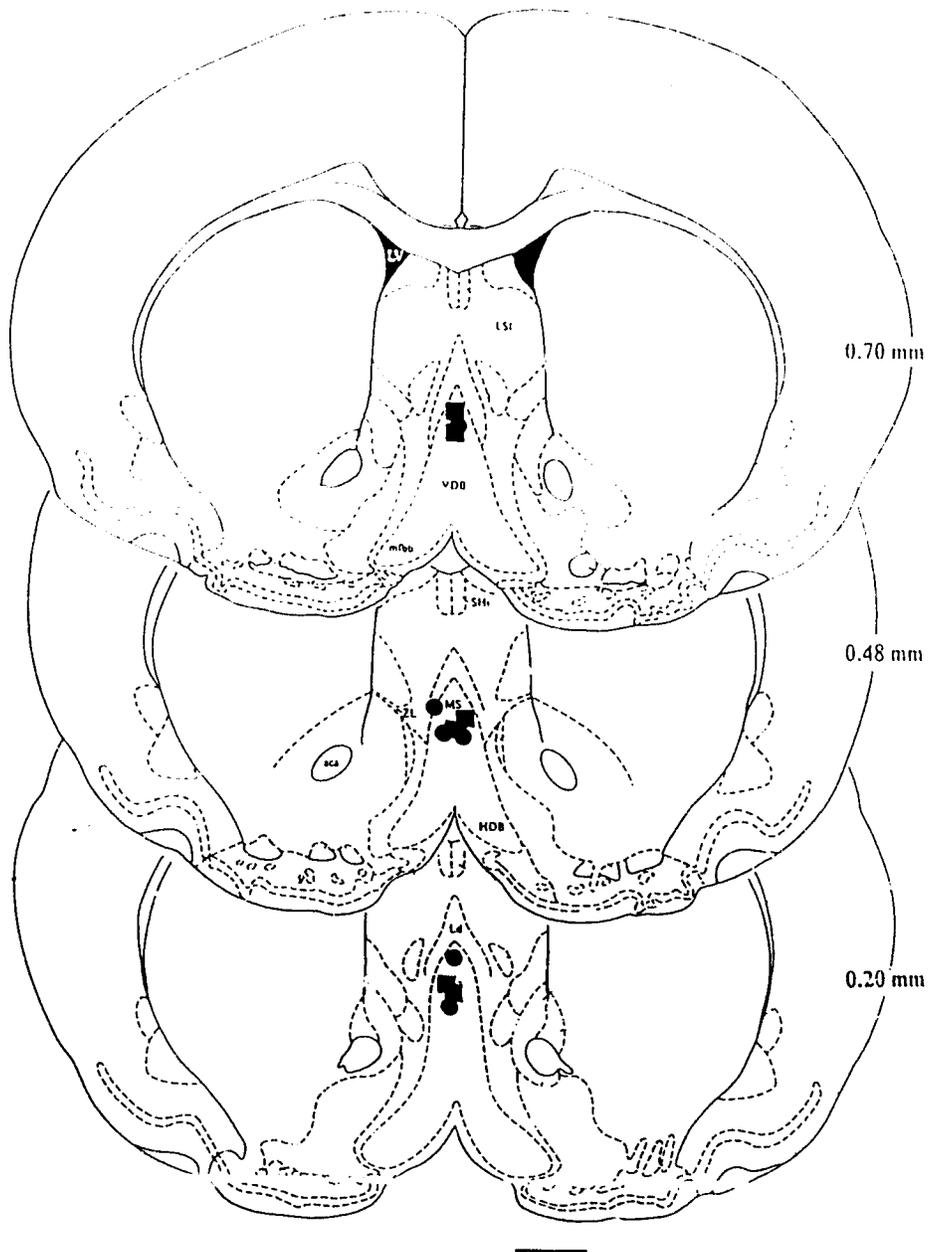
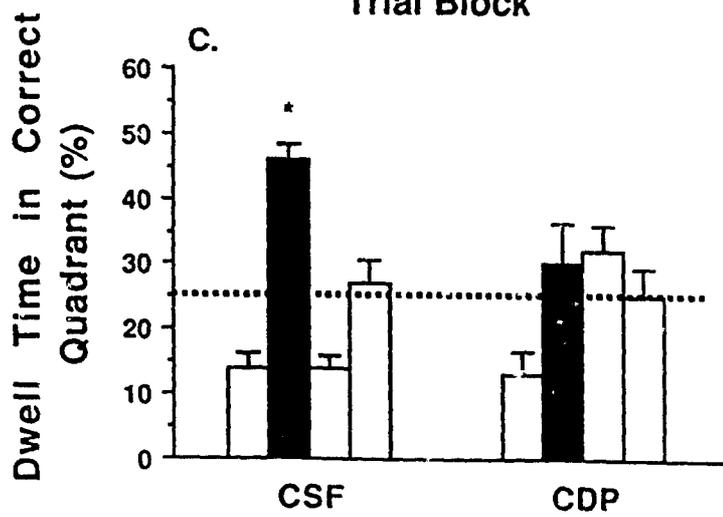
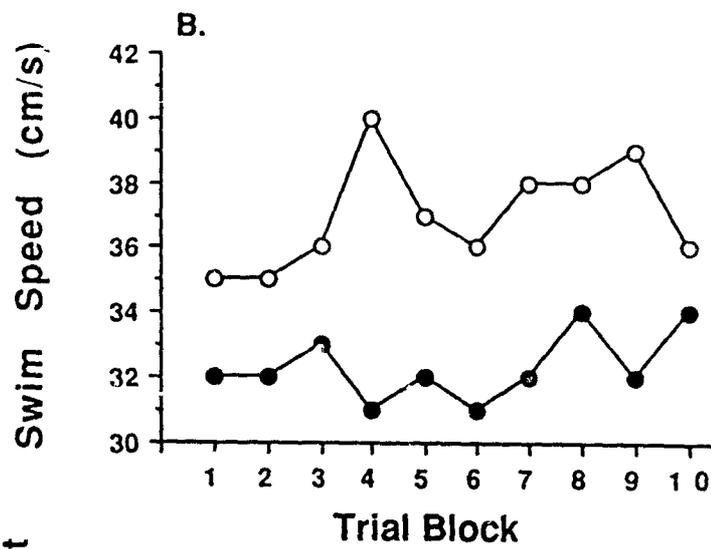
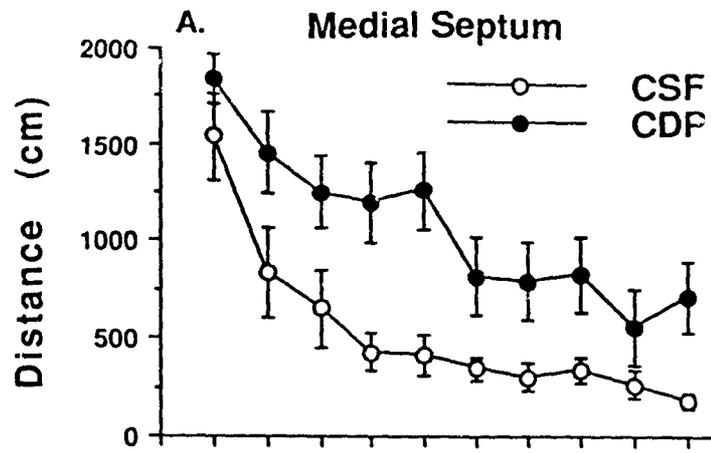


Figure 4.7: Effects of intra-septal infusions of chlordiazepoxide (60 nmol; CDP) or CSF on (A) the distance taken to locate the submerged platform on the twenty trials/day procedure, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note: 1) that the profound spatial learning deficit produced by the intra-septal infusions of chlordiazepoxide, and 2) the slowing of swim speed produced by intra-septal infusions of chlordiazepoxide. * $p < 0.01$ compared to chance level (25%-dotted line).



trial (Fig. 4.7C), only the CSF group demonstrated a significant bias for the correct quadrant ($p < 0.01$ compared to chance, 25%). On the visible platform task, the swim distances did not differ significantly between the CSF and CDP groups (CSF: 153 ± 15 cm vs. CDP: 147 ± 12 cm; $p > 0.05$).

The positions of the injection needle tips in the hippocampus were similar in the CDP and CSF groups with the majority of placements located in the dorso-medial aspect of the hippocampus and in the subiculum. Some needle tips terminated in the dentate gyrus as well. Analysis of dye diffusion revealed that the 0.5 μ l volume diffused only slightly in the medial-lateral direction but did spread some distance in the anterior-posterior direction. In many rats, the infusion failed to reach the lateral aspect of CA1, CA2, or CA3 fields. The effects of intra-hippocampal infusions on spatial learning is illustrated in Figure 4.9. Infusions of CDP into the dorsal hippocampus produced a subtle disruption of spatial learning during the initial phase of acquisition but had little effect by the end of training (Fig. 4.9A). An ANOVA on distance data revealed a significant trial-block difference, $F(9,198)=10.6$, $p < 0.001$, but not a significant group difference, $F(1,22)=3.2$, N.S., or group by trial-block interaction, $F(9,198)=1.3$, N.S. Analysis of swim speed data revealed that both groups swam at approximately the same rate over the course of training (Fig. 4.9B). An ANOVA on swim speed data revealed no significant group difference, $F(1,22)=0.07$, N.S., trial-block difference, $F(9,198)=1.32$, N.S., or group by trial-block interaction, $F(9,198)=0.33$, N.S. During the probe trial (Fig. 4.9C), both the CSF and CDP groups showed a significant bias for the correct quadrant ($p < 0.01$). The mean distance taken to reach the visible platform was not significantly different between the CSF group (155 ± 40) and the CDP group (173 ± 21).

The positions of the injection needle tips in the amygdala are illustrated in Figure 4.10. The majority of the needle tips from either group terminated in the region just dorsal-medial to the amygdaloid complex. In many cases, the needle tips terminated in the central nucleus. Analysis of dye diffusion revealed that central amygdaloid nucleus and the majority of the basolateral amygdaloid complex were reached by the infusions. The effects of intra-amygdala infusions on spatial learning are illustrated in Figure 4.11.

Figure 4.8: Illustration of injection needle tip placements in the dorsal hippocampus. Note that the placements are similar in each group. Closed circles = CSF infusions, closed squares = chlordiazepoxide infusions (bar = 1 mm)

Hippocampus

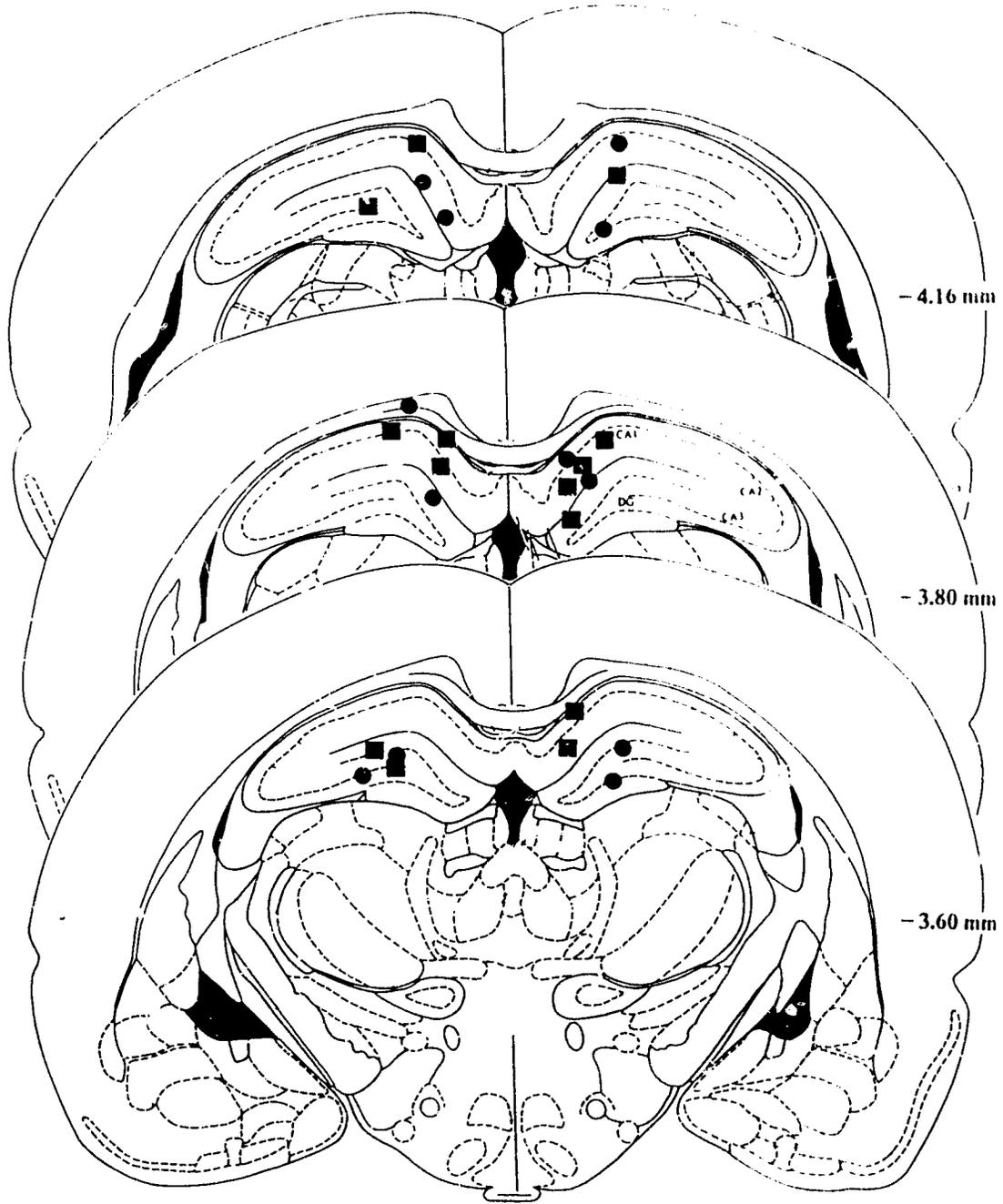


Figure 4.9: Effects of intra-hippocampal infusions of chlordiazepoxide (60 nmol; CDP) or CSF on (A) the distance taken to locate the submerged platform on the twenty trials/day procedure, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note that both CSF- and CDP-treated rats acquire the platform location quickly and show a bias for the target quadrant during the probe trial. * $p < 0.01$ compared to chance level (25%-dotted line).

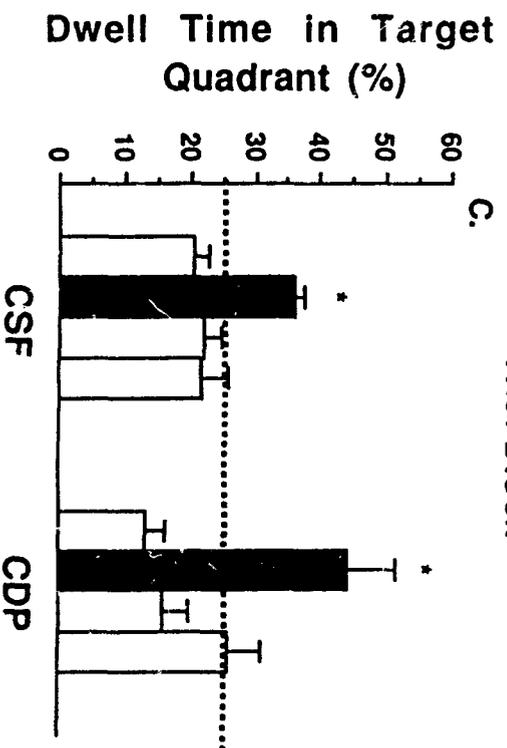
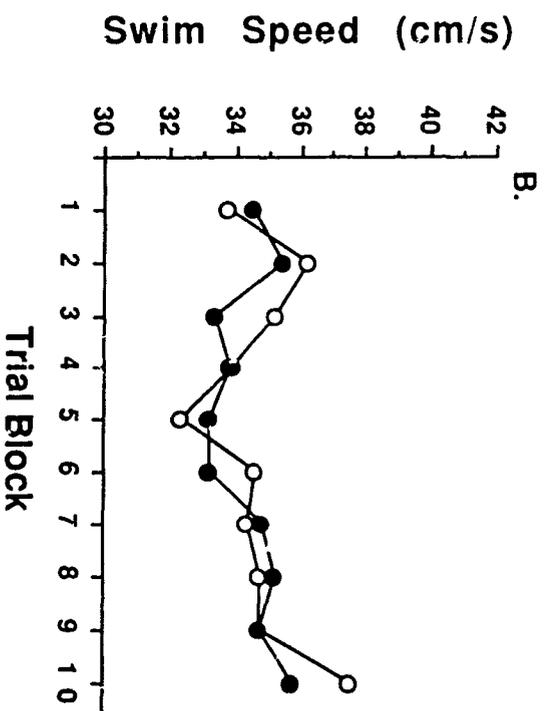
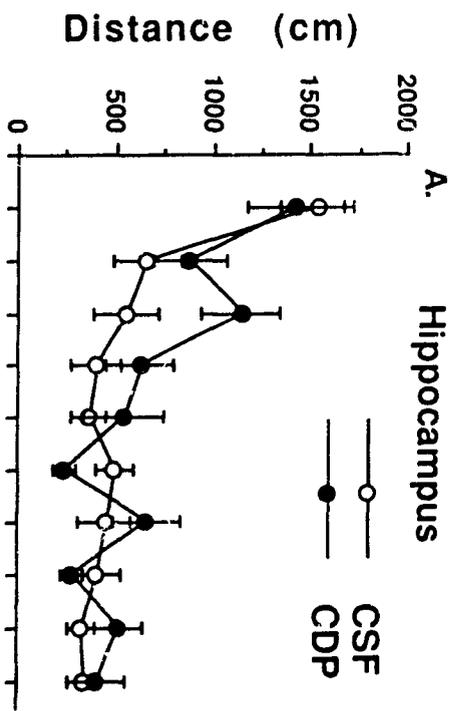


Figure 4.10: Illustration of cannula placements in the amygdala. Note that the needle tip placements are similar between treatment groups. Closed circles = CSF infusions, closed squares = chlordiazepoxide infusions. (BLA = basolateral amygdaloid nucleus; CeL = central amygdaloid nucleus, lateral aspect; CeM = central amygdaloid nucleus, medial aspect; bar = 1 mm).

Amygdala

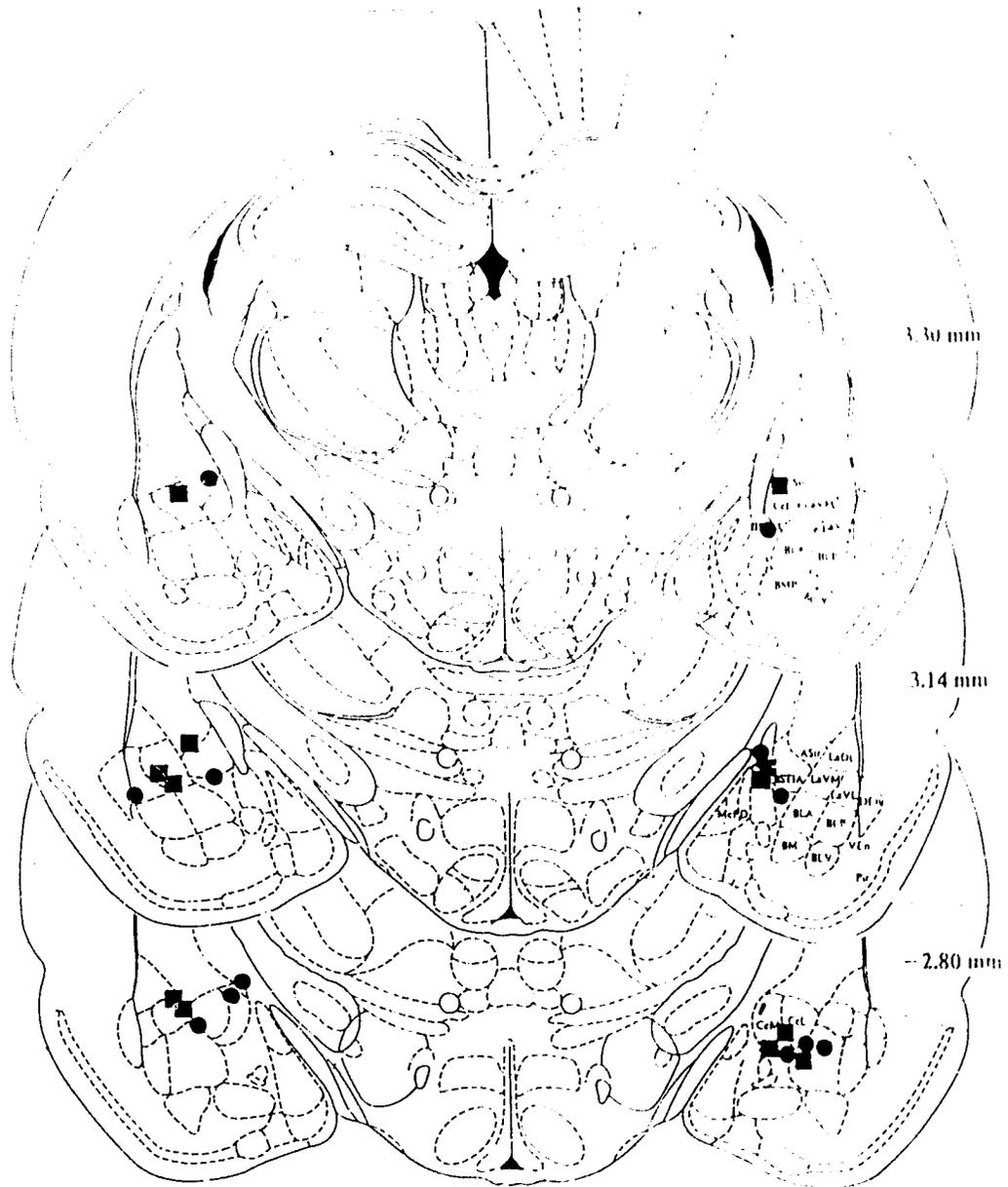
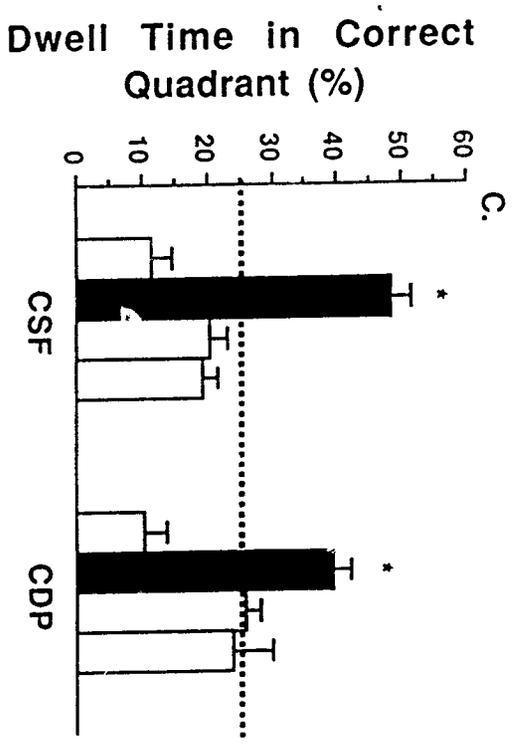
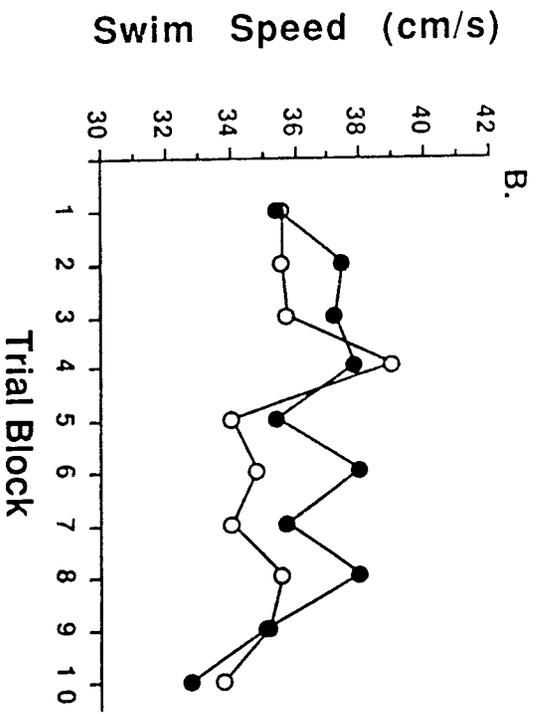
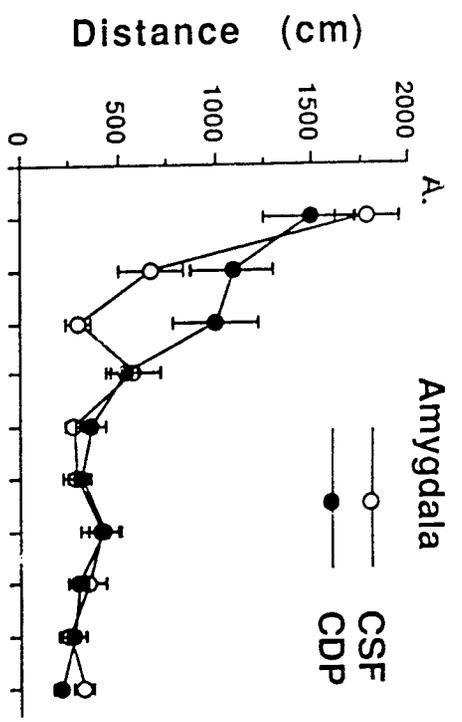


Figure 4.11: Effects of intra-amygdala infusions of chlordiazepoxide (60 nmol; CDP) or CSF on (A) the distance taken to locate the submerged platform on the twenty trials/day procedure, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note: 1) the slight disruption of acquisition over the first three trial blocks in CDP-treated rats, and 2) both CSF- and CDP-treated rats show a bias for the target quadrant during the probe trial. * $p < 0.01$ compared to chance level (25%-dotted line).



Assessment of the distance data reveals that the CDP group may have been mildly disrupted during the initial stages of training relative to the CSF group, but were otherwise unimpaired (Fig. 4.11A). An ANOVA on distance data revealed a significant trial-block difference, $F(9,198)=26.3$, $p<0.001$, group by trial-block interaction, $F(9,198)=2.94$, $p<0.003$, but not a significant group difference, $F(9,198)=1.6$, N.S. The CSF and CDP groups tended to swim at the same rate over the course of training (Fig. 4.11B). An ANOVA on swim speed data revealed a significant trial-block difference, $F(9,198)=1.9$, $p<0.05$, but not a significant group difference, $F(1,22)=0.6$, N.S. or group by trial-block interaction, $F(9,198)=0.6$, N. S. During the probe trial (Fig. 4.11C), both the CSF and CDP groups demonstrated a significant bias for the correct quadrant ($p<0.01$ compared to chance, 25%). The bias shown by the CDP group was significantly less than that shown by the CSF group ($p<0.05$). The mean distance taken to reach the visible platform did not differ between groups (CSF: 160 ± 27 cm vs. CDP: 169 ± 19 cm; $p>0.05$).

The positions of the injection needle tips in the cerebellum are illustrated in Figure 4.12. Most of the needle tips terminated in the region dorsal to the lateral and medial cerebellar nuclei. Analysis of dye diffusion revealed that in most cases, the infusion volume reached the region containing the medial and lateral cerebellar nuclei. In other cases, the infusion tended to spread to the cerebellar cortex dorsal to the deep nuclei. Indeed, in the majority of rats, the infusion volume tended to spread dorsally along the cannula track, thereby reaching aspects of cerebellar cortex. The effects of intra-cerebellar infusions on spatial learning are illustrated in Figure 4.13. On the submerged platform task, both the CSF and the CDP groups required only two trials to reach asymptotic levels (Fig. 4.13A). An ANOVA on distance data revealed a significant trial-block difference, $F(9,198)=26.5$, $p<0.001$, but not a significant group difference, $F(1,22)=1.7$, N.S., or a group by trial-block interaction, $F(9,198)=0.3$, N.S. The CDP group swam at a slower rate relative to the CSF group over the course of training (Fig. 4.13B). An ANOVA on swim speed data revealed a significant group difference, $F(1,22)=4.4$, $p<0.05$, and a significant trial-block difference, $F(9,198)=1.9$, $p<0.05$, but not a significant group by trial-block interaction, $F(9,198)=1.4$, N. S. During the probe trial (Fig. 4.13C), both the CSF and CDP

Figure 4.12: Illustration of cannula placements in the cerebellum. Note the similar placements of the two treatment groups. Closed circles = CSF infusions, closed squares = chlordiazepoxide infusions (Int = interpositus cerebellar nu.; Lat = lateral cerebellar nu.; medial cerebellar nu.; bar = 1 mm).

Cerebellum

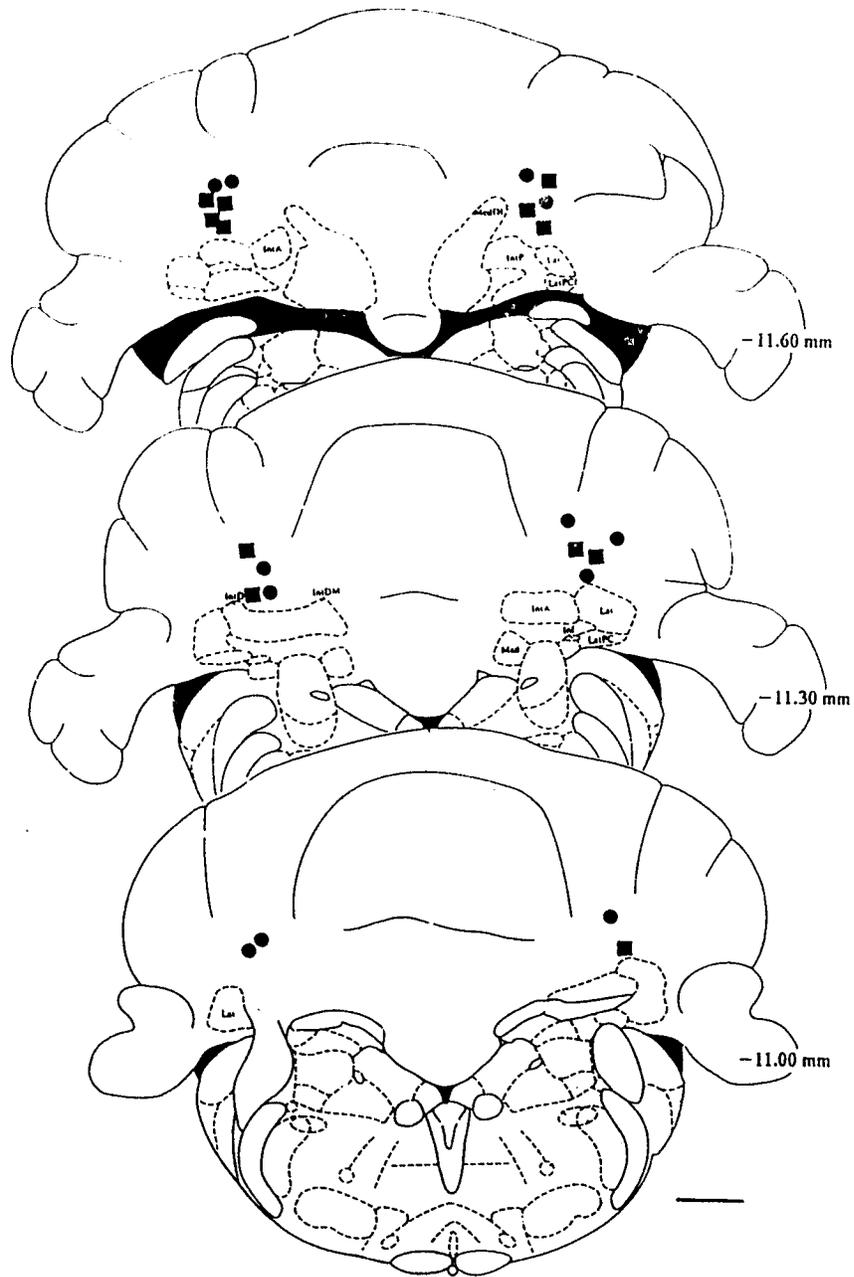
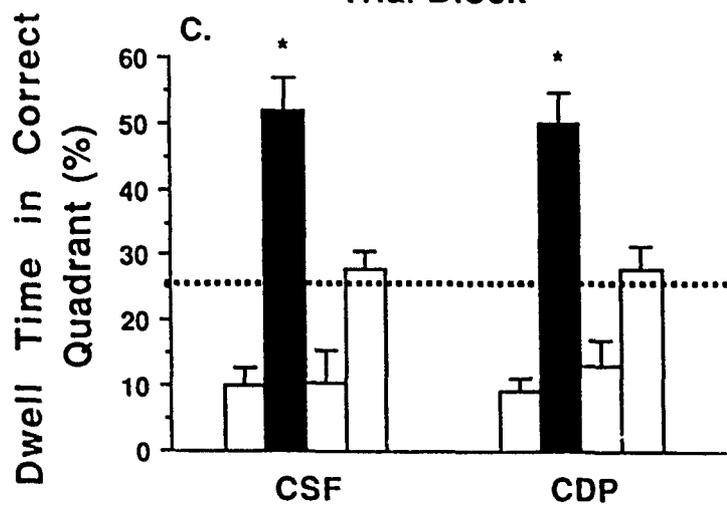
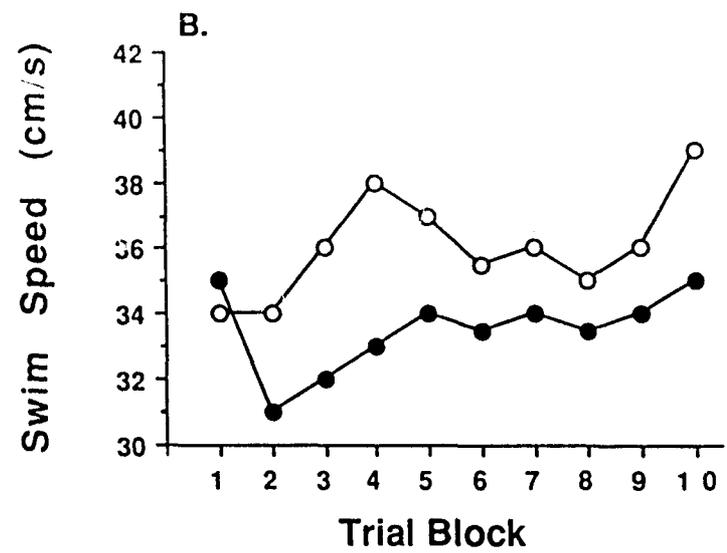
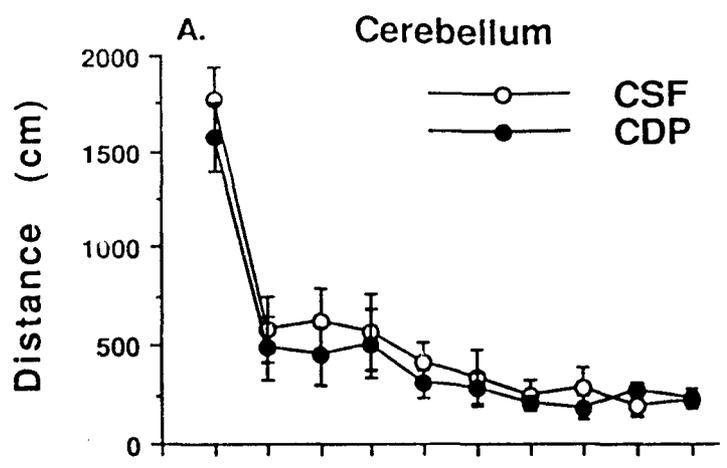


Figure 4.13: Effects of intra-cerebellar infusions of chlordiazepoxide (60 nmol; CDP) or CSF on (A) the distance taken to locate the submerged platform, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note: 1) that both CSF- and CDP-treated rats acquire the platform location by the second trial block and show a bias for the target quadrant during the probe trial, and 2) the swim speed reduction produced by intra-cerebellar infusions of chlordiazepoxide. * $p < 0.01$ compared to chance level (25%-dotted line).



groups demonstrated a significant bias for the correct quadrant ($p < 0.01$ compared to chance, 25%). On the CDP visible platform task, swim distances taken by the CSF and CDP groups did not differ significantly (CSF: 210 ± 21 cm vs. CDP: 191 ± 21 cm; $p > 0.05$).

DISCUSSION

The present study revealed that systemically administered CDP reduced thigmotaxia in the open field, without affecting total activity, and impaired spatial learning, without affecting swim speed. Hence, the present behavioral assays are sufficiently sensitive to detect the amnesic and anxiolytic properties of CDP. Infusions of CDP into the frontal cortex, NBM, hippocampus, or cerebellum failed to reduce thigmotaxia or total activity in the open field, suggesting that these regions, by themselves, do not mediate the anxiolytic effects of systemic CDP. Further, infusions of CDP into the frontal cortex, NBM, hippocampus, or cerebellum also failed to impair spatial learning in the MWM, suggesting that these sites do not mediate the amnesic actions of CDP. Infusions of CDP into the cerebellum did, however, reduce swim speed, suggesting that the BZ receptors in this region did influence behavior. Infusions of CDP into the medial septum impaired spatial learning and reduced swim speed, but had little effect on thigmotaxia or total activity levels. Hence, BZ receptors in the medial septum appear to mediate the amnesic, but not the anxiolytic, actions of systemically administered CDP. Conversely, intra-amygdaloid infusions of CDP reduced thigmotaxia but had little effect on total activity, spatial learning, or swim speed, suggesting that BZ receptors here mediate the anxiolytic actions of systemically administered CDP. Hence, these findings indicate that the amnesic and anxiolytic actions of BZs are independent processes mediated by different neuroanatomical sites. Moreover, several neuroanatomical sites that possess BZ receptors in high density do not, by themselves, mediate the amnesic, or anxiolytic, actions of BZs.

Infusions of CDP into the frontal cortex region failed to affect spatial learning in the present investigation. It is unclear why infusions of CDP into this region failed to impair spatial learning in the present study. Lesions of

the frontal cortex, most notably the medial aspect, impair spatial learning in the water maze as well as other tasks (Becker, Walker, & Olton, 1980; Kolb, Sutherland, & Wishaw, 1983; Sutherland, Kolb, & Wishaw, 1982; Thompson, 1983). Furthermore, the the frontal cortex region possesses a high density of BZ receptors (Young et al., 1980). Infusions of dye into this region revealed that the 1 μ l infusion did not saturate the entire frontal cortical region. Hence, it is possible that activation of a small number of BZ receptors in this region is insufficient to compromise the region's functional integrity or impair spatial learning. Indeed, those frontal cortical lesions that were effective in producing a spatial learning deficit included cingulate cortex (Becker et al., 1980), a region that was largely missed by drug infusions. However, infusions that were clearly proximal to cingulate cortex also failed to impair spatial learning. Alternatively, it is possible that lesions of the size found to produce an spatial learning impairment also disrupted activity in regions distal to the actual lesion site (e.g., hippocampus). Nonetheless, the present findings suggest that the activation of BZ receptors in the frontal cortex region does not impair spatial learning. Since activation of frontal cortex BZ receptors also failed to affect anxiety in the open field, the functional significance of these receptors remains unknown.

Intraseptal infusions of CDP had little effect on thigmotaxia, suggesting that the BZ receptors in this region do not mediated the anxiolytic effects of systemically administered CDF. This contrasts with previous reports implicating the septal region in emotionality. For example, lesions of the septum produce hyperreactivity (Lee, Lin, & Yin, 1988) and septal stimulation can produce behavioral freezing (Kaada, 1951). Further, septal lesions were found to attenuate the anxiolytic effects of BZs (Yadin, Thomas, & Strickland, 1987) and intra-septal infusions of muscimol (Drugan, Skolnick, Paul, & Crawley, 1986) or CDP (Grishkat, 1991) produce anxiolysis on conflict tasks. However, the recent evidence showing that septal lesions fail to attenuate the anxiolytic effects of diazepam on fear-potentiated startle (Melia & Davis, 1991), as well as the the present findings, argue against the notion that the anxiolytic effects of BZs are mediated through the septum.

A major finding of the present study is that intra-septal infusions of CDP impair spatial learning in the MWM. Whether this reflects a disruption of mnemonic-related activity occurring within the septum or activity in regions efferent to the medial septum (e.g., hippocampus) is unclear. Nevertheless, this finding is congruent with previous reports showing that BZ/GABA receptors in the medial septum are important for modulating mnemonic processes (Brioni et al., 1990; Chrobak, Stackman, & Walsh, 1989; Chrobak & Napier, 1991, 1992; Stackman & Walsh, 1992). For example, consolidation of spatial information was impaired when CDP was infused into the medial septum immediately but not 15 min following acquisition (Stackman & Walsh, 1992), suggesting that activation of BZ receptors in the medial septum can impede information storage even after the to-be-learned information has been experienced.

Infusions of CDP into the NBM failed to impair spatial learning in the present study. Since lesions of the NBM have been shown to impair spatial learning (e.g., Whishaw et al., 1985), the present finding suggests that intra-NBM infusions of CDP did not compromise the functional integrity of this structure. The present findings do contrast to some extent with the findings of Majchrzak et al. (1990) who demonstrated that intra-NBM infusions of GABA impaired both avoidance conditioning as well as spatial learning (radial arm maze). However, it is possible that GABAergic processes are not tonically active during spatial learning, rendering CDP neutral (recall that BZs enhance the actions of GABA). This latter notion is corroborated by the finding that cortical ACh activity, provided by the NBM, changes little after spatial acquisition, unlike hippocampal ACh activity (Wenk, Hepler, & Olton, 1984). Given that the NBM-cortex ACh projection is regulated by a GABAergic projection originating in the nucleus accumbens (Wood, 1986), the failure to observe an increase of cortical ACh activity suggests that this ACh projection is not required for spatial learning. Indeed, several studies have found that lesions of the NBM have little or no effect on spatial learning in the MWM (e.g., Hagen et al., 1988), creating a discrepancy that requires reconciliation. Nevertheless, the present findings suggest that neither the amnesic nor the anxiolytic actions of systemic CDP are mediated through the NBM.

Infusions of CDP into the hippocampus had little effect on open field behavior. Intra-hippocampal infusions of CDP did not affect overall activity levels or thigmotaxia, suggesting that the hippocampus does not mediate the anxiolytic actions of CDP. The similarity between the behavioral effects of BZs and hippocampal lesions, as well as septal lesions, prompted Gray (1983) to propose that the hippocampus and the septum, are responsible for withholding responses in fear-evoking situations and BZs, by acting on these regions, release behavior that would otherwise be inhibited by fear. The present results would suggest that the anxiolytic actions of BZs are not mediated by the hippocampus. However, given that only a small proportion of the hippocampus was reached by the infusions, assessment of the effects of larger infusion volumes is warranted.

Intra-hippocampal infusions of CDP failed to impair spatial learning. This was surprising given the vital role of the hippocampus in spatial learning (O'Keefe & Nadel, 1978) and the high density of BZ receptors within the hippocampus (Young et al., 1980). However, none of the infusions reached the entire hippocampus, and in some cases, only a small portion of the dorsomedial hippocampus was reached. Infusions of a larger volume (1 μ l), which clearly reached a greater proportion of the hippocampus, did impair spatial learning without producing the seizure-related activity that may have caused the impairment produced by CSF infusions. Hence, it would appear that a greater proportion of the hippocampus needs to be influenced by CDP before a spatial learning deficit is manifested.

The finding that intra-amygdala CDP reduces thigmotaxia supports the notion that activation of BZ receptors in the amygdala is sufficient to produce anxiolysis. The finding that intra-amygdala infusions of CDP reduced experimental indices of anxiety is consistent with the commonly held notion that the amygdala is involved in emotionality, most notably fear (e.g., Sarter & Markowitsch, 1985). This notion is corroborated by the finding that amygdala lesions reduce anxiety on a conflict punishment task (Shibita, Kataoka, Yamashita & Ueki, 1986). Moreover, the amygdala possesses a high density of BZ receptors (Niehoff & Kuhar, 1983; Niehoff & Whitehouse,

1983) and the degree of occupation of these receptors by [³H]diazepam correlates well with the degree of ensuing anxiolysis (Thomas, Lewis, & Iversen, 1985). Finally, intra-amygdala infusions of BZ agonists increase punished responses in conflict tasks (Hodges, Green, & Glenn, 1987; Nagy, Zambó, & Decsi, 1979; Shibita, Kataoka, Gomita, & Ueki, 1982; Thomas et al., 1985). However, amygdala lesions do not prevent BZ-induced increases of punished responding, suggesting that other sites may also mediate the anxiolytic effects of BZs (Yardin, Thomas, Strickland, & Grishat, 1991). It should also be noted that intra-amygdala infusions of CSF produced what appears to be an anxiogenic response in control rats. It is possible that amygdaloid excitation resulting from CSF infusion increased anxiety, suggesting that anxiety levels can be either increased or decreased via manipulations of activity in the amygdala.

In addition to its role in anxiety, the amygdala is also involved in certain forms of emotionally based memory (Sarter & Markowitsch, 1985). For example, the amygdala lesions impair fear conditioning (Davis et al., 1987) and avoidance conditioning (Liang, McGaugh, Martinez, Jensen, Vasquez, & Messing, 1982). Additionally, intra-amygdala infusions of the GABAergic agonist impaired the retention of avoidance conditioning (Brioni, Nagahara, & McGaugh, 1989) and lesions of the amygdala block the amnesic of muscimol, a GABA agonist, or diazepam on an inhibitory avoidance task (Ammassari-Teule, Pavone, Castellano, & McGaugh, 1991; Tomaz, Dickinson-Anson, & McGaugh, 1991). Thus, GABAergic and BZ receptors in the amygdala appear to be important modulators of memory formation of aversively motivated learning. However, amygdala lesions have little effect on spatial learning in the water maze (Sutherland & McDonald, 1990) or in a T-maze (Thompson, 1983). Hence, the failure of intra-amygdala CDP to impair spatial learning in the present study agrees with the lesion data to suggest that the BZ receptors in the amygdala do not modulate the acquisition of spatial information. It should be noted, however, that the smaller bias for the correct quadrant during the probe trial observed in CDP treated rats may reflect either suboptimal spatial learning or a facilitation of extinction. The previous finding that intra-amygdala infusions of BZ agonists increase (not decrease) punished responses would

argue against the facilitated extinction interpretation. Hence, intra-amygdala infusions of CDP may have reduced optimal spatial learning.

Infusions of CDP into the cerebellum failed to affect spatial learning. The role of the cerebellum in spatial learning is unclear. Lesions of the cerebellum do not impair left-right spatial discrimination learning (Thompson, 1983) but mutant mice with cerebellar degeneration show an acquisition deficit in the water maze (e.g., Lelonde & Botez, 1986). However, the mutant mice also show an impairment on the visible platform task, which is suggestive of a profound motorical impairment rather than a cognitive deficit (Lelonde & Botez, 1986). Nonetheless, lesions of the cerebellum do impair avoidance conditioning (Dahhaoui, Caston, Auvray, & Reber, 1990; Guillaumin, Dahhaoui, & Caston, 1991) and eyeblink conditioning (McCormick & Thompson, 1984), suggesting that this structure does subserve some mnemonic-related processes. Further, the cerebellum possesses a high density of BZ receptors (Young et al., 1980) and infusions of GABAergic antagonists into cerebellar deep nuclei or cerebellar cortex disrupts eyeblink conditioning in the rabbit (Mamounas, Thompson, & Madden, 1987). The failure of intra-cerebellar CDP to impair spatial learning suggests that either BZ receptors in this region are not related to the amnesic effects of BZs or that the cerebellum is not involved in spatial learning processes. Given that CL 218,872, which selectively interacts with the ω_1 receptor which predominates in the cerebellum, impaired spatial learning it seems more likely that the cerebellum is not intricate for spatial learning processes.

The infusion of CDP into the lateral aspects of the cerebellum failed to influence anxiety, suggesting that this region of the cerebellum is not involved in such processes, unlike the the vermal region which has been implicated in anxiolysis (Supple, Leaton, & Fanselow, 1987). Intra-cerebellar infusions of CDP did reduce the mean total distance travelled in the open field, though this difference was not statistically significant. Overall, it would appear that cerebellar BZ receptors are not related to either the anxiolytic or amnesic actions of BZs.

The results of the present study suggest that the different behavioral effects of BZs are mediated by different regions within the CNS. That is, the amnesic actions of CDP appear to be mediated by BZ receptors in the medial septum and the anxiolytic actions appear to be mediated by BZ receptors in the amygdala. This neuroanatomical dissociation of the anxiolytic and amnesic actions of BZs provides good evidence that the two processes are indeed distinct and independent. Determining the purpose of the BZ receptors located in these regions (frontal cortex, NBM, hippocampus, or cerebellum) will require further testing.

The present study, and others (Stackman & Walsh, 1992), indicate that the amnesic actions of BZs are mediated through BZ receptors located in the medial septum. Because the medial septum interacts with the hippocampus, and given the role of the hippocampus in mnemonic processes, the anatomical and neurochemical interactions between the septum and the hippocampus will be discussed in preparation for Experiment VII, which analyzes the neurochemical nature of the spatial learning deficits produced by intra-septal infusions of CDP. The electrophysiological interactions between the septum and the hippocampus will be discussed in Chapter 5.

THE SEPTOHIPPOCAMPAL SYSTEM

Research into the function of the septohippocampal formation has a long history and the behavioral effects of septal or hippocampal lesions have been thoroughly reviewed (Gray, 1983; Gray & McNaughton, 1982; O'Keefe & Nadel, 1978). While it is difficult to apply a single unifying theory to the function of the septohippocampal system, there is little doubt that one role of this system subserves learning and memory processes (O'Keefe & Nadel, 1978). The findings of the previous study suggest that the activation of BZ receptors in the medial septum is sufficient to produce amnesia. However, it is possible that intraseptal infusions of CDP impaired spatial learning by disrupting processes within the hippocampus. Experiment VII was conducted to assess this possibility. Firstly, however, it is important to understand the anatomical and neurochemical basis of the septohippocampal system.

Anatomy

The hippocampal formation is composed of the the hippocampus proper (CA1, CA2 and CA3 sub-fields), the dentate gyrus (fascia dentata), the subicular complex (subiculum, presubiculum and parasubiculum) and the entorhinal cortex. Polymodal sensory information enters the hippocampal formation via the perforant path, which originates in the entorhinal cortex (stellate cells in layer II; Hjorth-Simonsen & Jeune, 1972), although smaller alternative routes do exist (e.g., pyramidal cells in layer III of entorhinal cortex project to CA1; Germroth et al., 1989; Steward, 1976). The series of connections in the hippocampus form a tri-synaptic loop. The perforant path 'perforates' the pyramidal layer of the subiculum along its long axis and terminates on the molecular layer of the dentate gyrus (Hjorth-Simonsen & Jeune, 1972). The axons of dentate granule cells (mossy fibers), which collateralize in the polymorph layer (hilus) of the dentate gyrus, enter the CA3 and CA4 fields forming *en passant* synapses on the proximal apical dendrites of the CA3 pyramidal cells. These pyramidal cells in turn send axon collaterals axons (Schaffer collaterals) to other areas of the CA3 field, pyramidal cells of the CA1 field and the septum via the fimbria/fornix. The pyramidal cells of the CA1 field then project to neurons in the subicular complex which send axons back to the entorhinal cortex and to the retrosplenial cortex, septal nuclei, mammillary bodies, hypothalamus, thalamus (lateral & anterior nuclei) and nucleus accumbens (Groen & Wyss, 1990; Hjorth-Simonsen, 1973).

The septum lies anterior to the hippocampus and is bound on either side by the lateral ventricles, above by the corpus callosum, and below by the anterior commissure. The septal region has been subdivided into medial, lateral, posterior and ventral divisions (Swanson & Cowan, 1976). The medial septal division has been further divided into medial septal nucleus (dorsal) and the nucleus of the diagonal band (of Broca; ventral; hereafter collectively referred to as medial septum). Together, the latter septal nuclei form a broad mass of large neurons between which are smaller neurons. The lateral division has been further divided into dorsal, intermediate and

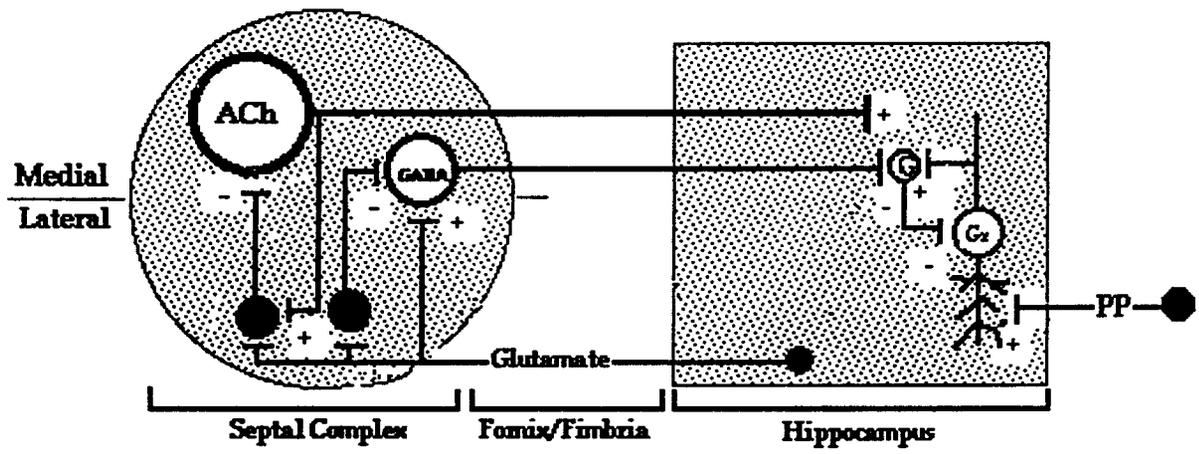
ventral based on the size of neurons in each region. The posterior division consists of the septo-fimbrial and the triangular nuclei. The ventral division is a heterogeneous group of neurons collectively termed the bed nucleus of the stria terminalis, as the entire ventral division receives afferents from the amygdala via the stria terminalis.

The efferent and afferent connections of septal nuclei have been described in detail by Swanson and Cowan (1976), some of which are illustrated in Figure 4.14. The lateral septal area receives input primarily from the dorsal hippocampus (Alonso & Frotscher, 1989). The lateral septal nucleus in turn sends projections to the medial septal nucleus, although the direction of this latter projection has been questioned (Leranth, Deller, & Buzsaki, 1992). The medial septal nucleus projects to the hippocampus (largely ipsilateral) via the dorsal fornix (ventral hippocampus) and fimbria (to dorsal hippocampus), entering at the level of the stratum oriens of the CA3 field. Fibers in the medial septum projecting to the hippocampus are topographically organized, with cells in the rostral portion of the medial septum projecting to the dorsal hippocampus and cells in the caudal portion to the ventral hippocampus (Yoshida & Oka, 1990). Once these fibers enter the hippocampus, they divide to innervate the stratum oriens (containing basilar dendrites of pyramidal cells) of fields CA1, CA2, CA4, the granular cell layer of the dentate gyrus, and the entorhinal cortex. Thus, the *septohippocampal system* consists of the following 'circuitry': medial septum → fimbria → hippocampus (CA1, CA3, & dentate gyrus → subicular complex → fimbria → lateral septum → medial septum.

Neurochemistry

Several different neurotransmitters have been detected in the septohippocampal system. The majority of the fibers originating in the medial septum have been identified as cholinergic, though a smaller GABAergic projection has also been confirmed (see below). In addition, several other neurotransmitter systems, including glutamate, dopamine and noradrenaline, serotonin, substance P and the opioids, interact with the septum. The majority of investigations of the interactions of these other neurotransmitter systems with the septum utilized hippocampal AChE.

Figure 4.14: Schematic diagram showing the connections and neurotransmitter systems of the septohippocampal system. Note: 1) the large ACh and the small GABAergic septohippocampal projections, 2) the ACh and GABA projection neurons located in the medial septum are inhibited by lateral septum GABAergic interneurons, 3) the septohippocampal GABAergic projection innervates GABAergic inhibitory interneurons in the hippocampus, 4) inhibition of the septohippocampal GABA projection would remove inhibition from interneurons in the hippocampus (dentate gyrus) thereby hyperinhibiting principle cells (i.e., granule cells) to afferent input from the perforant path. (G = GABAergic interneuron; Gr = granule cell (or pyramidal cells in CA1 & CA3); PP = perforant path; + = excitatory synapse; - = inhibitory synapse).



activity as an index of their effects on the septum. However, it should be kept in mind that the smaller, and less well documented, GABAergic projection may also be influenced.

Acetylcholine. The major source of hippocampal ACh originates in large neurons in the medial septum (Amaral & Kurz, 1985; Lewis & Shute, 1969; Shute & Lewis, 1963). Lesions of the medial septum produce a near complete ($\approx 90\%$) loss of acetylcholinesterase, a marker of ACh activity, in the hippocampus (McKinney et al., 1983; Szerb et al., 1977). Stimulation of the medial septum elicits ACh release in the dorsal hippocampus, an action that is abolished by fimbria transection (Dudar, 1975). Within the hippocampus, ACh muscarinic receptors are present in all layers but are more concentrated in the pyramidal and granule cells layers and in the hilus of the dentate (Houser et al., 1983). The iontophoretic application of ACh onto neurons in the hippocampus elicits excitatory post synaptic potentials (see below; Bland et al., 1974), which is mediated chiefly through muscarinic receptors (Storm-Mathisen & Ottersen, 1984). A relatively small number of nicotinic ACh receptors are also present in the hippocampus.

GABA. Glutamic acid decarboxylase (GAD), a marker for GABA, immunohistochemistry, has revealed large numbers of GABAergic interneurons in the medial septum with scattered cells in the lateral septum (Mugnaini & Oertel, 1985). In the hippocampus, GABAergic interneurons are located in all fields but are most concentrated in the dentate and CA1 regions (Storm-Mathisen & Ottersen, 1984). Additionally, GAD-positive neurons originating in the medial septum *project* to the hippocampus (Kohler et al., 1984), innervating most of the GABA-containing interneurons within the hippocampus (Freund & Antal, 1988). Glutamatergic hippocampo-septal fibers innervate GABAergic interneurons in the lateral septum (Leranth & Frotscher, 1989) which in turn synapse with septohippocampal ACh and GABA projection neurons in the medial septum (see Figure 4.14; Leranth & Frotscher, 1989; Onteniente, Geffard, Campsiron, & Calas, 1987).

The iontophoretic application of GABA to neurons in the medial septum has been shown to inhibit their discharge (Dutar et al., 1986; McLennan & Miller, 1974a) and intraseptal infusions of muscimol, a GABA_A agonist, reduces ACh activity in ventral, but not dorsal hippocampus (Blacker et al., 1984; Wood, 1986). Conversely, the intraseptal infusion of bicuculline, a GABA_A antagonist, increases hippocampal ACh activity (Zucker et al., 1987), suggesting that ACh neurons are in a state of tonic inhibition. Together, these results suggest that ACh neurons in the medial septum are regulated by GABAergic interneurons originating from either the lateral septum (Leranth & Frotscher, 1989; Onteniente et al., 1987) or collaterals arising from GABAergic projection neurons in the medial septum (Leranth et al., 1992). Hence, GABAergic neurons in the medial septum and hippocampus comprise an important component of the septohippocampal system.

Glutamate. The lateral septum is innervated by axon collaterals originating from the CA3 field of the hippocampus and employs the excitatory neurotransmitters aspartate and glutamate (Joels & Urban, 1984; Stevens & Cotman, 1986; Storm-Mathisen & Opsahl, 1978). Stimulation of the hippocampo-septal pathway produces a strong inhibition of medial septal neurons (Dutar et al., 1987; McLennan & Miller, 1974b), suggesting that this pathway activates GABAergic inhibitory interneurons originating in the lateral septum.

Serotonin. There are indications that serotonin (5-HT) regulates hippocampal ACh activity by interacting with neurons in the medial septum. For example, lesions of the median raphe nucleus, the prime source of forebrain 5-HT, increase ACh turnover rate in the hippocampus (Robinson, 1983) and intraseptal infusions of the 5-HT agonist MK-212 reduce hippocampal ACh activity (Zucker et al., 1987). These results suggest that 5-HT tonically inhibits medial septal neurons, thereby regulating hippocampal ACh activity. Whether 5-HT inhibits medial septum neurons directly or via the activation of GABAergic interneurons remains to be determined.

Catecholamines. Dopamine and noradrenaline interact with the septum to influence ACh activity in the hippocampus. Dopamine has been detected in the lateral septal nucleus (Brownstein et al., 1974) and the main dopaminergic projection to the lateral septum originates in the cell body group A₁₀, located in the ventral medial tegmentum (Lindvall, 1975). Lesions of area A₁₀ or the medial forebrain bundle completely abolishes dopamine in the lateral septum suggesting that dopamine projects from the medial tegmentum to the lateral septum via this band of fibers. Systemic injections of apomorphine, a dopamine agonist, reduces the turnover rate of ACh in the hippocampus (Costa et al., 1983), an effect that is blocked by intraseptal infusions of bicuculline (Robinson et al., 1979). Together, these results suggest that dopamine activates GABAergic interneurons in the lateral septum which then inhibit ACh neurons in the medial septum.

Like dopamine, noradrenaline has been detected in the both the lateral and medial aspects of the septal area (Brownstein et al., 1974). Noradrenergic projections to the medial septum arise mainly from the locus coeruleus (A₆) located in the brainstem and travel via the dorsal noradrenergic bundle (Lindvall, 1975). The lateral septum receives noradrenergic projections originating in the medulla oblongata noradrenergic system (A₁, A₂, A₃; Jones et al., 1977). Amphetamine, a noradrenergic agonist, increases the turnover rate of ACh in the hippocampus, an effect that is blocked by intraseptal infusions of the irreversible α -adrenergic blocker phenoxybenzamine (Costa et al., 1983). Hence, noradrenaline either disinhibits ACh neurons in the medial septum by inhibiting recurrent GABAergic inhibition or by direct excitation of medial septal ACh neurons.

Neuropeptides. Neuropeptides such as substance P and β -endorphin interact with the septum to modulate ACh activity in the hippocampus. Substance P has been detected in the lateral septum in abundance and, to a lesser extent, in the medial septum (Roberts et al., 1984; Leeman & Mroz, 1974). Intraseptal infusions of substance P decrease ACh turnover in the dorsal, but not ventral, hippocampus, an effect which is not blocked by bicuculline (Blacker et al., 1984; Wood et al., 1979). Hence, substance P

appears to have a direct inhibitory effect on ACh neurons in the medial septum.

Opioid peptides such as β -endorphin and met-enkephalin have been detected in the lateral septum (Atweh & Kuhar, 1977) and intraseptal infusions of β -endorphin or met-enkephalin reduce ACh activity in the hippocampus, though only the reductions produced by β -endorphin are blocked with bicuculline (Wood et al., 1979). The latter result suggests that β -endorphin activates GABA_Aergic interneurons in the lateral septum which inhibit medial septum neurons and reduce hippocampal ACh activity (Blaker et al., 1984). Interestingly, systemically administered morphine (Vallano & McIntosh, 1980) and intraventricular infusion of β -endorphin (Botticelli & Wurtman, 1979) both *increase* ACh activity in the hippocampus. The reason for this discrepancy is unclear.

In sum, several neurotransmitters and neuropeptides interact with the septal region, influencing hippocampal ACh activity. Hippocampal ACh activity is decreased by GABA, glutamate, 5-HT, dopamine, substance P, β -endorphin and met-enkephalin and increased by noradrenaline. These substances may reduce ACh activity in the hippocampus by interacting directly with ACh neurons in the medial septum, or indirectly, by activating interneurons that inhibit ACh neurons in the medial septum.

Conclusions

The septum is innervated by several major neurotransmitters and neuropeptides originating from distal regions (e.g., raphe nucleus in brain stem). Their interaction with the septum modulates the activity of medial septum neurons which project to the hippocampal formation via the fimbria/fornix. The neurons projecting from the medial septum to the hippocampus use either ACh or GABA as neurotransmitters. In the hippocampus, the net effect of medial septal activation is excitation. This excitatory effect increases the efficacy of signals entering the hippocampus by other routes, most notably the perforant path (see Chapter 5 for further discussion). The septum, then, serves as a modulatory point where several different brain regions can regulate the excitability of the hippocampus.

If we accept as a working hypothesis that the hippocampus is involved in the temporary storage of to-be-remembered, configural information, then the septum is in the position to regulate the degree to which that information is remembered. In other words, the septum can 'attach' a weight to incoming to-be-remembered information. The greater the weight (emotional significance?), the greater the likelihood that the information will be stored.

Experiment VII: Intraseptal infusion of BZs: neurochemistry

Experiment VII sought to better characterize the neurochemical effects of intraseptal BZ infusions on spatial learning in the MWM. The previous experiment showed that infusions of CDP (60 nmol) impaired spatial learning. In the present experiment, three different concentrations of CDP (10, 30 & 60 nmol) were infused into the MS prior to water maze training. This experiment also sought to verify that the effects of intraseptal CDP are due to an interaction with BZ receptors, rather than a non-specific disruption of MS activity. This was done by assessing the effects of systemically administered flumazenil, a BZ receptor antagonist, on the spatial learning deficit produced by CDP. To determine if the medial septum mediated the amnesic effects of systemically administered CDP, flumazenil was infused into the medial septum in an attempt to block the spatial learning deficits produced by systemically administered CDP.

The present experiment also sought to determine if the effects of intraseptal CDP on spatial learning are due to a reduction of hippocampal ACh activity. As described in the above discussion of septohippocampal neurochemistry that the source of hippocampal ACh is the medial septum (e.g., Woolf, 1991). For example, stimulation of the medial septum increases hippocampal ACh activity (Dudar, 1975) and lesions of medial septum reduce (~60 %) hippocampal activity (Szerb, Hadhazy, & Dudar, 1977). Further, infusions of muscimol (Brioni et al., 1990) or CDP (Stackman, Emerich, Taylor, & Walsh, 1989) into the medial septum reduces hippocampal ACh activity (as indexed by high-affinity choline uptake; HACHU) while flumazenil (Stackman et al., 1989) and bicuculline (Zucker,

Calkins, Zabawska, Lai, & Horita, 1987) increase hippocampal ACh activity. In previous experiments, THA has been found effective in blocking the amnesic effects of medial septal lesions (Riekkinen, Aaltonen, Sirvio and Riekkinen, 1991; Riekkinen, Sirvio and Riekkinen, 1990), scopolamine (Murray, Cross and Green, 1991) and hemicholinium-3-induced ACh depletion (Hagen et al., 1989). Thus, to assess whether intraseptal infusions of CDP impair spatial learning by reducing ACh activity, systemic tetrahydroaminoacridine (THA), a acetylcholinesterase inhibitor, was coadministered with intraseptal CDP. Thus, if intraseptal infusions of CDP are impairing spatial learning by reducing hippocampal ACh activity, then THA should attenuate or reverse the impairment. The effects of systemic scopolamine, an ACh muscarinic receptor blocker, was also assessed on spatial learning to: 1) verify that the protocol employed in the present study is sensitive to the spatial learning deficits associated with ACh hypofunction, and 2) compare with the amnesic effects of systemically administered CDP.

METHODS

Animals

Male Long-Evans rats served as subjects. They were housed in pairs and maintained on a 12:12 hr light-dark cycle. All testing was conducted during the light phase of the cycle. Rats weighed 350-450 g at the beginning of testing. Food and water were available ad libitum.

Apparatus and procedure

The MWM and procedure is the same as that used in Experiment VI except that rats were additionally required to learn a new platform location (northwest -> southeast; reversal) while undrugged on the day following initial acquisition. All rats were given 16 consecutive trials separated by a 60 s intertrial interval, 15 s on the submerged platform and 45 s in the holding cage. Escape latency and swim path lengths were measured with the tracking system. Following the sixteenth and final trial of the first day, a probe trial was given and the time spent in each quadrant was measured.

Surgery

Rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and given scopolamine methylnitrate (1 mg/kg, i.p.) to relieve respiratory congestion. A single stainless-steel guide cannulae (23 guage, 13.5 mm) was implanted into the dorsal portion of the medial septum with the tip of the injection cannula extending 1.0 mm beyond the tip of the guide cannula. The stereotaxic coordinates were: AP +0.5 mm from bregma; ML 0.0 mm (on midline); DV -5.0 from skull; the nose bar was set at -3.9 from the interaural line. The cannulae was anchored to the skull using four screws and dental acrylic. An obturator was inserted into the guide cannulae to prevent occlusion. One week recovery time was provided prior to behavioral testing.

Drugs

Systemic chlordiazepoxide hydrochloride (Hoffman-La Roche) was dissolved in saline (0.9% NaCl) in a concentration of 5 mg/ml. Systemic and intracranial flumazenil (Hoffman-La Roche) was suspended in saline with Tween 80 (1 drop/ 10 ml) in a concentration of 10 mg/ml. Scopolamine hydrobromide (Sigma) was dissolved in saline in a concentration of 1 mg/ml. Tetrahydroaminoacridine hydrochloride (Sigma) was dissolved in saline in concentrations of either 1 or 3 mg/ml. Control injections were either saline or saline plus Tween 80 (1 drop/10 ml).

For intraseptal infusions, chlordiazepoxide hydrochloride (Hoffman-La Roche) was dissolved in artificial cerebrospinal fluid (CSF in mM: NaCl 147, KCL 2.9, MgCl₂ 1.6, CaCl₂-2H₂O 1.7, NaHCO₃ 35.9, and dextrose 2.2) in either 10, 30 or 60 nmol concentrations. Controls were infused with CSF. Flumazenil was suspended in saline (0.9% NaCl) by a drop of Tween 80 (1 drop/10 ml).

Drug injection procedures

Systemic injections were given IP in the rat's home cage. THA and flumazenil were administered 15 min prior to intraseptal infusions; scopolamine was injected 30 min prior to training and CDP was injected 30 min prior to maze training.

During intraseptal infusions, the obturator was removed and the injection cannulae was gently inserted. The injection needle was attached to polyethylene tubing which was connected to a 10 μ l syringe driven by a minipump (Razel Scientific Instruments Inc.). CDP and CSF were infused in a volume of 1 μ l at a rate of 0.33 μ l/min. The injection needle was left in the guide cannulae for 60 s after the injection to facilitate passive drug diffusion. The obturator was replaced immediately after the injection needle was removed. Behavioral testing was conducted 3 min post-infusion. All rats received only one infusion over the course of training.

Group assignment

Rats were randomly divided into one of the following treatment groups:

Systemic test: systemic saline (1 ml/kg; n = 6), systemic CDP (5 mg/kg; n = 6), systemic scopolamine (1 mg/kg; n = 6).

Dose-response test: intraseptal CSF (1 μ l; n = 8), intraseptal CDP (10, 30 & 60 nmol; n = 8/concentration).

Systemic flumazenil antagonism test: intraseptal CSF + systemic saline (1 ml/kg; n = 6), intraseptal CSF + systemic flumazenil (10 mg/kg; n = 6), intraseptal CDP (60 nmol) + systemic saline (n = 6), intraseptal CDP (60 nmol) + systemic flumazenil (10 mg/kg; n = 6).

Intraseptal flumazenil antagonism test: intraseptal vehicle + systemic saline (1 ml/kg; n = 5), intraseptal vehicle + systemic chlordiazepoxide (5 mg/kg; n = 5), intraseptal flumazenil (60 nmol) + systemic saline, intraseptal flumazenil (10, 30 or 60 nmol; n = 3/dose) + systemic chlordiazepoxide (5 mg/kg).

THA antagonism test: CSF + systemic saline (1 ml/kg; n = 6), CSF + systemic THA (1 mg/kg; n = 6), CSF + systemic THA (3 mg/kg; n = 6), CDP (60 nmol) + systemic saline (1 ml/kg; n = 6), CDP (60 nmol) + systemic THA (1 mg/kg; n = 6), CDP (60 nmol) + systemic THA (3 mg/kg; n = 6).

Histology

After the completion of behavioral testing, cannulae placements were verified histologically. Rats were anesthetized with an overdose of sodium pentobarbital and perfused intracardially with saline (0.9%). Some rats were infused with 1 μ l of black ink prior to sacrifice to verify fluid distribution. Brains were extracted and stored in 10% formaldehyde for 2 weeks. Brains were then sliced in 80 μ m thick sections and mounted on slides for visual and microscopic inspection.

Statistics

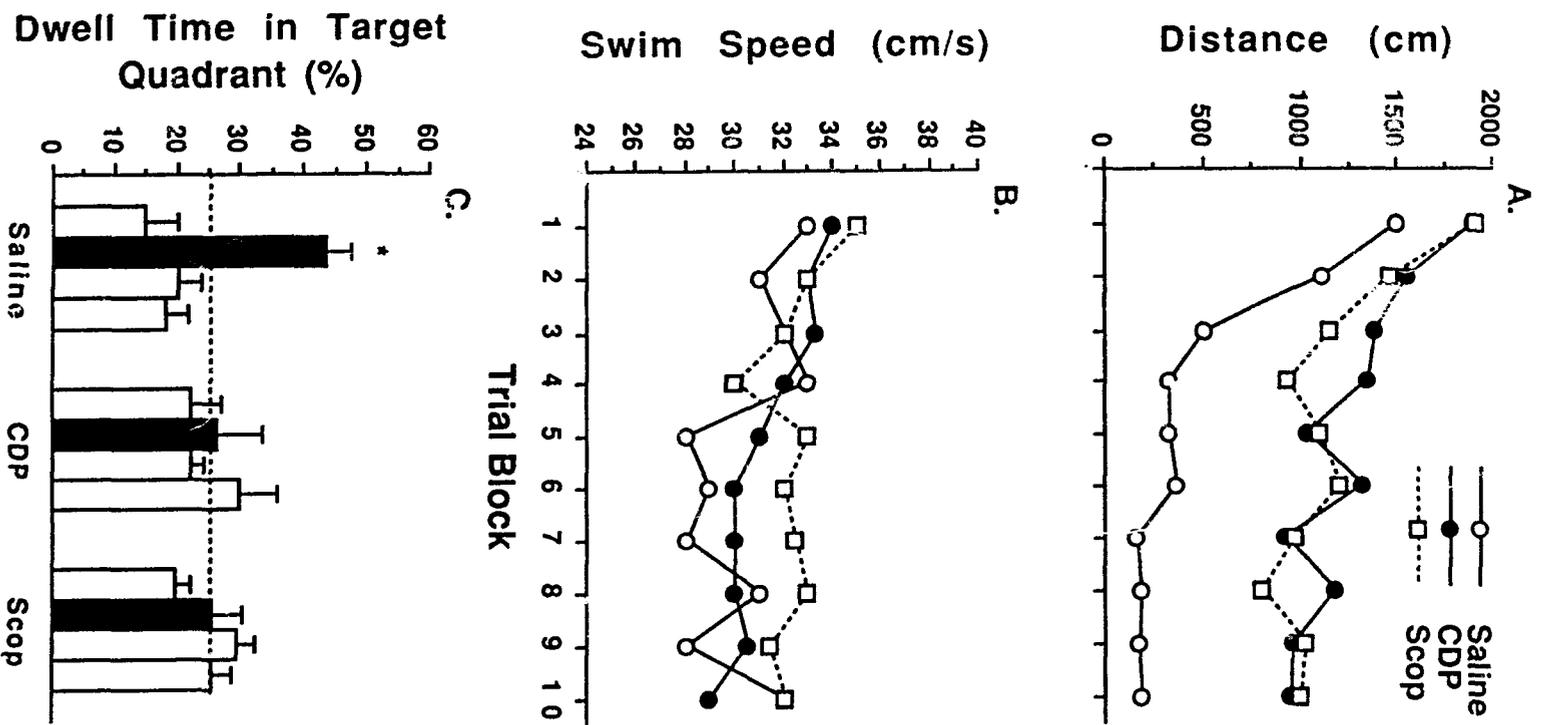
Escape latencies, swim path lengths and swim speeds over training were assessed using an analysis of variance (ANOVA) procedure with repeated measures. Post hoc comparisons were assessed using Dunnett's test. In every case the acceptable level of statistical analysis was $p < 0.05$.

RESULTS

Systemic test

The distance required by each treatment group to locate the submerged escape platform over the twenty trials is shown in Figure 4.15A. Rats treated with saline rapidly acquired the platform location, requiring only 8 trials to reach asymptotic levels. Rats treated with CDP or scopolamine took longer paths to locate the platform and attained asymptotic levels that were approximately ten times greater than control levels. Rats treated with CDP tended to swim thigmotaxically (hugging the wall) for a greater number of initial trials but did eventually begin to traverse the pool during the latter stages of training. Even then they tended to adopt inefficient search strategies that typically involved swimming around the pool at a distance from the wall sufficient to intersect the platform. Other rats treated with CDP appeared to give up searching after several trials into training and swam thigmotaxically for the remaining trials. Rats treated with scopolamine also demonstrated an impairment of spatial learning and, like rats treated with CDP, swam thigmotaxically, typically in the one direction (i.e., clockwise), and in some cases on all of the 20 trials. An overall ANOVA on swim path

Figure 4.15: Effects of scopolamine (1 mg/kg; Scop) and chlordiazepoxide (5 mg/kg; CDP) or saline on (A) the distance taken to locate the submerged platform on the twenty trials/day procedure, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note the spatial learning deficit produced by both scopolamine and chlordiazepoxide in the absence of swim speed reductions. * $p < 0.01$ compared to chance level (25%-dotted line).



lengths revealed a significant group difference, $F(2,31)=8.28$, $p<.001$, trial-block difference, $F(9,279)=20.5$, $p<.0001$, but not a significant interaction between groups and trial-block, $F(18,279)=0.53$, N.S. Post hoc tests revealed that rats treated with CDP or scopolamine had significantly longer swim path lengths than controls ($p<0.01$), and did not differ significantly from each other. Latency data (not shown) revealed a similar pattern of deficits and statistical significances.

The effects of systemic CDP and scopolamine on swim speed are presented in Figure 4.15B. Overall, the swim speeds of all groups differed little. Control rats swam at an overall average rate of 30.5 ± 0.5 cm/s but tended to swim slower during the last six trial blocks, when swim path lengths were asymptotic. Rats treated with CDP swam at an overall average rate of 31.3 ± 0.35 cm/s and showed a downward trend over the course of training. Rats treated with scopolamine swam at an overall average rate of 32.4 ± 0.4 cm/s which was maintained over training. An overall ANOVA on swim speeds revealed a nonsignificant group difference, $F(2,31)=1.26$, N.S, significant trial block difference, $F(9,279)=3.99$, $p<0.001$, but not a significant interaction between groups and trial block, $F(18,279)=1.55$, N.S.

Performance during the probe trial (Fig. 4.15C) tended to confirm the distance data in that only controls showed a bias for the quadrant that contained the escape platform during acquisition training ($p<0.01$ compared to chance levels, 25%). Rats treated with either CDP or scopolamine failed to show a quadrant bias, distributing their swims randomly about all four quadrants.

Unexpectedly, rats treated with scopolamine were impaired on the visible platform task (data not shown), with some rats swimming around the perimeter of the pool for the duration of the trial. During visible platform training, rats treated with scopolamine swam thigmotactically, as during the submerged platform task. These rats would on occasion break away from the pool wall to swim directly to the visible platform. Rats treated with CDP or saline learned quickly to escape onto the visible platform. The mean overall distance are as follows: saline, 157 ± 20.6 ;

CDP, 148 ± 13.7 ; scopolamine, 389 ± 90 . An overall ANOVA on the distances taken to reach the visible platform revealed a significant group difference, $F(2,23)=3.4$, $p<0.05$, but not a significant trial block difference, $F(3,69)=0.44$, N.S, or group by trial block interaction, $F(6,69)=0.56$, N.S. Post hoc tests confirmed that rats treated with scopolamine had significantly longer distances relative to rats treated with saline ($p<0.05$) and CDP ($p<0.05$).

Intraseptal infusions

During infusions, none of the rats demonstrated wet dog shakes, a phenomenon indicative of ictal activity and observed previously with intrahippocampal infusions of a 1 μ l volume (see results in Experiment VI).

Dose-response test. The positions of the needle tips in the medial septum are illustrated in Figure 4.16. The majority of placements were dorsal to the medial septum region with some variation in the anterior-posterior and dorsal-ventral directions. Overall, cannulae positions were similar for each of the treatment groups. Dye infusions revealed that the medial septum and diagonal band of Broca, as well as a small portion of the lateral septum, were reached by the infusions.

The intraseptal infusion of CDP produced a dose-dependent impairment of spatial learning (Fig. 4.17A). Rats infused with CSF, like rats treated systemically with saline, showed rapid acquisition, reaching asymptotic levels by the fifth trial block (9th and 10th trial). Rats treated with the 10 nmol dose of CDP also showed good acquisition, differing little from rats treated with CSF. Rats treated with either the 30 nmol and 60 nmol doses showed impaired acquisition, taking longer swim paths to the platform. Rats in the 60 nmol group with severe deficits would swim around the edge of the pool (thigmotaxia) on the majority of trials, only occasionally entering the center of the pool. Less severe deficits were associated with the adoption of large circular loops that tended to cross the center of each of the four quadrants. An overall ANOVA on swim distances revealed a significant group difference, $F(3,56)=10.9$, $p<0.001$, trial block difference, $F(9,504)=37.9$, $p<0.001$, but not a significant interaction between groups and trial blocks, $F(27,504)=0.54$, N.S. Post hoc tests revealed that rats receiving either the 30 or

Figure 4.16: Illustration of injection needle tip placements in the medial septum. Note the grouping of placements of the different treatment groups dorsal to the medial septum. Note the similar placement proximity for each treatment group. (open circles = CSF infusions, closed circles = CDP 10 nmol, closed squares = CDP 30 nmol, closed diamonds = CDP 60 nmol; bar = 1 mm).

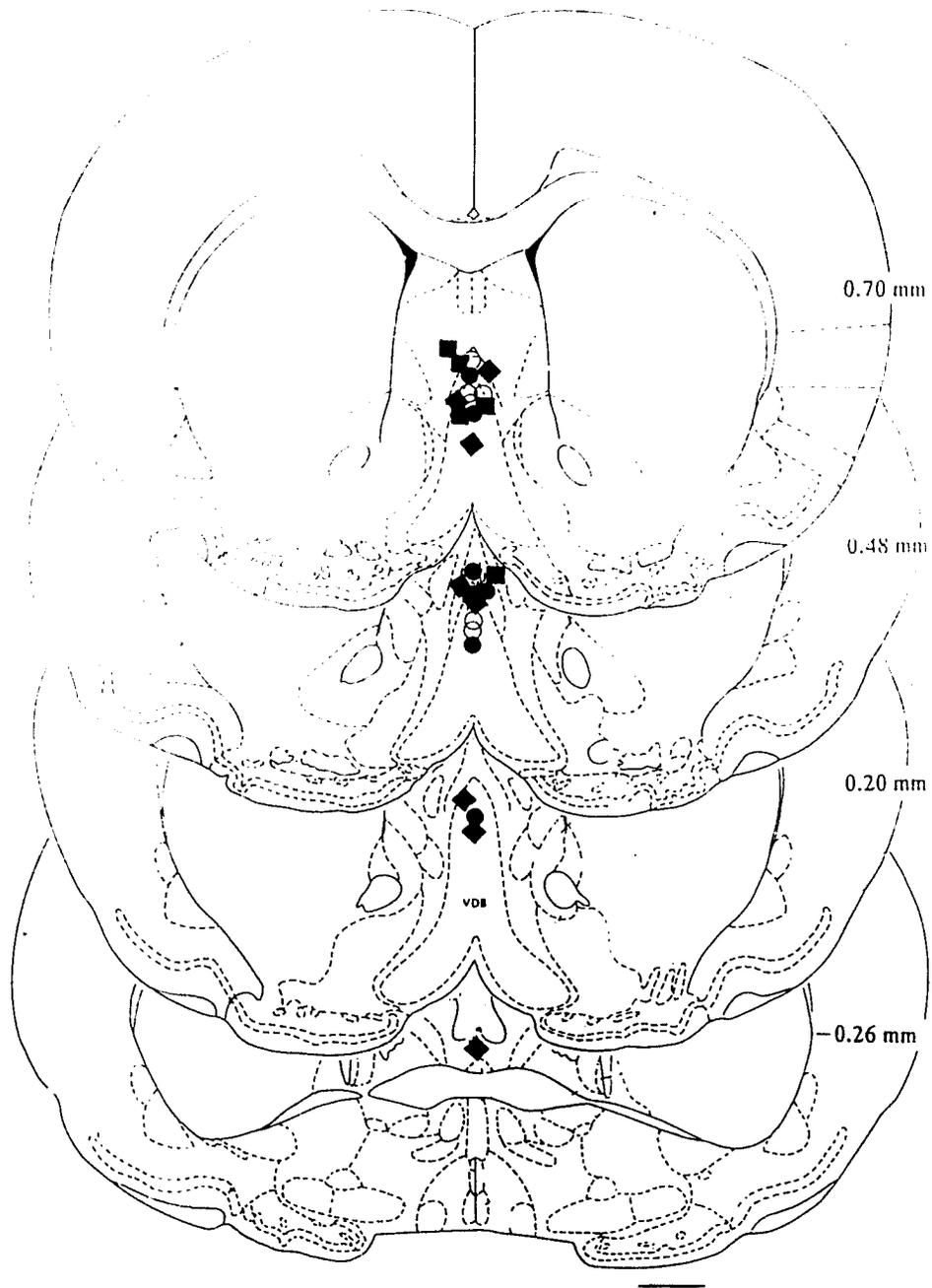
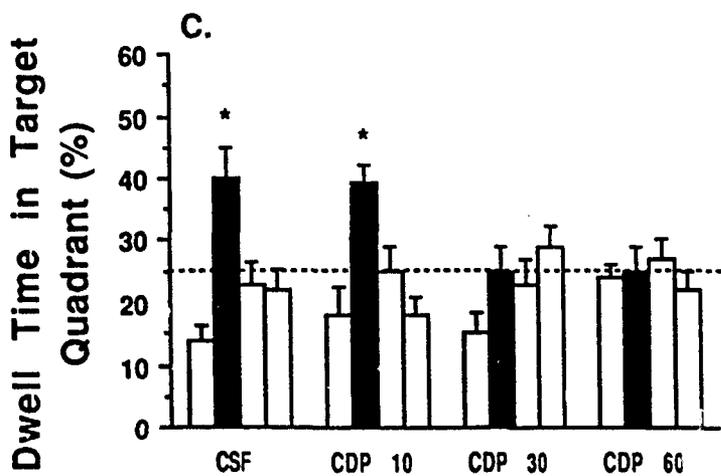
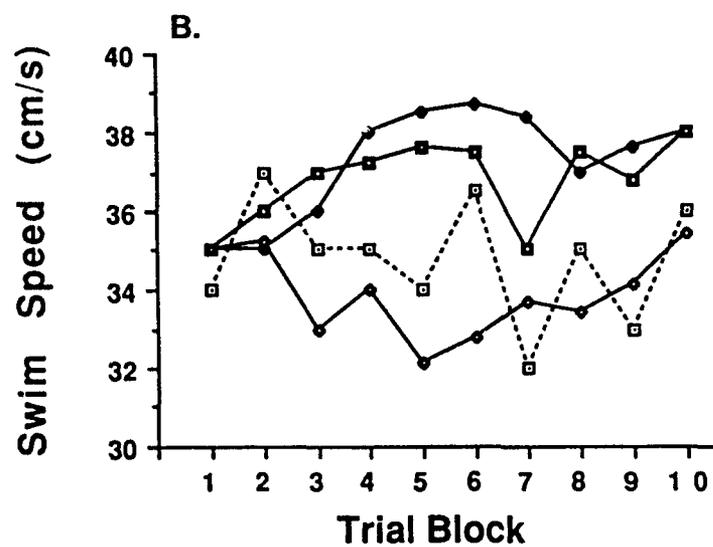
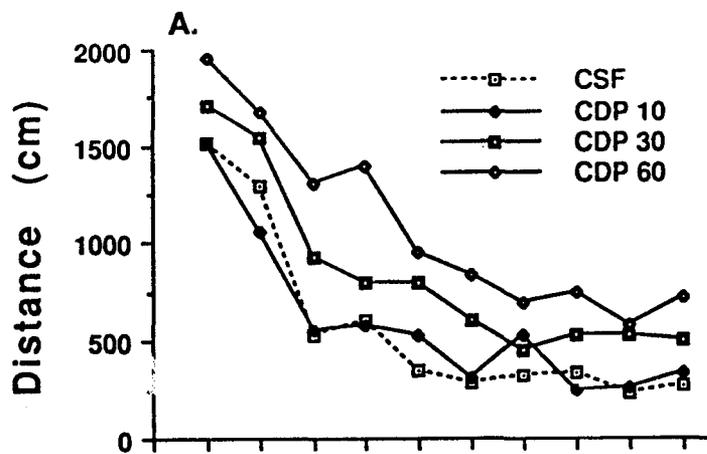


Figure 4.17: Effects of intra-septal infusions of chlordiazepoxide (10, 30, & 60 nmol) or CSF on (A) the distance taken to locate the submerged platform, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note the dose-dependent impairment of spatial learning produced by chlordiazepoxide. * $p < 0.01$ compared to chance level (25%-dotted line).



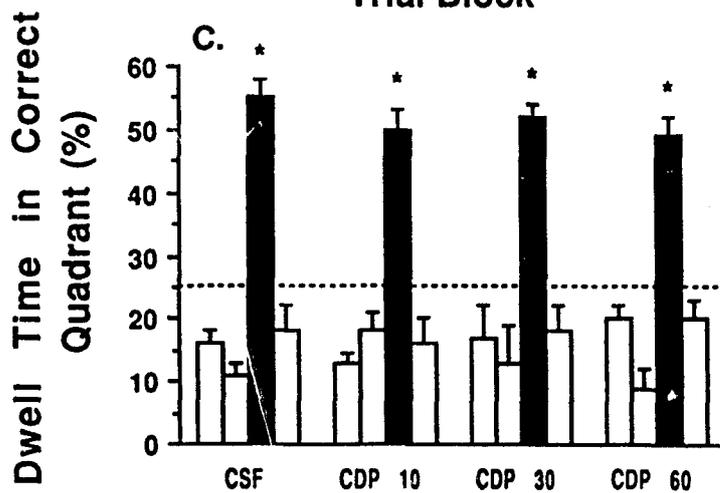
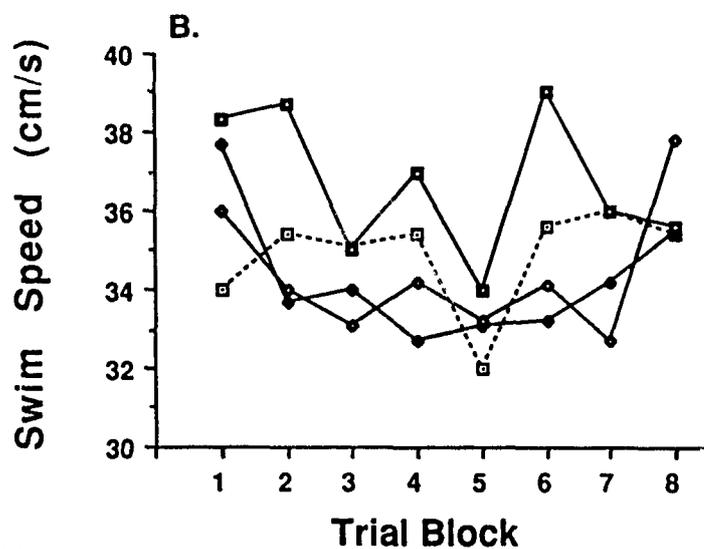
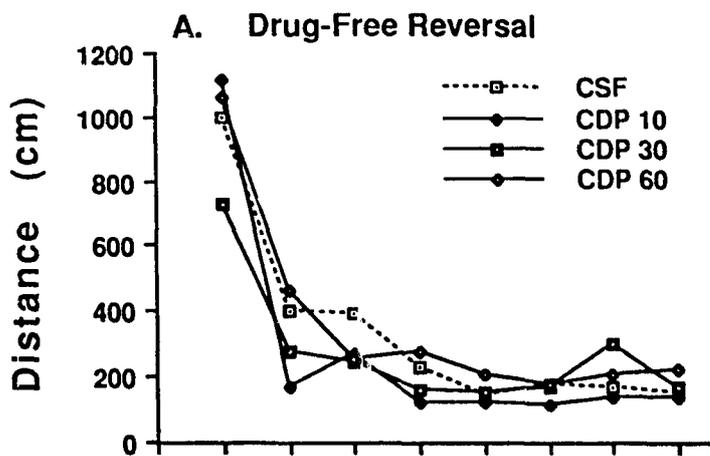
60 nmol dose had significantly longer distances relative to both rats treated with CSF ($p < 0.01$) or the 10 nmol dose ($p < 0.01$).

The effects of intraseptal infusions of CDP on swim speed over training is shown in Figure 4.17B. Rats receiving intraseptal CSF swam at a relatively constant rate across training with an overall mean of 34.8 ± 0.4 cm/s. Rats receiving the 10 or 30 nmol infusions tended to swim faster than controls with mean overall swim speeds of 37.4 ± 0.4 and 36.7 ± 0.3 cm/s respectively. Rats receiving the 60 nmol infusion tended to swim slower than controls with an overall mean of 33.8 ± 0.4 cm/s. An overall ANOVA on swim speeds revealed a significant group difference, $F(3,56)=3.73$, $p < 0.02$, but not a significant trial block difference, $F(9,504)=1.45$, N.S., or a group by trial block interaction, $F(27,504)=1.35$, N.S. Post hoc tests revealed that the only significant group difference was between the 10 nmol and 60 nmol concentrations.

Performance during the probe trial (Fig. 4.17C) confirmed the pattern of deficits found during acquisition, with rats treated with CSF and 10 nmol CDP showing a bias for the correct quadrant ($p < 0.01$ compared to chance) while rats treated with 30 nmol or 60 nmol doses of CDP failed to show a quadrant bias. None of the treatment groups differed on the visible platform task with the mean distances: CSF, 230.5 ± 25 ; CDP 10, 222 ± 28 ; CDP 30, 228 ± 27 ; CDP 60, 205 ± 27 .

When the platform was moved to the opposite quadrant (reversal training), all of the rats (now undrugged) showed fast acquisition (Fig. 4.18A), learning the platform location within the first two trial blocks, showing asymptotic performance over the remaining trials. An overall ANOVA on distances showed a non-significant group difference, $F(3,60)=0.9$, N.S., a significant trial block difference, $F(7,420)=33.4$, $p < 0.001$, and a non-significant group by trial block interaction, $F(21,420)=0.9$, N.S. Similarly, during the probe trial (Fig. 4.18C) each of the treatment groups showed a significant bias for the new correct quadrant ($p < 0.01$). During reversal training, all groups swam at the same rate over the course of training (Fig. 4.18B). An ANOVA on speed data confirmed this, revealing a non-significant group difference,

Figure 4.18: Performance of rats that received intra-septal infusions of chlordiazepoxide (10, 30, & 60 nmol; CDP) or CSF during initial acquisition on (A) infusion-free reversal acquisition, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note that all groups show rapid and accurate acquisition in with little differences in swim speeds. * $p < 0.01$ compared to chance level (25%-dotted line).



$F(3,50)=0.7$, N.S., and group by trial block interaction, $F(21,350)=0.9$, N.S., but a significant trial block difference, $F(7, 350)=2.6$, $p<0.01$.

Flumazenil antagonism test. The positions of the injection needle tips are illustrated in Figure 4.19. The majority of placements are accurately located dorsal to the medial septum with some variability in the anterior-posterior and dorsal-ventral directions. Dye infusions confirmed that the 1 μ l volume reached the medial septum.

The concurrent administration of systemic flumazenil blocked the disruptive effects of CDP (60 nmol) on spatial learning (Fig. 4.20A). Rats treated with either CSF + saline or CSF + flumazenil showed rapid acquisition over the five trial blocks. Rats treated with intraseptal CDP + saline had greater distances to locate the platform, replicating previous findings (as above). In contrast, rats treated with intraseptal CDP + flumazenil resembled controls, indicating that the systemic flumazenil blocked the impairment produced by intraseptal infusions of CDP. An overall ANOVA on distances revealed a significant group difference, $F(2,33)=16.3$, $p<0.001$, trial block difference, $F(9,297)=18.4$, $p<0.001$, but not a group by trial block interaction, $F(18,297)=0.81$, N.S. Post hoc tests revealed that the CDP + saline group had longer distances than either the CSF + saline, CSF + flumazenil group ($p<0.01$) or CDP + flumazenil groups ($p<0.01$). The distances of the CSF + flumazenil and CDP + flumazenil groups did not differ statistically.

Swim speeds differed little between treatment groups over the course of training (Fig. 4.20B). This was confirmed by an overall ANOVA which revealed a non-significant group difference, $F(3,39)=1.2$, N.S., trial block difference, $F(9,351)=1.5$, N.S., or group by trial block interaction, $F(27,351)=0.7$, N.S.

The blockade of spatial learning deficit produced by intraseptal infusions of CDP seen during acquisition was confirmed on the probe trial. During the probe trial, all treatment groups except the CDP + saline group showed a robust bias for the correct quadrant (Fig. 4.20C). The CSF + saline,

Figure 4.19: Illustration of injection needle tip placements in the medial septum. Note the similar placements for each of the different treatment groups. (open circles = intra-septal CSF + systemic saline; closed circles = intra-septal CSF + systemic flumazenil (10 mg/kg), closed squares = intra-septal CDP (60 nmol) + systemic saline, closed diamonds = intra-septal CDP (60 nmol) + systemic flumazenil; bar = 1 mm).

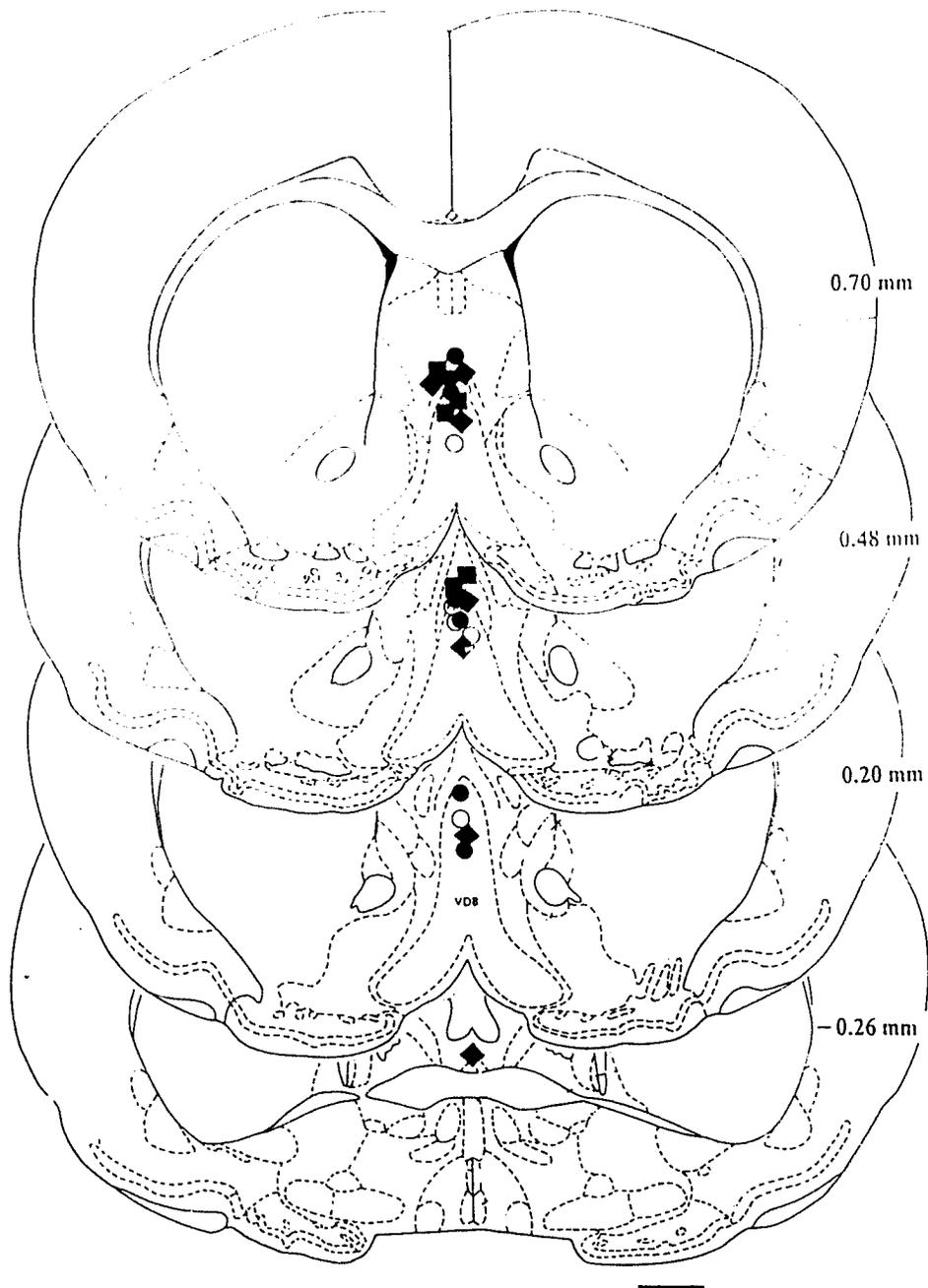
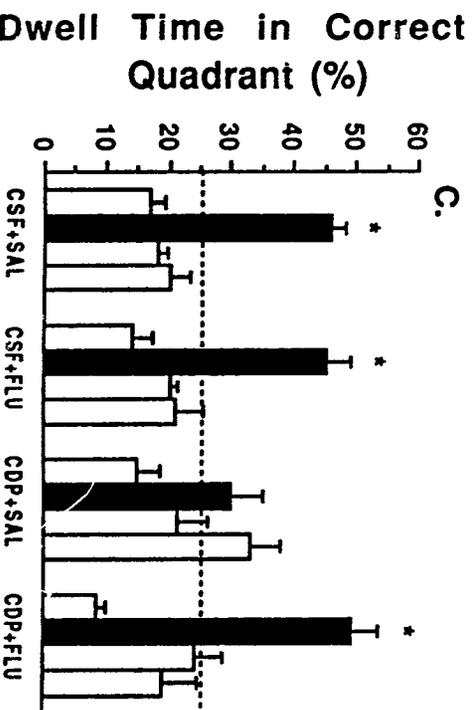
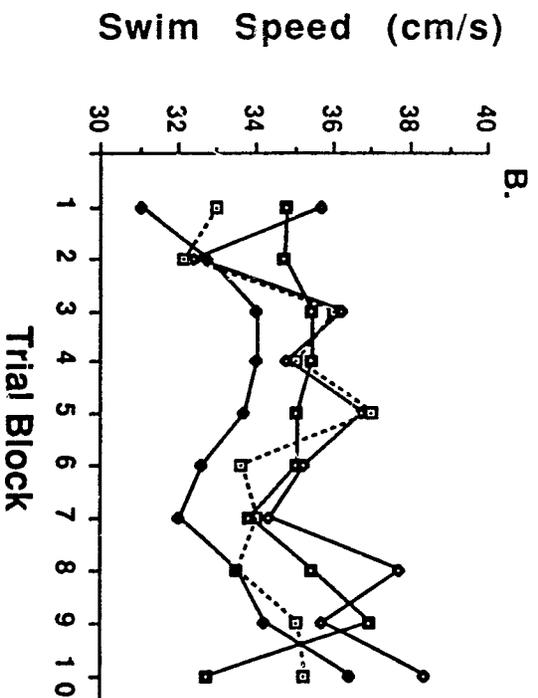
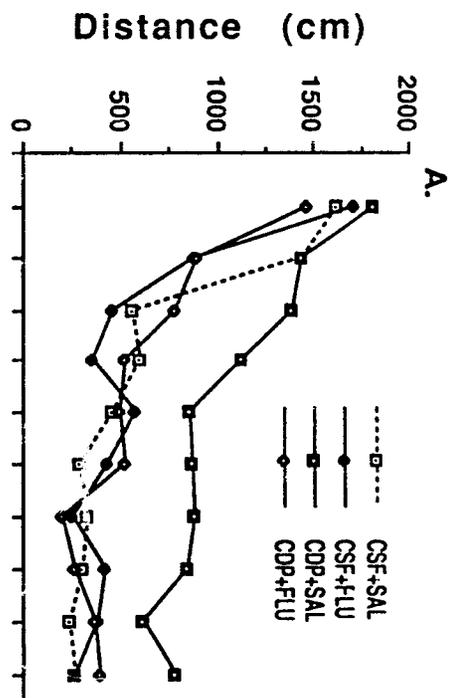


Figure 4.20: Interactions between intra-septal infusions of chlordiazepoxide (60 nmol; CDP) and systemic flumazenil (10 mg/kg; FLU) on (A) the distance taken to locate the submerged platform, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note that the systemic administration of flumazenil blocked the spatial learning deficit produced by intra-septal infusions of chlordiazepoxide. * $p < 0.01$ compared to chance level (25%-dotted line). (SAL = systemic saline).



CSF + flumazenil, and CDP + flumazenil all spent a significant proportion of their total distance in the correct quadrant ($p < 0.01$ compared to chance, 25%).

Intraseptal flumazenil antagonism test: The positions of the injection needle tips are illustrated in Figure 4.21. The needle tips of each group were similarly located dorsal to the medial septum. Assessment of dye distribution revealed that infusions were reaching the medial septum. The medial aspect of the lateral septum was also reached by the dye infusion.

Intraseptal infusions of flumazenil did not block the spatial learning deficit produced by systemic CDP. None of the three concentrations of flumazenil (10, 30 & 60 nmol) attenuated the spatial learning deficit produced by systemically administered CDP and the data was pooled (Fig. 4.22A). Rats in the vehicle + saline or flumazenil + saline groups acquired the platform location at comparable rates. In contrast, rats in the vehicle + CDP and flumazenil + CDP groups showed a severe spatial learning deficit. An overall ANOVA on the distance data revealed a significant group difference, $F(3,38)=33.9$, $p < 0.001$, trial-block difference, $F(9,342)=16.4$, $p < 0.001$, and group by trial-block interaction, $F(27,342)=1.5$, $p < 0.05$. Post hoc tests revealed that the vehicle + saline and the flumazenil + saline groups both had significantly shorter distances relative to both the vehicle + CDP and flumazenil + CDP groups ($p < 0.01$). The distances of the flumazenil + CDP group did not differ significantly from the vehicle + CDP group.

Swim speeds tended to be slower in the groups receiving CDP systemically (Fig. 4.22B). This was confirmed by an overall ANOVA which revealed a significant group difference, $F(3,38)=13.1$, $p < 0.001$, trial-block difference, $F(9,342)=2.9$, $p < 0.001$, and group by trial-block interaction, $F(27,342)=1.5$, $p < 0.05$.

The pattern of findings found during acquisition were confirmed in the probe trial. During the probe trial, all rats given systemic CDP failed to show a significant bias for the correct quadrant (Fig. 4.22C). The vehicle + saline and flumazenil + saline groups both spent a significant proportion of their total distance in the correct quadrant ($p < 0.01$ compared to chance, 25%).

Figure 4.21: Illustration of injection needle tip placements in the medial septum. Note the similar placements for each of the different treatment groups. (open circles = intra-septal vehicle + systemic saline; closed circles = intra-septal vehicle + systemic CDP (5 mg/kg), open squares = intra-septal flumazenil (60 nmol) + systemic saline, closed squares = intra-septal flumazenil (10, 30 or 60 nmol) + systemic CDP (5 mg/kg).

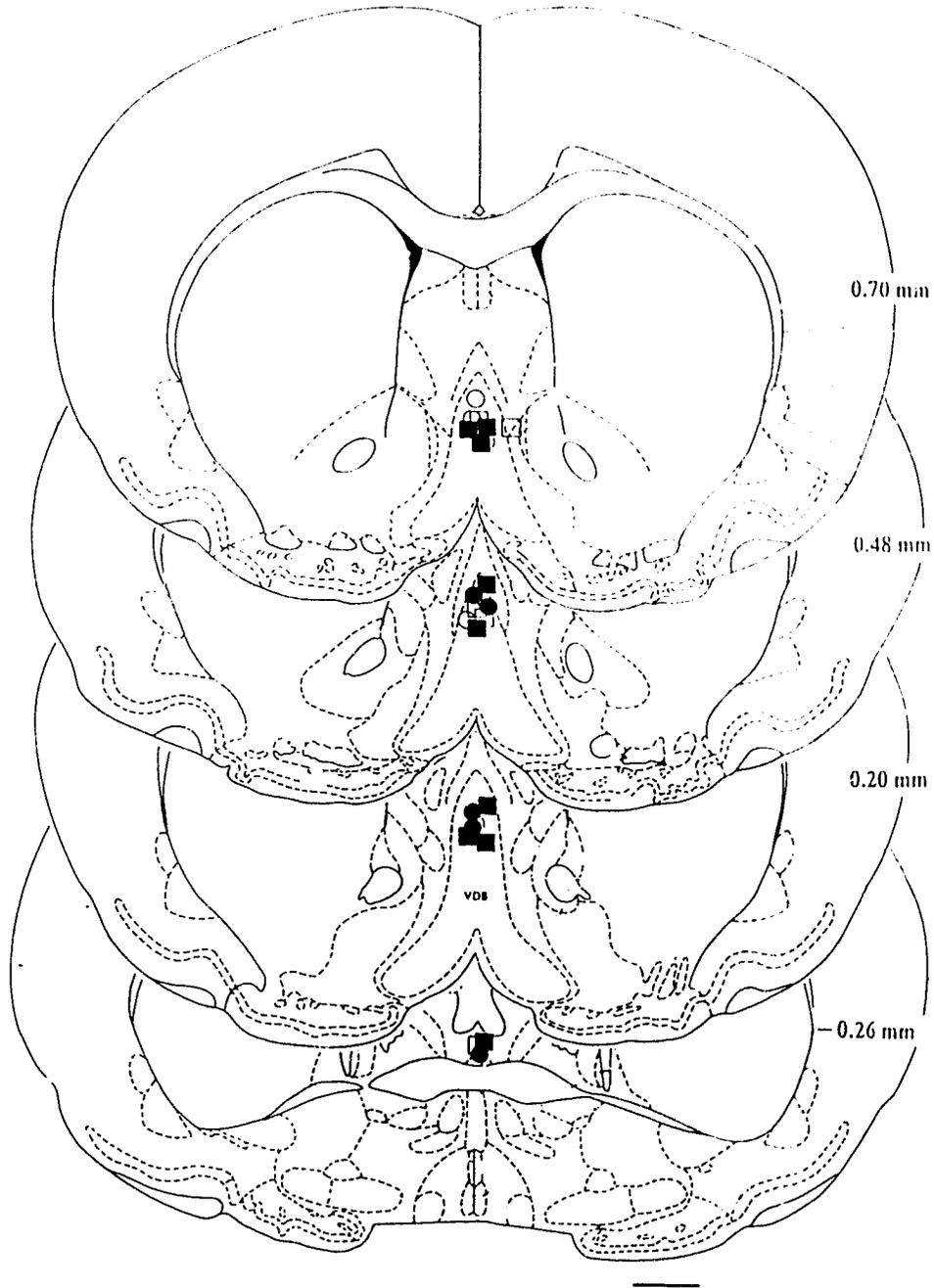
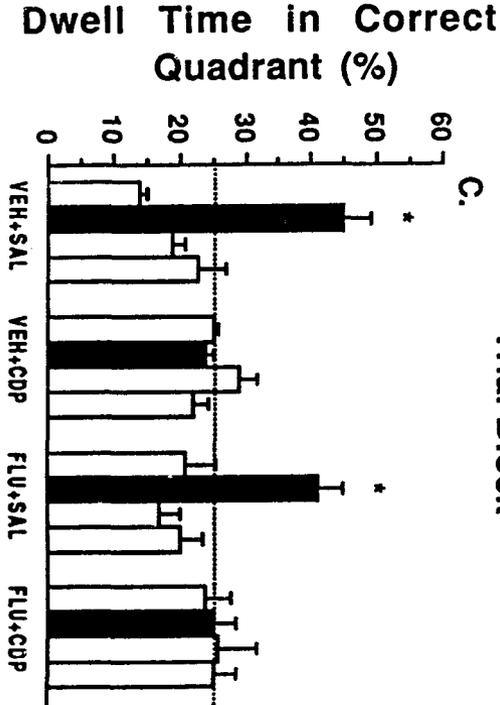
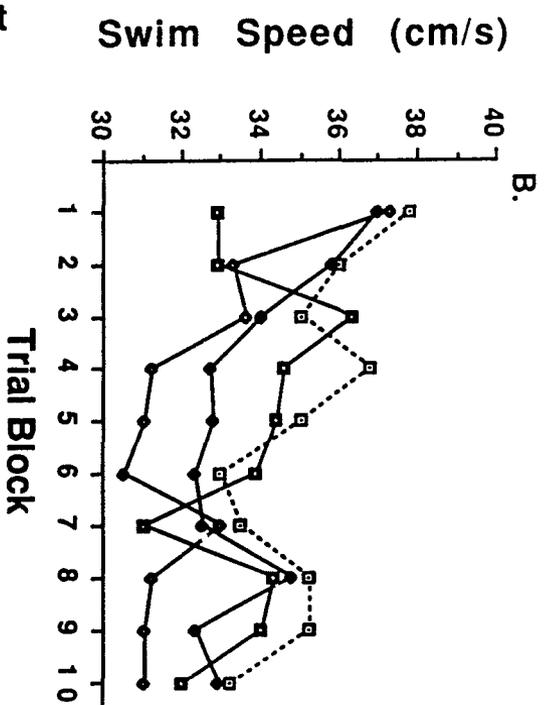
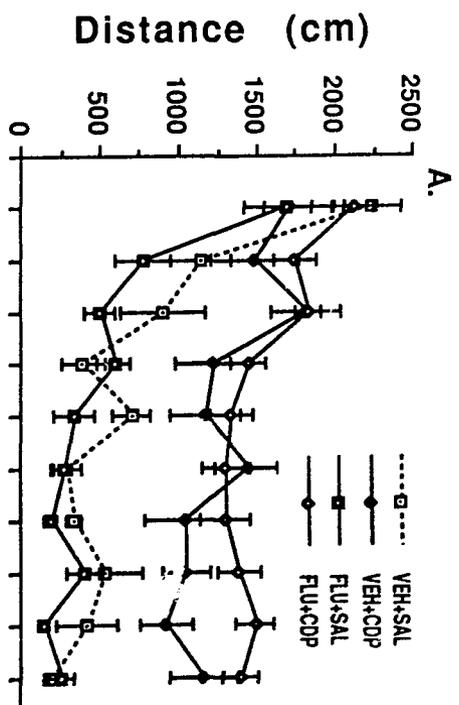


Figure 4.22: Interactions between intra-septal infusions of flumazenil (10, 30 & 60 nmol, data pooled; FLU) and systemic CDP (5 mg/kg) on (A) the distance taken to locate the submerged platform, (B) swim speed, and (C) percentage distance spent in the correct quadrant during the 30 s probe trial. Note that the failure of intra-septal flumazenil, at any dose, to attenuate the amnesic effects of systemically administered CDP. * $p < 0.01$ compared to chance level (25%-dotted line). (VEH = flumazenil vehicle).



The proportion of total distance spent in the correct quadrant by the vehicle + CDP or flumazenil + CDP groups did not differ significantly from chance levels ($p > 0.05$).

THA antagonism tests. The positions of the injection needle tips are illustrated in Figure 4.23. Once again, the tips of the cannulae are positioned dorsal to the medial septum with some variance in the anterior-posterior direction. Dye infusions confirmed that the infusion reached the medial septum.

Overall, THA, at both doses, failed to attenuate the spatial learning deficit produced by intraseptal infusions of CDP (Fig 4.24A). Rats in the CDP + saline group performed poorly compared to the CSF + saline group. Rats in the CDP + THA (1 mg/kg) group or CDP + THA (3 mg/kg) performed as poorly as the CDP + saline group. Rats in the CSF + THA (3 mg/kg) group were impaired relative to the CDP + saline group while rats in the CSF + THA (1 mg/kg) group were facilitated. An overall ANOVA on the distance data revealed a significant group difference, $F(5,58)=6.2$, $p < 0.001$, trial block difference, $F(9,522)=5.2$, $p < 0.001$, and group by trial block interaction, $F(45,522)=0.6$, $p < 0.001$. Post hoc tests revealed that the CDP + saline, CDP + THA (1 mg/kg), CDP + THA (3 mg/kg), and CSF + THA (3 mg/kg) groups all had longer mean distances relative to the CSF + saline group ($p < 0.05$) but did not differ significantly from each other. The CSF + THA (1 mg/kg) group had significantly a shorter mean distance compared to the CSF + saline group ($p < 0.01$).

The failure of THA to attenuate the spatial learning deficits produced by intraseptal infusions of CDP were confirmed in the probe trial. During the probe trial (Fig 4.24B), the only groups to show a significant bias for the correct quadrant were the CSF + saline and CSF + THA (1 mg/kg) groups ($p < 0.01$ compared to chance levels, 25%). The remaining groups failed to show a significant bias for the correct quadrant. None of the groups were impaired on the visible platform task (data not shown).

Figure 4.23: Illustration of cannula placements in the medial septum. (open circles = intra-septal CSF + systemic saline; closed circles = intra-septal CSF + systemic THA (1 mg/kg), closed squares = intra-septal CDP (60 nmol) + systemic THA (1 mg/kg), closed diamonds = intra-septal CSF + systemic THA (3 mg/kg), open squares = intra-septal CDP (60 nmol) + systemic THA (3 mg/kg), open diamonds = intra-septal CDP (60 nmol) + systemic saline; bar = 1 mm).

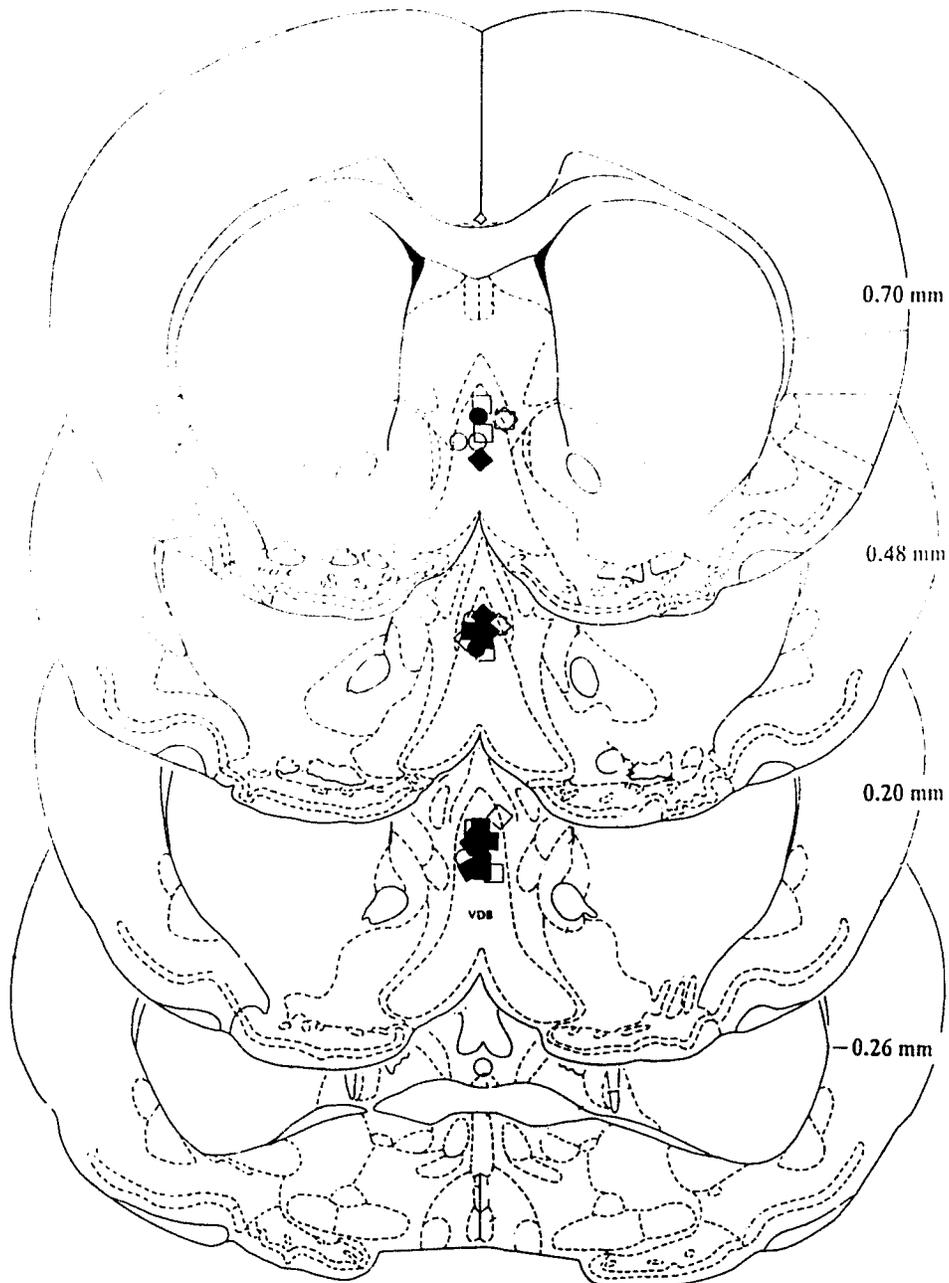
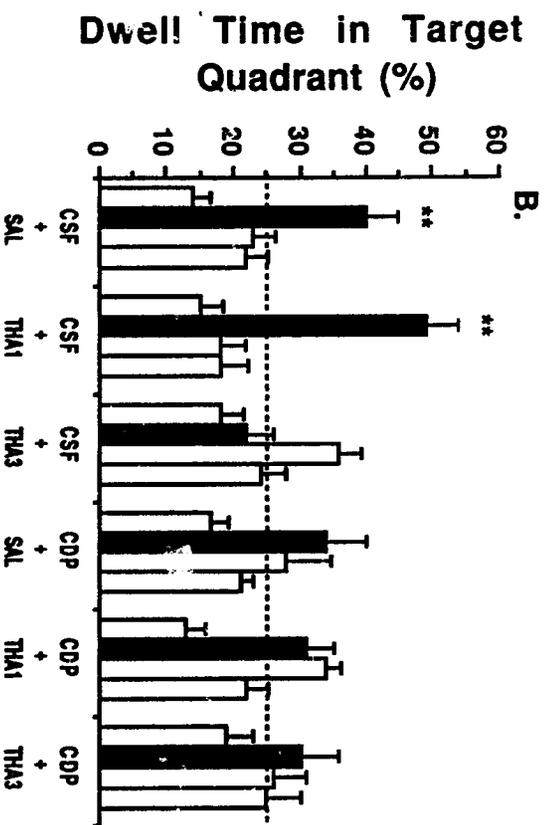
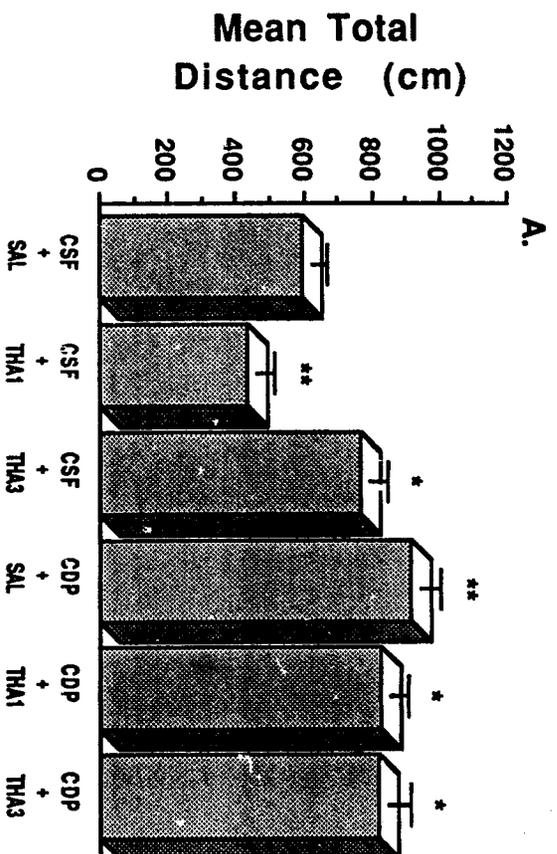


Figure 4.24: Interactions between intra-septal infusions of chlordiazepoxide (60 nmol; CDP) and systemic tetrahydroaminoacridine (1 or 3 mg/kg; THA) on (A) the distance taken to locate the submerged platform on the twenty trials/day procedure averaged over days, and (B) percentage distance spent in the correct quadrant during the 30 s probe trial. Note: 1) the significant enhancement of acquisition produced by the low dose of THA (CSF + THA1), 2) the deficit produced by the high dose of THA (CSF + THA3), and 3) the failure of either dose of THA to attenuate the spatial learning deficit produced by intra-septal infusions chlordiazepoxide. * $p < 0.01$ compared to chance level (25%-dotted line). (SAL = systemic saline).



DISCUSSION

The present investigation found that systemic scopolamine and systemic CDP both impair spatial learning in this task, that intraseptal CDP impairs spatial learning in a concentration-dependent and flumazenil-reversible manner, that intraseptal infusions of flumazenil fail to attenuate the amnesic effects of CDP, and that THA fails to attenuate the spatial learning deficit produced by intraseptal infusions of CDP. Together, these data suggest that CDP interacts with BZ receptors in the medial septum to impair spatial learning but that additional sites are involved, and that the spatial learning deficit produced by intraseptal CDP is not due to reductions of ACh activity.

The spatial memory deficit produced by CDP in the present experiment agrees with previous reports (Experiment VI; Arolfa & Brioni, 1991; McNaughton & Morris, 1987) which suggest that BZ receptor activation is detrimental to spatial learning processes. It is worth noting that the present task (20 trials in one day) placed little demand on retention processes, unlike the tasks used in previous reports in which daily training sessions were separated by 24 hour retention intervals. It is also worth noting that the spatial learning deficit produced by CDP was quantitatively similar to that produced by scopolamine but differed qualitatively. For example, rats given CDP were more flexible in their search strategies, swimming in more than one direction and reversing directions midswim.

The finding that systemic scopolamine impaired spatial learning confirms previous reports (Experiment V; McNaughton & Morris, 1987). The present findings further show that acute scopolamine administration impairs cue learning (visible platform performance), in addition to spatial learning, an effect also shown previously (Okaichi & Jarrard, 1982). An impairment of visible platform training was not observed in Experiment V, where rats had received scopolamine several times prior to visible platform training suggesting that tolerance developed to this impairment. This latter finding suggests that the deficits produced by scopolamine are due to either gross sensori-motor impairments or a general learning impairment rather

than a disruption of spatial learning per se. The swim patterns demonstrated by scopolamine-treated rats, in some cases swimming around the periphery of the pool for the duration of the trial, agree with this notion and suggest that scopolamine produces severe perseveration. Nevertheless, the present results show that this task is sensitive to the disruptive effects of cholinergic hypofunction. Therefore, if BZs do impair spatial learning by reducing ACh activity, it would be manifested on this task.

In the previous experiment, it was found that of several different anatomical regions tested, only intraseptal infusions of CDP (60 nmol) impaired spatial learning. The present investigation similarly found that intraseptal infusions of CDP impair spatial learning. Additionally, the present study revealed that the spatial learning deficit produced by intraseptal infusions of CDP is dose-dependent, with the lowest concentration (10 nmol) of CDP having little effect on spatial learning. The dose-response relationship found in the present study agrees well with a previous report which also found that a 30 nmol, but not 10 nmol, concentration impaired retention of spatial information in the radial arm maze (Stackman et al., 1992). Hence there appears to be a critical concentration of CDP to impair spatial learning, possibly relating to receptor occupation or duration of action.

Flumazenil, a specific and potent BZ receptor antagonist (Hunkeler et al., 1981), blocked the spatial learning deficit produced by intraseptal infusions of CDP. This result provides strong evidence that the spatial learning deficit was produced by an interaction of CDP with BZ receptors, rather than a non-specific disruption of normal septal functioning. Moreover, the finding that previously infused rats could acquire a reversed platform position on the following day in the absence of CDP infusions also suggests that the deficit observed during initial training was not due to damage in medial septum caused by the cannula. This is an important consideration given that medial septum lesions impair spatial learning in the MWM (Hagen et al., 1988; Kelsey & Landry, 1988; Miyamoto et al., 1987). Finally, routine histological inspection failed to reveal gross cell loss in the septal region.

Intraseptal infusions of flumazenil (10, 30, or 60 nmol) failed to attenuate the spatial learning deficit produced by systemically administered CDP (5 mg/kg). This finding contrasts with Stackman et al. (1992) who found that intraseptal infusions of flumazenil (10 nmol) prevented the spatial learning impairment produced by systemic CDP (5 mg/kg) on the radial arm maze. The reason for this discrepancy is not obvious and will require further experimentation. Nevertheless, the present findings suggest that the medial septum is not the only site mediating BZ-induced amnesia. Given that CDP infusions into several other neuroanatomical sites fail to impair spatial learning (Experiment VI), it is difficult to predict which site(s) is responsible.

The findings of the present study suggest that intraseptal infusions of CDP do not impair spatial learning by inhibiting septohippocampal ACh activity. As described above, the medial septum sends a large cholinergic projection to the hippocampus via the fimbria/fornix (Woolf, 1991). Medial septal lesions (Riekkinen et al., 1990, 1991) and intraseptal infusions of CDP (Stackman et al., 1989) have been shown to reduce hippocampal ACh activity by ~50 percent. Further, systemically administered THA, at doses comparable to those used in the present study, has been shown to attenuate the amnesic effects produced by medial septal lesions (Riekkinen et al., 1990, 1991). Hence, the failure of THA to attenuate the spatial learning deficit produced by intraseptal CDP strongly suggests that the deficit was not due entirely to reductions of ACh activity. Moreover, this finding adds further support to the notion that hippocampal ACh activity is not correlated well with spatial learning ability (Dunnet et al., 1987). For example, intracerebroventricular (ICV) and intraseptal infusions of the neurotoxin colchicine were both found to reduce hippocampal ACh activity (~ 50 percent) but only the ICV infusions of colchicine impaired spatial learning (Barone, Nairy, Mundy, McGinty, & Tilson, 1991). Finally, the failure of THA to attenuate the spatial learning deficits produced by intraseptal CDP is consistent with the finding that the spatial learning deficit produced by systemically administered CDP is not attenuated by doses of physostigmine that completely blocked the spatial learning deficit produced by scopolamine (Experiment V). Together these

results suggest that CDP impairs spatial learning independently of cholinergic systems.

Rather, the present results suggest that intraseptal infusions of CDP produce amnesia by interacting with the septohippocampal GABAergic projection (refer to Fig. 4.14). The GABAergic projection neurons originating in the medial septum are innervated by GABAergic interneurons originating in the lateral septum (Onteniente et al., 1987) and innervate GABAergic interneurons in the hippocampus (Freund & Antal, 1988; Gulyas et al., 1991). Hence, infusions of CDP into the medial septum would exacerbate inhibition of the GABAergic projection neuron, thereby disinhibiting inhibitory interneurons in the hippocampus producing a state of hyper-inhibition of principle cells. Hence, this model could account for the failure of THA to attenuate the spatial learning deficit produced by intraseptal CDP as well as the failure of intraseptal flumazenil to block the amnesic effects of systemically administered CDP (i.e., CDP acting on hippocampal neurons would still prolong recurrent inhibition). However, the finding that intra-hippocampal infusions of CDP failed to impair spatial learning (Experiment VI) suggests that the enhancement of recurrent inhibition in the hippocampus is by itself insufficient to impair spatial learning. Hence, the precise reason why intraseptal infusions of CDP impair spatial learning will require a more refined, possibly electrophysiological, investigation into the role of the septohippocampal GABAergic projection.

In sum, the present study replicated an early observation that intraseptal infusions of CDP impair spatial learning in the MWM and further revealed that this deficit is concentration dependent and flumazenil reversible, suggesting mediation via BZ receptors, and not due to lesioning or a non-specific disruption of septal activity. Intraseptal flumazenil failed to block the amnesic effects of CDP, suggesting that the medial septum is not the only site mediating the impairment. Finally, THA failed to attenuate the amnesic effects of intraseptal CDP, suggesting that the impairment is independent of cholinergic systems. A tentative model is proposed such that intraseptal CDP inhibits GABAergic projection neurons which, in turn,

disinhibit GABAergic neurons in the hippocampus, which then inhibit principle cells in the hippocampus.

CHAPTER 5: Electrophysiology

As discussed in the previous section, BZs are able to impair mnemonic processes through an interaction with BZ receptors in the medial septum. Further, the medial septum sends two major projections to the hippocampus, one cholinergic and one GABAergic (e.g., Leranth & Frotscher, 1989). The failure of THA and physostigmine to attenuate the spatial learning deficits produced by intraseptal or systemic CDP, respectively, implicates GABAergic projection neurons in the amnesic effects of BZ. The role of this septohippocampal GABAergic projection has been further elucidated using electrophysiological techniques. The following section will describe three electrophysiological phenomena, namely theta rhythm, signal amplification, and LTP, that highlight the important contribution the septum makes to hippocampal functioning.

Theta rhythm. Theta rhythm, or rhythmical slow activity (RSA), is an approximately sinusoidal extracellular recorded potential of 4-9 Hz. Theta rhythm amplitudes are maximal in the CA1 and the molecular layer of dentate gyrus. These two regions have therefore been termed theta 'generators' and are 180° out of phase with each other (Bland et al., 1975; Bland & Whishaw, 1975). These theta generators depend on the inputs of the medial septum, since lesions (Andersen et al., 1979; Green & Arduini, 1954; Sainsbury & Bland, 1981) or intraseptal infusions of a local anaesthetic (Stumpf, 1965) or GABA_A agonists (Allen & Crawford, 1984) abolish hippocampal theta rhythm. The septal influences of hippocampal theta appear to be topographically organized. Lesions of the lateral septum disrupt theta production in the CA1, but not in the dentate gyrus (Rawlins et al., 1979; Sainsbury & Bland, 1981; Stewart & Vanderwolf, 1987). Moreover, fimbria lesions abolish theta in the ventral, but not dorsal hippocampus; dorso-medial fimbria lesions abolished theta in the dorsal, but not ventral hippocampus (Rawlins et al., 1979).

The functional significance of hippocampal theta has prompted several theories, none of which appear capable of fully accounting for the data (see Bland, 1986 for review). Theta rhythm has been attributed to

arousal/attentional processes (Green & Arduini, 1954), motivation (Pond & Schwartzbaum, 1970), voluntary movement (Vanderwolf, 1969), behavioral inhibition (Gray, 1970), vibrissae movement (Komisaruk, 1970), learning and memory (Nicholas et al., 1976), and spatial map construction (O'Keefe & Nadel, 1978, p. 220). It has also been speculated that theta rhythm is the response of an inactive (idling?) hippocampus (Gray, 1982, p.195). Thus, hippocampal theta activity has eluded a unitary functional hypothesis and may reflect several different sensory-motor processes.

Hippocampal theta has been dissociated into two types, type 1 and type 2 (e.g., Vanderwolf, 1983). Type 1 theta usually has a higher frequency (7-12 Hz), is correlated with so-called 'voluntary' movements (i.e., walking/swimming), is independent of ACh (atropine/scopolamine resistant) but blocked by systemic GABA_A agonists (Gray, 1970; McNaughton & Coop, 1991; McNaughton & Sedgwick, 1978; McNaughton et al., 1986). Type 2 theta has a lower frequency (4-7 Hz), is not correlated with movement (occurring during alert immobility/arousal; see Sainsbury & Montoya, 1984), is dependent on cholinergic activity (atropine/scopolamine sensitive), and is not blocked by systemic GABA_A agonists (Barnes & Roberts, 1991; Bland et al., 1984; Kramis et al., 1975; Vanderwolf, 1975). As mentioned above, septohippocampal pathway has both cholinergic and GABAergic components (Kohler et al., 1984). It is therefore possible that the GABAergic component of the septohippocampal projection controls type 1 theta while the cholinergic component controls type 2 theta.

Glutamate/aspartate and noradrenergic systems are also involved in theta regulation. For example, the competitive NMDA receptor blocker APV blocks theta in area CA1, suggesting that glutamate, aspartate, or both are required for theta production (Leung & Desborough, 1988). This contrasts with finding that hippocampal theta is not attenuated by the noncompetitive NMDA receptor antagonist MK-801 (Whishaw & Auer, 1989). The latter discrepancy has not yet been resolved. α -noradrenergic systems also appear to participate in hippocampal theta regulation (Heynen & Sainsbury, 1991; Sainsbury & Partlo, 1991). For example, intrahippocampal injections of α_2 -agonist detomidine in urethane-anesthetized rats attenuates type 2 theta

collected from the molecular layer of the dentate gyrus, an effect blocked by the α_2 -antagonist tolazoline (Heynen & Sainsbury, 1991). Unlike intrahippocampal infusions, systemic administration of detomidine in awake rats produced type 2 theta and attenuated type 1 theta (Sainsbury & Partlo, 1991). Thus, like the septum (see above), hippocampal theta rhythm involves several different neurotransmitter systems.

Evidence suggests a relationship exists between theta and LTP in the hippocampus. Firstly, APV blocks both theta (Leung & Desborough, 1988) and the induction of LTP (Morris et al., 1986) in the hippocampus. Secondly, trains of high-frequency stimulation applied at an interval that is 'patterned' after theta frequency produces greater potentiation than shorter intervals (Larson et al., 1986). Similarly, trains of HFS applied at theta rhythm peaks induced LTP, while the same stimulation applied at the theta rhythm 'troughs' failed to induce LTP (Pavlidis et al., 1988). This has led to the speculation that exploration-induced theta induces LTP (Larson et al., 1986; Pavlidis et al., 1988). However, during immobility, when theta does not occur, evoked potentials (Brankack & Buzsaki, 1986; Leung, 1980) and LTP induction (Hargreaves et al., 1990) are facilitated. Further, theta rhythm, but not exploration-induced LTP, are abolished by medial septum lesions (Green et al., 1990). Finally, a dose of MK-801 that impaired spatial learning failed to suppress hippocampal theta (Whishaw & Auer, 1989). Thus, although the theta pattern favours LTP induction, it does not appear to be a necessary requirement.

In sum, the septohippocampal projection subserves rhythmical activity related to ongoing sensorimotor processes. The projections from the medial septum are important for theta production in the hippocampus such that lesions of the medial septum or fornix/fimbria abolish it. Theta rhythm is modulated by cholinergic, GABAergic, glutamatergic and noradrenergic systems. Theta frequency is ideally suited for the induction of LTP, although the relationship between theta and LTP requires further investigation.

Signal amplification. Although electrical stimulation of the medial septum does not by itself generate substantial field responses in the hippocampus

(Alvarez-Leefmans & Gardner-Medwin, 1975), it does enhance the responses elicited in the dentate gyrus via perforant path (PP) stimulation (Fantie & Goddard, 1982) and in CA1 pyramidal cells via commissural stimulation (Krnjevic & Ropert, 1981, 1982). Despite a similar net effect, the facilitations produced by medial septal stimulation in the CA1 and dentate gyrus appear to be mediated by different mechanisms. Facilitation in the CA1 results from ACh-induced (atropine-sensitive) inhibition of inhibitory interneurons as well as a direct ACh-induced depolarization of principle cells (Krnjevic & Ropert, 1981; Ropert & Krnjevic, 1982; Rovira et al., 1983). Facilitation in the dentate gyrus is atropine-resistant, suggesting that it is ACh independent (Fantie & Goddard, 1982; Freund & Antal, 1988; Gulyas et al., 1990, 1991; Robinson & Racine, 1986), and may depend on the GABAergic projection. The GABAergic projection innervates GABAergic interneurons in the dentate gyrus (e.g., Freund & Antal., 1988) and infusions of picrotoxin, a GABA_A antagonist, into the dentate region block the facilitating effect of septal stimulation on PP-dentate responses (Bilkey & Goddard, 1985). The latter effect may be attributed to a blockade of the GABAergic projection neuron-GABAergic interneuron synapse. Finally, medial septal conditioning pulses blocked recurrent inhibition in the dentate elicited by PP stimulation in a paired pulse task, suggesting that the recurrent inhibition mediated by GABAergic interneurons was blocked by septal stimulation (Bilkey & Goddard, 1985). Thus, septal projections to the hippocampus facilitate responses elicited by other hippocampal afferents and this effect is mediated by GABAergic processes.

Long-term potentiation. Few electrophysiological phenomenon have received as much attention in the context of information storage as long-term potentiation (LTP). LTP was first described in the rabbit hippocampus where it was demonstrated that brief (tens of milliseconds) high frequency (100 - 400 Hz) stimulation applied to the PP produced an enduring enhancement of synaptic efficacy (Bliss & Lomo, 1973). For example, a single test pulse that initially elicited a 2 mV response will, after high-frequency stimulation, produce a 6 mV response. This increase in synaptic efficacy has been shown to persist for weeks and months (Brown, Chapman, Kairiss, & Keenan, 1988) and has been dissociated from a phenomenon termed post-

tetanic potentiation which decays within minutes (1 - 10 min). LTP can be obtained in other forebrain structures but is most easily induced in the hippocampal formation (Brown et al., 1988). This is of particular importance given the role of the hippocampus in learning and memory (Milner, 1972; Zola-Morgan, Squire, & Amaral, 1986).

Another important property of LTP is that it is associative. For example, when weak and strong synapses to a neuron are stimulated at approximately the same time, the weak synapse becomes strengthened (McNaughton, Douglas, & Goddard, 1978). Associative LTP has been demonstrated between septal and entorhinal inputs to the dentate gyrus (Robinson, 1988; Robinson & Racine, 1982; see also McNaughton & Miller, 1984). For example, high-frequency stimulation of the septo-dentate pathway produces only short-term potentiation of population spikes elicited by PP stimulation. However, concurrent high-frequency stimulation of both septo-dentate pathway and PP enhanced the entorhinal-dentate population spike to levels greater than that produced by high-frequency stimulation of the PP alone; there was no comparable increase in septo-dentate LTP amplitude as a result of the combined high-frequency stimulation (Robinson & Racine, 1982). The largest increase in LTP was found when septal trains were applied less than 100 ms prior to PP trains; if septal trains followed PP trains, there are no additional increases in LTP magnitude (Robinson, 1988). Further, high-frequency stimulation of the medial septum 10 min prior to PP high-frequency stimulation suppresses LTP in the Schaffer collateral pathway (Newlon, Goldberg, & Hayes, 1991). Thus, cooperative septal stimulation increases the magnitude of LTP in the the hippocampus in a temporally specific manner.

Septal stimulation may facilitate LTP induction in the dentate gyrus by reducing GABA-mediated inhibition. Medial septal stimulation produced a disinhibitory effect in the dentate gyrus by inhibiting GABA release from local inhibitory interneurons (as described above; Bilkey & Goddard, 1985; Freund & Antal, 1988). Further, medial septal stimulation does not produce a notable signal in the dentate gyrus (Alvarez-Leefmans & Gardner-Medwin, 1975), suggesting that this pathway has a modulatory function rather than an

information processing function. Thus, medial septal stimulation reduces GABA release in the dentate gyrus, in turn increasing the excitability of granule cells to perforant path input. Such a disinhibitory mechanism receives support from the finding that homosynaptic LTP induction in the dentate gyrus is facilitated by reducing GABA_A mediated inhibition with picrotoxin (Wigstrom & Gustafsson, 1983) and blocked by enhancing inhibition with BZ drugs (Del Cerro, Jung, & Lynch, 1992).

The molecular and neurochemical properties of LTP are becoming well understood. LTP has been dissociated pharmacologically into an initial induction phase and a maintenance phase. The induction of LTP is controlled by the glutamate receptor subtype NMDA (Collingridge, Kehl, & McLennan, 1983). For example, antagonists of the NMDA receptor, such as AP5, block the induction of LTP but have little effect on LTP that has already been induced and do not impair ordinary synaptic transmission (Collingridge et al., 1983). The NMDA receptor is coupled to Ca²⁺ channel which is thought to require two signals simultaneously before it becomes permeable to Ca²⁺: glutamate must be bound to the NMDA receptor and the membrane must be sufficiently depolarized to evict a Mg²⁺ 'plug' (Brown et al., 1988). High-frequency stimulation is sufficient to evict the Mg²⁺ plug, allowing the glutamate bound to the NMDA receptor to cause the Ca²⁺ to open. If Ca²⁺ is sequestered, LTP cannot be induced (Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983; Smith, 1987; Williams & Johnston, 1989). The maintenance of LTP now appears to result from Ca²⁺-activation of protein kinases in both pre- and postsynaptic regions (Lovinger, Wong, Murakami, & Routtenberg, 1987; Lynch & Baudry, 1984). Activation of these protein kinases induces morphological alterations of the synapse (Desmond & Levy, 1983), increases the number of glutamate receptors (Baudry & Lynch, 1979) and a prolonged increase in glutamate release (Dolphin, Errington, & Bliss, 1982).

Although the properties of LTP are suggestive of a mnemonic role, it is necessary to clearly establish a link between LTP and memory storage in the behaving animal. As Bliss and Lomo (1973) stated in their seminal description of LTP: "Whether or not the intact animal makes use in real life

of a property which has been revealed by synchronous, repetitive volleys to a population of fibers the normal rate and pattern along which are unknown, is another matter" (p. 355). To date, links between LTP and learning have been provided by three lines of evidence: 1) LTP 'saturation' impairs learning, 2) LTP accompanies learning, and 3) pharmacological blockade of LTP impairs learning. Each of these links will be discussed briefly.

The saturation of LTP refers to the driving of synaptic responses to their maximal capacity (ceiling) such that any further HFS could not increase responsiveness. It is hypothesized that LTP saturation exhausts surplus synaptic plasticity, leaving no modifiable synapses to store to-be-learned information (McNaughton, Barnes, Rao, Baldwin, & Rasmussen, 1986). In an early study, LTP saturation in the PP impaired spatial learning but not spatial recall (McNaughton et al., 1986), suggesting that LTP saturation is detrimental to spatial learning but not spatial recall. Subsequent analysis has revealed that spatial learning can occur once synaptic responsiveness returns to pre-saturation levels (Castro, Silbert, McNaughton, & Barnes, 1989). Thus, the LTP saturation approach has provided support for an interaction between LTP and learning. It should be noted, however, that several attempts to replicate this effect have been unsuccessful, with maximal LTP having little effect on spatial learning (e.g., Cain, Hargreaves, Boon, & Dennison, 1992).

Perhaps the most crucial demonstration of a link between LTP and learning is the finding that LTP accompanies learning and memory. To date learning-induced LTP has been demonstrated in the hippocampus following training across a variety of behavioral tasks: 1) during sessions of classical conditioning of the nictitating membrane response (Weisz, Clark, & Thompson, 1984), 2) 4 and 24 hours after training in a passive avoidance task (Matthies, Ruethrich, Ott, Matthies, & Matthies, 1986), 3) several days after the exposure to a novel, complex environment (Sharp, McNaughton, & Barnes, 1985), and during operant conditioning (Skelton, Scarth, Wilkie, Miller, & Phillips, 1987). Hence, there does exist evidence from a variety of different learning tasks that LTP accompanies learning.

As mentioned, the induction of LTP requires the activation of NMDA receptors. Blockade of NMDA receptors with AP5 (competitive antagonist), MK-801, and ketamine (noncompetitive) are known to block the induction, but not maintenance, of LTP (e.g., Collingridge et al., 1983). Thus, it would be predicted that these drugs would also impair learning, but not *memory* processes. Indeed, continuous infusion of AP5 into the cerebroventricles was found to produce a profound impairment of spatial learning in the rat (Morris, Anderson, Lynch, & Baudry, 1986). Similarly, spatial learning is impaired by the noncompetitive NMDA receptor antagonists MK-801 and ketamine (Alessandri, Battig, & Welzl, 1989; Whishaw & Auer, 1989). Neither AP5 nor MK-801 impair *memory* or performance of a previously acquired spatial problem (Heale & Harley, 1990). Together these results suggest that preventing the activation of the NMDA receptor, which blocks the induction of LTP, impairs learning but not memory.

In sum, several important links have been made between LTP and mnemonic processes. That is, LTP saturation prevents the acquisition of new information while having little effect on previously acquired information. Further, endogenous LTP has been found to accompany learning on several learning tasks and NMDA receptor antagonists block both the induction of LTP as well as spatial learning. Together these data add strength to the notion that LTP induction is required for information to be stored in the CNS. Indeed, LTP reflects a long-term increase of synaptic efficacy that might increase the probability that a given stimulus, or stimulus configuration, in the animals environment will be detected and recognized out of a larger array. Nevertheless, if LTP is the only mechanism by which spatial information is stored in the hippocampus, then BZs, because of their effects on spatial learning, should block LTP induction.

Experiment VIII: BZs and long-term potentiation

There are several reasons why it might be thought that BZs impair mnemonic processes by blocking the induction of LTP: 1) BZs and NMDA receptor antagonists have common behavioral effects. For example, BZs impair learning but not memory in a manner comparable to NMDA receptor antagonists (Heale & Harley, 1990; McNamara & Skelton, 1991A) and NMDA

receptor antagonists possess anxiolytic properties (Bennett & Amrick, 1986; Brandao, Fontes, & Graeff, 1980; Clinschmidt, Williams, Witoslawski, Bunting, Risley, & Totaro, 1982; Sanger, Perrault, Morel, Joly, & Zivkovic, 1991). 2) Diazepam inhibits depolarization-induced release of [³H]glutamate in hippocampal slices (Baba, Okumura, Mizouo, & Iwata, 1983). 3) Maximal LTP is induced at the theta frequency (5 - 7 Hz; Larson, Wong, & Lynch, 1986; Pavlides, Greenstein, Grudman, & Winson, 1988; Staubli & Lynch, 1987) and BZs abolish the theta frequency band (Caudarella, Durkin, Galey, Jeantet, & Jaffard, 1987; McNaughton, Richardson, & Gore, 1986). 4) BZs may preempt a series of stimulations from producing maximal excitation by prolonging recurrent inhibition (Adamec, McNaughton, Racine, & Livingston, 1981; Albertson & Joy, 1989; Lee, Dunwiddie, & Hoffer, 1979; Rock & Taylor, 1986; Tsuchiya & Fukushima, 1978; Wolf & Haas, 1977). 5) GABA antagonists facilitate the induction of LTP in the hippocampus and neocortex (Artola & Singer, 1987; Wigstrom & Gustafsson, 1983). 6) Postsynaptic inhibition, induced either through the injection of hyperpolarizing current (Malinow & Miller, 1986), focal application of GABA (Scharfman & Sarvey, 1985) or commissural afferent stimulation (Douglas, Goddard, & Riives, 1982) blocks the induction of LTP in the hippocampus. 7) The induction of LTP has been correlated with reductions of GABA release (Bliss, Douglas, Errington, & Lynch, 1986) and GABA-mediated inhibition (Kanda, Maru, Ashida, Tatsuno, & Takatani, 1989; Maru, Ashida, & Tatsuno, 1989; Stelzer, Slater, & Bruggencate, 1987), although the sensitivity of receptors does not change (Scharfman & Sarvey, 1985). 8) BZ receptors are in high density in both area CA1 and dentate gyrus (Young & Kuhar, 1980). Together, these data provide strong evidence that a reduction in GABA-mediated inhibition is a critical requirement for the induction of LTP and enhancing GABA-mediated inhibition will suppress LTP induction.

To date, few studies have assessed the effects of BZs on the induction of LTP and those that have produced inconsistent results. For example, diazepam, triazolam (Del Cerro et al., 1992), and lorazepam (Brown, Riches, Cairns, & Smithson, 1988) were found to block the induction of LTP in the Schaffer collateral pathway (CA3 → CA1) in hippocampal slices. Further, diazepam (5 & 10 mg/kg) was found to suppress post-tetanic potentiation in

the Schaffer collateral pathway (10 - 30 Hz, 3 - 5 s train duration; Matthews & Connor, 1976) or in the CA1 region after tetany delivered to the medial septum of urethane-anaesthetized rats (8 stimuli at 8 Hz; Clement-Cormier, DeFrance, Divakaran, Stanley, Taber, & Marchand, 1980). However, midazolam failed to block LTP in CA1 in hippocampal slices (4 trains, 100 Hz; Birnstiel & Haas, 1991) and diazepam failed to block LTP in CA1 in urethane-anaesthetized rats (1 train of 25 stimuli at 50 Hz; Stringer & Guyenet, 1983). The effect of BZs on LTP induced in the PP -> dentate pathway in the unanaesthetized rat has not been tested. Further, in the slice studies it is difficult to determine whether the concentration of BZs is sufficient to impair mnemonic processes in the behaving animal. The present experiment sought to examine the effects of amnesic doses of CDP, diazepam, and CL 218,872 on the induction of LTP in this pathway in the awake and freely moving rat.

METHOD

Animals

Male Long-Evans hooded rats served as subjects. They were housed in pairs in shoebox cages and maintained on a 12:12 h light-dark cycle. Testing was conducted during the light phase of the cycle. Food and water were continuously available. The rats weighed approximately 400 g at the time of surgery.

Surgery

Under pentobarbital anesthesia (60 mg/kg), chronic bipolar electrodes were implanted into the dentate gyrus (-3.5 mm AP; 2.0 mm ML; ~ 4.0 mm DV from dura) for the recording of EEG and evoked potentials and into the PP (-7.9 mm AP; 4.2 mm ML; 2.4 mm DV from dura) for the delivery of single pulses and high-frequency stimulation. Recording electrodes consisted of a single strand of stainless-steel wire, 76 μm in diameter, coated with enamel to a total diameter of 114 μm . Two skull screws, connected to uninsulated stainless-steel wire, served as current-return and ground-reference electrodes. Gold-plated pins (Amphenol 220-S02) were soldered to the ends of the electrodes, inserted into a connector (McIntyre), and affixed to

the skull using dental acrylic and two additional anchoring skull screws. Both PP and dentate electrodes were implanted under electrophysiological guidance. While PP and dentate gyrus electrodes were lowered, a storage oscilloscope displayed field potentials recorded from the electrode in the dentate gyrus, evoked by single square wave pulses delivered to the PP electrode (0.1 ms, 0.2 Hz, 250 μ A). The positions and depths of the electrodes were adjusted to produce a dentate gyrus evoked potential with a population spike of a maximum amplitude and minimum threshold, on the rising edge of the EPSP.

High-frequency stimulation

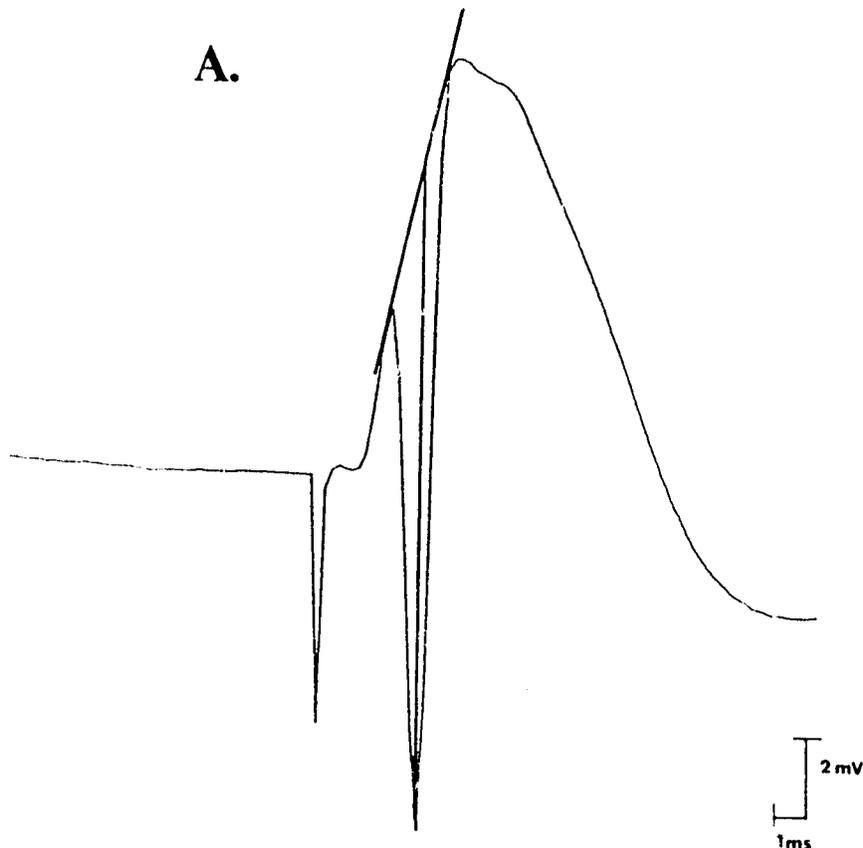
After one week recovery, individual rats were placed in a grounded Faraday cage and connected to the electronics. A series of input/output (I/O) curves were recorded with 5 passes at each of the following set intensities: 50, 100, 250, 500, 750 μ A. After two baseline I/Os were complete, and 30 min after drug administration, a third, post-drug I/O was recorded. After the post-drug I/O, 10 trains consisting of 10 pulses at 200 Hz were applied at 750 μ A. I/Os were recorded immediately, 30 min, 60 min, 24 h, and 1 week after high-frequency stimulation. Brain stimulation was controlled by, and evoked potentials were collected with, Brainwave hardware and the data were analysed using Brainwave software. Population spike amplitude was determined by fitting a line tangent to the response waveform at two points, corresponding to the onset and offset of the spike, and measuring the distance of a vertical line drawn from the tangent line to the trough (see Fig. 5.1A). For each of the sampling periods, an I/O curve was constructed for each rat by plotting the five population spike amplitudes against the five current intensities (see Fig. 5.2). Each curve was then reduced to a single number by calculating the area between it and the x-axis by using a geometric formula (Durrant, Kingston, Sharp, & Kerr, 1961).

Behavioral testing

Ten days after the tetanization, rats that received high-frequency stimulation were administered the same drug and dose administered during high-frequency stimulation and trained in the MWM. During initial acquisition, the submerged escape platform was located in the center of the

Figure 5.1: Illustration of (A) how the population spike amplitude was measured and (B) a representative evoked potential before and after high-frequency stimulation. (arrow pointing the potentiated population spike). Note that the line drawn from the trough of the population spike to the tangent in (A) would increase substantially after high-frequency stimulation in (B).

A.



B.

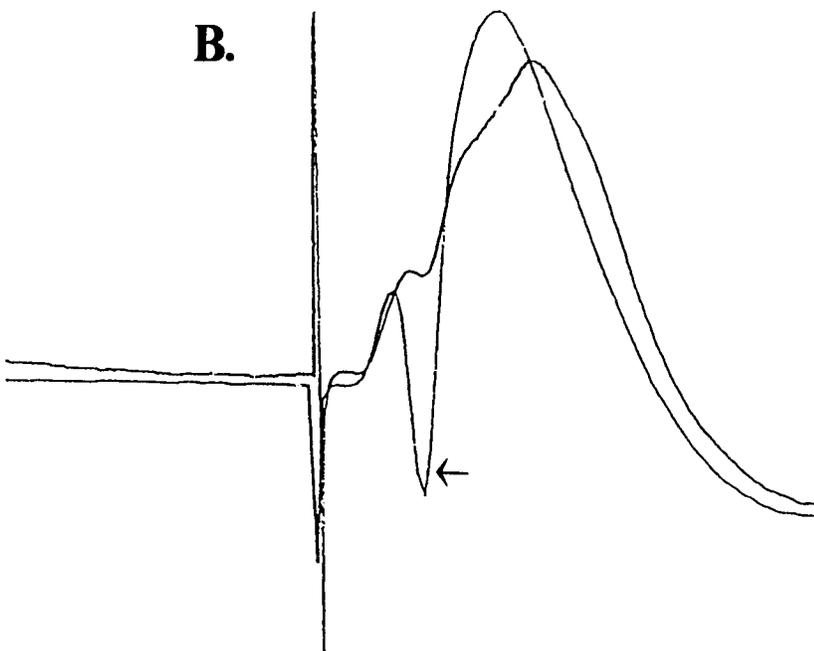
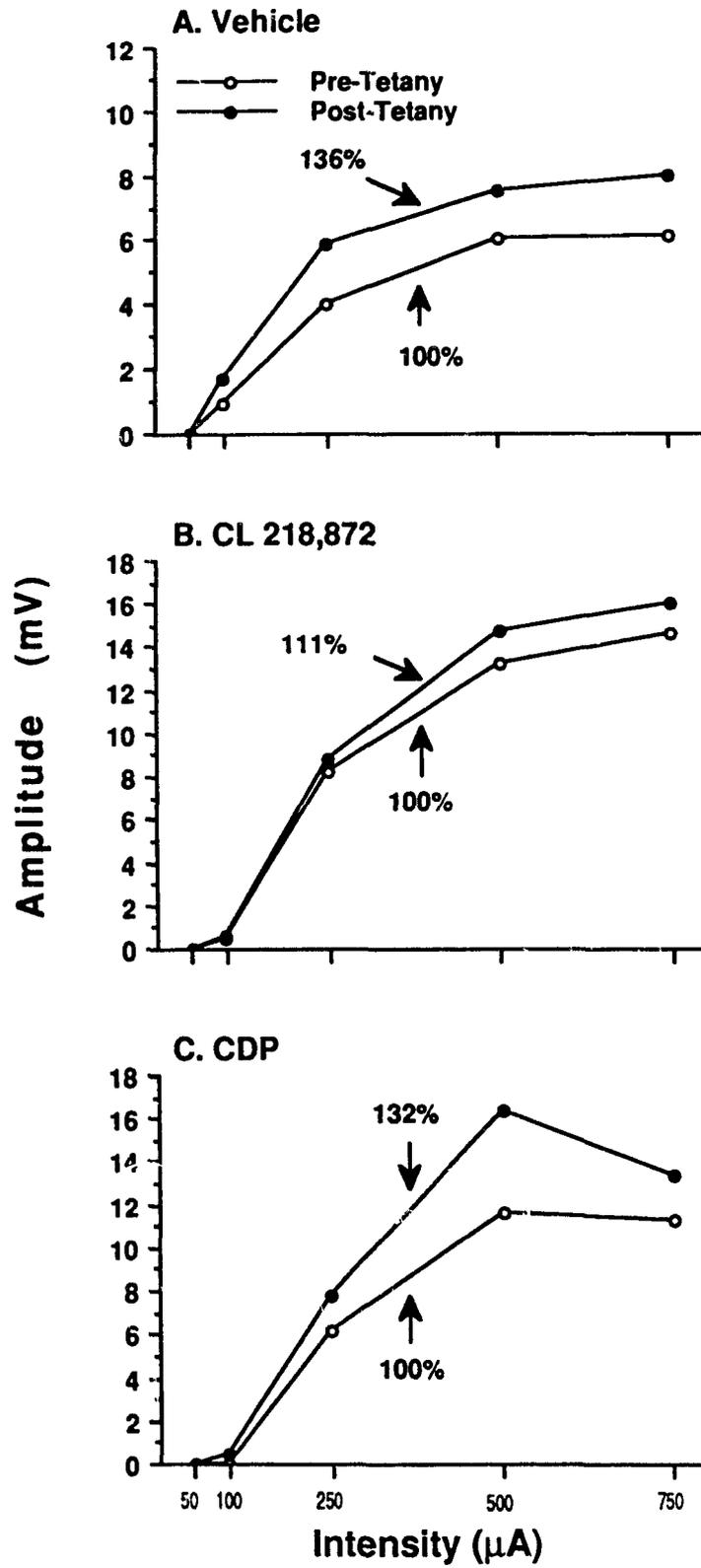


Figure 5.2: Representative input/output curves from the (A) vehicle group, (B) CL 218,872 group, and (C) chlordiazepoxide group.



northwest quadrant. Training consisted of 20 consecutive trials followed by a probe trial and visible platform training (4 trials). On the day following initial acquisition, undrugged rats were required to learn the location of the platform now located in the diagonally opposite quadrant. The latter reversal was conducted to determine if previously drugged rats could indeed demonstrate spatial learning in the absence of drugs. Rats were trained individually. During each trial, the rat's swim path length and escape latency were recorded with a video tracking system (Chromotrak Inc.). Swim speed was calculated for each trial as the distance divided by escape latency (cm/s). Once the rat located the platform, it was permitted to remain on it for 15 s. If the rat did not locate the platform within 60 s, it was guided to it and allowed to remain on it for 15 s. After each trial, the rat was returned to a holding cage positioned 90 cm under a 250 W brooding lamp (for warmth) and allowed to remain there for the 45 s intertrial interval. Total run time for 20 trials was approximately 30 min. Soon after training, rats were perfused transcardially and brains were examined histologically to determine the location of electrodes.

Drugs

Chlordiazepoxide hydrochloride (5 mg/kg; Hoffman-La Roche Inc.) was dissolved in saline (0.9% NaCl) and administered in a volume of 1 mg/ml. Diazepam (5 mg/kg; Hoffman-La Roche) and CL 218,872 (10 mg/kg; Lederle) were suspended in saline with Tween 80 (1 drop/10 ml). Control injections consisted of either saline or saline plus Tween 80 (1 drop/10 ml). All injections were administered IP 30 min prior to both high-frequency stimulation and behavioral testing.

Data analysis

Differences of I/O area, swim path lengths and swim speeds over training were assessed using an analysis of variance (ANOVA) procedure with repeated measures. Post hoc comparisons were assessed using Dunnett's test. In every case the acceptable level of statistical analysis was $p < 0.05$.

RESULTS

Electrode placements in the PP and dentate gyrus are illustrated in Figure 5.3. PP electrodes were concentrated in the angular bundle and dentate recording electrodes tended to be concentrated in the upper leaf of the dentate gyrus granule cell layer. The electrophysiological and behavioral responses of rats treated with saline and saline plus Tween 80 were very similar and their data were therefore pooled. CDP and CL 218,872 had little effect on the size of population spike prior to high-frequency stimulation. The mean post-drug population spike amplitudes (percent of baseline) were $99 \pm 3.5\%$ (saline; $n = 8$), $98.5 \pm 3.0\%$ (CDP; $n = 6$), $99 \pm 1.3\%$ (CL 218,872; $n = 6$), and $91 \pm 4.9\%$ (diazepam; $n = 6$). The degree of LTP obtained in the dentate gyrus after systemically administered saline or CL 218,872 is illustrated in Figure 5.4A. Rats treated with saline demonstrated a robust increase in the population spike amplitude that persisted for up to 24 hours. However, rats treated with CL 218,872 showed only a small increase in population spike amplitudes, the magnitude of which did not match that seen in saline controls. An overall ANOVA on the data indicated a significant treatment effect, $F(1, 13)=4.3$, $p<0.05$, time effect, $F(6,78)=13.7$, $p<0.001$, and treatment by time interaction, $F(6,78)=2.9$, $p<0.01$. During each of the sampling phases after high-frequency stimulation, the population spike amplitudes of the vehicle and CL 218,872 groups differed significantly from baseline levels ($p<0.05$). CL 218,872 also impaired spatial learning (Fig. 5.4B). Rats treated with the vehicle rapidly acquired the location of the platform while rats treated with CL 218,872 showed a severe impairment. An ANOVA in distance data revealed a significant group difference, $F(1,18)=30.3$, $p<0.001$, trial-block difference, $F(9,162)=15.2$, $p<0.001$, as well as a significant group by trial block interaction, $F(9,162)=2.2$, $p<0.05$. Performance on the probe trial confirmed CL 218,872 deficit, with rats treated with CL 218,872 failing to show a bias for the correct quadrant (Fig. 5.4C) unlike controls who did ($p<0.01$ compared to chance levels). Rats treated with CL 218,872 were not impaired on the visible platform task (data not shown). Further, rats treated with CL 218,872 during initial training acquired the reversed platform location at control levels when tested undrugged the following day ($p>0.05$; data not shown).

Figure 5.3: Representative placements of recording electrodes in the dentate gyrus (top two coronal sections) and stimulating electrodes in the perforant path (bottom sections). (closed circles = vehicle group; closed squares = drug group).

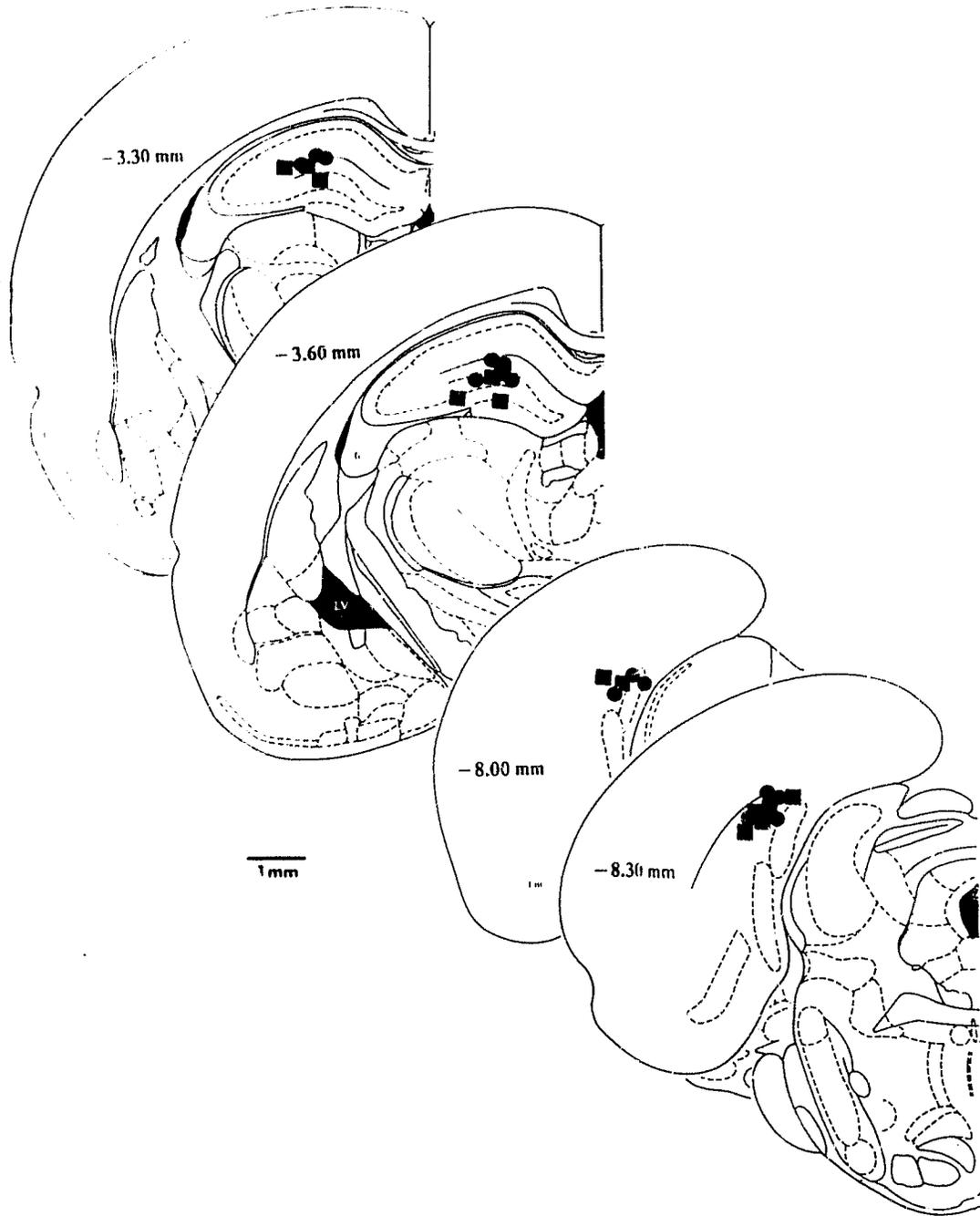
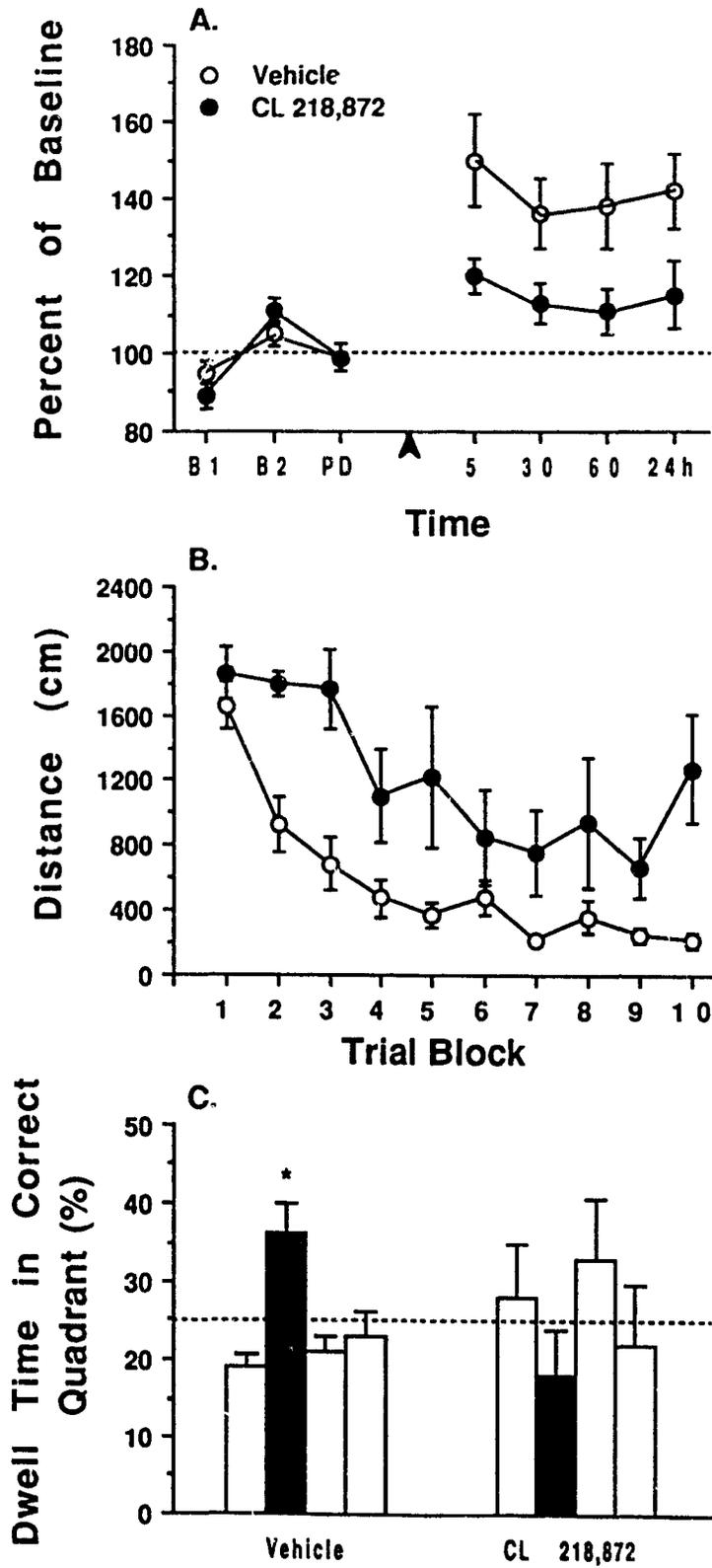


Figure 5.4: Effects of systemic CL 218,872 (10 mg/kg) on (A) LTP induction, (B) the distance taken to locate the submerged platform, and (C) performance during the probe trial. Note that CL 218,872 suppresses LTP induction and impairs spatial learning. (arrow = point at which high-frequency stimulation applied; * $p < 0.01$ compared to chance).



The LTP obtained in the dentate gyrus after systemically administered saline or CDP is illustrated in Figure 5.5A. CDP did partially suppress LTP, though the magnitude of the potentiation was not significantly different from controls. An ANOVA on population spike amplitude data revealed a significant time difference, $F(6,78)=15.2$, $p<0.001$, but not a significant group difference, $F(1,13)=1.1$, N.S., or group by time interaction, $F(6,78)=0.9$, N.S. At all sampling phases, the population spike amplitudes of the CDP group differed significantly from baseline levels ($p<0.01$). CDP did, however, impair spatial learning (Fig. 5.5B). Rats treated with CDP continued to take longer swim paths than controls over the course of training. An ANOVA on distance data revealed a significant group difference, $F(1,16)=70.8$, $p<0.001$, trial-block difference, $F(9,144)=9.5$, $p<0.001$, and group by trial-block interaction, $F(9,144)=2.3$, $p<0.01$. The probe trial confirmed the CDP impairment (Fig. 5.5C). Rats treated with vehicle, but not CDP, showed a significant bias for the correct quadrant ($p<0.01$). Rats treated with CDP performed at control levels on both the visible platform task when undrugged (data not shown) and reversal training when undrugged (data not shown).

The effects of diazepam on LTP and spatial learning are illustrated in Figure 5.6. Diazepam partially suppressed LTP, though the magnitude of the potentiation did not differ significantly from control levels. An ANOVA on population spike data failed to reveal a significant group difference, $F(1,13)=2.4$, N.S., or group by time interaction, $F(6,78)=3.1$, N.S., but there was a significant time difference, $F(6,78)=12.3$, $p<0.001$. Comparison with baseline levels revealed that the diazepam group's population spike amplitudes were significantly greater than baseline levels only over the first three sampling phases ($p<0.05$). However, the diazepam group's population spike amplitude did not differ significantly from baseline levels at the 24 h sampling phase. Rats treated with diazepam showed a spatial learning deficit, taking longer swim paths to reach the escape platform (Fig. 5.6B). This was confirmed by an ANOVA which revealed a significant group difference, $F(1,16)=40.1$, $p<0.001$, trial-block difference, $F(9,144)=10.6$, $p<0.001$, and group by trial interaction, $F(9,144)=2.3$, $p<0.01$. This impairment was confirmed during the probe trial (Fig. 5.6C) where only rats treated with vehicle showed a bias for the correct quadrant ($p<0.01$ compared to chance). Rats treated with diazepam

Figure 5.5: Effects of systemic CDP (5 mg/kg) on (A) LTP induction, (B) the distance taken to locate the submerged platform, and (C) performance during the probe trial. Note that CDP only partially suppresses LTP induction but severely impairs spatial learning. (arrow = point at which high-frequency stimulation applied; * $p < 0.01$ compared to chance).

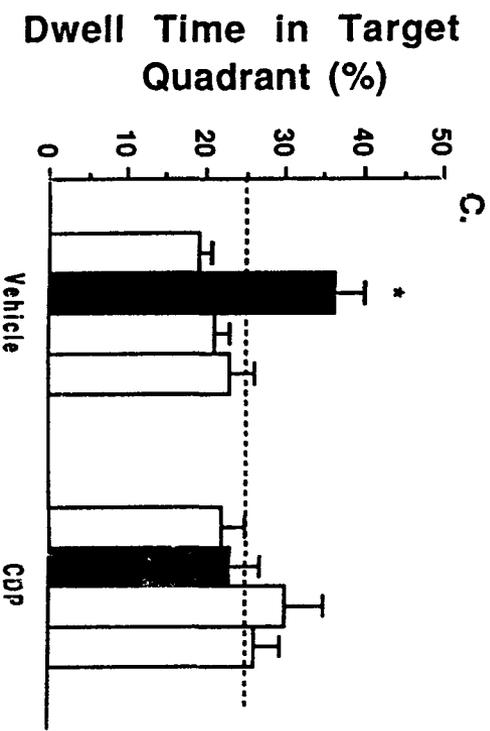
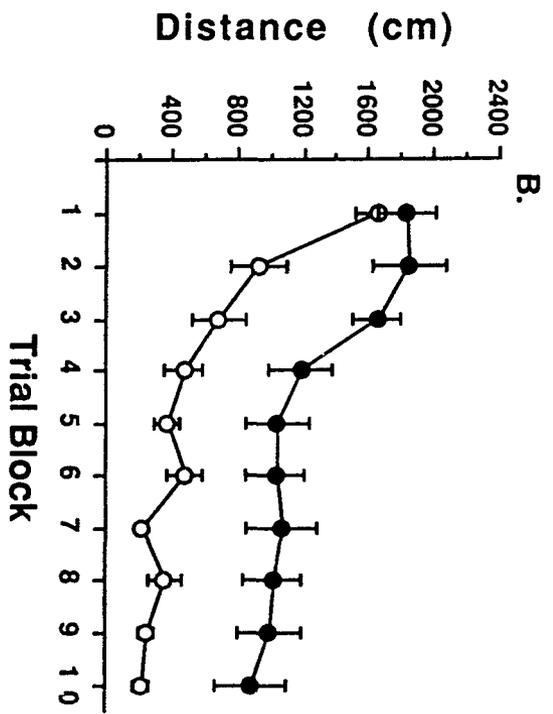
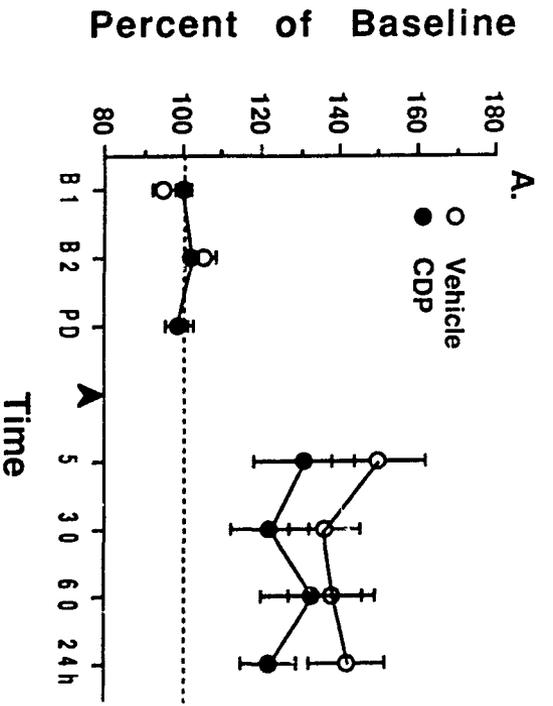
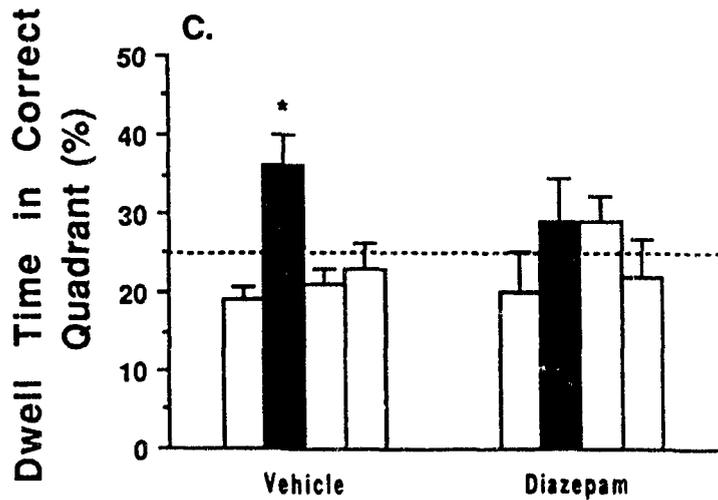
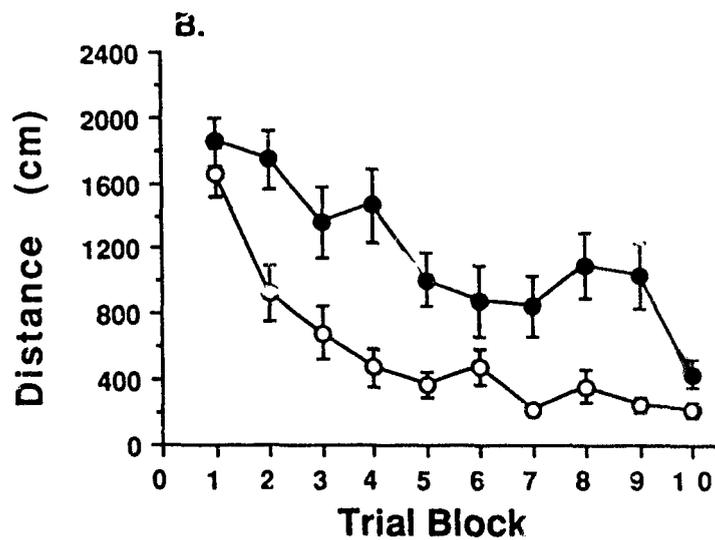
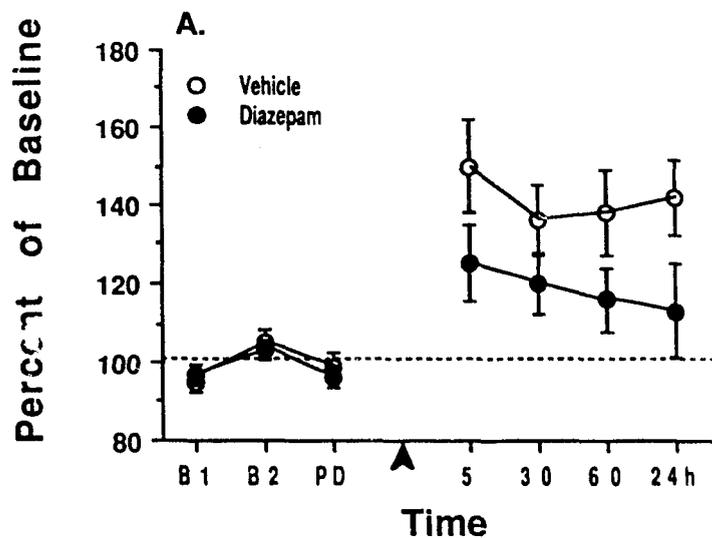


Figure 5.6: Effects of systemic diazepam (5 mg/kg) on (A) LTP induction, (B) the distance taken to locate the submerged platform, and (C) performance during the probe trial. Note that diazepam partially suppresses LTP induction but severely impairs spatial learning. (arrow = point at which high-frequency stimulation applied; * $p < 0.01$ compared to chance).



performed at control levels on both the visible platform task (data not shown), as well as reversal learning in an undrugged state (data not shown).

DISCUSSION

The present study found that acute treatment with CL 218,872, but not CDP or diazepam, significantly suppresses the induction of LTP at doses that impair spatial learning in the MWM. While rats treated with either CDP or diazepam showed potentiation only slightly lower than that observed in rats treated with the control vehicle, rats treated with CL 218,872 showed only minimal potentiation. Rats treated with any of the drugs failed to show evidence of spatial learning over the course of training, as evidenced by the failure of a quadrant bias during the probe trial. This latter finding suggests that drug-treated rats adopted an inefficient response strategy such as swimming in large circles at a certain distance from the pool wall. The absence of an impairment on the visible platform task further suggests that drug-treated rats are motivated to escape from the water as well as capable of swimming in a coordinated manner. Additionally, when these rats were undrugged they could acquire the reversed platform location at rates comparable to controls, suggesting that the impairment observed during initial acquisition was not due to electrodes or the effects of high-frequency stimulation interacting with the drug. Hence, the drug doses tested in the present study reversibly impair spatial memory in the absence of sensorimotor disruption.

The finding that CL 218,872, a triazolopyridazine with a selective affinity for the BZ ω_1 receptor, impairs both LTP induction as well as spatial learning suggests that PP-DG LTP is required for spatial learning. This finding provides support for the notion that LTP is related in some manner with spatial learning (Morris et al., 1986). However, both CDP and diazepam impaired spatial learning without significantly affecting LTP, indicating that spatial learning deficits can occur in the absence of PP-DG LTP suppression. Moreover, it was recently shown that AP5, an NMDA receptor blocker, failed to prevent spatial learning at a concentration that completely blocked LTP induction in the PP (Davies, Butcher, & Morris, 1992). Together, these

findings suggest that LTP suppression in the PP-DG is not a prerequisite for a spatial learning impairment in the MWM.

The failure of BZs to block LTP in the hippocampus contrasts with previous reports (Brown et al., 1988; Del Cerro et al., 1992) but is consistent with other reports in which neither midazolam (hippocampal slice; Birnstiel & Haas, 1991) nor diazepam (anaesthetized rats; Stringer & Guyenet, 1983) blocked LTP-induction. This discrepancy does not appear to be related to pathway stimulated, with BZs failing to block LTP in both the Schaffer collateral pathway (Birnstiel & Haas, 1991) and the PP (present findings). However, Mathews and Connor (1976) found that systemic diazepam dose-dependently facilitated post-tetanic potentiation in the PP while suppressing it in the Schaffer collateral pathway. Moreover, differences in drug concentration and high-frequency stimulation parameters may also contribute to the discrepancy.

The reason why CL 218,872 suppressed LTP and CDP and diazepam did not is unknown. CDP and diazepam bind with a similar affinity to both ω_1 and ω_2 receptor subtypes, suggesting that selective ω_1 activation by CL 218,872 is somehow distributed differently from the ω_2 receptor. One possibility is that in the dentate gyrus, the ω_1 receptor is located exclusively postsynaptic while the ω_2 receptor is located both pre- and postsynaptically. Presynaptic GABA_B receptors have been identified on GABAergic terminals and reduce GABA release when activated (Baumann, Wicki, Stierlin, & Waldmeier, 1990). Moreover, selective activation of GABA_B receptors facilitates LTP in the dentate gyrus (Mott, Lewis, Ferrari, Wilson, & Swartzwelder, 1990). Thus, it seems possible that if CDP and diazepam stimulated the presynaptic ω_2 receptor, which in some manner activates the GABA_B receptor, then GABA release would be reduced and their GABA-enhancing effects at the postsynaptic receptor (ω_1 & ω_2) would be neutralized. However, selective activation of the postsynaptic ω_1 receptor by CL 218,872 would augment only the postsynaptic actions of GABA. Although this account of the different effects of CDP, diazepam, and CL 218,872 on LTP is highly speculative, it does accommodate the findings of the present study. However, since CDP, diazepam, and CL 218,872 impair spatial learning in a similar manner, it may

be that CDP and diazepam impair spatial learning by suppressing LTP in other regions of the hippocampus (e.g., CA1; De' Cerro et al., 1992).

In sum, the present experiment found that CL 218,872, a selective BZ ω_1 receptor agonist, suppressed, while CDP and diazepam had little effect on, the induction of LTP in the PP-DG pathway. The same doses tested on LTP were also found, in the same rat, to severely impair spatial learning in the MWM. Together these findings suggest that suppression of PP-DG LTP is not required to produce a spatial learning deficit in the MWM. Further, these results suggest that triazolopiridazine CL 218,872 and the classic BZs impair spatial learning by different mechanisms.

CHAPTER 6: General summary and discussion

There can be little doubt that since the introduction of BZs onto the sedative/anxiolytic market, they have had a great impact on both clinical and experimental communities. Indeed, BZs are among the most widely prescribed drug in the world. Chapter 1 reviewed the evidence that BZs have amnesic properties and the clinical implications of this property. Briefly, in humans, BZs selectively impair the transfer of information from short-term memory to long-term storage; BZs do not impair immediate recall (short-term memory) or the recall of information that has already been acquired. Moreover, BZs selectively impair the encoding of declarative (e.g., factual information) while having little effect on the acquisition of procedural skills. In animal studies, a similar pattern of impairment is manifested when the animal is required to acquire spatial information (egocentric space). In the MWM, for example, BZs selectively impair new acquisition while having little effect on the use of previously acquired spatial information. Moreover, BZs do not impair the development and use of response strategies to locate the platform (praxis strategy) or the ability to navigate to a single cue (taxon strategy). In short, BZs produce anterograde amnesia in both humans and animals (see Cole, 1986 for a review) and the MWM is a useful tool for further expanding our understanding of how information is stored in the CNS.

Also in Chapter 1, the neurochemical basis of BZ actions was reviewed. BZs produce their effects through an interaction with the inhibitory neurotransmitter GABA. BZs allosterically modulate the postsynaptic GABA_A-Cl⁻ ionophore complex to increase the affinity of GABA_A receptors for GABA, increasing the frequency that GABA opens the Cl⁻ ionophore. The net effect of this enhancement of GABA by BZs is to increase the duration of IPSPs. Conversely, BZ inverse-agonists decrease the affinity of GABA_A receptors for GABA, decreasing the frequency that GABA opens the Cl⁻ ionophore thereby reducing IPSP durations. Finally, BZ receptor antagonists do not affect the affinity of the GABA_A receptor for GABA, but rather occupy the BZ receptor, preventing its activation by either BZ drugs or endozepines.

In Chapter 2, the role of endogenous BZ ligands in spatial learning was assessed, as was the contribution of the BZ receptor ω_1 subtype. In Experiment I, flumazenil and CGS 8216, two BZ receptor antagonists, enhanced spatial learning in the MWM, suggesting that an endogenous BZ ligand (e.g., endozepine-2) is activated during testing in the water maze which prevents optimal learning. Further, the enhancing effect of β -carboline, an inverse-agonist with a selective affinity for the BZ ω_1 receptor subtype (Braestrup & Nielson, 1981), suggests that directly reducing the inhibitory actions of GABA benefits the encoding process. Together, the results of Experiment I confirm that endogenous BZs are released during stressful (anxiety provoking) situations in quantities sufficient to influence mnemonic processes. In Experiment II, the BZ ω_1 receptor subtype was further implicated in spatial learning by the finding that CL 218,872, a selective agonist at the ω_1 receptor (Lippa et al., 1979), impaired spatial learning to the same degree as diazepam, which interacts with both ω_1 and ω_2 receptors equally (Benavides et al., 1988). Importantly, the spatial learning deficits produced by both CL 218,872 and diazepam were blocked by flumazenil, indicating that BZ receptors mediate their amnesic effects. In sum, the amnesic actions of BZ drugs are mediated by the same endogenous receptors that mediate the modulatory action of endozepines. These receptors are heterogeneous and differentially distributed through out the CNS.

Chapter 3 revealed that several neurotransmitter systems are important for spatial learning in the MWM. Since GABA interacts with all of these neurotransmitter systems, it is possible that BZs exert their amnesic actions by interacting with other neurotransmitter systems, such as the opioids or ACh. In Experiment III, it was found that morphine impaired spatial learning in the MWM, though the effect was attenuated by increasing escape motivation. Morphine also impaired cue learning and spatial recall. Thus, although opioidergic activation is detrimental to spatial learning, the pattern of impairment produced by morphine is indicative more of a motivational deficit than a pure amnesic effect. Because BZs (Wuster et al., 1980), as well as the cold pool water (Barta & Yashpal, 1981), increase opioidergic activity,

Experiment IV assessed the possibility that BZs impair spatial learning by increasing opioidergic activity. The results of Experiment IV showed that BZs impair spatial learning independently of opioidergic systems. For example, BZs and morphine produce dissimilar impairment patterns; BZs do not impair performance or cue learning, and naloxone, at doses that block the spatial learning deficit produced by morphine, do not attenuate the spatial learning deficit produced by BZs.

A second neurotransmitter system that is regulated by GABA and influenced by BZs is ACh. In the MWM, ACh hypofunction is detrimental to spatial learning and biochemical studies have revealed that BZs reduce ACh activity (Sarter et al., 1990). However, Experiment V revealed that enhancing ACh activity does not attenuate the spatial learning deficit produced by BZs, even at doses that completely reverse the spatial learning deficit produced by scopolamine, a muscarinic ACh receptor antagonist. It was further shown that ACh systems interact with BZ systems in some manner to impair spatial learning; doses of flumazenil that are effective in blocking the spatial learning deficit produced by BZs also attenuate the spatial learning deficit produced by scopolamine. This finding suggests that ACh receptor blockade by scopolamine is partially due to an interaction with BZ receptors. The above results suggest that BZs do not impair spatial learning by reducing ACh activity, a notion recently corroborated by Moore, Bernston, Sarter, and Bruno (1991) who found that systemically administered CDP had little effect on ACh release in the frontal cortex using *in vivo* microdialysis. Moreover, the patterns of impairment produced by scopolamine and CDP are different; scopolamine impaired cue learning (Experiment VII) suggesting that ACh blockade produces profound sensorimotor impairments that are not shared by CDP.

As described in Chapter 4, BZ receptors are located throughout the CNS, though their densities differ between regions (e.g., Young & Kuhar, 1979). Importantly, the highest densities of BZ receptors are located in regions implicated in learning and memory processes. For example, BZ receptors are in high density in septum and hippocampus and lesions of these regions impair spatial learning in the MWM (Hagen et al., 1988; Morris et al. 1982).

Therefore, it seems likely that BZs impair spatial learning by interacting with receptors in these regions. In Experiment VI it was found that direct infusions of BZs into the medial septum, but not into the frontal cortex, NBM, amygdala, or cerebellum, impairs spatial learning. Infusions of CDP into the hippocampus also failed to impair spatial learning. In Experiment VII, it was further revealed that intraseptal infusions of CDP impaired spatial learning in a dose-dependent and flumazenil reversible manner. Additionally, increasing ACh activity with THA failed to attenuate the deficit produced by intraseptal infusions of BZs, suggesting that reductions of the septohippocampal ACh projection did not mediate this effect. The septum does not, however, appear to be the only region mediating the amnesic effects of BZs because infusions of flumazenil into the septum did not block the amnesic effects of systemically administered BZs.

In Chapter 5, the effect of BZs on LTP, a synaptic model of information storage, was assessed. LTP is believed to subserve associative learning because: 1) it possesses the characteristics of a mnemonic device, it has associative properties and persists for long durations, 2) pharmacological blockade of LTP induction (Morris et al., 1986) or the saturation of surplus plasticity (Castro et al., 1989) in the hippocampus impairs spatial learning, 3) endogenously produced LTP accompanies some forms of learning (Skelton et al., 1987). Hence, it appears that the induction of hippocampal LTP is a prerequisite for spatial learning. In Experiment VIII, it was found that CL 218,872, an ω_1 receptor agonist, suppressed LTP at a dose that impaired spatial learning. However, CDP and diazepam failed to affect LTP at doses that impair spatial learning in the MWM. Together, these findings provide only partial support for the notion that LTP in the PP is related to information storage processes.

In summary, the findings of this thesis offer a reasonably clear picture of how BZs impair spatial learning. For example, BZ drugs, as well as endozepines, interact with BZ receptors, possibly just the ω_1 subtype, located in the medial septum. Activation of these BZ receptors inhibits the septohippocampal GABAergic projection, disinhibiting inhibitory interneurons and exaggerating inhibition of principle neurons in the

hippocampus. Note that intrahippocampal infusions of CDP would not be expected to produce comparable inhibition since the interneurons in the hippocampus would themselves be inhibited by CDP. The exaggerated hippocampal inhibition would hamper normal hippocampal activity, such as theta rhythm, and spatial learning would be compromised. The failure of CDP or diazepam to impair LTP induction at doses that impair spatial learning suggests that LTP in PP-DG synapses does not subservise spatial learning, though LTP in other areas of the hippocampus (CA1) may mediate BZ-induced spatial learning deficits. This notion is corroborated by the finding that reductions of GABA-mediated inhibition with BZ receptor inverse-agonists enhance both LTP magnitude in CA1 (Sorensen, Zwolshen, & Humphreys, 1990) as well as spatial learning (Experiment I). Overall, the findings presented in this thesis advance our current knowledge of how BZs impair mnemonic processes and suggest that the modulation of GABA-mediated inhibition is an important component mediating information storage. These findings also add to the growing body of evidence implicating, firstly, the septohippocampal system in spatial learning processes and, secondly, the septohippocampal system as the region mediating the amnesic actions of BZs. Finally, the findings of this thesis are testimony to the utility of the MWM as an important tool for deciphering the neurochemical substrates of spatial learning in the rat.

An issue of central importance is the relationship between anxiety and learning and memory processes. While moderate levels of anxiety may be required to motivate acquisition, high levels of anxiety can be detrimental (Eysenck, 1979). Since BZs are potent anxiolytics, it seems reasonable to attribute their amnesic actions to reductions of anxiety. However, three findings from this thesis strongly suggest that the anxiolytic and amnesic effects of BZs are distinct processes: 1) BZs do not impair escape to a visible platform, suggesting the rat is motivated to escape from the water, 2) there is a poor relationship between anxiogenic and memory enhancing effects of BZ agonists and inverse-agonists (Fig. 2.6), and 3) the anxiolytic and amnesic actions of BZs were dissociated neuroanatomically (Table III). Hence, reduced anxiety and anterograde amnesia are independent consequences of BZ drug action.

In addition to providing insight into the mechanisms subserving information storage in the CNS, understanding how BZs impair information storage also has several clinical implications. The widespread use of BZ drugs for various illnesses may be more problematic than therapeutic. This is particularly true for the elderly. The elderly are the greatest consumers of BZs (Balter et al., 1984), despite an enhanced sensitivity to their amnesic effects (Pomora et al., 1984). Similarly, aged rats demonstrate mnemonic deficits at doses below that required to impair memory in young rats (Komiskey, Cook, Lin, & Hayton, 1981). Biochemical analysis revealed that the increased sensitivity found in aged rats is not due to alterations on BZ receptor binding affinity or total receptor number but, rather, to a reduction of pharmacokinetic processes (decreased elimination half-lives; Barnhill, Greenblatt, Miller, Gaver, Harmatz, & Shader, 1990). Therefore, for any given dose of BZ, plasma levels will remain higher for longer a period of time in the aged. Although BZ and GABA_A receptors do not decrease with age, there is a loss of NMDA receptor binding in various regions including the hippocampus (Wenk, Walker, Price, & Cork, 1991), as well as significant reductions of cortical and striatal ACh activity (Gallagher, Burwell, Kodsi, McKinney, Southerland, Vella-Rountree, & Lewis, 1990). The cognitive decline associated with glutamate and ACh reductions may be exacerbated by BZs. For example, BZs have been reported to inhibit glutamate release (Baba et al., 1983) and ACh activity (Phillis, Siemans, & Wu, 1980) in cortical tissues. Therefore, drugs that reduce GABA-mediated inhibition, disinhibiting glutamate and ACh release, may be of some therapeutic value. Indeed, preliminary evidence has revealed that the BZ receptor inverse-agonist, ZK 93 426, significantly increases ACh release in aged rat frontal cortex (Moore et al., 1991) and picrotoxin, a GABA_Aergic antagonist, was found to enhance mnemonic processes in aged rats (Flood & Morley, 1990). Hence, blockade of BZ/GABA-mediated inhibition may prove to be an important remedy for age-related cognitive decline. However, caution should be noted given the potential for these GABA-antagonists to elicit seizures.

The senile dementia associated with Alzheimer's disease, unlike that associated with aging (as above), has been linked to reductions of GABA-mediated inhibition. For example, there is a significant reduction of presynaptic GABA terminals (Hardy, Cowburn, Barton, Reynolds, Dodd, Wester, O'Carroll, Lofdahl, & Winblad, 1987) and postsynaptic GABA_A receptors (Chu, Penny, & Young, 1987) in the cerebral cortex and hippocampus of Alzheimer's patients. Further, there is a significant reduction of GABA in the CSF of Alzheimer's patients (Zimmer, Teelken, Trieling, Weber, Weihmer, & Lauter, 1984). Thus, it is possible that a loss of GABA-mediated inhibition in the cortex and hippocampus of Alzheimer's patients accelerates cholinergic and glutamatergic cell death in a manner analagous to the excitotoxicity induced by excitatory amino acids (Rothman & Olney, 1987). Therefore, early diagnosis and treatment with GABA-enhancing agents, like diazepam, may actually reduce the neural pathology and severe dementia associated with the latter stages of Alzheimer's disease (e.g., Hyman, Van Hoesen, Damasio, & Barnes, 1984). By this reasoning, it would be counter-productive to administer Alzheimer's patients BZ receptor inverse-agonists, a strategy that has recently been proposed (Sarter, Schneider, & Stephens, 1988). Thus, unlike the hyperinhibition proposed to impair mnemonic processes in the aged, hypoinhibition may in fact precipitate the neural pathology and ensuing cognitive decline associated with dementia of the Alzheimer's type.

There is little doubt that the GABA-mediated inhibition is a critical element in the balance of neuronal excitation and inhibition. The findings presented in this thesis provide some insight into how manipulations of GABA-mediated inhibition can affect information storage processes. Further enquiry into the neurochemical, neuroanatomical, and electrophysiological mechanisms responsible for information storage will undoubtedly lead to both a greater understanding of how the brain functions in general as well as treatments for pathologies associated with the loss of this most valuable cognitive capacity. Indeed, the increasing popularity of so called "smart drugs" may reflect a future role of pharmaceuticals as important tools for influencing human cognition.

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