Molecular Neurobiology

ISSN0893-7648/92/6(4): 377–386/$2.00

Benzodiazepines in the Brain

Their Origin and Possible Biological Roles

J. H. Medina,[1,*] C. Peña,[2] M. Piva,[2] C. Wolfman,[1] M. L. de Stein,[1] C. Wasowski,[2] C. Da Cunha,[3] I. Izquierdo,[3] and A. C. Paladini[2]

[1]Instituto de Biología Celular, Fac. Medicina, Buenos Aires, Argentina; [2]IQUIFIB, Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina; and [3]Centro de Memoria, Departamento de Bioquímica, Instituto de Biociencias, UFRGS, Porto Alegre, Brazil

Contents

Abstract
Benzodiazepines in the CNS
Origin of Natural Brain BDZs
  Exogenous Origin of Natural BDZs
  Endogenous Origin of Natural BDZs
Biological Role of Natural BDZs
  Natural BDZs and Pathophysiology of Hepatic Encephalopathy
  Modulation of Memory Storage by $GABA_A$/BDZR Mechanisms
  Regional Changes of BDZ-Like Molecules After Habituation and Avoidance Learning
  Effect of Localized Intracerebral Injections of Flumazenil on Memory
  Anxiety, Stress, and $GABA_A$/BDZ-R–Mediated Memory Modulation
Acknowledgment
References

*Author to whom all correspondence and reprint requests should be addressed.

Abstract

Great progress has been made in the last 5 yr in demonstrating the presence of benzodiazepines (BDZs) in mammalian tissues, in beginning studies on the origin of these natural compounds, and in elucidating their possible biological roles. Many unanswered questions remain regarding the sources and biosynthetic pathways responsible for the presence of BDZs in brain and their different physiological and/or biochemical actions. This essay will focus on recent findings supporting that: (1) BDZs are of natural origin; (2) mammalian brain contains BDZs in concentrations ranging between 5.10^{-10}–$10^{-8}M$; (3) dietary source of BDZs might be a plausible explanation for their occurrence in animal tissues, including man; (4) the formation of BDZ-like molecules in brain is a possibility, experimentally supported; (5) BDZ-like molecules including diazepam and *N*-desmethyldiazepam are elevated in hepatic encephalopathy; and (6) natural BDZs in the brain are involved in the modulation of memory processes. Future studies using the full range of biochemical, physiological, behavioral, and molecular biological techniques available to the neuroscientist will hopefully continue to yield exciting and new information concerning the biological roles that BDZs might play in the normal and pathological functioning of the brain.

Index Entries: Brain benzodiazepines; endogenous/exogenous origin; memory processes; consolidation; flumazenil.

Benzodiazepines in the CNS

During the last 15 yr, considerable efforts have been made to elucidate the nature of endogenous ligand(s) for the central-type benzodiazepine receptor (BDZ-R) (De Robertis et al., 1988). In a novel approach to identify this elusive molecule(s), De Blas and colleagues raised monoclonal antibodies (MAb) to BDZ, and used one of them to fractionate by immunoaffinity chromatography extracts from bovine brain (Sangameswaran and De Blas, 1985). A low-mol-wt substance that specifically interacts with the BDZ-R was isolated and identified as *N*-desmethyldiazepam (Sangameswaran et al., 1986). In addition, these authors were able to detect BDZ-like immunoreactivity in human brains stored since 1940. This date has important implications, because the first chemical synthesis of a BDZ was performed in the late 1950s and the first BDZ, chlordiazepoxide, was introduced to the market in 1960.

During the last 4 yr several groups have confirmed and expanded the aforementioned findings (Wildmann, 1989; Medina, 1990; Basile, 1991; Klotz, 1991). For instance, using conventional methods of extraction and purification of small molecules, we were able to purify to homogeneity three BDZ-like molecules from bovine and rat brain. One of these substances was identified as diazepam. Subcellular fractionation of rat cerebral cortex revealed that BDZ-like molecules are mainly located in synaptic vesicles and synaptosomal cytosol (Medina et al., 1988).

Confirming the original finding of De Blas and colleagues, (Sangameswaran et al. 1986), it has been recently demonstrated the occurrence of diazepam in human brains stored from 1957 to 1959 (Unseld et al., 1989a). By using a sensitive gas chromatography/mass spectrometry method with a stable isotope dilution technique, Unseld et al. (1989a) have found that the concentration of diazepam ranges from 0.15 to 0.34 ng/g wet wt according to the neural structure analyzed. Because of the fact that these levels are much lower than those achieved in brain after the administration of a pharmacologically effective dose of diazepam (3–30 ng/g wet tissue), it has been emphasized that the clinical significance of the presence of BDZs in brain is uncertain. However, it has to be kept in mind that the possibility that BDZs might be unevenly distributed in dif-

ferent neural structures (De Blas and Sotelo, 1987; Medina et al., 1988; Medina, 1990) and that this kind of compartmentalization could perhaps increase several-fold the levels of BDZs at a specific locus in the neuronal membrane, for example, the BDZ-R. In fact, recent studies from our laboratory revealed that BDZ-like molecules are unevenly distributed in brain, the highest concentration being detected in the septum, amygdala, and hippocampus (Basile et al., 1990a; Wolfman et al., 1991).

An important consequence of the aforementioned findings is that they provide strong evidence against environmental pollution by synthetic BDZs and its subsequent intake by mammals. In this context, our recent results showing the presence of different BDZs, including diazepam, in milk from drug-free women (Medina et al., 1990; Peña et al., 1991) and the occurrence of lorazepam and other BDZs in plasma and brain of BDZ-free rodents (Wildman et al., 1987; Izquierdo et al., 1990a; Wolfman et al., 1991) further support the hypothesis that BDZs are natural products whose origin needs to be elucidated.

Until now, several BDZs, or incompletely characterized BDZ-like molecules, were found in brain, peripheral organs, plasma, and cerebrospinal fluid (CSF) of BDZ-free humans and animals. The amounts of BDZ found in biological tissues and fluids varied between 0.01 ng/g wet frog brain to 600 ng/g wet bovine brain. However, an average value of 1–10 ng/g wet tissue was found in brains of drug-free rats and cattle by three different research groups (Basile et al., 1989; Basile et al., 1990b; Medina et al., 1988; Unseld et al., 1989a; Wildmann, 1989).

Origin of Natural Brain BDZs

Since it seems well established that BDZs are naturally occurring compounds present in several mammalian tissues, an important question to be answered is their origin (Basile et al., 1990b; De Robertis et al., 1988; Wolfman et al., 1991). On discussing this point, two main hypotheses, that are not mutually exclusive must be considered: BDZs are exogenous compounds biosynthesized by microorganisms and/or plants and are incorporated in mammals via the food chain; and BDZs are totally or partially biosynthesized endogenously in mammalian tissues.

Exogenous Origin of Natural BDZs

This hypothesis is supported by several recent studies showing the presence of BDZs in cow milk (Medina et al., 1988), grains (Unseld et al., 1989b; Wildmann et al., 1988), vegetables (Medina et al., 1989; Unseld et al., 1989b; Wildmann et al., 1988), egg white (Unseld et al., 1989b), and in several plants, some of them used as tranquilizers or sedatives in folkloric medicine (Medina et al., 1989). The majority of BDZs found in vegetable products used as foods by several animals and humans possess chemical structures identical to those of synthetic BDZs used in the current therapy of insomnia, epilepsy, and anxiety. Others are novel since they are not metabolites of BDZ drugs in use or alternatively, are identical to BDZs never introduced to the market (Wildmann et al., 1988).

The amount of pharmacologically active BDZs contained in the diet ranges from 10–20 ng/Kg of wheat to 10–20 μg/Kg of potato tuber (Wildmann et al., 1988) or 0.5–2 μg/L of milk (Medina et al., 1988). Hence, the pool of BDZs from those sources seems to be well below the pharmacologically active levels reached after an oral dose of 1–20 mg of BDZs. However, since BDZs have a low clearance rate (giving rise to half-life times greater than 100 h), the chronic intake of trace amounts of these natural compounds could lead to their accumulation in the brain, thus providing a dietary influence on brain function and behavior (De Robertis et al., 1988).

The BDZs contained in plants could be totally synthesized by the vegetable cells or through the action of the ever-present contaminating microorganisms. In support of the first hypothesis,

Wildmann et al. (1988) has found that in wheat grains and potato tuber there is a five- to eight-fold increase in the content of BDZs after 6 d of germination. These indirect evidences indicate that biosynthesis of BDZs by plants might occur and favors the idea that natural BDZs are of vegetable origin. Another evidence supporting the formation of BDZs in plants is based on their existence in potato marrow, which is considered to be sterile (Wildmann et al., 1988).

It is well known that the 1,4-benzodiazepine nucleus is synthesized by certain microorganisms such as *Penicillium cyclopium*. This fungus produces an antibiotic, called cyclopeptine, with a BDZ structure, from L-phenylalanine, L-methionin, and the tryptophan metabolite anthranilic acid (Luckner, 1984; Gerlach et al., 1985). However, we have found that cyclopeptine does not interact with BDZ-R (unpublished observations). In fact, it is generally accepted that electronegative substitution of C-7 and/or C-2' and C-5 phenyl substitution of 1,4-BDZ are prerequisites for high-affinity BDZ receptor binding. Not any 1,4-BDZ synthesized by microorganisms is known to fulfill one of these criteria.

In spite of these results and based on the occurrence of BDZs in bovine brain and milk, we decided to carry out a series of in vivo and in vitro experiments using bovine rumen as a biosynthetic reactor, where the putative BDZ precursors could be provided by the ingested plants. The rationale of these experiments is mainly based on two findings from our laboratory: BDZs are present in the ruminal content; and at least two particular forages (*Festuca arundinacea* and *Trifolium repens*) contain nondetectable or ten times lower amounts of BDZ—like molecules than those observed in the ruminal content a few hours after their ingestion (Medina et al., 1991).

The relevant findings in our study can be summarized as follows: The in vitro incubation of ruminal microorganisms with the abovementioned forages during 4 h in anaerobic conditions produced a ninefold increase in the initial content of BDZ-like molecules. Three new BDZ-like substances appeared during incubation. Purification of these compounds by high-pressure liquid chromatography (HPLC) yielded homogenous peaks that share physicochemical and immunological properties with BDZs. In addition, an in vivo experiment in a cow with a rumen fistula gave similar results. Before feeding, the concentration of BDZ-like molecules in an almost emptied rumen was 0.07 ng/g of ruminal content. After free grazing on *Festuca* and *Trifolium* for 4–10 h, the values increased up to eleven times (0.65–0.78 mg/g of ruminal content). This increase is mainly the result of an enhanced production of existing BDZ-like molecules. However, modifications of existing less active BDZ-like molecules or "demasking" of BDZ-conjugates by ruminal enzymes cannot be ruled out. These findings indicate that BDZ-like molecules are synthesizeable by ruminal microorganisms. The presence of BDZs in brain and milk may simply reflect the absorption and distribution through the body of these newly formed molecules.

Endogenous Origin of Natural BDZs

The hypothetical endogenous origin of natural BDZs present in mammalian brains is suggested by two different pieces of evidence: BDZ-like molecules were detected in the neuroblastoma of glioma cell line NG 108-15 even after these cells were grown for 3 mo in a serum-free medium (Blas et al., 1987). Also, the incubation of rat brain or cerebral cortex homogenates, or slices, during 2–4 h at 37°C with a nutritive BDZ-free medium, increased five to nine times the basal content of BDZ-like molecules (Table 1). One of these substances, which on reversed-phase HPLC on C_{18} and C_4 columns eluted just before diazepam, inhibited the binding of ^3H flunitrazepam to BDZ-R and the specific antibenzodiazepine MAb 21-7F9. It had a low-mol wt (< 1000) as determined by gel permeation HPLC, but the low amount of purified compound precluded its chemical identification (Piva et al., 1991). The basal levels of BDZ-like compounds found in control experiments (rat brain incubated at 0°C,

Table 1
Levels of BDZ-Like Molecules
in HPLC Eluates of Control Rat Brain or Cerebral Cortex Homogenates

Tissue	*n*	DE ng/g wet wt	%
Control	14	0.13 ± 0.02	100
Incubated brain	7	0.6 ± 0.10[b]	460
Inncubated cerebral cortex	4	0.89 ± 0.12[b]	680
Incubated cortical slices	3	0.6 ± 0.15[b]	460

[a]After incubation at 37°C during 2 h in BDZ-free GIBCO Dulbecco's modified Eagle's medium.

[b]$p < 0.001$ Student's *t*-test. Data are expressed as mean ±SEM. DE = diazepam equivalents in ng based on extrapolation from standard displacement curves of ^{3}H FNZ binding to MAb 21-7F9 generated using diazepam.

nonincubated brain, use of preboiled brain, or use of hepatic tissue instead of brain) were remarkably low and constant: 0.13 ± 0.02 ng diazepam equivalents/g of wet wt tissue (± SEM; $n = 14$).

So far, the possible synthesis of BDZs by mammalian tissues still remains a problem, but one has to recall the similar case of the plant alkaloid morphine, which has been found in brain and milk, and that enzymatic systems able to synthesize morphine and codeine were demonstrated in mammalian tissues (Weitz et al., 1987). Our recent findings strongly favors the argument of a dual origin of brain BDZs. A neuronal or glial biosynthetic pathway would exist for BDZs, and these molecules would be ingested directly with food or indirectly through the action of enteric flora. Therefore, exogenous BDZs could regulate their biosynthesis to keep a constant endogenous pool (Piva et al., 1991).

Biological Role of Natural BDZs

Natural BDZs and Pathophysiology of Hepatic Encephalopathy

Based on recent electrophysiological, neurochemical, and behavioral evidences, it has been postulated that brain BDZ-like compounds are probably involved in the pathogenesis of hepatic encephalopathy (HE) (Basile et al., 1989; Basile et al., 1990a,b; Basile et al., 1991; Olasmaa et al., 1990). Using rat and rabbit models of this disease, these authors have clearly demonstrated that brains of animals with HE contained five to ten times more BDZ-like molecules than control animals (Olasmaa et al., 1990; Basile et al., 1991). Furthermore, analysis of human brain, CSF, and serum from patients suffering from HE revealed a six- to tenfold increase in the content of BDZ-like molecules in comparison with control samples (Olasmaa et al., 1990; Basile et al., 1991). Two of the purified peaks were positively identified as diazepam and *N*-desmethyldiazepam (Basile et al., 1991). In conclusion, the enhancement of GABAergic neurotransmission observed in HE is probably caused by the increase in the levels of naturally occurring BDZ-like molecules, suggesting that these compounds could be behaviorally relevant and may contribute to the pathogenesis of this syndrome.

Modulation of Memory Storage by $GABA_A$/BDZ-R Mechanisms

Recent studies from our laboratories suggest that brain BDZs could play a modulatory role in memory processes (Izquierdo et al., 1990a,b; Izquierdo et al., 1991; Wolfman et al., 1991). The earliest evidence for GABA/BDZ receptor-mediated regulation of memory storage came from experiments published 30 yr ago showing

retrograde memory facilitation by picrotoxin, a blocker of the Cl^- channel activated by GABA, and anterograde amnesia caused by diazepam and other BDZ (Breen and McGaugh, 1961; Randall et al., 1961). A great body of evidence has recently emerged on the regulatory role of the GABA/BDZ-R complex in learning and memory (Brioni et al., 1989; Da Cunha et al., 1991a,b,c; File and Pellow, 1988; Izquierdo, 1989; Izquierdo et al., 1990a,b,c; Izquierdo and Medina, 1991; Lister, 1985; McGaugh, 1988,1989; McGaugh et al., 1990; Pereira et al., 1989; Thiebot, 1985; Wolfman et al., 1991).

The BDZ-R antagonist flumazenil has behavioral effects of its own that, depending on the behavioral test or measure used (Izquierdo et al., 1990a,b; Lal et al., 1988; Pereira et al., 1989), may be viewed as being opposite to those of BDZs or inverse agonists of BDZ-R such as β-carbolines (Lal et al., 1988; Pereira et al., 1989). Before the discovery of endogenous brain BDZs and other putative ligands for BDZ-R (De Robertis et al., 1988), it was speculated that flumazemil might have an intrinsic action (File and Pellow, 1986). Today, the most parsimonious explanation of the effects of flumazenil on its own is that it antagonizes either endogenous agonist or inverse agonist ligands of BDZR (Izquierdo et al., 1990a,b; Lal et al., 1988; Pereira et al., 1989).

Low, nonanxiogenic doses of flumazenil (5 mg/kg or less, ip) given prior to training enhance retention only to mildly stressful or anxiogenic behaviors such as active avoidance (Lal et al., 1988), inhibitory avoidance (Izquierdo et al., 1990c; Pereira et al., 1989), habituation to a buzzer (Pereira et al., 1989), and spatial learning in a water tank (Brioni et al., 1991). On the other hand, systemic flumazenil did not affect retention of other less stressful tasks (Izquierdo et al., 1990c). These experiments lent support to the hypothesis that flumazenil enhances retention by antagonizing the influence of endogenous BDZ-like agonists on brain $GABA_A$/BDZ-R complex. As will be seen later, data obtained using intracerebral flumazenil microinjections strongly support this contention (Izquierdo et al., 1990a,b; Wolfman et al., 1991).

Regional Changes of BDZ-Like Molecules After Habituation and Avoidance Learning

The amygdala is 100 times more sensitive to the amnesic effect of the $GABA_A$ agonist muscimol when it is injected posttraining than when it is given pretraining (Da Cunha et al., 1991a; Izquierdo et al., 1990a). This suggests that $GABA_A$/BDZ-R in the amygdala becomes sensitized during acquisition. When this information is put together with that on the effect of systemically administered flumazenil, mentioned above, it suggests that brain BDZs are released during and/or very shortly after training. This led us to measure the effect of training procedures on brain BDZs levels in several neural structures including the amygdala (Izquierdo et al., 1990a).

The simple, free exploration of a novel, restricted environment during 1 min, a procedure that caused habituation (Izquierdo et al., 1990b; Wolfman et al., 1991), was followed by a 20–40% decrease in the content of BDZ-like molecules in the cortex, amygdala, and medial septum. Inhibitory avoidance training in the same apparatus was followed by a more pronounced decrease of BDZ-like molecules level (60–90%) in the cortex, amygdala, medial septum, and hippocampus but not in the cerebellum (Wolfman et al., 1991).

The depletions are best explained by release of BDZs contained in synaptic vesicles (Medina et al., 1988), followed by rapid diffusion (Izquierdo et al., 1990b). The possible biosynthesis of BDZs (Medina et al., 1991) and/or the catabolism of these compounds take from minutes to hours; thus, a sudden inhibition of BDZ synthesis or catabolism can be ruled out as a factor in the regional BDZ-like molecules depletions caused by training. Therefore, it can be concluded that inhibitory avoidance training is accompanied by some degree of BDZ release in amygdala, septum, and cortex during acquisition and by a much greater release both in these structures and in the hippocampus immediately after acqui-sition, e.g., at the time of consolidation (Izquierdo et al., 1990a) (Fig. 1). In this context, it

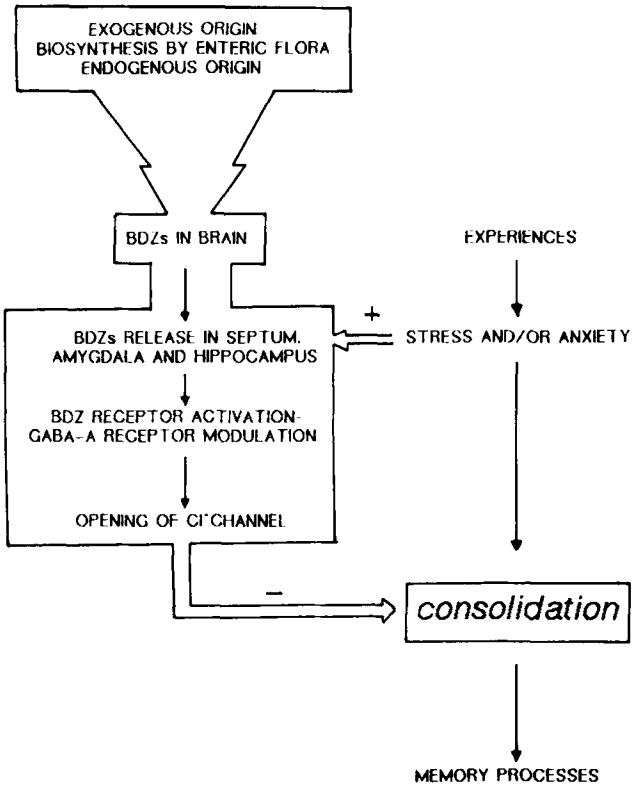

Fig. 1. Learning modulates the consolidation of memory through the influence of the level of stress and/or anxiety. Anxiety could release benzodiazepine-like compounds in several neural structures. By acting on BDZ-R, these compounds enhance $GABA_A$ receptor-mediated opening of Cl^- channels and downregulates memory consolidation.

is important to mention that an acute swim-stress provokes the release of BDZ-like compounds (Trullas et al., 1987).

Effect of Localized Intracerebral Injections of Flumazenil on Memory

Having thus obtained evidence that a differential regional release of brain BDZs occurs during and after two different forms of training, we decided to study the effect of immediate posttraining bilateral injection of flumazenil (5 nmol on each side) into the rat amygdala, hippocampus, and medial septum on the retention of habituation to the inhibitory avoidance box and of step-down inhibitory avoidance acquired in the same apparatus. The microinjection of flumazenil into the three structures enhanced retention of the avoidance task, but only intrahippocampal flumazenil administration enhanced retention of habituation to exploration of the training apparatus (Da Cunha et al., 1991c; Wolfman et al., 1991).

These effects of flumazenil on the inhibitory avoidance task correlated well with the training-induced decline of 60–90% observed in the content of BDZ-like molecules in the three structures (Wolfman et al., 1991), suggesting that flumazenil exerts its action by antagonizing BDZs released during and/or after training in the amygdala, hippocampus, and medial septum. Taken together, our recent findings strengthen the hypothesis that posttraining storage processes are normally downregulated by a $GABA_A$/BDZ-R–mediated mechanism in these structures, that it is modulated by brain BDZs, and that flumazenil facilitates memory through an influence on these mechanisms.

Anxiety, Stress, and $GABA_A$/BDZ-R–Mediated Memory Modulation

The $GABA_A$/BDZ mechanisms in the amygdala and septum downregulate the retention of inhibitory avoidance; in the hippocampus, these mechanisms are also involved in the modulation of memory processes of habituation to a novel environment (Fig. 1). The amygdala and medial septum are involved in the processing of attending or aversive behaviors (Cahill and McGaugh, 1990; Gray, 1982), and the hippocampus is involved in behaviors requiring the analysis of spatial cues and/or working memory (Izquierdo, 1989). In addition, it has been suggested that the medial septum and hippocampus are related to the perception of or reaction to anxiety (Gray, 1982), which may be superimposed to the role they have in memory modulation. It is tempting to suggest that BDZs released in the hippocampus appear to downregulate posttraining

memory processing of habituation to a novel environment and avoidance training, and that BDZs released in the septum and amygdala appear to be involved only in the modulation of consolidation of shock-motivated and more stressful avoidance tasks.

It is likely that different amounts of BDZs are released in various brain regions in response to different levels of anxiety or stress. The release was larger after the more stressful avoidance procedure than after the less stressful habituation task in all structures. Thus, it is reasonable to think that brain BDZ release may represent a simple, general response of the amygdala, septum, and hippocampus to anxiety and/or stress, proportional to the level of anxiety and/or stress (Fig. 1).

It is important to mention that a certain degree of arousal, anxiety, or stress is thought to be necessary for learning and memory (McGaugh, 1988,1989; Izquierdo, 1989) and that too much anxiety or stress hinders learning in animals and in humans. Furthermore, it has been claimed that memory modulation depends on the analysis by limbic structures of the emotional "content" or anxiogenic value of the experiences (Squire, 1987; Izquierdo, 1989). Then, the modulation of mem-ory storage processes by brain $GABA_A$/BDZ mechanisms in different brain regions would be a by-product of the perception of or reaction to diverse levels of anxiety or stress.

Acknowledgment

We would like to thank A. De Blas for kindly providing the antibenzodiazepine MAb 21-7F9.

References

Basile A. S. (1991) The contribution of endogenous benzodiazepine receptor ligands to the pathogenesis of hepatic encephalopathy. *Synapse* **7**, 141–150.

Basile A. S., Gammal S. H., Jones E. A., and Skolnick P. (1989) Characterization of the GABA-benzodiazepine receptor supramolecular complex in thioacetamide-treated rats: evidence for the presence of endogenous ligands for the benzodiazepine receptor in hepatic encephalopathy. *J. Neurochem.* **53**, 1057–1063.

Basile A. S., Jones E., and Skolnick P. (1991) The pathogenesis and treatment of hepatic encephalopathy: evidence for the involvement of of benzodiazepine receptor ligands. *Pharmacol. Rev.* **43**, 28–71.

Basile A. S., Ostrowski N. L., Gammal S. H., Jones E. A., and Skolnick P. (1990a) The $GABA_A$ receptor complex in hepatic encephalopathy: autoradiographic evidence for the presence of elevated levels of a benzodiazepine receptor ligand. *Neuropsychopharmacology* **3**, 61–71.

Basile A. S., Pannell L., Jaouni T., Gammal S., Fales H. M., Jones E., and Skolnick P. (1990b) Brain concentration of benzodiazepines are elevated in an animal model of hepatic encephalopathy. *Proc. Natl. Acad. Sci. USA* **87**, 563–567.

Breen R. A. and McGaugh J. L. (1961) Facilitation of maze learning with posterial injection of picrotoxin. *J. Comp. Physiol. Psychol.* **54**, 498–501.

Brioni J. D., Arolfo M. P., Jerusalinsky D., Medina J. H., and Izquierdo I. (1991) The effect of flumazenil on acquisition, retention and retrieval of spatial information. *Behav. Neural. Biol.* **56**, 329–335.

Brioni J. D., Nagahawa A., and McGaugh J. L. (1989) Involvement of the amygdala GABAergic system in the modulation of memory storage. *Brain Res.* **96**, 505–510.

Cahill L. and McGaugh J. L. (1990) Amygdaloid complex lesions differentially affect retention of tasks using appetitive and aversive reinforcement. *Behav. Neurosci.* **104**, 532–543.

Da Cunha C., De Paula L., Medina J. H., and Izquierdo I. (1991a) The amnesic effect of intraamygdala muscimol administration. *Com. Biol.* **9**, 219–225.

Da Cunha C., Huang C. H., Walz R., Dias M., Koya R., Bianchin M., Pereira M. E., Izquierdo I., and Medina J. H. (1991b) Memory facilitation by post-training intraperitoneal, intraventricular and intraamygdala injection of R0 5–4864. *Brain Res.* **544**, 133–136.

Da Cunha C., Wolfman C., Huang C. H., Walz R., Koya R., Bianchin M., Medina J. H., and Izquierdo

I. (1991c) Effect of posttraining injection of flumazenil into the amygdala, hippocampus and septum on retention of habituation and of inhibitory avoidance in rats. *Braz. J. Med. Biol. Res.* **23,** 301–306.

De Blas A. L., Park, D., and Friedrich, P. (1987) Endogenous benzodiazepine-like molecules in the human, rat and bovine brains studied with a monoclonal antibody to benzodiazepines. *Brain Res.* **413,** 275–284.

De Blas A. L. and Sotelo C. (1987) Localization of benzodiazepine-like molecules in the rat brain. A light and electron microscopy immunocytochemistry study with an anti-benzodiazepine monoclonal antibody. *Brain Res.* **413,** 285–296.

De Robertis E., Peña C., Paladini A. C., and Medina J. H. (1988) New developments on the search for the endogenous ligand(s) of central benzodiazepine receptors. *Neurochem. Int.* **13,** 1–11.

File S. E. and Pellow S. (1988) Intrinsic action of the benzodiazepine receptor antagonist R015-1788. *Psychopharmacology* **88,** 1–11.

File S. E. and Pellow S. (1988) Low and high doses of benzodiazepine receptor inverse agonists respectively improve and impair performance in passive avoidance but do not affect habituation. *Behav. Brain Res.* **30,** 31–36.

Gerlach M., Schwelle N., Lerbs W., and Luckner M. (1985) Enzymatic synthesis of cyclopeptine intermediates in Penicillium cyclopium. *Phytochemistry* **24,** 1935–1939.

Gray J. A. (1982) *The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-Hippocampal System.* Clarendon, Oxford, pp.

Izquierdo I. (1989) Different forms of posttraining memory processing. *Behav. Neural. Biol.* **51,** 171–202.

Izquierdo I., Da Cunha C., Huang C.H., Walz R., Wolfman C., and Medina J. H. (1990a) Posttraining down-regulation of memory consolidation by a $GABA_A$ mechanism in the amygdala modulated by endogenous benzodiazepines. *Behav. Neural. Biol.* **54,** 105–109.

Izquierdo I., Da Cunha C., and Medina J. H. (1990b) Endogenous benzodiazepine modulation of memory processes. *Neurosci. Biobehav. Res.* **14,** 419–424.

Izquierdo I., Pereira M. E., and Medina J. H. (1990c) Benzodiazepine ligand influences on acquisition: suggestion of an endogenous modulatory mechanism mediated by benzodiazepine receptors. *Behav. Neural. Biol.* **54,** 27–41.

Izquierdo I. and Medina J. H. (1991) Brain Benzodiazepine/$GABA_A$ modulation of memory. *TIPS* **12,** 260–265.

Izquierdo I., Medina J. H., Da Cunha C., Jerusalinsky D., Wolfman C., and Ferreira M.B.C. (1991) Memory modulation by brain benzodiazepines. *Braz. J. Med. Biol. Res.* **24,** 865–882.

Klotz U. (1991) Occurrence of "natural" benzodiazepines. *Life Sci.* **48,** 673–682.

Lal H., Kumar B., and Forster M. J. (1988) Enhancement of learning in mice by a benzodiazepine antagonist. *FASEB J.* **2,** 2707–2711.

Lister R. G. (1985) The amnesic action of benzodiazepines in man. *Neurosci. Biobehav. Rev.* **9,** 87–94.

Luckner M. (1984) *Secondary Metabolism in Microorganisms, Plants and Animals,* 2d ed., Springer, Berlin, pp. 272–276.

McGaugh J. L. (1988) Modulation of memory storage processes, *Perspectives of Memory Research,* Solamin P. R., Goethals G. R., Kelley C. M., and Stephens B. R., eds., Springer, New York, pp. 33–64.

McGaugh J. L. (1989) Dissociating learning and performance: drug and hormone enhancement of memory storage. *Brain Res. Bull.* **23,** 339–345.

McGaugh J. L., Castellano C., and Brioni J. D. (1990) Picrotoxin enhances latent extinction of conditioned fear. *Behav. Neurosci.* **104,** 264–267.

Medina J. H. (1990) Natural benzodiazepines in the brain; possible biological roles. *Com. Biol.* **8,** 217–234.

Medina J. H., Danelon J. L., Wasowski C., Levi de Stein M., and Paladini A. C. (1991) Production of benzodiazepine-like compounds in bovine rumen. *Biochem Biophys. Res. Comm.* **181,** 1048–1055.

Medina J. H., Peña C., Levi de Stein M., Wolfman C., and Paladini A. C. (1989) Benzodiazepine-like molecules, as well as other ligands for the brain benzodiazepine receptors, are relatively common constituents of plants. *Biochem. Biophys. Res. Comm.* **165,** 547–553.

Medina J. H., Peña C., Piva M., Paladini .C., and De Robertis E. (1988) Presence of benzodiazepine-like molecules in mammalian brain and milk. *Biochem. Biophys. Res. Comm.* **152,** 534–539.

Medina J. H., Peña C., Piva M., Wasowski C., Levi de Stein M., Wolfman C., and Paladini A. C. (1992) Evidences for a dual origin of naturally occurring

benzodiazepines in the mammalian brain, *Proc. Biol. Psych.*, G. Racagni G., ed., Elsevier, in press.

Medina J. H., Peña C., Rubio M., Wikinski S. and Paladini A. C. (1990) Benzodiazepine-like molecules in human milk. *Lancet* **336,** 1379.

Olasmaa M., Rothstein J. D., Guidotti A., Weber R. J., Paul S. M., Spector S., Zeneroli M. L., Baraldi M., and Costa E. (1990) Endogenous benzodiazepine receptor ligands in human and animal hepatic encephalopathy. *J. Neurochem.* **55,** 2015–2023.

Peña C., Medina J. H., Piva M., Diaz L. E., Dimilowicz C., and Paladini A. C. (1991) Naturally-occurring benzodiazepines in human milk. *Biochem. Biophys. Res. Comm.* **175,** 1042–1050.

Pereira M. E., Izquierdo I., and Medina J. H. (1989) Effect of pretraining flumazenil administration on the acquisition of three different tasks in rats. *Braz. J. Med. Biol. Res.* **22,** 1501–1505.

Piva M., Medina J. H., De Blas A. L., and Peña C. (1991) Formation of Benzodiazepine-like molcules in rat brain. *Biochem. Biophys. Res. Comm.* **180,** 972–981.

Randall L. O., Heise G. A., Schallek W., Bagdon R. E., Banziger R. F., Boris A., Moe R. A., and Abrams W. E. (1961) Pharmacological and clinical studies on valium; a new psychotherapeutic agent of the benzodiazepine class. *Current Res.* **3,** 405–425.

Sangameswaran L. and De Blas A. L. (1985) Demonstration of benzodiazepine-like molecules in the mammalian brain with monoclonal antibody to benzodiazepines. *Proc. Natl. Acad. Sci. USA* **82,** 5560–5564.

Sangameswaran L., Fales H. M., Friedrich P., and De Blas A. L. (1986) Purification of a benzodiazepine from bovine brain and detection of benzodiazepine-like immunoreactivity in human brain. *Proc. Natl. Acad. Sci. USA* **83,** 9236–9240.

Squire L. R. (1987) *Memory and Brain*, Oxford Univesity Press, Oxford, UK.

Thiebot M. H. (1985) Some evidence for amnesic-like effects of benzodiazepines in animals. *Neurosci. Biobehav. Rev.* **9,** 95–100.

Trulles R., Havoundjian H., and Skolnick P. (1987) Stress induced changes in t-[^{35}S] butyl-bicyclophosphorothionate binding to -aminobutyric acid-gated chloride channels are mimicked by in vitro occupation of benziazepine receptors. *J. Neurochem.* **49,** 968–974.

Unseld E., Fischer C., Rothemud E., and Klotz U. (1989a) Occurance of "natural" diazepam in human brain. *Biochem. Pharmacol.* **39,** 210-212.

Unseld E., Krishaa D. R., Fischer C., and Klotz U. (1989b) Detection of desmethyldiazepam and diazepam in brain of different species and plants. *Biochem. Pharmacol.* **38,** 2473-2478.

Weitz C. J., Faull K. F., and Goldstein A. (1987) Synthesis of the skeleton of the morphine molecule by mammalian liver. *Nature* **330,** 674–677.

Wildmann J. (1989) Heterocycles as physiological ligands for the benzodiazepine receptor and for other binding sites. *Pharmacol. Res.* **21,** 673-682.

Wildmann J., Möhler H., Vetter W., Ranalder U., Schmidt K., and Maurer R. (1987) Diazepam and N-desmethyldiazepam are found in rat brain and adrenal and may be of plant origin. *J. Neural. Transm.* **70,** 383–398.

Wildmann J., Vetter W., Ranalder U., Schmidt K., Maurer R., and Möhler H. (1988) Occurence of pharmcological active benzodiazepines in trace amounts in wheat and potato. Biochem. Pharmacol. **37,** 3549–3559.

Wolfman C., Da Cunha C., Jerusalinsky D., Levi de Stein M., Viola H., Izquierdo I., and Medina J. H. (1991) Habituation and inhibitory avoidance training alter brain regional levels of benzodiazepine-like molecules and are affected by intracerebral flumazenil microinjections. *Brain Res.* **584,** 74–80.