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Chronic Administration of Flumazenil Increases Life Span and Protects Rats From Age-Related Loss of Cognitive Functions: A Benzodiazepine/GABAergic Hypothesis of Brain Aging

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MARCZYNSKI, T. J., J. ARTWOHL AND B. MARCZYNSKA. *Chronic administration of flumazenil increases life span and protects rats from age-related loss of cognitive functions: A benzodiazepine/GABAergic hypothesis of brain aging.* NEUROBIOL AGING **15**(1) 69–84, 1994.—Under barrier condition and with ad lib access to food and water, 20 Fischer-344 rats were chronically treated for 10 months with the benzodiazepine (BDZ) antagonist, flumazenil (FL; 4 mg/kg/day in drinking water acidified to pH = 3.0), beginning at the age of 13 months, while the group of 20 control age-matched rats received plain acidified water. The life span of the first 8 deceased rats treated with FL was significantly longer than that of the first 8 deceased rats in the age-matched control group. In tests for spontaneous ambulation and exploratory behavior in the Holeboard apparatus, conducted during the 3rd and the 8th month of treatment, the FL group, relative to controls, had significantly higher scores for the ambulation and exploratory behavior. In tests for unrewarded spontaneous alternation in the T maze, conducted at days 7, 39, 42, and 47 through 54 after drug withdrawal, i.e., at the age of 24–25 months, the FL-exposed group, compared to age-matched controls, showed a significantly higher percent of alternating choices, a behavior that was statistically comparable to that of the "young" 6-month-old controls. In the Radial Maze tests conducted 2 months after drug withdrawal, the FL group made significantly less "working memory" errors and "reference memory" errors, relative to the age-matched 25-month-old control group, a performance that was comparable to that of the young 7-month-old control group. In conclusion, chronic FL significantly protected rats from age-related loss of cognitive functions. It is postulated that the age-related alterations in brain function may be attributable to the negative metabolic/trophic influences of the "endogenous" benzodiazepine (BDZ) ligands and/or those ingested with food. A BDZ/GABAergic hypothesis of brain aging has been formulated which assumes that age-related and abnormally strong BDZ/GABAergic influences promote neurodegeneration by suppressing trophic functions of the aminergic and peptidergic neurons through opening of chloride channels in soma membrane and axon terminals, causing excessive hyperpolarizing and depolarizing inhibition, respectively. The review of human clinical and animal data indicates that FL has nootropic actions by enhancing vigilance cognitive and habituation processes.

Flumazenil Aging Rats Radial maze T maze Working memory Reference memory

IN MAMMALS, factors that influence the time-course of brain aging are poorly understood. One of the most influential hypothesis of the etiopathogenesis of premature aging of the brain, including presenile dementia or Alzheimer's disease (AD), postulates that the degenerative processes do not begin in the forebrain where they are most conspicuous but are triggered by dysfunction and/or degeneration of the brainstem ascending aminergic system (BAAS) whose main function is to regulate metabolic processes in the forebrain (10,43,103). The BAAS is composed of neurons containing norepinephrine and the Vasoactive Intestinal Peptide (VIP; 65), serotonin and dopamine, and this system appears to be indispensable for metabolic and trophic glial-neuronal relationships (43). Such a function is implied by the morphology of aminergic axons and their terminals which, in most instances, do not form true synaptic junctions but show "varicosities" from which the amines (and the co-localized peptides) reach their targets by diffusion (20).

In early stages of AD, attempts at correlating the forebrain

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degenerative changes with those in the BAAS neurons have been only partially successful (92,134). Moreover, in Fischer-344 rats between the age of 12 through 32 months, the numbers of neurons in the locus coeruleus do not change (34), despite that at the age of 17–20 months Fischer rats develop significant cognitive and memory deficits, as measured by acquisition and retention of passive avoidance behavior (60). These observations cast doubts on the heuristic value of the BAAS degeneration hypothesis of brain aging. Hence, we hypothesize that it is the age-related and/or abnormally strong benzodiazepine/gamma-amino butyric acid (BDZ/GABA) - induced postsynaptic (hyperpolarizing) and presynaptic (depolarizing) inhibition of the BAAS and peptidergic neurons that is responsible for promoting degenerative changes in the forebrain. Because the distinction between AD and the "physiologic" aging of the brain is essentially quantitative rather than qualitative (111), our hypothesis encompasses AD and predicts that chronic disinhibitory modulation of the BAAS and the peptidergic system will retard or forestall the progress of AD.

The BDZ receptor agonists which have been found in mammalian brain, once regarded as exogenous, are now believed to be "endogenous," as they are normally present in mammalian brain even in subjects who have never been treated with BDZ drugs (8,48). The source of the endogenous BDZ agonist ligands, such as diazepam, N-desmethyldiazepam and oxazepam, may be the food stuffs and/or BDZ compounds synthesized de novo by intestinal flora (8,48). The levels of these BDZ ligands may increase with diet rich in milk, soybeans, and meat, particularly in humans and animals with cirrhotic liver damage; this condition may elevate the levels of BDZ ligands to a degree resulting in hepatic encephalopathy and coma which is most effectively reversed by IV or oral administration of the BDZ receptor antagonist, flumazenil (FL; see ref. 8.)

Physiologic concentrations of endogenous BDZ-receptor ligands in mammalian brain have a potentiating action on the postsynaptic (hyperpolarizing) inhibition elicited by GABA acting on the BDZ-$GABA_A$-(chloride ionophore) receptor complex (8, 17,115,132); this may be revealed by disinhibition of neuronal activity following local microinjections of $GABA_A$-receptor antagonists, and by behavioral effects of systemic and topical administration of FL (see Discussion). In the mammalian brain, the BDZ/$GABA_A$ receptors are ubiquitous and their negative metabolic-trophic influences on the forebrain are likely to be mediated by a two-pronged action: (a) a postsynaptic (hyperpolarizing) inhibition of neuronal bodies of the BAAS and those of the basal forebrain cholinergic neurons that contain the nerve growth factor peptide (NGF; 55); (b) a presynaptic (depolarizing) inhibition of axon terminals whose "swellings" or "varicosities," particularly those of peptidergic neurons, are endowed with BDZ-$GABA_A$ receptors coupled to chloride ionophores (132); the latter inhibitory mechanisms my also be operational at the axon terminals of the BAAS (see Discussion).

Here we describe experiments showing that the BDZ/GABAergic hypothesis of brain aging is plausible, because chronic administration of FL to aging Fischer-344 rats (4 mg/kg/day in drinking water for 10 months), between the age of 13 through 23 months, not only increased the life span but also significantly retarded the age-related loss of cognitive functions. The latter was ascertained by behavioral tests conducted 1–2 months following drug withdrawal, i.e., at the age of 24–25 months, to make sure that the behavioral differences between the FL-exposed group and the age-matched control group, were not caused by drug withdrawal nor presence of its metabolites, but were caused by enduring differences in function and morphology that have accrued over the time period of 10 months due to FL's interference with aging processes. A preliminary report of the results has been published in the abstract form (76)

METHOD

Animal Maintenance and Drug Administration

Initially, 40 barrier-reared Fischer-344 male rats, 13 months of age, were obtained from the Charles River colony of the National Institute on Aging (Sprague Dawley Company, Indianapolis, IN). On the basis of their weight and behavioral scores in the Hole-Board Test (26) that electronically measured ambulation and exploratory behavior (Table 1, first test), the animals were divided into two "balanced" groups, the drug (FL) group ($n = 20$) and the control age-matched group (CA; $n = 20$). The experiments comprised 10 months of treatment and 2 months of drug/vehicle withdrawal, which was followed by one month of behavioral testing. In each group, 12 rats survived to the age of 25 months, when they were killed. The FL group received the drug dissolved in tap drinking water (3–4 mg/kg/day). To dissolve the drug, water was acidified with hydrochloric acid to pH = 3.0. The control age matched group received plain acidified tap water. Toward the end of the vehicle/drug treatment of the aging rats, a 3rd group of 5-month-old "young" control rats (CY; $n = 10$) was obtained from the same Fischer-344 rat breeding colony. They were kept for 4 weeks in the same laboratory environment prior to behavioral tests which were subsequently run in parallel, i.e., in the same days, with tests conducted on the two aged animal groups, FL and CA.

Under barrier condition, microisolator rat polycarbonate cages (Laboratory Products, Maywood, NJ), corncob bedding and bottles with drinking tap water were sterilized by autoclaving, and changed once a week. The Purina Red Chow 5012 was sterilized through gamma-radiation. Once a week the amount of water consumed was ascertained, and if necessary, the drug concentration was adjusted to make sure that the daily doses of FL ranged between 3 to 4 mg/kg. Over the time period of 10 months, the drug treatment was intermittent, as every 4 weeks of treatment were followed by 1 week of a drug "holiday". FL was generously provided by Dr. Peter Sorter (Hoffmann-LaRoche Company, Nutley, NJ). The animals were housed singly in an air-conditioned room, with a 14L:10D cycle (lights on at 1800 h). To prevent pulmonary infections, each cage was equipped with an air filter. The general condition of the animals was checked every 12 h, and they were allowed to live until they became moribund and unable to move; such rats were euthanized with carbon dioxide and necropsied; critical organs were preserved for histopathological examination by a board-certified veterinary pathologist to ascertain the cause of death. Serology panels were performed for viral and mycoplasmal titers and they were negative.

All experiments were conducted between 0900 and 1600 h. The mean (±SD) daily intake of acidified water with the dissolved FL, and the mean intake of plain acidified water per 200 g of body weight by the two control groups, CA and CY, were comparable and equaled 16.8 (±1.8) ml, 16.0 (±2.0) ml, and 17.2 (±2.0) ml, respectively, $F(2, 31) = 2.2$; $p = 0.1$; ANOVA repeated measures.

Mortality and Related Anatomo-Histopathology

Out of 40 animals, 16 (8 in CA; 8 in FL group) died prior to completion of behavioral tests. The percent incidence of pathologic alterations in the control aging group (CA) and in the aging FL exposed group did not significantly differ from published comprehensive statistical reports on barrier-reared aging Fischer-344 male rats (14,107). The remaining 34 rats (12 CA; 10 CY; 12 FL)

TABLE 1

AMBULATION AND HOLE-BOARD TEST (MEAN VALUES ± SE)

Time of Test in Relation to Treatment	Treatment Group		Ambulation	Head Dipping Into Plain Holes With No Objects (HD–pl)	Head Dipping Into Holes With Objects (HD–obj)
Test 1					
Prior to treatment	Vehicle	$n = 20$	78.8 ± 8.0	7.4 ± 0.4	11.5 ± 4.9
	Vehicle	$n = 20$	76.5 ± 5.5	8.0 ± 0.6	12.6 ± 5.1
Test 2					
3 months and following	Vehicle	$n = 20$	83.4 ± 9.8	3.1 ± 0.3	5.1 ± 3.6
a 7 day drug 'holiday'	FL	$n = 20$	103.9 ± 10.3	5.5 ± 0.9	8.9 ± 2.3
			$p = 0.04$*		
Test 3					
5 months and during	Vehicle	$n = 20$	58.5 ± 10.1	9.0 ± 8.0	10.6 ± 7.5
treatment	FL	$n = 20$	98.1 ± 12.6	10.5 ± 5.0	27.5 ± 8.0†
			$p = 0.02$*		$p = 0.02$*
Test 4					
8 months and following	Vehicle	$n = 18$	45.7 ± 11.8†	5.5 ± 2.8	9.2 ± 2.3
a 7 day drug 'holiday'	FL	$n = 20$	88.6 ± 7.5	6.7 ± 1.3	19.3 ± 2.8‡
			$p = 0.01$*		$p = 0.01$*

* Comparison between Vehicle group and FL group; †Significantly lower than in test 1 ($p = 0.02$; Mann-Whitney test); ‡Significantly higher than in test 1 ($p = 0.01$).

that continued to live for 15–21 days after completion of the behavioral tests, were anesthetized with an IP injection of 50 mg/kg sodium Nembutal and their brains were intracardially perfused with heparinized saline, followed by a buffered solution of paraformaldehyde and picric acid; after washing with buffer and 30% sucrose solution, the brains were stored at −70°C for future morphometric and immunohistochemical evaluation of the neurotransmitter systems.

Ambulation and the Hole-Board Test

This test was carried out in an apparatus of File and Wardill (26) in which the infra-red light beams electronically measured ambulation (horizontal movements) and exploratory behavior by monitoring the number and duration of head-dipping into floor holes were combined for statistical evaluation. Two holes provided a view of various objects placed underneath, while the other two holes did not have any objects underneath. Although the apparatus allowed separate monitoring of the number and duration of head-dipping, these two measures were combined. Each animal was placed in the apparatus for 5 min.

T-Maze Test for Spontaneous Choice Alternation

The rationale for the use of this test is based on the fact that spontaneous and unrewarded choice alternation depends on the integrity of the cholinergic system and short-term memory (86,123). Thus, this behavior reflects, at a rudimentary level, the cognitive status of the animal. As described by Meyers and Domino (86), the T maze consisted of a stem alley (50 cm long, 12 cm wide, 12 cm high) which ended in left and right symmetrical alleys of the same size. The performance on any given day was determined by one, and only one, preceding trial. In a dimly lit room, each rat was placed in the start alley and was allowed to explore the maze and make a left or right choice in the maximum allotted time of 3 min. After a complete entry into one of the arms, the rat was confined there by a guillotine door for 5 s, then the rat was removed and placed in a holding cage. A second trial was initiated after 30 s, and based on the directional choice (right or left), each rat's behavior was classified as alternating (choosing the opposite arm from trial 1) or not alternating (choosing the same arm as trial 1). The percent differences between the animal groups showing alternating behavior were evaluated using the Mann–Whitney U test.

Radial Arm Maze

In a six arm radial maze, the animals were tested for short-term "working memory" and longer term "reference memory". The maze was a down-sized version of the previously used 12 arm maze (74) and consisted of a center platform (50 cm in diameter) and 6 symmetrically arranged arms (each 60 cm long, 12 cm wide, 20 cm high). Prior to tests, the animals were kept for 5 days on a restricted diet (80% of normal consumption of Purina Rat Chow), habituated to the maze and handling; they were also introduced to novel food, small "loops" of sugar-coated cereal (Fruit Loops; Kellogg Co., Battle Creek, MN) which were randomly placed in the radial maze. During actual trials, a single bait was placed at the end of each of 5 alleys, while the 6th alley was never baited and served for testing the animal's "reference memory" (90).

In daily trials, each animal was placed in the center platform and allowed to explore the radial maze for 10 min or to collect all 5 baits, whichever came first. The animals' performance was observed from a distance and simultaneously scored by two investigators, one whom did not know whether a particular animal belonged to the drug or the vehicle group. A Working Memory Error (WMError) was scored when the animal reentered an alley from which the bait had already been collected; the Reference Memory Error (RMError) was scored when the animal entered the arm which was never baited. Because there were large differences in the animals' agility, particularly between the aged animals and the young controls, the animal's performance was expressed as the ratio between the number of errors and the number of collected baits during the maximum allotted time of 10 min, whichever came first.

RESULTS

Survival

An equal number of rats died in the control (n = 8) and the FL-treated group (n = 8) during the time period between their arrival at the age of 13 months and the age of 25 months, when the behavioral tests were completed. In the control group, the life-span (mean ± SEM) of the first 8 rats that died prior to completion of behavioral tests was significantly shorter (22.3 months ± 0.7), compared to the first 8 rats that died in the FL group (24.0 months ± 0.6; p = 0.04 Mann–Whitney test), i.e., the 8 FL exposed rats, on the average, lived 1.7 months longer than the 8 age-matched control rats.

Pathology

The following causes of death were identified on the basis of necropsies and histopathologic examination. In the control group, there were 4 cases of pituitary chromophobe adenoma, 2 cases of terminal chronic nephropathy, 1 case of testicular mesothelioma, 1 case of salivary gland carcinoma. In the FL treated group, there were 3 cases of pituitary adenoma, 3 cases of mesothelioma, 1 case of terminal nephropathy, and 1 case of mononuclear leukemia.

Necropsies of surviving 34 rats killed after the completion of behavioral tests, i.e., at the age of 25 months, did not reveal any significant macroscopic changes. Also, no significant gross pathologic changes were observed in the control "young" 7-month-old group. None of the 50 rats studied (20 CA, 20 FL, 10 CY) had lesions suggestive of chronic infectious disease. All serologic tests for mycoplasmal and viral pathogens were negative.

Ambulation and "hole-board" exploration. As shown in Table 1, Test 1, one week prior to vehicle/drug treatment, there were no significant differences between the two age-matched groups (FL, CA) in ambulation and the level of curiosity as indexed by duration and frequency of head dipping into holes with objects (HD-obj) placed underneath, relative to head dipping into plain holes (HD-pl). In Test 2, after 3 months of vehicle/drug treatment and at Day 6 of the 7-day drug "holiday," the ambulation score for the FL group was increased, relative to the control group (p = 0.04; Mann–Whitney U test). In Test 3, following 5 months of vehicle/drug exposure and 1 day before a 7-day drug "holiday," the scores for ambulation and head dipping into holes with objects (HD-obj) were significantly higher in the FL group, compared to controls (p = 0.02); the same was true for test number 4 conducted 8 months after vehicle/drug exposure and at Day 7 of a drug "holiday" (p = 0.01). Throughout the 8-month time period, i.e., comparing Test 1 with Test 4, the ambulation scores for the FL group remained unchanged, while in the control group these scores declined (p = 0.01). In the FL group, the HD-obj scores increased from 10.6 in Test 1, to 19.3 in Test 4 (p = 0.01), meanwhile, there were no significant alterations in the scores for HD-pl.

T-Maze

After 11 daily trials (Fig. 1), conducted at Days 7, 39, 42, and 47 through 54 after FL withdrawal, the mean percent alternation score was highest in the CY group (65.4%) and somewhat smaller for the FL group (53.7%; p = 0.04). On the other hand, for the CA group, this score equaled only 30.7%, i.e., was 19.3% below the 50% chance level. The mean alternation score for the CA

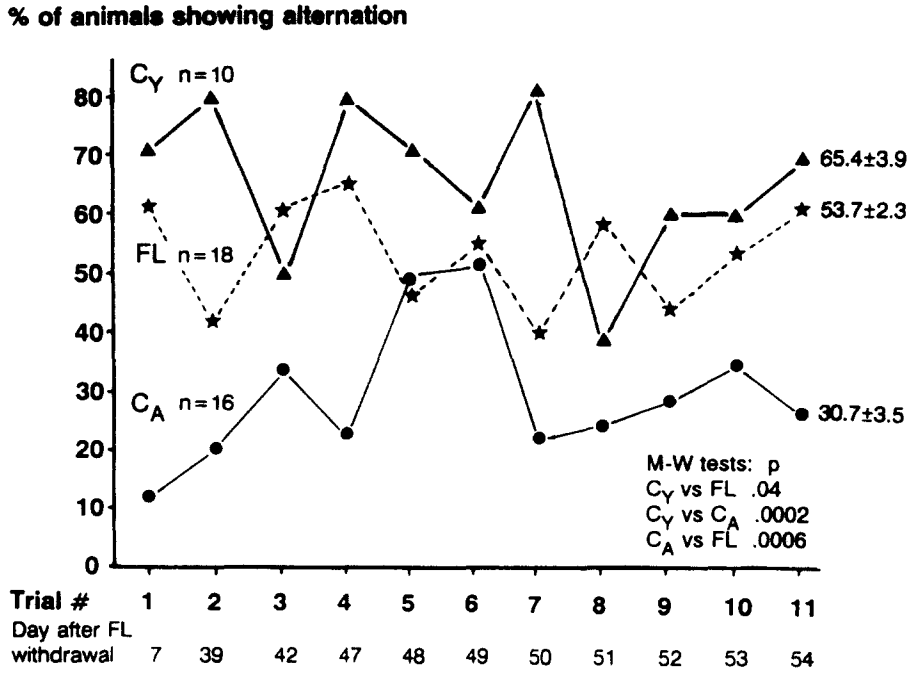

FIG. 1. Effect of 10-month administration of flumazenil (FL) on percent of rats choosing alternate arms in the T maze (ordinate), as tested in 11 nonreinforced double daily trials, separated by 30 s intertrial intervals, conducted at days 7, 39, 42, and 47 through 54, following drug/vehicle withdrawal (abscissa). The percent of animals choosing alternate arms was highest (65.4%) in the control young group (CY; 7 months of age) and was slightly lower in the 25 months old FL group (53.7%; p = 0.04 Mann–Whitney test), while in the control aged group (CA) there was a significant decline in the alternating behavior (30.7%), relative to FL group (p = 0.0006) or to CY group (p = 0.0002).

group was significantly lower, relative to the FL group (p = 0.0006), and also was lower than the score for the CY group (p = 0.0002), thus indexing a strong tendency of the CA rats toward perseverative behavior which is linked to dysfunction of the cholinergic system and characterizes aged rats (85).

In 11 daily trials, a significant percentage of both control and drug-exposed animals failed to make two choices within the allotted 3 min time for each run (Fig. 2). In the CA group, there were 46.6% such failures, and this percentage was significantly greater, relative to FL group (23.4%) and to CY group (14.5%; p = 0.002 and p = 0.0003, respectively).

Radial Arm Maze

After 10 daily trials, the differences between the three groups in the mean daily numbers of working memory errors (WMErrors) were highly significant [Fig. 3; $F(2, 31) = 15.1$; $p < 0.0001$; repeated measures analysis of variance (ANOVA)]; the mean error per one collected bait for the FL group equaled 0.71 and was significantly smaller ($p < 0.008$; Scheffe's posthoc test), relative to the age-matched controls (CA) which equaled 1.24; most important, the mean score of 0.71 for the FL group was not significantly different (p = 0.08) from that for the young controls (CY) which equaled 0.58.

Also, there were significant differences among the three groups in scores for the Reference Memory errors (RMErrors), i.e., the animals' ability to remember and to disregard the location of the unbaited alley [Fig. 4; $F(2, 31) = 12.98$, $p < 0.0001$]. For the FL group, the mean RMError of 0.26 per one collected bait was significantly smaller than that for the CA group (0.43; $p < 0.005$), and was comparable to that of CY group; 0.20; p = 0.16; Scheffe's post hoc test).

DISCUSSION AND THE BDZ/GABA HYPOTHESIS OF BRAIN AGING

Presynaptic (Depolarizing) Actions of BDZ/GABAergic System

It has long been known that GABA, acting via $GABA_A$ receptors, inhibits the release of transmitters from nerve terminals throughout the nervous system, including mammalian spinal cord and the hippocampus (for literature, see ref. 132). The explanation of GABA actions on the presynaptic membrane was based on extrapolations from observations on postsynaptic receptors and differences between the intracellular and extracellular chloride ion concentrations that determine whether the opening of chloride channels would hyperpolarize or depolarize the membrane.

Recently, using slices of the rat posterior pituitary and the whole cell voltage clamp technique and outside-out membrane patches, it was shown that GABA activates $GABA_A$ receptors, opens chloride channels and depolarizes the membrane of peptidergic nerve terminals. Moreover, the BDZ agent, chlordiazepoxide, markedly enhanced GABAergic responses, while the $GABA_A$ receptor antagonists, bicuculline and picrotoxin blocked the responses; on the other hand, the GABAB agonist, baclofen, which was expected to increase K^+ conductance, had no effects. Most important, the peptidergic axon terminals, depolarized by GABA or by BDZ/$GABA_A$ agonists, failed to propagate action potentials into the thousands of swellings (varicosities) that emanate from an axon of a peptidergic neuron (132).

The gross morphology of the BAAS terminals resembles that of the peptidergic neurons, and the release of amines is blocked by systemic administration of BDZ agents (29), or in vitro by micromolar concentrations of BDZ agents and GABA acting on rat hippocampal synaptosomes containing norepinephrine (28). Thus, the inhibitory (depolarizing) BDZ/GABAergic mechanisms control both the peptidergic and BASS terminals, and may even con-

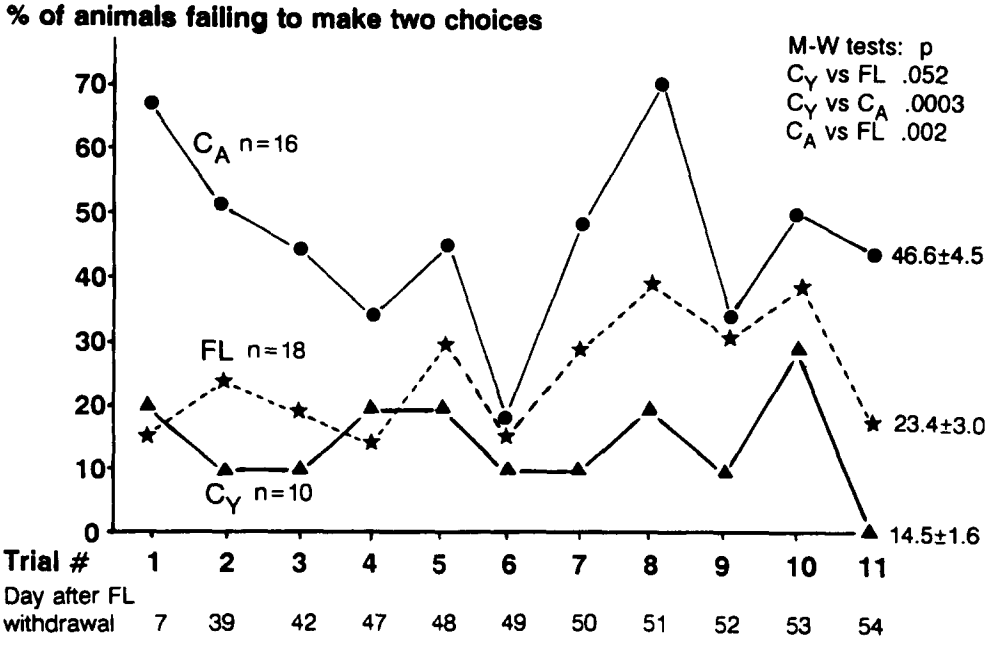

FIG. 2. Effect of the 10 month administration of flumazenil (FL) on percent of animals (ordinate) that failed to make two choices in the T maze in a maximum 3-min time period allotted for each run. The tests were conducted at days 7, 39, 42, and 47 through 54 after drug/vehicle withdrawal (abscissa). The highest percent of failing animals was in the control aged group (CA; 46.6%) which was significantly higher than in the age-matched FL group (23.4%; p = 0.002, Mann–Whitney test) and in the control young group (CY; 14.5%; p = 0.0003). The performance of the FL group was comparable to that of the CY group (p = 0.052).

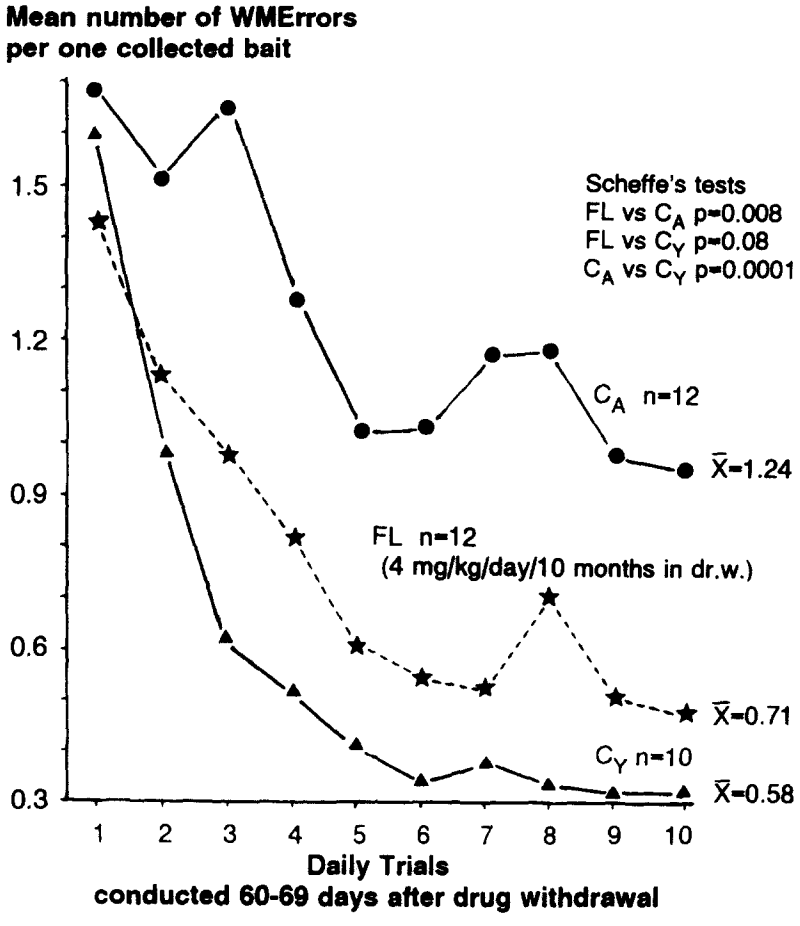

FIG. 3. Effect of 10 month administration of flumazenil (FL) to rats on the Working Memory Errors (WMErrors) in the radial arm maze, as ascertained in 10 daily trials conducted two months after drug/vehicle withdrawal (abscissa). The mean WMErrors are expressed by the ratios between numbers of errors and numbers of collected baits per trial (ordinate). There were significant differences between the three groups, $F(2, 31) = 15.1$; $p = 0.0001$; ANOVA repeated measures. The mean number of errors made by the 25 months old FL-exposed rats was much smaller than in the control age-matched group (CA; $p = 0.008$; posthoc Scheffe's test) and was comparable to that of the control young group (CY; $p = 0.08$).

trol the terminals containing acetylcholine and co-localized peptides.

From the functional point of view, chronic blockade of aminergic and peptidergic axon terminals may be equated with axotomy and interference with both anterograde and retrograde transport, the latter being of critical importance for the nerve growth factor (NGF) whose retrograde transport to neuronal soma appears to be necessary for survival of the basal forebrain cholinergic neurons (31,41,42,97,110,126).

The glycolytic enzymes of mitochondria are concentrated in dendrites and axon terminals (9) where ion fluxes are induced by synaptic events. Chronic depolarization block of axon terminals by excessive tone of the BDZ/GABAergic system is bound to disturb the function of the Na^+/K^+ - transporting ATPase, causing prolonged opening of voltage-dependent Ca^{++} channels and thereby decreasing the protective, voltage-dependent Mg^{++} block of NMDA channels activated by glutamate. The inward movement of Na^+ and Ca^{++} through the high-conductance Ca^{++} permeable NMDA channels would be enhanced, and the toxic intracellular Ca^{++} concentration would increase dramatically, leading to activation of proteases, lipases and endonucleases resulting in autodestruction of the cell (9).

Postsynaptic (Hyperpolarizing) BDZ/GABAergic Control of the BAAS Neurons

The BDZ drugs and GABA inhibit the function of catecholamine neurons (29,30), and GABAergic terminals are present on the somata of the BAAS neurons (27,30). The iontophoretic application of the GABAA receptor antagonist, bicuculline, disinhibits the BAAS neurons, particularly those in the locus coeruleus, in which bicuculline may increase neuronal firing by 70–80% (21,90), thereby showing the tonic character of the BDZ/GABAergic influences. Also, the nucleus raphe neurons (27,32, 35,37), and the dopaminergic neurons that project to meso-limbic and meso-cortical regions (16), remain under tonic BDZ/GABAergic control.

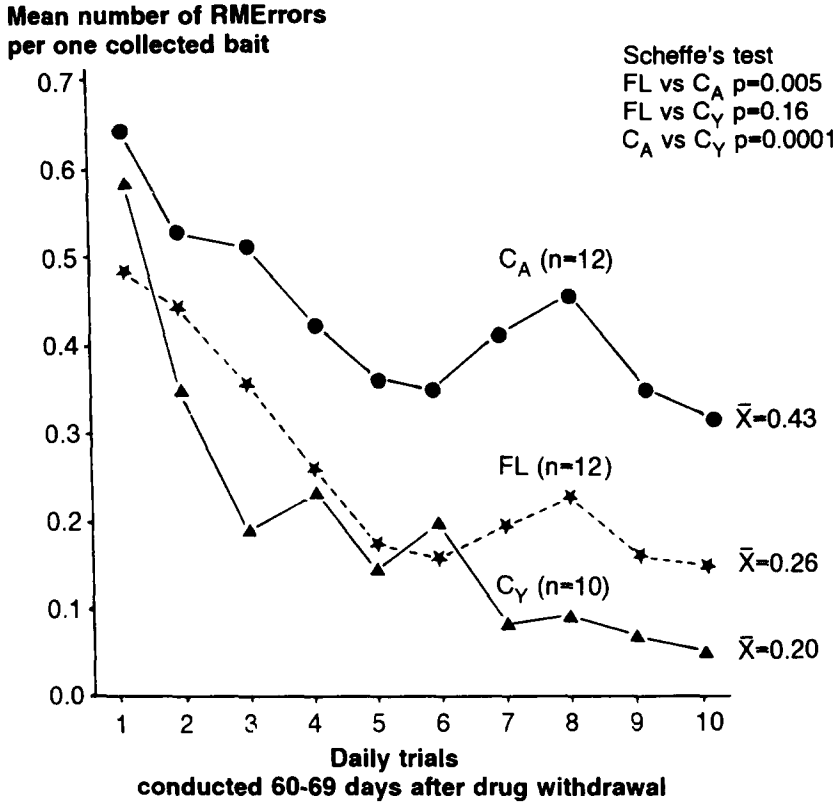

FIG. 4. Effect of the 10-month administration of flumazenil (FL) to rats on the mean numbers of Reference Memory Errors (RMErrors) in the radial arm maze, as ascertained in 10 daily trials conducted 2 months after drug/vehicle withdrawal (abscissa). The mean numbers of RMErrors are expressed by the ratios between the numbers of errors and the numbers of collected baits per trial (ordinate). There were highly significant differences between the 3 groups, $F(2, 31) = 13.2$; $p = 0.0001$; ANOVA repeated measures. The 25 months old FL group was much more efficient than the age-matched control group (CA; $p = 0.005$), and was comparable to that of the 7 months of age control "young" group (CY; $p = 0.08$; Scheffe's test).

Postsynaptic (Hyperpolarizing) BDZ/GABA Modulation of the Ascending Cholinergic and Glutamatergic Systems.

Cholinergic neurons in the ponto-mesencephalic and the laterodorsal tegmentum that project their axons to the thalamus and basal forebrain (128,129,130), like other nonspecific sensory ascending systems that modulate the sleep-wake cycle (68,87), remain under tonic BDZ/GABAergic control exerted by neurons of the substantia nigra pars reticulata and other GABAergic neurons located in the ventral tegmentum (37). Also, neurons of the periaqueductal gray matter that project glutamatergic axons to the forebrain and convey nonspecific sensory information that regulates subjective states of emotional tension and anxiety, are controlled by the BDZ/GABAergic system (36). The tonic character of this control in tegmental regions was revealed by iontophoretic application or microinjection of the BDZ/$GABA_A$ receptor antagonists, picrotoxin and bicuculline, which increase neuronal activity in these regions (68,87) and cause aversive behavior (36).

GABAergic terminals are also present on cell bodies of cholinergic neurons of the basal forebrain and septum which project to the neocortex, mesolimbic cortex, amygdala, and the hippocampus (47,118,127,129,131). These GABAergic terminals exert tonic inhibitory influences on cholinergic neurons and, if these influences are excessive, they may interfere with cognitive processes, as shown by microinfusion of GABA into the rat basal forebrain (66) or by increasing the brain GABA levels (79). Microinjection of the GABA agonist, muscimol, into the nucleus basalis blocks in the cortex the release of acetylcholine and the sodium-dependent high-affinity choline uptake (43). Iontophoretic application of GABA or procaine to the nucleus basalis inhibits the cholinergic neurons (58) and blocks the conditioned cue-elicited neuronal responses in the rat frontal cortex to which the cholinergic neurons project (100).

Is There an Age-Related Increase in BDZ/GABAergic Tone?

Intracerebroventricular administration of monoamines stimulates, while GABA agonists depress respiratory reflexes in rats (40). Aging humans and particularly those with AD, show sleep-related phasic depression of respiratory reflexes that may cause collapse of upper airways and occlusive sleep apnea (45), a syndrome which is dramatically aggravated by relatively small hypnogenic doses of BDZ drugs (84,85). With advancing age, humans show increasing sensitivity to BDZ drugs (22). Compared to mature 4-month-old controls, the Sprague–Dawley rats, at the age of 26–28 months, showed increased BDZ binding to neocortical, cerebellar, and hippocampal synaptosomes (12,83). Moreover, the synaptosomes from aging rats, compared to those from younger

control rats, showed a more efficient coupling between the BDZ and the $GABA_A$ recognitions sites, as indexed by stronger BDZ potentiation of GABA binding to neocortical and hippocampal synaptosomes (12). On the other hand, chronic administration of FL reverses this age-related alteration by partially uncoupling the allosteric link between the BDZ and the $GABA_A$ recognition sites, as indexed by decline in GABA potentiation of [^{3}H]flunitrazepam binding to neocortical synaptosomes (74,119). This uncoupling occurs despite the fact that chronic FL treatment increases the number of neocortical and hippocampal BDZ receptors (72,74,82, 119), an effect that may be interpreted as a compensatory response.

GABAergic Functions in Patients With AD

The age-related degeneration and/or functional depression of the BAAS neurons and those in the cholinergic-somatostatin system is well documented in AD patients (98,111). In contrast, the GABAergic system remains intact or even shows markedly increased activity. Proper evaluation of the GABA system function is difficult in aging individuals and particularly in patients with AD, because the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), is very sensitive to agonal conditions, such as anoxia, reduced blood flow, and prolonged comatous states (89). Thus, to enable proper selection of AD patients for comparison with age-matched controls, a premortem severity index was introduced which excludes, in a semiquantitative manner, patients with history of long agonal states, premortem hypoxia, and hypovolemia (89). Using this index, Reinikainen et al. (98) selected 10 AD patients and 11 controls, most of whom died after a short illness from myocardial infarction, pulmonary embolism or pneumonia. As summarized in Fig. 5, the brain tissues of AD patients with relatively low premortem severity indexes of 0–2, when compared to age-matched controls, showed elevated GAD activity in the substantia nigra, thalamus, striatum, and pons by 105%, 86%, 75%, and 27%, respectively. Although there were no significant alterations in the GAD activity in the frontal and temporal cortex, parahippocampal gyrus, and the hippocampus proper, in all these areas, Reinikainen et al. and other investigators (5,6,111) found large deficits of monoaminergic and cholinergic neurotransmitters: the norepinephrine levels were reduced in the frontal and temporal cortex, hippocampus, and putamen. Serotonin levels were lower in the parahippocampal gyrus, hippocampus, caudate nucleus, and putamen, while the concentrations of the serotonin metabolite, 5-hydroxyindoloacetic acid (5–HIAA), were significantly reduced in the cortex, thalamus, and putamen. Moreover, the choline acetytransferase (CAT) activity was reduced in the frontal, temporal, parietal, parahippocampal, and hippocampal cortex by

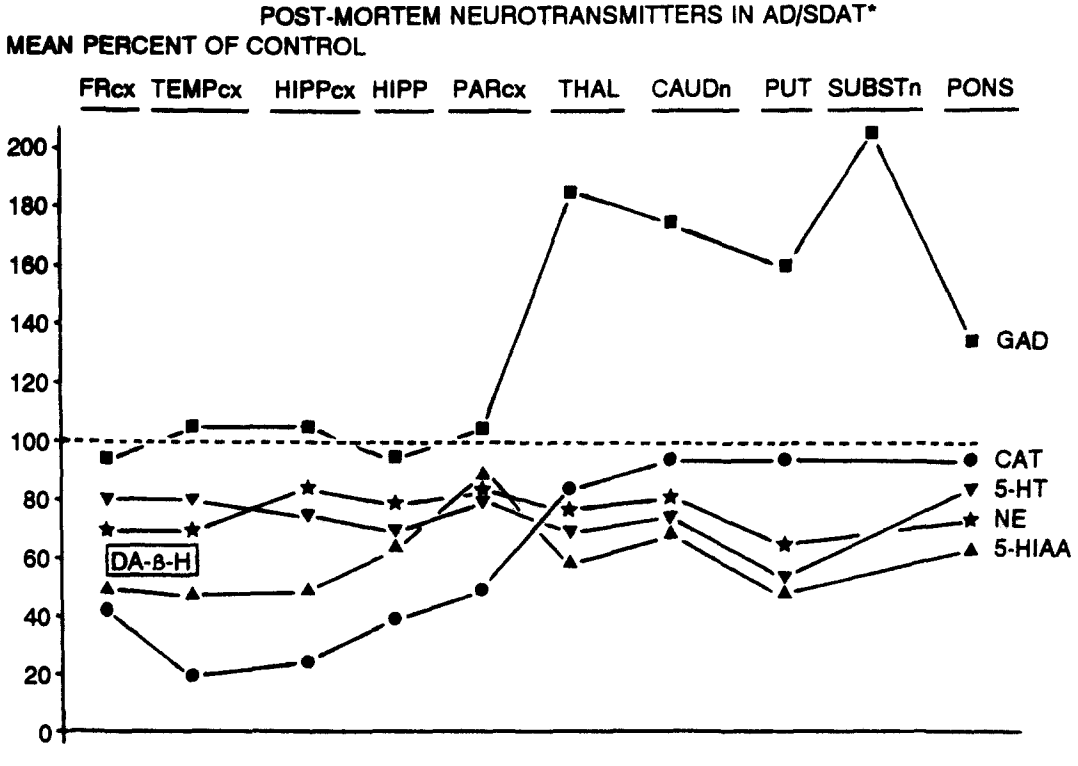

FIG. 5. Relative dominance of GABA system over other transmitter systems in patients with AD. Ordinate: Concentrations of norepinephrine (NE), serotonin (5–HT), 5-hydroxy-indoleacetic acid (5–HIAA), and activities of choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) in brain tissues from AD/SDAT patients expressed as percent of values from age-matched control subjects [the data based on Reinikainen et al. (98)]. The mean percent values are plotted for 10 brain regions (abscissa). Because GAD activity is sensitive to agonal states, the selected subjects in both groups had comparable agonal PreMortem Severity Indexes (PMSI), as defined by Monfort et al. (89). Note the low values for NE, 5–HT, 5–HIAA, and CAT in the presence of normal or markedly increased GAD activity. The boxed "DA-β-H" indicates the approximate values for reduced dopamine-β-hydroxylase activity found in AD patients, as reported by Cross et al.; for literature, see ref. 97). Note that the distribution of the mean values for the AD–ASDAT patients is significantly different from control values ($p = 0.025$; Kolmogorov-Smirnov test). Abbreviations: FRcx = frontal cortex; TEMPcx = temporal cortex; HIPPcx = parahippocampal cortex; HIPP = hippocampus proper; PARcx = parietal cortex; THAL = thalamus; CAUDn = caudate nucleus; PUT = putamen; SUBSTn = substantia nigra.

55%, 82%, 52%, 77%, and 52%, respectively. Interestingly enough, other investigators (5) reported three-fold increases in activity of the GABA inactivating enzyme, aminobutyrate amino transferase in brains of AD patients. The latter finding, if considered with the increases in GAD activity found by Reinikainen et al., suggests an increased GABA turnover in AD.

BAAS Metabolic/Trophic Influences

In nonhuman primates, cortical regions most adversely affected by age-related metabolic/trophic disturbances are the prefrontal and premotor areas known to be involved in short-term spatial working memory; in these regions, the density of dopaminergic "varicosities," relative to other cortical regions and other monoamines is most significantly reduced with age (6).

The involvement of monoamines in etiopathogenesis of psychotic disorders and the discovery of several families of dopamine receptors (112), indicate that the dopaminergic system supports a uniquely large spectrum of metabolic/trophic functions. Such a role is consistent with the known functions of dopamine receptors that may be positively or negatively coupled to the G protein-mediated adenyl and guanyl cyclases, synthesis of 2nd messengers, cAMP and cGMP, and activation of protein kinase C. The dopamine receptors also may be positively or negatively coupled to the 3rd messenger protein, DARPP-32, a phosphatase inhibitor present in dopaminoceptive cells. Each of these signal transductions initiates several cascades of metabotropic reactions essential for normal function of the neocortex and mesolimbic cortex (92).

Astrocytes respond to norepinephrine or to the β-receptor agonist, isoproterenol, with increased glycogenolysis. The β-adrenergic agonists increase phosphorylation of certain proteins and elevate the concentration of free intracellular calcium which plays a key role as an intracellular messenger (9,43). Astrocytes have higher rates of oxygen consumption than neurons, and in vitro they respond to monoamines by increasing the rate of lactate formation from glucose. Lactate and pyruvate are released from astrocytes, and may be required for normal metabolism and neuronal survival, as purified brain neuronal cultures survive for a limited period of time (weeks), whereas neurons cultured with astrocytes can be maintained for many months (43).

Glycogen is the single largest energy reserve of the brain and is predominantly localized in astrocytes, and it undergoes a fast turnover, while the enzymes for glycogen synthesis and degradation are regulated by intracellular messengers, cAMP, and Ca^{++} (9,43). In cortex, norepinephrine containing terminals, and the co-localized Vasoactive Intestinal Peptide (VIP), act synergistically in stimulating glycogenolysis via α(1)-adrenergic receptors (65). These actions remain under a tonic negative control exerted by the BDZ/GABAergic systems at the brainstem level (16,29).

Age, Alzheimer's Disease, and Depression of Glucose Utilization by the BDZ/GABAergic System

Glucose utilization is reduced in AD patients, and this appears to be one of the first measurable events in the clinical profile of AD (9,46). The reductions are most severe in the association cortical and subcortical areas (49), and the degree of cognitive dysfunctions correlates with metabolic deficits (81). Also, the cortex of AD patients shows accumulation of glycogen granules (43), suggesting excessive storage and impaired glycogenolysis.

The most direct in vivo demonstration of the tonic inhibitory role of the BDZ/GABAergic system on glucose utilization is the observation that FL disinhibits brain metabolic functions, as shown by single dose of FL (0.03–3 mg/kg, IV) administered to rats in which glucose utilization increased in most forebrain regions, particularly, if the utilization had been previously lowered by administration of the BDZ receptor agonist, diazepam (99).

BDZ/GABAergic Block of Retrograde Transport of Nerve Growth Factor (NGF)?

Production of this peptide is enhanced by β-adrenergic stimulation (43). The NGF receptor and choline acetyltransferase are co-localized in cholinergic neurons of the nucleus basalis of AD patients (55), and NGF is a trophic peptide for the basal forebrain cholinergic neurons both in vivo and in vitro (41,42,97,126). Because in vivo NGF reaches neuronal soma by retrograde transport from neocortical axon terminals (110), exogenous supply of NGF can prevent the dysfunction of axotomized central cholinergic neurons in the adult brain (31,97,126). One can plausibly conjecture that: (a) in brain regions with age-compromised blood supply and/or impaired BAAS support of trophic glial/neuronal relationships, systemic administration of FL may be expected to prevent the BDZ/GABAergic depolarization block of axon terminals, to improve neuronal function and to restore retrograde transport of NGF, thus promoting neuronal survival; (b) at some point, the mismatch between the growth stimulating peptides and the trophic/metabolic deficiencies caused by an excessive tone of the BDZ/GABA system blocking the somata of the BAAS neurons and those of the basal forebrain cholinergic neurons, could cause the neurons to acquire entirely novel and abnormal morphologic features characteristic of aging and AD, which Woolf and Butcher (130) called dysdifferentiation.

Premature Brain Aging and the β-Amyloid Precursor Protein (β-APP)

Repeated head trauma incurred by boxers or a single strong but closed-head injury may induce, within several weeks, a demented state and profuse depositions of the amyloid (33,104). Also, a seemingly negligible brain injury, such as that caused by a needle stab wound, causes accumulation of the β-APP along the needle tract (93). Although the post-traumatic features of these deposits are more diffuse, relative to more compacted amyloid deposits in plaques typical of AD, the fact that such different circumstances trigger excessive production of the amyloid suggests that its overproduction is not the cause of neuronal degeneration but the result of insult. Hence, we propose that the posttraumatic accumulation of the amyloid is a neuroprotective response to damage of the unmyelinated and fine axons and varicosities of the BAAS neurons that are most likely sheared by sudden traumatic tissue displacements, resulting in multifocal loss of metabolic/trophic functions of the BAAS and peptidergic neurons. This view is supported by recent observations made by Mattson and his colleagues (78): (a) the normally secreted forms of the βAPP, in micromolar concentrations, have potent neuroprotective actions against hypoglycemic/metabolic damage in cultured rat hippocampal and septal neurons and in human cortical neurons; (b) this protection is linked to prevention by βAPP of the toxic rise in intracellular Ca^{++} that mediates the hypoglycemic and/or excitotoxic (glutamatergic) damage caused by breakdown of mitochondrial energy metabolism which fuels ion pumps responsible for maintaining critical voltage and ion gradients across membranes (9). The critical nature of this process is shown by the fact that the cytoplasmic free Ca^{++} concentration is maintained at a level about 1000 times less than outside the cell by means of ATPases that move Ca^{++} out of the cell and/or into the cell's endoplastic reticulum. The breakdown of this energy consuming homeostatic process and elevation of free internal Ca^{++} are bound to activate proteases, lipases, and endonucleases, causing cell autodestruction (9).

FL-Induced Enhancement of REM Sleep: An Index of Anti-Aging Action?

There is a gradual age-related or a more precipitous AD-related decline in REM sleep; the latter is presumably caused by degeneration of the cholinergic and somatostatin systems (11,122). A single dose of FL (10 mg/kg) given orally to dogs increases REM sleep, and to a lesser extent, deep SWS (124). Chronic administration of much smaller FL doses (3–4 mg/kg/day in drinking water) gradually increases REM sleep by up to 100%–130%, without significantly affecting SWS. This effect was still present for several days after drug withdrawal (88), despite that the FL's half-life in the rat brain is shorter than 16 min, and 80 min after IP injection of 10 mg/kg, FL is no longer detectable using high performance liquid chromatography (62). Because the FL enhancement of REM sleep outlasted the drug presence, these increases most likely reflect the FL-induced and ongoing metabolic cascades triggered by disinhibition of the BAAS, cholinergic and the somatostatinergic neuronal systems. This conclusion is compatible with the fact that REM sleep is associated with increases in brain protein synthesis and energy metabolism (114).

For unknown reasons, the increases in REM sleep in dogs and rats have not yet been replicated in healthy human volunteers. Following a single 10 mg IV administration of FL (133), within the 60-min period the total sleep time and stage 4-SWS were moderately reduced ($p = 0.04$), while stage 1-SWS was increased ($p = 0.04$). However, the time spent in REM sleep was not significantly affected. The whole night record following a single 10 mg injection of FL failed to reveal a significant effect of the drug. It is possible that replication in humans of the REM increases observed in rats and dogs would require chronic FL administration in relatively small doses to avoid potential agonist action on BDZ receptors of larger FL doses (25,26).

Considering the neurotransmitter systems involved in generating REM sleep and the critical role of the cholinergic system (7,50,51,56,67,69,80), one can tentatively explain the FL's enhancement of REM sleep by disinhibition of cholinergic neurons in the laterodorsal tegmentum (LDT) and the pontine-mesencephalic tegmentum (PMT), as their activity is tonically controlled by GABA acting on GABAA receptors (37). The PMT neurons generate ponto-geniculate-occipital (PGO) discharges, which, via descending axon collaterals, activate the pontine medullary nuclei, whose glutamatergic axons activate spinal inhibitory interneurons, causing relaxation of the skeletal muscles, one of the hallmarks of REM sleep (7,56,113).

Acetylcholine is a secretagogue for somatostatin (101), a peptide that is critical for generation of REM sleep (18). Thus, the FL-induced disinhibition of the LDT-PMT cholinergic neurons and those of the basal forebrain may be expected to participate in generation of REM sleep. The levels of somatostatin in the basal forebrain and cortex decline with age, particularly in AD patients (18,45,122).

In aged rats, intracerebroventricular administration of somatostatin, or systemic administration of its synthetic analogues that cross the blood–brain barrier, promptly restored REM sleep to the level seen in young rats (18). As discussed above, activation of BDZ/GABAergic receptors on peptidergic axon terminals causes their depolarization block (132), and the tone of the GABAergic system, relative to other transmitter systems, increases with age and particularly in AD patients (98). Hence, it is tempting to ascribe the FL-induced increases in REM sleep to disinhibition of somatostatinergic terminals and maximizing their physiological function, the implication being that in healthy rats and dogs the mechanisms for generating REM sleep does not function at a full capacity, remaining under a tonic moderating control of the BDZ/GABAergic system.

REM Sleep Contributions to Cognitive and Brain Recuperative Functions.

Animals that are required to learn various tasks, such as the operantly conditioned bar pressing, show increases in the frequency and duration of the REM sleep episodes (114). The "place cells" in the hippocampal formation of behaving rats, if activated during the animal's exploration of environment, show increased firing rates during subsequent SWS and REM sleep, relative to other place neurons that had not been "used" during exploratory behavior (94).

Analysis of "words" composed of action potential trains, specifically organized in time domain, showed that REM sleep, selects, replays, and amplifies information the cat acquires prior to sleep, e.g., during appetitively motivated exploration of manipulanda while learning to associate milk delivery with bar pressing (71,75). The novel "information" is not encoded in alteration of the mean firing rate which often remained unchanged but in emission of novel firing patterns. Patterns that occurred during the animal's successful operation of the manipulanda were augmented by 120% to 500% during subsequent 2 to 3 REM sleep episodes. However, 10–15 min later, during subsequent SWS, patterns that were excessively emitted and linked to bar pressing and REM, developed deficits with reference to the random model, i.e., they were virtually eliminated from the otherwise active neuronal firing repertory, a change that suggests an accomplished memory consolidation process (75).

There were additional aspects of the above inversions in statistical distributions of patterns that implicate REM sleep in memory processing: (a) the inversions in distributions of patterns were not random but graded and correlated, i.e., the emission magnitudes were followed by proportionally deep deficits; therefore, the inversions were probably regulated by graded alterations in densities and/or affinities of neuronal receptors used in generation of these patterns; (b) this orderly process implies that patterns are involved in associative learning and recuperative processes (71,75).

In conclusion, one can argue that: (a) the well-known age- and/or Alzheimer's disease-related loss of REM sleep (11,122) is likely to have strong negative consequences on recuperative quality of sleep and cognitive processes; (b) REM sleep may be regarded as an index of subject's cognitive capacity; (c) the FL-induced increases in REM sleep in rats and dogs may be tentatively interpreted as a regression to a more "youthful" brain function; (d) the potential FL enhancement of REM sleep by FL in normal humans, and particularly in patients with emerging signs of AD, is of clinical and theoretical importance

Paradox of Flumazenil's Antiepileptic Action

Although FL in moderate doses is a BDZ antagonist and therefore should be expected to lower seizure threshold, FL has actually antiepileptic actions in animals (38,102). Also, in humans oral and IV administration of FL for up to 42 months had significant antiepileptic actions (109): in 19 out of 27 patients, FL suppressed the EEG spike-wave patterns which were refractory to conventional drugs. Most important, none of the patients developed tolerance to therapeutic action of FL, and in this group, 9 patients showed a moderate, but consistent mood elevation, suggesting an increased tone of the BAAS neurons. However, in 3 patients, FL administration had to be discontinued, because of aggravation of seizures (2 subjects) and emergence of aggressive behavior (1 subject).

Another example of therapeutic action of FL is its ability to control seizures occurring in the Lennox-Gastaut syndrome (39) characterized by generalized repetitive fast spike-wave EEG patterns that have a tendency to occur at the onset of or during slow wave sleep. The electrophysiologic and biochemical etiopathogenesis of this syndrome is not understood, and the EEG patterns are regarded as a malignant derivative of the petit mal spike-wave epilepsy, which according to the conventional view, is caused by breakdown of the GABAergic hyperpolarizing inhibitory functions. This view appears to be incorrect, since this syndrome may be aggravated by BDZ receptor agonists and barbiturates, while IV injections of FL were able to control the EEG ictal discharges (39).

We believe that the antiepileptic actions of FL are brought about by modulation of the BDZ/GABAergic tone and disinhibition of the BAAS and peptidergic neurons and their axon terminals, and the resulting improvement of the glial/neuronal trophic relationships, more efficient energy supply, causing restoration of normal neuronal membrane potentials in epileptogenic foci. This view is supported not only by the FL's increases in oxygen utilization (99) but also by in vitro observations that serotonin and its agonist, acting on 5–HT1A receptors, increases membrane K^+ conductance and blocks the posthyperpolarization rebound action potential bursts of brainstem neurons, particularly if they are triggered by BDZ/$GABA_A$ hyperpolarization and resulting activation of voltage sensitive low-threshold Ca^{++}-conductances (63; see also reference 51).

Chronic Treatment of Hepatic Encephalopathy

Although several successful treatments of hepatic encephaly (HE) with FL have been reported (for a review, see ref. 8), the best documented case is that of a woman with severe chronic encephalopathy, refractory to standard therapy, and which was caused by extensive liver resection and construction of a portacaval shunt (23). Before treatment with FL, the patient was encephalopathic and experienced 12 attacks of coma within 2 years. When treated with FL (25 mg per day orally), all signs of encephalopathy abated in spite of an unrestricted dietary intake of protein. Two days after FL withdrawal she became comatous again, remained chronically encephalopathic and had four further episodes of deep coma during the subsequent 3 months. Following reinstitution of chronic FL treatment (25 mg of FL b.i.d.) and despite of unrestricted protein diet, she has been well for 14 months without any signs of encephalopathy, and she was able to lead a normal life.

The above case is interesting not only because of FL's ability to control gross clinical signs of encephalopathy, but also because, in addition, FL fully restored cortical event-related P300 evoked potentials. The amplitude of these endogenous potentials, normally generated after the subject recognizes a "relevant" tone pitch in the background of "irrelevant" tones, is known to index the subject's cognitive capacity, in contrast to short-latency "exogenous"-evoked potentials that reflect the passive process of impulse conduction (70). Two hours after the subject ingested 25 mg of FL, the amplitude of the P300 potentials were comparable to those of healthy subjects.

FL and Subacute Neurodegenerative Processes

In humans, single IV administration of FL was shown to dramatically but temporarily ameliorate the comatose state and dysautonomia associated with degeneration of thalamic nuclei (64). A single IV FL injection also dramatically improved resolution of cortical visual evoked potentials in patients suffering from a fatal spongiform encephalopathy, the Creutzfeldt-Jacob disease (1). These effects of FL are indicative of pathologic increases in the tone of the BDZ/GABAergic system, which in theory, could be a sufficient factor contributing to neurodegenerative process. Whether or not chronic treatment of these diseases with FL would be able to retard or even forestall their progress remains to be investigated.

Conflicting Results of Repeated FL Administration to Healthy Humans

In a cross-over-double-blind design, Lavie et al. (61) administered orally every 4 h a 30 mg FL tablet or a look-alike placebo (vitamin C tablet) to healthy young subjects after they had been sleep-deprived for one night; the same untreated subjects were also studied. The working hypothesis was that, if FL has any effects on sleep, the subjects would be affected in their efforts the resist sleep when asked to lie down for 7 min in bed in a dark room, close their eyes and resist sleep. Such six 7-min trials were spaced at 4 h intervals; at the onset of each trial, the subject was given a FL tablet or placebo. Light snacks and soft drinks were available ad lib during the interim period.

Repeated FL administration significantly decreased the ability of subjects to resist sleep, relative to the placebo trials. Most of the hypnotic effects occurred 80 to 100 min after drug ingestion, and were associated with increased stage 2–SWS (EEG spindle activity). Therefore, the authors interpreted these effects as resulting from FL's agonist rather then antagonist action on BDZ receptors. This interpretation is, however, challenged by the fact that, after each of six 7-min sleep-resist trial, the FL treated subjects, when asked to perform a coordinated one-hand and a two-hand task, were significantly more skillful and faster than subjects who received placebo. This suggests an improved sleep quality and motor coordination, the latter arguing against the BDZ receptor agonist action of FL, as the BDZ agonists are known to impair speed of motor reflexes and coordination (22).

The improved motor performance of the FL group is reminiscent of motor and cognitive improvements in rats receiving FL 4 mg/kg/day in drinking water, doses known to have antagonist actions at BDZ receptors resulting in compensatory receptor upregulation (74,82,119). These animals, if challenged to solve a difficult swim-escape task of exploring and selecting the over-head ropes that varied in their climbability, were much more skillful, relative to controls, and needed only 1/8 of the time needed by the placebo group (121). Taken together, the improved motor skills observed by Lavie et al. in humans argue against the agonist actions of FL on BDZ receptors and in favor of the antagonist actions, probably linked to disinhibition of the BAAS neurons and their axon terminals, and resulting facilitation of glucose utilization (99) and other metabolic and recuperative functions of sleep.

Unfortunately, the above sleep study is flawed by the fact that 100 mg vitamin C tablets were used as placebo which could have influenced the results; this is indicated by the large differences between the mean values of sleep parameters in the untreated and the placebo/vitamin C condition. The mean values for stages 1–4 SWS in the FL group were much higher than in the untreated condition, the amount of REM sleep in the FL group being 2.75 times higher. The increases in REM sleep relative to the untreated condition, seem to parallel the increases in stages 1, 2, and 3 of SWS monitored between 7 and 22 h. Thus, the conclusion made by the authors that FL treatment had no effect on REM sleep appears to be questionable.

FL Administration to Healthy Humans: Combined Enhancement of Vigilance and Habituation

To delineate differences between the electrophysiological and psychophysiological effects of diazepam and FL, Higgitt et al.

(44) studied the effects of four treatments: two oral doses of FL (100 mg and 30 mg), diazepam (5 mg) and placebo; healthy volunteers, 6 men and 6 women, aged 20–39 years, participated in this study. At six 30-min periods, the EEG and psychometric data were collected before and after drug/placebo administration.

The low and high dose of FL, relative to diazepam and placebo, clearly promoted habituation of subjects to inconsequential environmental stimuli, without compromising the level of vigilance and cognitive functions. The following observations support this view:

1. Cortical potentials evoked by inconsequential stimuli (series of 32 clicks) which did not require subject's attention, declined over the 6 tests conducted at 30-min intervals in subjects treated with FL, and the pace of this decline was significantly accelerated, relative to placebo and diazepam treatment;
2. This effect of FL was paralleled by physiologically moderate but significant decline in the mean systolic and diastolic blood pressure and pulse rate, while there were no such cardiovascular changes after placebo or diazepam;
3. FL treatment, relative to placebo and diazepam, stabilized the autonomic system functions over the 180-min time of testing, as indexed by significant reductions in skin conductance fluctuations and finger tremor;
4. FL treatment stabilized the vigilance level of subjects over 6 measurements at 30-min intervals, as indexed by unchanged cognitive performance in the Digit Symbol Substitution Test, while this performance over time, was depressed by diazepam and placebo;
5. FL had no depressant effect on visual input processing, as measured by the Critical Flicker Fusion Threshold which is known to be sensitive to BDZ agonists.

The above observations, taken together with increases by FL of the EEG alpha mean frequency, and decreases in slow wave activity (108,133), are consistent with the view that FL increases vigilance in humans, while stabilizing the functions of the autonomic system and promoting habituation. The animal work confirmed and significantly extended these observations (see below).

Another observation made by Higgitt et al. that differentiates FL from diazepam was that the FL-treated subjects, when asked to self-rate their mood along three states, alertness, contentedness, and calmness, significantly rated themselves as discontent, while diazepam and placebo had no such effects. One can suggest that this mood change may be related to the FL-induced feeling of "pressure" to move and explore the environment reported following a single 5 mg IV injection of FL (108). Such a feeling would be rather incompatible with a 3-hour long confinement of subjects to the laboratory and the elaborate experimental protocol, and could have contributed to this shift in mood. This subjective pressure to move seems to correspond to surges of unusual exploratory behavior observed in adult 5 months old rats chronically treated with FL (74; see below) and such a pressure might have caused surges of exploratory activity of rats in the Hole Board apparatus described in the present study (Table 1).

Habituation as a Basis for FL's Nootropic ("Toward the Mind") Action

The habituation to inconsequential environmental stimuli, is the first learning process to emerge in human infants and is an excellent predictor of subsequent development of cognitive abilities. For instance, the ability of 1-year-old infants to habituate to inconsequential presentations of a visual stimulus was found to correlate well with various measures of intelligence obtained at 4 years of age (52). In contrast to FL, the BDZ agonists, and β-carboline "inverse" BDZ agonists (15,105,106,) and psychostimulants of the amphetamine group (3,4,19) block habituation. Moreover, the latter two drug groups may distort cognitive processes and cause perseverative or even psychotic behavior (2,3).

Intuitively, simultaneous enhancement of vigilance and habituation is often regarded as incompatible, mainly because vigilance is often conceptualized as a state of moderate arousal which is thought to be antithetical to habituation (2,3,4,19). However, the habituation process is known to be predominantly cholinergic in nature and is antagonized by scopolamine (13), while psychostimulants of the amphetamine group block habituation by increasing arousal mainly through activation of monoaminergic systems which, in addition, may have endocrinologic consequences similar to those of emotional stress (2,3,4,19).

Although the precise mechanisms of the habituation process are poorly understood, it is apparent that this process is contingent upon analysis of novel environment and subsequent feedback inhibition of input judged to be irrelevant (52,54). The benefits of such a process are thought to be essential for cognitive functions and are brought about by an active process of suppressing arousal, thereby relaxing functional neuronal connectivities generated when the subject first confronted, analyzed, and classified novel stimuli. Thus, in parallel with the habituation process, the previously committed neuronal assemblies become available to form novel associations needed for alternative cognitive functions (54).

In cats, the dynamics of these processes, if studied using behavioral, EEG, and neuronal firing patterns, showed that, among a large population of cats those that were unable to habituate to a novel environment had difficulties in acquiring a novel operantly conditioned behavior, such as bar pressing for milk reward. This learning deficit was clearly related to the animal's reduced flexibility and resourcefulness in exploring manipulanda and, as a result, such animals perseverated in stereotyped and ineffective motor modes (70). These cats, compared to the resourceful ones, showed: (a) absent or reduced cortical P300-like potentials normally triggered by reinforced bar press; (b) unchanging 'frozen' spectra of neuronal firing patterns in the thalamic association nuclei, presumably reflecting self-perpetuating activation of neuronal assemblies that often endured, without major changes, the animal's transitions from wakefulness to slow wave sleep and REM sleep (70, and unpublished).

The ability of FL to simultaneously promote vigilance and habituation may be contingent upon two mechanisms: (a) improved cognitive functions (73,120,121), and (b) block of access to BDZ receptors of the endogenous agonists (anxiolytic) and the anxiogenic "inverse" agonists which are normally present and released in the brain (8,25,48); such a protective block of BDZ receptors may be expected to make the subjects "immune" to emotional and autonomic system responses, when challenged by environmental stimuli, as suggested by File and Pellow (25).

In rats, such a protective action of FL was observed in two experimental paradigms: in the radial maze (74) and in the social interaction test (24). In radial maze, after 15 daily runs, guillotine doors were introduced to confine the animal for 10 s to the center platform. This novel procedure greatly disturbed the control rats which in the subsequent trials made significantly more working memory errors than during the preceding trials. On the other hand, rats chronically treated with FL (4 mg/kg/day in drinking water) remained virtually undisturbed and performed much better than the controls (74). Also, in the social interaction tests, introduction of a novel auditory stimulus (tone) significantly blocked "friendly" interactions among pairs of control rats, while animals chronically treated with FL (3.5 mg/kg/day for 10 days), following a brief

pause, resumed normal friendly interactions whose scores were comparable to those prior to introduction of the auditory stimulus (73).

Nootropic-Like Actions of Chronic FL in Animals

In tests for acquisition and retention of passive avoidance behavior, chronic oral administration of FL (4 mg/kg/day in drinking water for 21 days) protected 6-month-old rats from the amnesic action of scopolamine hydrobromide given 15 min before the test (73). Similar observations were made in mice following a single dose of FL (57). Perhaps the most impressive effect of chronic FL treatment of adult rats was the already mentioned astonishing performance in the water maze, where the FL group was more than 8 times faster than the control group in resolving and retaining 24 hours later a complex swim-escape behavior that forced the animals to explore and discriminate between the over-head escape ropes that differed in their climbabilities depending on whether or not they were anchored to the bottom of the water tank (121).

The FL-induced increased capacity for associative motor learning is not only consistent with disinhibition of the BAAS and cholinergic/peptidergic functions but also consistent with the in vitro observations that a transient but precisely timed block of GABAA receptor mediated hyperpolarization of neuronal membrane determines an associative plastic change which is expressed as a long-term potentiation between paired sensory inputs in the rat piriform cortex (53). One can conjecture that the threshold for such a process may be lowered by FL.

In the social interaction test in the Vogel's test of punished drinking, and in the elevated Plus-Maze, chronic FL had anxiolytic effects which was associated with increased vigilance (120). Consistent with the present results in the Holeboard Tests (Table 1), we have previously found that chronic administration of FL strongly increases the animals' curiosity and exploratory behavior in the Radial Maze, an effect that emerged at day 3–5 of drug treatment, peaked at Day 14, and continued for at least 3 days after drug withdrawal (74); longer effects were not investigated. This behavior was unusual for three reasons: (a) the animals displayed unseen before resourcefulness in climbing over or underneath the guillotine gaits and alley walls to gain the view of objects located outside of the radial maze; (b) this behavior was not food motivated but instead was driven by the animals' curiosity, as it occurred in well-lit maze alleys that were never baited, but faced the well-lit room area "enriched" with furniture, as opposed to the darker and "dull" room corner; (c) the behavior was strikingly different from that of amphetamine treated rats, as it was neither stereotypic and nor aggressive when the animals were handled by the investigator (73,74,120; and unpublished observations).

Chronic Administration of FL and the Life Span

In the present study, the mean life span of eight FL-exposed rats was marginally longer than that of the eight age-matched control rats suggesting that FL had beneficial actions not only on the brain but also on peripheral organs. In theory, the FL's protective action on internal organs could be mediated via FL's actions on the BAAS neurons and central peptidergic, cholinergic systems that, in turn, could favorably influence the autonomic/endocrinologic and restorative processes in internal organs. The question of whether the stabilizing effect of FL on the autonomic/endocrinologic systems contributed to the increase in the life span, remains to be investigated.

Does FL Prevent Aging by Antagonizing Endogenous BDZ Agonist Ligands and/or Those Ingested With Food?

Food restriction has beneficial effects on longevity of humans and animals, including rats (77,125). It is tempting to conjecture that these beneficial effect may not be related to caloric restriction per se, but to lower consumption of BDZ agonists and/or their decreased synthesis by the intestinal flora (8). Thus, in the present study, in which there was no food restriction, the beneficial effects of chronic FL administration could have mimicked the beneficial effects of food restriction by "protecting" the BAAS and the cholinergic/peptidergic neurons from excessive levels of the "endogenous" BDZ agonist ligands, diazepam, N-desmethyldiazepam and oxazepam (8). Interestingly, a chronic 9-month treatment of aging rats with pentylenetetrazol, a drug known to interfere with the inhibitory functions of the BDZ/GABA/(chloride ionophore) receptor complex (116), significantly retarded the behavioral and histopathologic signs of brain aging (59). However, the epileptogenic and anxiogenic effects of pentylenetetrazol (116) precludes its use in humans.

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