Reviews in the Neurosciences, 6, 221-258 (1995)

GABAergic Deafferentation Hypothesis of Brain Aging and Alzheimer's Disease; Pharmacologic Profile of the Benzodiazepine Antagonist, Flumazenil

Thaddeus J. Marczynski

Department of Pharmacology, University of Illinois, College of Medicine Chicago, Illinois 60612, USA

SYNOPSIS

Recent experiments have shown that:
1) A chronic 10 month daily administration to rats of the benzodiazepine (BDZ) receptor antagonist, flumazenil (FL; 4 mg/kg in drinking water), from the age of 13 through 22 months, significantly retarded the age-related loss of cognitive functions, as ascertained by the radial arm maze tests conducted two months after FL withdrawal.
2) An equal number of 8 rats died in the control and FL-treated group before the behavioral tests were completed and the animals were sacrificed; the life span of the FL-treated 8 rats equaled 24.0 (±0.6 SEM) months, while that of the control 8 rats equaled 22.3 months (+0.7 SEM), and the group difference was marginally significant (p=0.04 Mann-Whitney test).
3) In rats sacrificed 3 months after FL withdrawal and behavioral testing, the protective action of FL, relative to age-matched controls, was revealed by a significant reduction in the age-related loss of neurons in the hippocampal formation.
4) In the time period of 3 months between the drug withdrawal and sacrificing of the animals, stress experienced by the aging rats during behavioral testing, related to excessive daily handling of the animals and partial food deprivation to motivate them to perform in the radial arm maze, apparently had excitotoxic effects on the hippocampal neurons, as indexed by the presence of 30% neurons in a state of moderate pyknosis found both in the FL group and the age-matched controls. In the 6 months "young" control group, the number of pyknotic neurons equaled only 3.5%. It was concluded that the drug withdrawal and stress of behavioral testing unleashed the previously FL-controlled age-related degeneration.

On the basis of these results and the literature, showing that the tone of the GABAergic system increases with age, and particularly in Alzheimer's disease (AD), the hypothesis of brain aging was formulated. It postulates that

Reprint address:
Dr. T.J. Marczynski
Department of Pharmacology M/C 868
University of Illinois
College of Medicine
835 S. Wolcott St.
Chicago, IL 60612, USA

Abbreviations: Ach = acetylcholine or cholinergic; AD = Alzheimer's disease; βAPP = beta-amyloid precursor protein; ATP = adenosine triphosphate; BAAS = brainstem ascending aminergic system; BDNF = brain derived nerve growth factor; BDZ = benzodiazepine; bfAChS = basal forebrain cholinergic system; c-GMP = cyclic guanylate monophosphate; cGMP-S-PDE = cGMP-stimulated cyclic nucleotide phosphodiesterase; ChAT = choline acetyl transferase; DA = dopamine; EAA = excitatory amino acid transmitters; endozepines = endogeneous BDZ compounds; FGF = fibroblast growth factor; FL = flumazenil; GABA = gamma-amino butyric acid; GAD = glutamic acid decarboxylase; Gcs = glucocorticoids; GL = glutamate or glutamatergic; hiaffChT = high affinity choline transport; LTP = long-term potentiation; NE = norepinephrine or adrenergic; NGF = nerve growth factor; nbM = nucleus basalis of Meynert; NPD = neuronal packing density; PET = positron emission tomography; PTZ = pentylenetetrazol; REM = rapid eye movement sleep; 5-HT = serotonin; SST = somatostatin.

in mammals, with growing age, and prematurely in humans with AD, the increasing tone of the BDZ/GABAergic system interferes with antero- and retrograde axonal transport through a chronic depolarizing block of preterminal axon varicosities of the ascending aminergic and cholinergic/peptidergic systems, which are indispensable for normal metabolic/trophic glial-neuronal relationships. Such a state leads to discrete anatomic deafferentation of forebrain systems, and particularly of the neocortex, where block of the anterograde axonal transport results in induction of the cortical mRNA responsible for synthesis of the β-amyloid precursor protein (βAPP). The simultaneous block of retrograde transport from chronically depolarized preterminal axon varicosities may account for toxic accumulation in cortex of the nerve growth factor (NGF) and other trophins, without which the basal forebrain cholinergic neurons degenerate.

The general pharmacologic profile of FL has been discussed on the basis of FL administration to animals and healthy and diseased humans. This profile shows that FL:

1) increases brain metabolic functions;

2) reduces emotional responses, thereby stabilizing the functions of the autonomic system in both humans and animals challenged by adverse environmental stimuli;

3) improves cognitive and coordinated motor functions in both humans and animals;

4) uniquely combines anxiolytic, vigilance and cognitive enhancing, i.e. nootropic, properties, which may, in part, stem from FL-induced emotional imperturbability (ataraxy);

5) facilitates habituation of healthy humans and animals to novel but inconsequential environmental stimuli, and promotes non-aggressive interactions among animals;

6) in single i.v. doses, and administered chronically to humans, FL has antiepileptic actions in the Lennox-Gastaut syndrome and other forms of epilepsy characterized by "spike-and-dome" EEG patterns; these actions are likely to depend on FL's disinhibition of the serotonin system;

7) administered in single i.v. doses to healthy humans, or chronically to 3-4 month-old rats in drinking water, FL causes restless, curiosity-driven and non-aggressive exploratory behavior, an effect which is probably related to increased brain metabolic functions, which in rats is indexed by increased time the animals spend in REM sleep without disturbing slow wave sleep EEG patterns.

KEY WORDS

brain, aging, Alzheimer's disease, rats, flumazenil, GABA, benzodiazepine

INTRODUCTION

In mammals, factors that influence the time-course of brain aging are poorly understood. The most influential hypothesis of the etiopathogenesis of premature aging of the brain, including presenile dementia or Alzheimer's disease (AD), is the hypothesis put forward by Rossor /126/ 13 years ago and later elaborated by Blass and Zemcov /13/ and Hertz /60/. This hypothesis postulates that the degenerative processes do not begin in the forebrain, where they are most conspicuous, but are triggered by "metabolic system degeneration" represented by the isodendritic core, i.e. the brainstem ascending aminergic system (BAAS) whose main function is to regulate metabolic processes in the forebrain /13, 60,126/. The BAAS is composed of neurons containing norepinephrine (NE) and a powerful metabolic stimulant, the vasoactive intestinal peptide (VIP) /89/, serotonin and dopamine. This system is indispensable for metabolic and trophic glial-neuronal relationships, and therefore its malfunction, could, in theory, lead to *anterograde degeneration* of forebrain systems involved in cognitive functions /60/. Such a function of the BAAS neurons is implied by the morphology and pharmacology of aminergic axons and their numerous preterminal varicosities from which the amines and the colocalized peptides reach their targets by diffusion /32/. This morphologic and functional criterion equally applies to abundant preterminal axon varicosities of the ascending cholinergic (ACh) system /48,110/,

composed of the brainstem and basal forebrain components, which may be regarded as an elongated syncythium of interacting neuronal clusters, by virtue of extensive ascending and descending axon collaterals /159/.

In early stages of age-related cognitive dysfunctions in animals and humans, and particularly in humans with AD, attempts at correlating the forebrain degenerative changes with those in the BAAS neurons have only been partially successful /25,33, 159,164/. In rats from the age of 12 through 32 months the numbers of neurons in the locus coeruleus do not change /50/, despite that, at the age of 17-20 months, rats develop significant cognitive and memory deficits, as measured by acquisition and retention of passive avoidance behavior /83/. All these observations cast doubts on the heuristic value of the original concept of the brainstem *ascending neurodegeneration process*, and instead suggest *that the degenerative process begins at the axonal preterminal varicosities of the ascending* systems, resulting in degeneration of preterminal axonal varicosities, *with axotomy-like consequences*, one caused by deafferentation of target systems, e.g. of the cortex, and the second caused by block of retrograde transport of postsynaptically synthesized trophins, without which the ascending systems degenerate (see below). This scenario is supported by two observations: a) cognitive deficits in Alzheimer's disease (AD) often contrast with relatively small neuropathologic findings /29,41/; and b) cortex of AD patients shows toxic accumulation of neurotrophins /27/. Hence, in early stages of AD, and in physiologic brain aging, disturbances in transport of transmitters, their release and re-uptake by chronically depolarized axon varicosities, and the block of retrograde transport of trophins, may all equally contribute to cognitive deficits and neurodegenerative processes.

Hence, we hypothesized /97,99/ that the age-related and/or genetically determined abnormally increasing tone of the neuronal system that uses benzodiazepine/gamma-amino butyric acid (BDZ/GABA) as transmitter/modulators induces excessive presynaptic (depolarizing) inhibition of the BAAS/peptidergic and cholinergic axon varicosities. These actions result in suppression of the normal glial-neuronal trophic relationships, thereby causing pathologic hypometabolic conditions that trigger a cascade of both ascending and descending degenerative alterations characteristic of aging. Because the distinction between AD and the "physiologic" aging of the brain is essentially quantitative rather than qualitative /137/, the GABAergic hypothesis also encompasses AD.

Recently, clinicians studying brain metabolism using positron emission tomography (PET) claim to have found topographic differences between the hypometabolic foci of physiologic aging and those seen in AD /41/; nonetheless, the GABAergic hypothesis encompasses both alterations and predicts that chronic disinhibition of the BAAS and the ascending peptidergic/cholinergic systems will retard the progress of brain aging and AD.

Chronic flumazenil (FL) protects rats from age-related loss of cognitive functions

We have shown that the BDZ antagonist FL, when administered in drinking water (4 mg/kg/day) for 10 months to Fisher 344 rats, from the age of 13 through 22 months, significantly retarded, relative to age-matched controls, the age-related loss of cognitive functions, as ascertained by the animals' performance in the radial arm maze /97, 99/ (Fig. 1).

Since the behavioral tests were conducted 2 months after drug withdrawal, and lasted about 3 weeks, it was concluded that the behavioral differences between the two aging groups were not determined by drug withdrawal or presence of the drug and/or its metabolites, but by the protective actions of FL against the age-related neurodegeneration.

Histologic morphometric correlates of chronic FL treatment

This analysis was carried out in animals sacrificed at the age of 24.5 months, approximately 3 weeks after completion of behavioral tests which were conducted two months after FL withdrawal. The following is a summary of the analysis, as published in abstract form /98/:

1) In the hippocampal formation of the control aged (CA) animal group, the mean neuronal

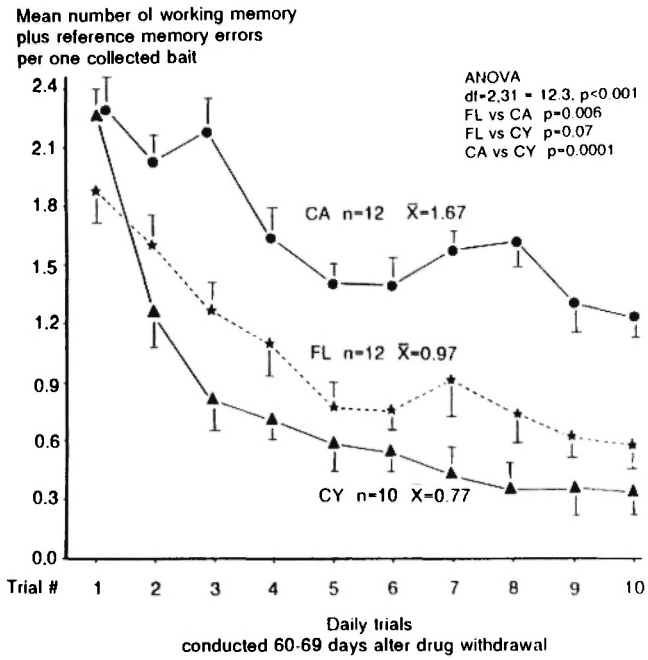


Fig. 1: Prevention of age-related loss of cognitive functions by chronic oral administration of flumazenil 4 mg/kg/day in drinking water) for 10 months to rats, beginning at the age of 13 months; the prevention was indexed by the animals' performance in the radial arm maze in 10 daily trials conducted 2 months after drug withdrawal. Over 10 daily trials (abscissa), the group mean numbers (±S.E.M) of "working memory" plus "reference memory" errors (ordinate) remained high in the control aged group (CA), while the FL exposed group showed a sharp reduction in errors. The performance of the FL group was comparable to that of the control young (CY) group as shown by ANOVA (upper right inset).

packing densities (NPDs) of the control aged (CA) group, relative to control young (CY) group, 6 months of age, were reduced in the hilus and the blades of the dentate gyrus by 25% and 56%, respectively, and in the hippocampal fields CA4, CA3 and CA1, by 77%, 43%, 45%, respectively; in the age matched FL treated group, these neuronal losses were significantly cut to 10%, 9%, 24%, 18% and 17%, respectively ($p < 0.005$; ANOVA).

2) The mean NPDs of all three animal groups inversely correlated with the mean numbers of errors each group made over 10 daily trials in the radial arm maze. In declining order, the strongest correlations were for CA4, CA1, CA3 hippocampal fields and the dentate gyrus blades ($r = -0.71$; $r = -0.68$; $r = -0.64$; $r = -0.60$, respectively; $p < 0.001$), while the lowest correlation was for the hilus of the dentate gyrus ($r = -0.42$, $p < 0.02$).

3) Across all 5 anatomic regions studied (two in the dentate gyrus and three in the hippocampus proper), 3.5% of neuronal somata in the CY group, and 30% in the control aged (CA) and 30% in the aged FL groups, sacrificed 3 months after drug/vehicle withdrawal, revealed an early stage of degeneration, characterized by shrunken somata whose nuclei and enlarged nucleoli were still discernible, despite these neurons staining much darker than the "healthy" ones, and their nuclear chromatin condensed at the nuclear margins (/98/, and Marczynski *et al.* unpublished observations).

The morphologic uniformity of the incipient neuronal degeneration seen in the FL and CA groups sacrificed 3 months after drug/vehicle withdrawal, indicates that:

1) the degenerative process was triggered by factors acting within a recent relatively narrow time span, most likely by stress of daily behavioral trials, lasting about one month, including excessive handling and partial food deprivation to motivate the animals' performance in the radial arm maze;

2) both aging groups, CA and FL, were equally 8 times more vulnerable to emotional stress than the CY group, which showed only 3.5% of pyknotic neurons.

It was concluded that FL protected hippocampal neurons against neurodegenerative processes and age-related loss of cognitive functions, but this protection waned shortly after drug withdrawal. Thus, despite the fact that the FL group showed significantly higher mean neuronal packing densities, relative to the control aged group, FL failed to alter the inherent genetic "clock" of the aging process, because during a 3 month period of drug withdrawal and stress of behavioral trials, the previously controlled aging process was apparently unleashed (/98/, and unpublished observations).

Support for the GABAergic hypothesis from an independent laboratory

Landfield *et al.* /76/ showed that a 9 month daily treatment of rats, from 18 through 27 months

of age, with subconvulsive daily doses of pentylenetetrazol (PTZ) significantly protected the animals from age-related decline in cognitive functions, as indexed by behavioral scores of the animals' learning to reverse their previously acquired performance in a T-maze. Neuronal densities in the hippocampal CA1 field were significantly greater in PTZ treated rats, compared to age matched controls. The protective mechanism of PTZ was loosely interpreted by the authors as resulting from chronic stimulation of brain funtions /76/. PTZ is known to bind to the picrotoxin receptor located in the chloride ionophore of the benzodiazepine/ GABA receptor complex, and PTZ displaces the convulsant agent, S-butyl-bicyclophosphorothionate (TBPS), as well as the physiologic ligands that open chloride ionophores to protect animals from epileptic convulsions /cf 89a/; PTZ may also induce a state of anxiety by antagonizing the inhibitory tone of the GABAergic system /cf 143/. Despite these gross similarities between FL and PTZ actions in reducing the function of the BDZ/$GABA_A$ (chloride ionophore) receptor complex, chronic FL has the advantage of antiepileptic and anxiolytic action (see below). The epileptogenic and anxiogenic effects of PTZ /143/ preclude its use in humans. Initially, while formulating the GABAergic hypothesis of brain aging, we were not aware of the work of Landfield and colleagues /76/.

DERIVATION OF THE BDZ/GABA HYPOTHESIS OF BRAIN AGING

Presynaptic (depolarizing) actions of the 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 /cf 162/. 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 which determine whether the opening of chloride channels would hyperpolarize or depolarize the membrane.

Using slices of rat posterior pituitary and the whole cell voltage clamp technique and outside-out membrane patches, Zhang and Jackson /162/ (Fig. 2) showed that in peptidergic axonal varicosities GABA activates $GABA_A$ receptors, opens chloride

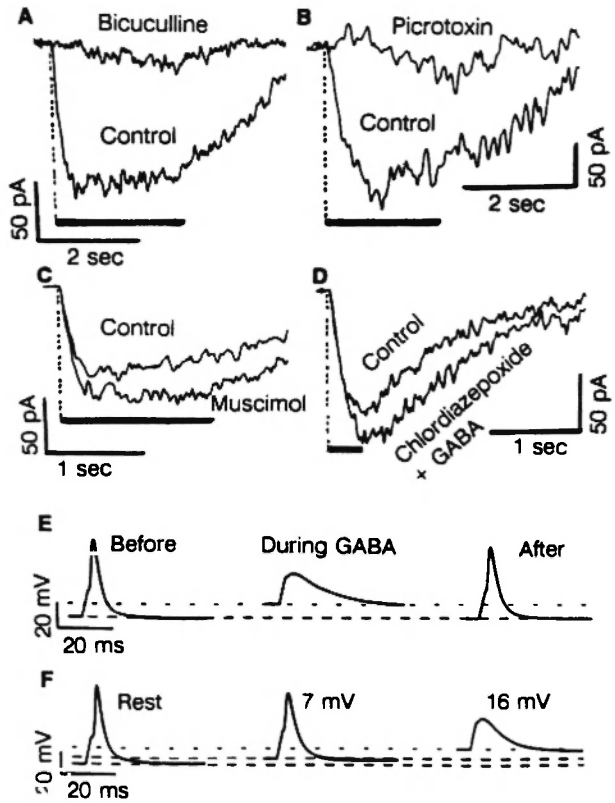


Fig. 2: Whole cell voltage clamp recordings of GABA responses of a nerve terminal (varicosity) of the posterior pituitary. (A) Bicuculline- and (B) picrotoxin blocked responses to GABA application from pressure pipet (horizontal bars). (C) Muscimol produced a response similar to the GABA response but 28% larger. (D) Chlordiazepoxide applied with GABA produced a response 27% larger than the response of the nerve ending to the concentration of GABA alone. (E) Whole cell current-clamp recording: GABA induced a depolarization of 15.9 mV; a 23-msec, 250-pA current pulse generated an action potential before GABA application, but not while GABA was present; after GABA removal, the membrane potential recovered, and current injections once again generated action potentials. (F) Depolarization alone prevented action potential generation. The membrane was depolarized under current clamp by injection of steady positive current. A depolarization of 7 mV did not block action potentials, but a depolarization of 16 mV did (based on Zhang and Jackson /162/).

channels and depolarizes the membrane. The BDZ agent, chlordiazepoxide, markedly enhanced GABAergic responses, while the $GABA_A$ receptor antagonists, bicuculline and picrotoxin, blocked the responses. Contrary to expectation, the $GABA_B$ agonist, baclofen, the conventional presynaptic inhibitor, expected to increase K^+ conductance, had no effect. The depolarization of the axon preterminal varicosities by GABA, through current injection or by application of $BDZ/GABA_A$ agonists, interfered with propagation of action potentials into more distal axonal varicosities /162/.

The gross morphology of the BAAS and ACh terminals resembles that of the peptidergic neurons, and the *in vivo* release of amines is blocked by systemic administration of BDZ agents (44); *in vitro* the release of NE from rat synaptosomes is blocked by micromolar concentrations of BDZ agents and GABA /43/. The inhibitory (depolarizing) BDZ/GABAergic mechanisms control both the peptidergic and BAAS preterminal varicosities /162/, and are likely to exert a similar inhibitory control over the axonal preterminal cholinergic/peptidergic varicosities.

Chronic blockade of aminergic/peptidergic preterminal varicosities may be equated with functional axotomy, interfering with anterograde and retrograde transport, the latter being of critical importance for the function of nerve growth factor (NGF) and other trophins whose retrograde transport to neuronal somata appears to be necessary for survival of the basal forebrain cholinergic system (bfAChS) /58,59,85,133-135,156/.

The deleterious effects of axotomy-induced block of retrograde transport or other conditions that interfere with survival of the bfAChS, such as ischemia, are all improved by application of NGF /45,58,85,115,122,128,130,144,156/ or fibroblast growth factor (FGF) /5/, if the application bypasses the locus of axotomy. In addition, the NGF-mediated increase of chohne acetyltransferase activity in neonatal rat forebrain /49/ and adult rat /59/ provides evidence for the physiologic role of NGF /49/.

The retrograde transport of trophic substances, initiated by specific receptors in preterminal axon varicosities, may be vulnerable to even minor alterations in membrane potential, as shown by recordings from single varicosities /162/ (Fig. 2); thus, an abnormal increase in GABA concentration could be deleterious. The glycolytic enzymes of mitochondria are concentrated in dendrites and axon terminals /12/ where ion fluxes are induced by synaptic events. A chronic depolarization block of axon terminals by excessive tone of the BDZ/GABAergic system is bound to disturb the function of the Na^+/K^+-ATPase, causing prolonged opening of voltage-dependent Ca^{2+} channels, thereby decreasing the protective, voltage-dependent Mg^{2+} block of NMDA channels activated by glutamate. The inward movement of Na^+ and Ca^{2+} through the high-conductance Ca^{2+} permeable NMDA channels would be enhanced, and the intracellular Ca^{2+} concentration would increase dramatically, leading to activation of proteases, lipases and endonucleases, resulting in auto-destruction of the cell /12/.

BDZ/GABAergic block of preterminal axon varicosities and retrograde transport leading to accumulation of nerve growth factor (NGF) in cortex

Production of NGF is enhanced by β-adrenergic stimulation /60/. The NGF receptor and choline acetyltransferase are co-localized in cholinergic neurons of the nucleus basalis of AD patients /99/. One can plausibly conjecture that: (i) 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, thereby improving neuronal function, restoring retrograde transport of NGF, and promoting neuronal survival; and (ii) at some point of the retrograde transport block, the mismatch between the NGF accumulating in neocortex and limbic system may cause the neurons to acquire abnormal morphologic features characteristic of aging and AD /65/, which Woolf and Butcher /cf. 159/ called dysdifferentiation. The GABAergic interference with retrograde transport would account for more than two-fold elevation of NGF-like activity in the frontal and occipital cortex of AD patients /27/, as shown in Fig. 3.

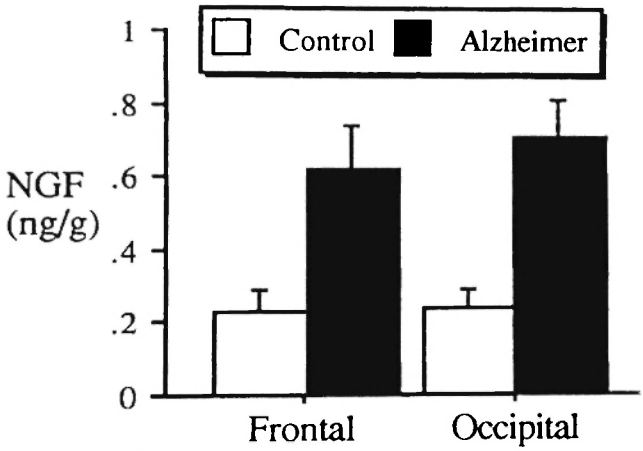


Fig. 3: NGF-like activity in frontal and occipital cortex samples from patients with AD and age-matched controls. In both brain regions there was much more NGF-like activity in AD samples, relative to controls (unpaired two-tailed t-test; frontal, $p = 0.003$; occipital, $p = 0.0002$). Modified from Crutcher *et al.* /27/.

Postsynaptic (hyperpolarizing) BDZ/GABAergic control of ascending systems

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 /39,43/. The iontophoretic application of the $GABA_A$ receptor antagonist, bicuculline, disinhibits the BAAS neurons, particularly those in the locus coeruleus /35, 119/, in which bicuculline may increase neuronal firing by 70-80%, thereby showing the tonic character of the BDZ/GABAergic influences. Also, the Raphe nucleus neurons /39,46,47,51/, and the dopa-minergic neurons that project to meso-limbic and meso-cortical regions /cf. 93/, remain under tonic BDZ/GABAergic control /92a,93/.

Ascending cholinergic and glutamatergic systems. Cholinergic neurons in the ponto-mesencephalic and the latero-dorsal tegmentum that project to the thalamus and basal forebrain /159/, like other non-specific sensory ascending systems that modulate the sleep-wake cycle /93,94/, remain under tonic BDZ/GABAergic control exerted by neurons of the substantia nigra pars reticulata and its vicinity /53/ and GABAergic neurons located in the ventral tegmentum /cf. 93,94/. Also, neurons of the periaqueductal grey that project glutamatergic axons to the forebrain and convey non-specific sensory information that regulates aversive behavior and subjective states of emotional tension and anxiety, are controlled by the BDZ/GABAergic system /52/. 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 and may cause aversive behavior /52/.

GABAergic terminals are present on cell bodies of cholinergic neurons of the basal forebrain and septum which project to the neocortex, meso-limbic cortex, amygdala and the hippocampus /81, 145,159,160/. 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 or by increasing the brain GABA levels /cf. 99/. Microinjection of the GABA agonist, muscimol, into the nucleus basalis blocks the release of acetylcholine in the cortex and the sodium-dependent high-affinity choline transport (hi-affChT) in the hippocampal axon terminals /cf. 99,153/. Iontophoretic application of GABA or procaine to the nucleus basalis inhibits the cholinergic neurons and blocks the conditioned cue-elicited neuronal responses in the rat frontal cortex to which the cholinergic neurons project /cf. 99/.

Medial septum is the source of cholinergic projections to the hipppocampus /81/, and microinjections into the medial septum of GABA and/or BDZ agonists block the hi-affChT normally observed in hippocampal synaptosomes, while microinjections of the $GABA_A$ receptor antagonist, bicuculline, or the BDZ receptor antagonist, FL, significantly increase the hi-affChT in a dose-dependent manner /153/, suggesting the presence of tonic BDZ/GABAergic influences.

BAAS metabolic/trophic influences

These complex relationships have been extensively reviewed by Hertz /60/ to support the hypothesis that AD is an anterograde degeneration, originating in the brain stem and disrupting metabo-

trophic glial-neuronal interactions in the forebrain. Here, only select and novel aspects are discussed.

In non-human 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 preterminal axon "varicosities", relative to other cortical regions and other monoamines, is highly significantly reduced with age /8/.

The involvement of monoamines in the etiopathogenesis of psychotic disorders and the discovery of several families of dopamine receptors /139/, 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 are positively or negatively coupled to the G protein-mediated adenyl and guanyl cyclases, synthesis of second messengers, cAMP and cGMP, and activation of protein kinase C. Also, the dopamine receptors are known to be positively or negatively coupled to the third messenger protein, DARPP-32, a phosphatase inhibitor present in dopaminoceptive cells. Each of these signal transductions initiates several cascades of metabotropic reactions essential for the normal function of the neocortex and mesolimbic cortex /121/.

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 /12, 60/. 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 /cf. 60/.

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^{2+} /12,60/. In cortex, norepinephrine containing terminals, and the co-localized vasoactive intestinal peptide (VIP), act synergistically in activating glycogenolysis via α_1-adrenergic receptors /89/. All these actions remain under a tonic negative control exerted by the BDZ/GABAergic system at the brainstem level /cf. 92a, 93/, and could be vulnerable to increased levels of GABA present in the vicinity of axon varicosities/terminals.

Age-related increase in the tone of the benzodiazepine/GABAergic system

Intracerebroventricular administration of monoamines stimulates, while GABA agonists depress, respiratory reflexes in rats /57/. Aging humans, and particularly those with AD, show sleep-related phasic depression of respiratory reflexes that causes collapse of upper airways and occlusive sleep apnea /62/, a syndrome which is dramatically aggravated by relatively small hypnogenic doses of BDZ drugs /109/. With advancing age, humans show increasing sensitivity to BDZ drugs /cf 99/. Fisher 344 rats 26-28 months of age, relative to young adult 3-4 month-old rats, show up to 30% increases in BDZ receptor affinity, while GABA concentration in brain extracts is increased by 22% /111/. Compared to mature 4 month-old controls, Sprague-Dawley rats at the age of 26-28 months show increased BDZ binding to neocortical, cerebellar and hippocampal synaptosomes /17,108/. Moreover, the synaptosomes from aging rats, relative to younger rats, show a more efficient coupling between the BDZ and the $GABA_A$ recognition sites, as indexed by stronger BDZ potentiation of GABA binding to neocortical and hippocampal synaptosomes than in young rats /17/. Chronic administration of FL may be expected to reverse this age-related alteration by uncoupling the allosteric link between the BDZ and the $GABA_A$ recognition sites, as indexed by a significant decline in GABA potentiation of [^{3}H] flunitrazepam binding to neocortical /102,146/ and to brainstem synaptosomes (Fig. 4). This uncoupling occurs despite the fact that chronic 2-3 weeks FL treatment (4 mg/kg/day in drinking water) increases the number (B_{max}) and affinity of neocortical and hippocampal BDZ receptors /102,107,

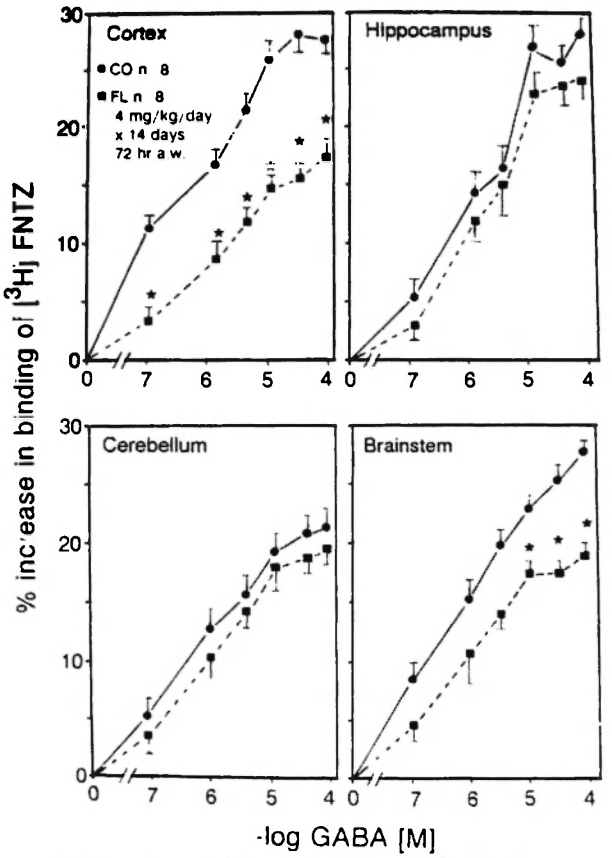


Fig. 4: Effect of GABA on binding of [^{3}H]flunitrazepam to cortical, hippocampal, cerebellar and brainstem synaptosomal membranes from control adult 4 month-old rats (filled circles) and rats chronically treated with flumazenil, 4 mg/kg per day for 14 days (filled squares), sacrificed 72 h after drug/vehicle withdrawal. The binding assays were performed without or with GABA (0.1-100 μM) and in the presence of 0.6 nM [^{3}H]flunitrazepam. Results are expressed as percent increases above basal values, i.e. in the absence of GABA. In the cortical membranes from flumazenil-exposed rats, the effect of GABA was significantly reduced ($F(1,12) = 24.6$; $p < 0.0005$, ANOVA), while in the hippocampus and cerebellum there were no significant effects of GABA ($p > 0.2$). In the brain stem, significant ($p<0.01$) reductions of GABA effects were observed only at the highest GABA concentrations. (Based partially on Urbancic and Marczynski /146/, and on unpublished observations.)

146/, an effect that may be interpreted as a compensatory response to FL chronic occupation of BDZ receptors.

Increased GABAergic functions in AD patients

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 /114/. In contrast, the GABAergic system remains intact or even shows 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 /114/. Thus, to enable proper selection of AD patients for comparison with age-matched controls, a premortem severity index must be used to exclude, in a semiquantitative manner, patients with a history of long agonal states, premortem chronic hypoxia and hypovolemia /114/. Using this index, Reinikainen *et al.* /124/ 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 /8, cf. 99, 137/ 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-hydroxyindoleacetic acid (5-HIAA), were significantly reduced in the cortex, thalamus and putamen. Moreover, the choline acetyltransferase (CAT) activity was reduced in the frontal, temporal, parietal, parahippocampal and hippocampal cortex by 55%, 82%, 52%, 77%, and 52%, respectively. Interestingly, other investigators /3/ reported three-fold increases in activity of the

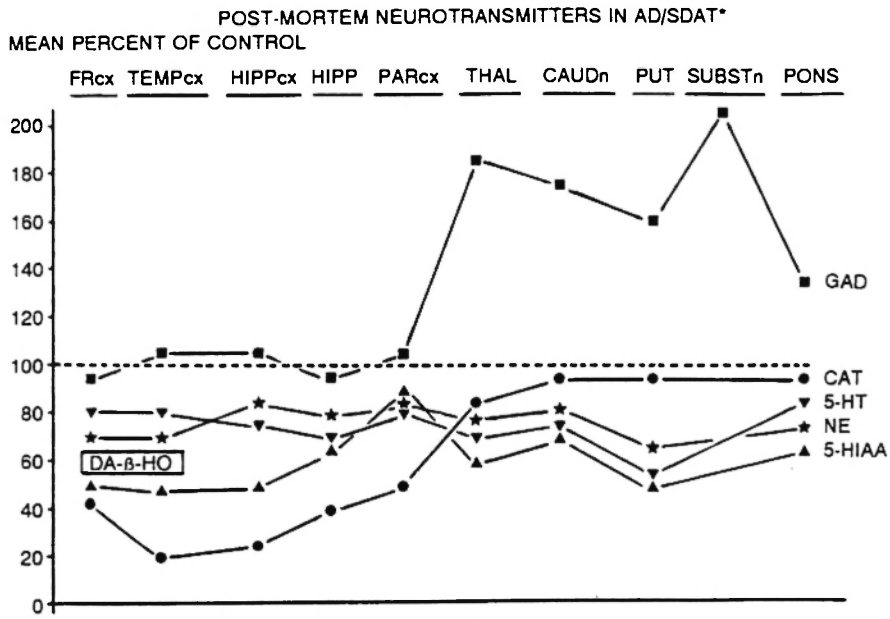


Fig. 5: Relative dominance of the GABA system over other transmitter systems in patients with AD and senile dementia of Alzheimer's type (SDAT). Ordinate: concentrations of norepinephrine (NE), serotonin (5-HT), 5-hydroxy-indoleacetic acid (5-HIAA), activities of choline acetyltransferase (CAT) and glutamic acid decarbolxylase (GAD) in brain regions from properly selected AD patients expressed as percent of values from age-matched control subjects. The mean percent values are plotted for 10 brain regions (abscissa). The boxed DA-β-OH shows the approximate activity level of dopamine β-hydroxylase. Abbreviations: FRcx = frontal cortex; TEMPcx = temporal cortex; HIPPcx = parahippocampal cortex; PARcx = parietal cortex; THAL = thalamus; CAUDn = caudate nucleus; PUT = putamen; SUBSRn = substantia nigra. Based on data from Reinikainen *et al.* /124/.

GABA inactivating enzyme, aminobutyrate amino transferase, in brains of AD patients. The last finding, if considered with the increases in GAD activity found by Reinikainen *et al.*, suggests a strongly increased GABA turnover in AD.

Trauma as a deafferentation factor causing excessive synthesis of the β-amyloid precursor protein (βAPP)

Repeated head trauma as incurred by boxers or a single strong but closed-head injury may induce, within several weeks, a demented state and profuse depositions of amyloid /cf. 99/. Also, a seemingly negligible brain injury, such as that caused by a needle stab wound, causes accumulation of βAPP along the needle tract /cf. 99/. 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 factors trigger excessive production of amyloid suggests that its overproduction is not the cause of neuronal degeneration, but the result of a metabolic insult. Hence, we proposed /99/ that the post-traumatic accumulation of amyloid is a neuroprotective response to damage of the unmyelinated fine axons and varicosities of the BAAS neurons that are most likely sheared by sudden traumatic tissue displacements, resulting in multi-focal loss of metabolic/trophic functions of the BAAS and peptidergic neurons. This view is supported by the following observations /cf. 99/: (i) the normally secreted forms of βAPP, in micromolar concentrations, have potent neuroprotective actions against hypoglycemic/metabolic damage in cultured rat hippocampal and septal neurons and in human cortical neurons; and (ii) this protection is linked to prevention by βAPP of the toxic rise in intracellular Ca^{2+} 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 /12/. The critical nature of this energy "expensive" process is shown by the fact that the cytoplasmic free Ca^{2+}

concentration is maintained at a level about 1000 times less than outside the cell by means of ATPases that move Ca^{2+} 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^{2+} are bound to activate proteases, lipases and endonucleases, causing cell autodestruction /12/.

Experimental deafferentation of cortex induces β-amyloid protein (βAP) synthesis

In adult rats, a selective lesion of the basal forebrain cholinergic system (bfAChS), or of the ascending noradrenergic (NE) bundle or the dorsal raphe nucleus that provide ACh, NE and serotonergic (5-HT) input to cortex, each resulted in highly significant activity increases of the cortical mRNA responsible for synthesis and *ex vivo* secretion of the β-amyloid precursor protein (βAPP). Each induction of the βAPP gene was rapid (maximal at 1 h after loss of transmitter function) and lasted as long as the transmitter deficit /151,152/ (Fig. 6). Similar, though temporary, βAPP-mRNA gene inductions were generated by injection of a local anesthetic, lidocaine, into either bfAChS or the ascending NE axonal bundle or the Raphe nucleus. The lidocaine induction of βAPP-mRNA was observed 90 min after injection, and returned to the low preinjection level of βAPP secretion after 7 days (Fig. 7). Importantly, the gene induction seemed to be specific for cortical deafferentation, because the following three different metabolic stress conditions failed to mimic the effects of cortical deafferentations: i) a two-week daily s.c. treatment with physostigmine; ii) a 7-day systemic administration of RU 28362, a potent neuro-excitotoxic glucocorticoid receptor agonist; and iii) systemic administration of a diabetogenic compound, streptozotocin, for 42 days, resulting in 47% reduction of brain energy metabolism /151,152/.

Hence, the induction of the cortical βAPP-mRNA gene resulted from loss of functional aminergic or cholinergic innervations, i.e. deficits most frequently observed in AD. These observations undermine the hypothesis that genetically

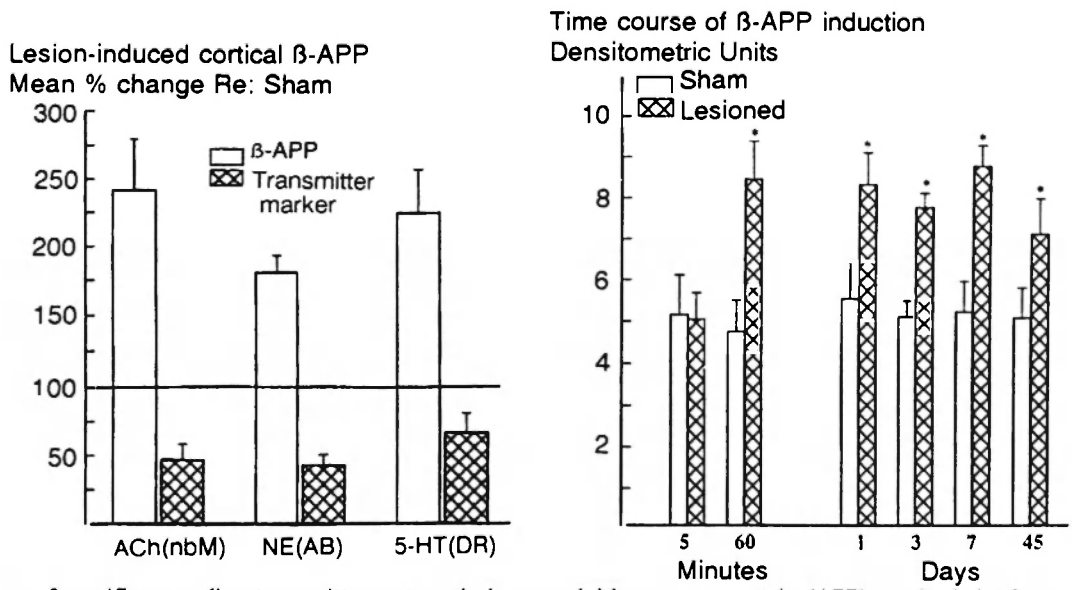


Fig. 6: Lesions of specific ascending transmitter systems induce amyloid precursor protein (APP) synthesis in the rat neocortex. Left: Lesions of the nucleus basalis of Meynert (nbM; cholinergic system), ascending norepinephrine bundle (NE-AB), and 5-HT dorsal raphe (DR) system, each significantly decreased the transmitter markers in the cortex, while increasing the levels of newly synthesized β-APP, expressed as a mean percent change from sham lesion. Right: Time course of β-APP induction which was assayed by immunoprecipitation of newly synthesized β-APP, followed by densitometric quantification of the resulting fluorograms. Note the significant increases in β-APP 60 min after lesion, and a persistent induction during 1 through 45 days. Asterisks mark significant alterations (p values < 0.002). Modified from Wallace and Haroutunian /151/.

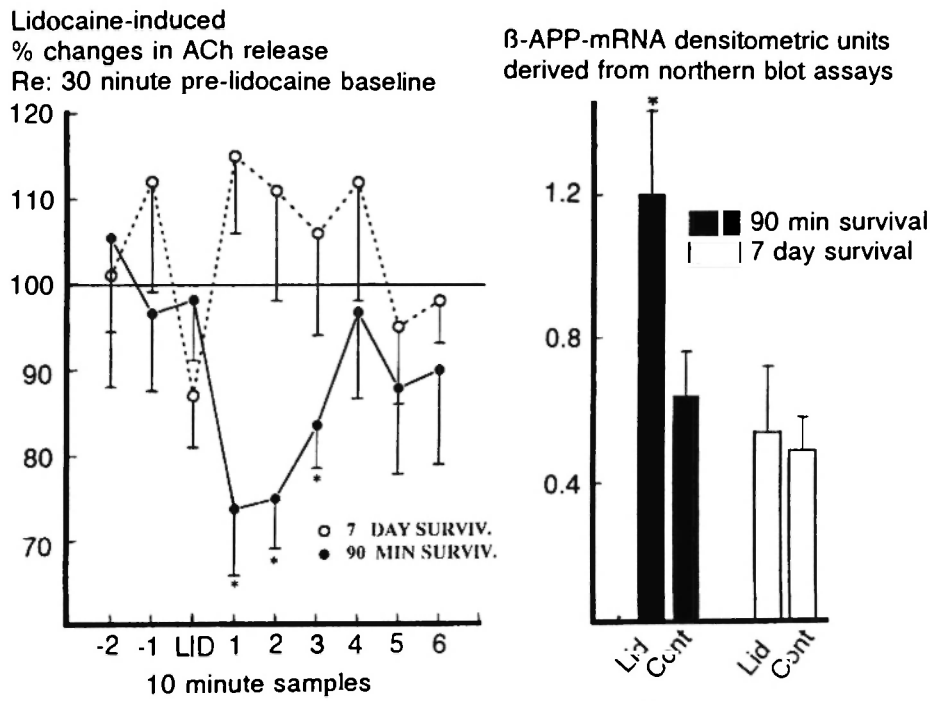


Fig. 7: Transient, i.e. functional, lidocaine-induced suppression of subcortical afferents to cortex decreased ACh release from cortex at 90 min after local injection of lidocaine; 7 days later, the ACh release fully recovered (left panel). At the time of the suppressed ACh release, there was a significant increase in the cortical levels of β-APP-mRNA (right panel). Lidocaine was infused unilaterally into nbM. The released ACh was assayed by *in vivo* microdialysis. Note the short latency and transient effect of lidocaine. (Modified from Wallace and Haroutunian, /151/.)

determined induction of the βAPP-mRNA is likely to be the main etiopathogenic factor in AD.

GABAergic deafferentation of subcortical nuclei?

The above consequences of permanent (lesion-induced) or temporary (functional) cortical deafferentation seem to represent a novel principle which may be applicable to deafferentation of subcortical nuclei and GABA action in view of the findings that in advanced stages of AD higher than 100% elevations of glutamic acid decarboxylase (GAD) activity, responsible for GABA synthesis, occur in the brainstem, thalamus and basal forebrain ganglia /124/. Thus, chronic BDZ-GABAergic depolarization of the ascending brainstem ACh, aminergic and glutamatergic axon terminals that synapse with and/or provide preterminal varicosities to the bfAChS neurons /19/ could, in theory, lead to deafferentation degeneration of the bfAChS neurons. Also, a GABAergic deafferentation could play a role in the hippocampal formation, since *in vitro* or *in vivo* blockade of GL receptors and/or stimulation of the BDZ/GABA receptors by muscimol or diazepam, significantly reduces the levels of NGF-mRNA and brain derived nerve factor-mRNA (BDGF-mRNA) in the rat hippocampus and medial septum, and reduces the synthesis of the respective trophins /161/.

GABA, benzodiazepines, age, Alzheimer's disease (AD) and brain energy hypometabolism

Glucose utilization is significantly reduced in AD patients, and this is one of the first measurable events in the clinical profile of AD /cf. 99/. Positron emission tomography (PET) with fluorodeoxyglucose showed that AD is associated with a global decline in brain metabolism, but the largest decline in glucose utilization is evident in the cerebral cortex, thalamus, caudate and putamen /41,42/, i.e. regions that showed high 75%-105% postmortem increases in GABA synthesis, as measured by activity of glutamic acid decarboxylase (GAD)

/124/. The hypometabolic foci are most severe in the polysensory, i.e. association cortical and subcortical, areas /49/, and the degree of cognitive dysfunction correlates with metabolic deficits /cf. 99/. The cortex of AD patients shows accumulation of glycogen granules /cf. 60/, suggesting excessive storage and impaired glycogenolysis.

In agreement with the GABAergic hypothesis, the initial stages of the aging process are functional and are revealed by cognitive dysfunctions and/or alterations in receptor functions. For instance, PET revealed that an i.v. injection of diazepam to AD patients causes an unusually diffuse, compared to age-matched controls, decline in glucose utilization throughout the cerebral cortex, as if triggering a diffuse cortical deafferentation /41/. Also, in adult rats, the most direct *in vivo* demonstration of the role of the BDZ/GABAergic system in hypometabolic states is the fact that glucose utilization can be readily depressed by the systemic administration of diazepam, and this can be reversed by FL, which increases glucose utilization /125/ (Fig. 8).

Glial cell responses to GABA and benzodiazepine agonists

Astrocytes, the main providers of lactate, the main neuronal energy substrate /34,60/, like neuronal axon varicosities, are depolarized by GABA and BDZ agonists through outward movement of Cl^-, while bicuculline and FL, but not the "inverse" BDZ receptor agonists, prevent these GABA-BDZ actions /10/. Chronic depolarization of astrocytes by excessive levels of GABA or endogenous BDZ agonists /cf. 11/ could, in theory, result in depolarization inactivation or less efficient production of neurotrophins, thus contributing to the overall hypometabolic state of brain structures and neuronal death /cf 24,122/.

Indirect evidence for BDZ/GABA impairment of axon terminal functions

As already mentioned, chronic BDZ/GABAergic depolarization of axonal varicosities and terminals of the BAAS, peptidergic and ACh neurons is bound to interfere with conduction of action potentials /162/, as well as with anterograde axonal transport and transmitter release /cf. 162/ and reuptake /4,64/ and with retrograde transport of trophic peptides, e.g. NGF and the fibroblast growth factor (FGF), that are indispensable for normal functions of the bfAChS, such as the hiaffChT, synthesis and activation of choline acetyltransferase (ChAT) and synthesis of ACh. All these functions could be suppressed by GABA, as

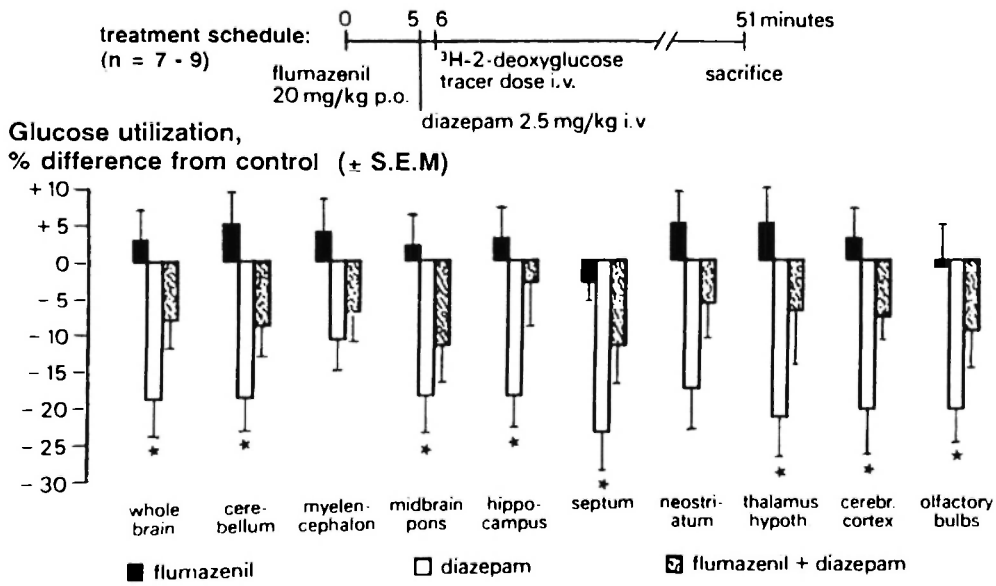


Fig. 8: Effects of flumazenil, diazepam and flumazenil + diazepam on glucose utilization in the whole rat brain and in nine specific brain regions. Note that flumazenil (20 mg/kg p.o.) strongly antagonized the depressant action of diazepam (2.5 mg/kg i.v.). Flumazenil increased glucose utilization in the whole rat brain and seven specific regions above the control levels (modified from Richards *et al.* /125/).

indicated by the following observations /153/: a) GABA and/or BDZ agonists, if microinjected *in vivo* to rat medial septal cholinergic neurons projecting to the hippocampus, block hi-affChT by hippocampal cholinergic synaptosomes; and b) the $GABA_A$ receptor antagonist, bicuculline, or the BDZ antagonist, FL, increase hi-affChT in a dose-dependent manner, while the "conventional" $GABA_B$ presynaptic receptor agonist, baclofen, or receptor antagonist, saclafen, have no significant effects.

The retrograde transport of as yet unknown trophin(s) may also be important for survival and function of BAAS neurons, because: a) FGF has protective actions on dopaminergic neurons, an effect which depends on the presence of glial cells, suggesting that FGF acts via induction of a glial cell-derived neurotrophic molecule(s) /120/; b) BAAS axon varicosities/terminals in the forebrain of aged animals, relative to young adults, show reduced NE uptake /64/; and c) in the advanced stage of AD, the dopamine (DA) uptake by forebrain axon terminals is reduced by 65% /4/, particularly in the putamen where GAD activity was found to be elevated by 60% to 75%, relative to age-matched control subjects /124/.

Identifying the earliest morphologic signs of neuronal degeneration in Alzheimer's disease (AD)

Extensive light and electron microscopic studies of cortex from AD patients showed that the earliest and dominant morphologic features of cortex are not intraneuronal fibrillary tangles and amyloid plaques, but very subtle and consistent signs of degeneration of axon terminals and preterminal varicosities which become swollen and/or distended, containing swollen transmitter vesicles, many of them of the type representing aminergic terminals /103/. These early features of cortical deafferentation were found to be associated with up to 50% loss of synapses /103/. Such a previously not realized deafferentation process is bound to result in glial-neuronal trophic-metabolic disturbances and decline of neuronal ability to utilize glycogen and lactate, which is normally produced, stored and released by astrocytes /10,34,60/.

Select aspects of genetic risk for premature brain aging and AD

The dominant view of the neuropathological processes leading to AD is that this disease is simply a premature aging of the brain and that the differences between the two are quantitative rather than qualitative /137/. Genetic studies identified three groups of AD patients: 1) those accounting for about 3% of cases in which autosomal genetic transmission appears to be unequivocal; 2) those with familial history, but with insufficient evidence for autosomal dominant genetic transmission, accounting for about 35% of cases; and 3) those with no apparent genetic risk, accounting for 60% of cases /cf. 116/.

Statistical considerations of etiologic risk factors related to AD have undermined the relevance of the above genetic classification of AD, because low education turned out to be about four times more influential than family history, and about 20 times more influential than head trauma /cf. 116/.

Studies of apolipoprotein E (apoE) as a potential "chaperone" protein factor in causation of AD /157/ led to the observation that the gene on chromosome 19 encoding the synthesis of apoE has alleles encoding one of three possible apoE isoforms. In contrast to apo2 and apo3, the apo4 isoform was found to be associated with approximately two-thirds of the late onset familial and sporadic cases of AD. Thus, the apoE4 allele may be a major risk factor for AD /140/.

ApoE is primarily produced by astrocytes and oligodendroglia, and is present in pathologic structures typical of AD, such as senile plaques and neuronal fibrillary tangles. AD patients who are homozygous for apoE4, compared with those homozygous for apoE3, and matched for duration of AD, show significantly greater and more numerous depositions of the β-amyloid peptide (βAP) /141/. However, amyloid deposits are known to occur in brain tissues with or without neuronal abnormalities /15,25,31,33/, and, contrary to "the amyloid cascade hypothesis" of brain aging /137/, amyloid depositions are clearly not the earliest markers of neurodegenerative process, because, as already mentioned, in the human cortex these markers are preceded by deafferentation, as indexed by about

50% loss of synaptic contacts, pathologic distensions/swellings of axon terminals/varicosities and transmitter vesicles /103/. These pathologic findings are compatible with the GABAergic deafferentation hypothesis /97-99/ and with evidence discussed above that transient cortical deafferentation induced by subcortical microinjection of a local anesthetic, or by permanent deafferentation induced by lesion of the aminergic or cholinergic afferents, each elicits a strong and virtually instantaneous βAPP-mRNA induction in cortex /151,152/. Moreover, it is not the amyloid plaques, but the neurofibrillary tangles that parallel the duration and severity of brain aging and AD /15/.

Is there an etiopathologic common ground for GABAergic deafferentation of neuronal systems, development of fibrillary tangles, interference with tau protein functions and protective actions of FL?

Compared to the βAPP hypothesis of brain aging, the *in vitro* interactions of apoE with tau protein are the basis for a more plausible hypothesis of brain aging. The intraneuronal fibrillary tangle is a defining lesion in AD and is formed from paired helical filaments whose main constituent is tau protein /26/. The latter binds tubulin, the protein which undergoes polymerization and is used for assembling microtubules that subserve bidirectional transport of molecules along the entire length of the axons and dendrites /78/. *In vitro*, apoE3 binds tau, while apoE4 does not /140/. This suggests that apoE3 may afford protection from clinical expression of AD by supporting development and function of microtubules. At this junction, the presumed protective effects of apoE3 /cf. 140/ and the GABAergic deafferentation hypothesis and the presumed protective action of chronic FL, all seem to converge to a common ground. Thus, the pressing question to be answered is whether or not genes that regulate synthesis of GABA and BDZ/$GABA_A$ receptors are all part of the polygenic influences that determine the dynamics and time course of brain aging and clinical expression of AD.

Age-related growing tone of GABAergic system as potential factor in genetically programmed cell death (apoptosis)?

The developmentally necessary neuronal death, the neuronal axon-target interactions with post-synaptic glial and neuronal elements and the discovery of the whole family of neurotrophins /cf. 24/, all led to the proposal that the developmentally important apoptosis and cell necrosis caused by physical or chemical insults are two different phenomena /122/. This view is supported by the fact that activation of the apoptotic program intrinsic to each cell is relatively slow, as it requires changes in RNA, synthesis of new proteins and enzymes capable of "altruistic" self-removal of the cell, without tissue inflammation and its potentially damaging consequences for the functioning of remaining cells /122/. It is believed that delineation of apoptosis from acute excitotoxic neuronal death may further our understanding of the aging process /cf. 24,38,122/, but such an effort may not be easy.

The relentless, age-related growth of the two-pronged GABAergic inhibitory control of energy utilization and neuronal survival /cf 99/, suggests that: i) this control is genetically programmed, and the protective effect of FL seems to act at the last cascades of the aging process; and ii) increase in this process should already be detectable in young mature organisms.

Indeed, if one scrutinizes the function of the septal cholinergic system, the evidence for apoptosis becomes evident. The GABAergic system in the lateral septum projects to and synapses on cholinergic neurons in the medial septum which are endowed predominantly with $GABA_A$ receptors coupled to BDZ recognition sites /81/. The lateral septum exerts a powerful inhibitory influence on cholinergic cells in the medial septum which innervate the hippocampus, in which the terminals of this cholinergic system show efficient hi-affChT. It turned out that the BDZ/GABAergic and tonic negative control of the hi-affChT is already present in rats 4-5 months of age; this was revealed by intraseptal microinjection of FL which increased the hi-affChT by 30% and in a dose-dependent manner

/153/. The GABA receptor agonist, muscimol, and the antagonist, bicuculline, injected into the medial septum, suppressed and enhanced the hippocampal hi-affChT, respectively /153/.

At the age of 18 months, rats still retain the capacity for utilizing exogenously supplied NGF, as indicated by the presence of NGF receptors on cholinergic neurons of the bfAChS, even after this system had been axotomized by fimbrial transection /128/. However, rats at 22-23 months of age cease to respond to exogenous NGF, as judged by failure of NGF to restore the lesion-induced loss of hi-affChAT and acetylcholinesterase activity in axotomized neurons /128/. The progressive failure of retrograde trophic support may be due to age-related loss of NGF receptors, leading to degeneration of the bfAChS and deterioration of cognitive functions /38/. The fact that GAD activity in the brain stem, thalamus and basal forebrain ganglia of AD patients is much higher, relative to properly selected age-matched controls /124/, is compatible with the notion that the age-related increasing GABAergic influences subserve the apoptotic process which, to some degree, may be controlled by FL.

FL as a potential upregulator of the guanosine-triphosphate-binding proteins (G-proteins)

G-proteins constitute a family of homologous, heterotrimeric (α, β and γ) proteins that convey signals from membrane receptors to intracellular effector proteins which, in turn, generate second messengers that control ion channels /91,123,149/. About 80% of more than 100 known hormones, neurotransmitters and neuromodulators induce cellular responses through G-proteins coupled to cellular effectors, such as adenylyl cyclase, phospholipase$_2$ and several ion channels. Receptors coupled to G-proteins include those for catecholamines, serotonin, acetylcholine, various peptides, such as vasopressin, substance P /cf. 91/, all of which remain under a powerful, negative and tonic control of the BDZ/GABAergic system /cf. 93,99/. Therefore, administration of FL may be expected to increase the G-protein functions in virtually all brain regions, particularly in hypometabolic regions depressed by excessive tone of the BDZ/GABAergic system. This action of FL, as indexed by cGMP levels, may be expected to exceed that found in the cerebellum /125/ (Fig. 9).

The critical role of G-proteins in cognitive functions is implied by the "strategic" distribution of the cGMP-stimulated cyclic nucleotide phosphodiesterase (cGMP-S-PDE) gene /123/. In decreasing order, the highest levels of this gene expression have been found in the hippocampus, neocortex, entorhinal cortex, habenula and nucleus accumbens, while the last three areas are known to provide a reciprocal interface between the forebrain limbic system, association neocortex, and the motor systems /cf. 93,123/. Since virtually all transmitter/modulators act through G-proteins, such a distribution of the cGMP-S-PDE is compatible with the hypothesis that FL's nootropic effects are brought about by disinhibition of all inherent physiologic brain functions, a mode of action qualitatively different from those of "conventional" pharmacologic interventions acting at specific BAAS/peptidergic and cholinergic transmitter systems, an approach that often disturbs the physiologic homeostatic relationships between the transmitter/modulator

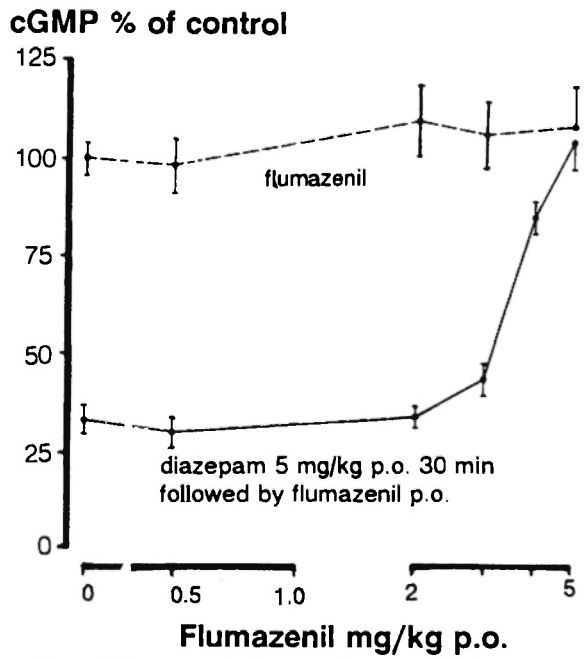


Fig. 9: Effect of oral flumazenil alone (dashed line) and oral diazepam followed by flumazenil (continuous line) on percent changes of cGMP levels in rat cerebellum (modified from Richards *et al.* /125/).

systems, clinically expressed as untoward cognitive/behavioral consequences.

Receptors coupled to G-proteins are prototypic "metabotropic" receptors, for they initiate cascades of biochemical events through modulation of intracellular second messengers which, through phosphorylation of membrane and cytosolic proteins, regulate the ion channel functions and the G-proteins themselves /cf. 91/. Perhaps the best example is that from Woody *et al.* /158/, in which intracellular injection of the cGMP-dependent protein kinase into neurons of precruciate cortex of the awake cat mimicked the actions of extracellularly applied ACh and intracellularly applied cGMP by temporarily increasing the membrane resistance and neuronal firing. The most critical attribute of these functions is that the cGMP-protein kinase activation generates only a "permissive" environment, because the persistent plastic changes in membrane and firing patterns are implemented only when the neuron concurrently receives a barrage of excitatory (depolarizing) input. Thus, these mechanisms represent a single neuronal model of Pavlovian-like conditioning /cf 158/.

FL-induced increases in cGMP levels are likely to impart flexibility to sculpturing plastic neuronal alterations and cognitive functions

A relevant aspect of the G-protein mediated responses is that the G-protein subtypes have the capacity to stimulate or inhibit adenyl cyclase, and some G-protein subtypes may activate phosphodiesterases that reduce the levels of previously increased cyclic nucleotides. Moreover, calpain, a calcium-activated protease, may differentially inactivate select subunits of G-proteins by proteolysis, a mechanism by which one type of experience- and learning-related plastic change may be actively "erased" and replaced by another plastic change in the same single neuron; this affords an almost instantaneous flexibility to neuronal functions, not provided by other signal transduction systems /91,123/.

Classical concepts of learning are based on the formation of neuronal assemblies, and their cooperative, i.e. correlated in time, interactions that must be abruptly terminated by an active disassembly process aimed at "freeing" neurons to make them available for formation of modified assemblies /93/. One can label these dual functions of the G-proteins and their target enzymes as subserving the Hebbian and anti-Hebbian processes which must occur side-by-side sharing the same neuronal populations. The assemblies seem to be generated by synchronous oscillation of activity, resulting in up- or down-regulation of synaptic strength between neurons as determined by in- or out-of-phase firing patterns /93,95,136/. Considering calculations that take into account the numbers of neurons available, the virtually endless possibilities of sensory input combinations, their interpretation, and selection of goal-directed behavior, would all not be possible without anti-Hebbian processes that are likely to occur during both wakefulness and sleep /cf. 95,96/.

In cGMP-rich substrates, such as the hippocampus and cerebellum, the long-term potentiation (LTP) of synaptic efficacy can rapidly change to long-term depression (LTD) of the same synapses /136/. In theory, even a slight malfunction of this process may be a factor in cognitive deterioration that could precede the morphologic alterations in aging brain, particularly in AD in which the G-protein stimulated adenyl cyclase activity was found to be reduced, whereas the G-protein inhibition of adenyl cyclase activity remained unchanged, relative to age-matched controls /118/. This indicates that in early stages of AD the anti-Hebbian processes become "over-zealous", a change that may not be expressed morphologically even at the electron microscopic level.

Adult rats, 3-4 months of age, chronically treated with FL show an unusual flexibility and resourcefulness in exploratory behavior relative to controls /102,148/. In order to avoid perseveration, a process of removing "spurious" plastic changes in the neuronal membrane, i.e. those that become useless for development of the animal's adaptive behavior, may be implemented by the cGMP-stimulated phosphodiesterase whose activity may be enhanced 50-fold by increasing cGMP concentrations; this enzyme breaks down both cAMP and cGMP, but it prefers cAMP /cf. 123/. In theory, the physiologic "sculpturing" of plastic alterations generated

by the BAAS, cholinergic and glutamatergic terminals, may be markedly enhanced by FL's concerted disinhibition of these systems that are known to upregulate the levels of cGMP /91,93/ and which are subject to the age-related tone of the BDZ/GABAergic system.

FL-induced REM sleep: an index of anti-aging and metabotrophic action?

In mammals and primates and humans, there is a gradual age-related decline in time spent in REM sleep /cf. 109/. The brainstem ACh and GL systems, and their phasic interplay with the aminergic systems are essential for the occurrence of REM sleep /cf 67,86,92,94,109/ and oral administration of FL to rats /117/ and dogs /154/ significantly increases REM sleep occurrence, most likely by disinhibiting the brainstem Ach/somatostatin systems /28/. The time spent in REM sleep by healthy humans and other mammals is gradually reduced with age and may be virtually eliminated in AD, even before full clinical expression of the disease /cf. 14,62,109/.

The loss of REM sleep is likely to be detrimental to the recuperative sleep functions that include memory processing, as indicated by REM-slow wave sleep (SWS) correlated shifts in statistical distribution of single neuronal firing patterns of feline non-specific, i.e. association, thalamic nuclei, and by the fact that patterns emitted during REM episodes mirrored those that had been recorded during the animal's most recent learning experiences while "spurious" patterns that were not related to actual bar pressing were eliminated from the neuronal firing repertory /95/.

The AD-related decline in REM sleep /14,62, 150/ is most likely caused by both pre- and post-synaptic GABAergic inhibition of cholinergic/somatostatin systems known to play a role in the emergence of REM sleep /28/. These systems are negatively modulated by a GABAergic input from local neurons of the pedunculo-pontine and particularly neurons in the lateral-dorsal tegmentum and those located in the vicinity of the substantia nigra /53,68,86/. In dogs, a single oral dose of FL (10 mg/kg) increases REM sleep and, to a lesser degree, deep SWS /154/. We have shown that chronic administration of smaller FL doses (3-4 mg/kg/day in drinking water) to 5-6 month-old rats gradually increases REM sleep by 100-155%, without significantly affecting SWS (Fig. 10). This effect was still present for several days after drug withdrawal /117/, despite FL's half-life in the rat brain being shorter than 16 minutes, and 80 minutes after i.p. injection of 10 mg/kg, FL is no longer detectable using high performance liquid chromatography /84/. Because the FL enhancement of REM sleep must have outlasted the drug presence, the REM sleep increases most likely reflect the FL-induced metabolic cascades triggered by disinhibition of the BAAS and the cholinergic/somatostatin neuronal systems, a conclusion compatible with the known REM sleep increases in brain protein synthesis and energy metabolism /138/.

For unknown reasons, the FL-induced increases of REM sleep in dogs and rats have not yet been

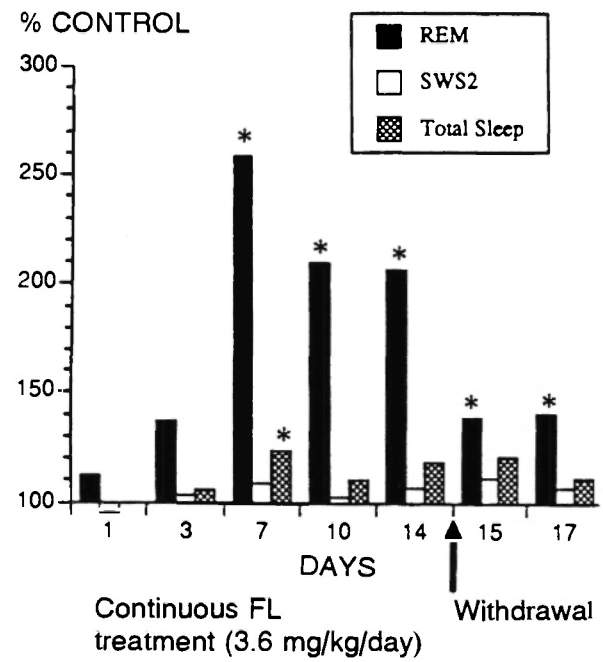


Fig. 10: Effect of continuous flumazenil administration (3.6 mg/day in drinking water) on sleep patterns of adult 4 month-old male rats. Note a gradual increase in REM sleep over the first 14 days of treatment, without major alterations in deep slow wave sleep (SWS-2) and total sleep. The REM sleep increases outlasted the drug administration (modified from O'Connor *et al.* /117/).

replicated in healthy humans. Following a single 10 mg i.v. administration of FL /132/, 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.

There are two possible explanations for the failure of FL to increase REM sleep in healthy humans: i) in rats and dogs, FL may have stronger disinhibitory effects on the cholinergic/somatostatin system promoting REM sleep, relative to such actions on the aminergic/peptidergic neurons, which are involved in terminating the REM episodes /67,92,94,109/, while in humans the presumed FL-induced disinhibition of the ACh system may be counterbalanced by a proportionally increased tone of the aminergic system involved in terminating REM sleep episodes /67,94/; and ii) considering the dose-dependent dual intrinsic actions of FL /36/, FL should be administered chronically in small doses, because a single 10 mg i.v. dose used in the above sleep studies might have induced dual effects, antagonist and agonist, at the BDZ receptors.

Acetylcholine is a secretagogue for somatostatin /cf. 7/, a peptide that is critical for generation of REM sleep /28/. Thus, the FL-induced disinhibition of the pontine tegmental cholinergic neurons, both at their somata and particularly at axonal varicosities, may be the most logical explanation of REM sleep enhancement in rats and dogs. The levels of somatostatin in the basal forebrain and cortex decline with age, particularly in AD patients /cf. 28,62,108/.

In aged rats, intracerebroventricular administration of somatostatin, or i.m. administration of its synthetic analogues that cross the blood-brain barrier, promptly restores REM sleep to the level seen in young rats /28/. If activation of BDZ/GABAergic receptors on peptidergic axon varicosities causes their depolarization block /162/, and if the tone of the GABAegic system, relative to other transmitter systems, increases with age, and particularly in AD patients whose brain stem shows more than 100% increase in GAD activity /124/, then it would be most logical, as already mentioned, to ascribe the FL REM sleep increases to disinhibition of the ACh/somatostatin terminals. Such an action could also take place in young adult animals /117,154/, as mentioned above, if the mechanisms for generating REM sleep are tonically moderated by the GABAergic system and do not function at full capacity.

REM sleep contributions to cognitive and brain recuperative functions

Animals that are required to learn various tasks, such as operant conditioned bar pressing, show increases in the frequency and duration of REM sleep episodes /139/. If the assumption is made that specific patterns of action potentials represent various aspects of the animal's behavior /93,95/, then one can say that REM sleep selects, replays and amplifies information acquired prior to sleep, e.g. during appetitively motivated exploration of manipulanda, while learning to associate, e.g., milk delivery with bar pressing /93,95/. The 'relevant' information is not encoded in the mean firing rate which often remains unchanged, but in emission of specific firing patterns. Pattern emissions during the animal's successful operation of manipulanda were augmented by 120 to 500% during REM sleep episodes. However, 10-15 min later, during subsequent SWS, the relevant patterns showed significant deficits with reference to the random model, i.e. certain patterns were virtually eliminated from the otherwise active neuronal firing repertory, suggesting an accomplished memory consolidation process /95,96/. All these phasic orderly alterations in neuronal firing patterns could not be observed in felines which had deficits in REM sleep, could not learn or had difficulties in acquiring operant conditioned behavior /95/ and perseverated in inefficient exploratory mode when confronted with manipulanda /93/.

The inversions in statistical distributions of patterns implicate REM sleep in memory processing because: (i) 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 must have been regulated by graded alterations in densities and/or affinities of neuronal

receptors used in generation of these patterns; (ii) this orderly process implies that patterns, most likely reflecting activity of neuronal assemblies, are involved in associative learning and recuperative processes /95,96/; and iii) it is inconceivable that the aforementioned diametric shifts in statistical distribution of single neuronal firing patterns, reflecting flexibility in neuronal connectivities, could occur without active processes of "erasing" and amplifying select plastic changes in neuronal membranes, implemented by transmitters coupled to cGMP stimulated cyclic nucleotide.

In conclusion, one can argue that: (i) the age- and/or AD-related loss of REM sleep /14,150/ is likely to have strong negative consequences on the recuperative quality of sleep and cognitive processes; (ii) REM sleep may be regarded as an index of a subject's cognitive capacity; iii) FL-induced increases in REM sleep in rats and dogs may be tentatively interpreted as a regression to a more "youthful" brain function; and (iv) the potential FL enhancement of REM sleep in healthy humans, and in humans with emerging signs of AD, is of clinical and theoretical importance.

Paradox of flumazenil's antiepileptic action; a preferential disinhibition of aminergic systems?

Although FL in moderate doses is a BDZ antagonist and therefore should be expected to lower the seizure threshold, FL actually has antiepileptic actions in animals /55,59/. Also, in humans oral and i.v. administration of FL for up to 42 months had significant antiepileptic actions /134/: in 19 out of 27 patients, FL suppressed the EEG spike-wave patterns which were refractory to conventional drugs. Most importantly, none of the patients developed tolerance to the therapeutic action of FL, and in this group, nine patients showed a moderate, but consistent, mood elevation, suggesting an increased tone of the BAAS neurons.

Another example of FL's therapeutic action is its ability to control seizures in the Lennox-Gastaut syndrome /56/ 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 is contradicted by the fact that the syndrome is aggravated by BDZ receptor agonists and barbiturates, while i.v. injections of FL were able to control the syndrome /56/ (see Fig. 11).

The antiepileptic actions of FL are likely to depend on disinhibition of the BAAS/peptidergic systems and their preterminal axon varicosities, and the resulting improvement of the glial/neuronal trophic relationships and energy supply, which may be expected to restore normal neuronal membrane potentials in epileptogenic foci. This view is supported by the fact that: i) FL increases oxygen utilization /1,125/, particularly following its depression by prior diazepam administration /125/; and ii) *in vitro*, serotonin acting on 5-HT_{1A} receptors, increases membrane K^+ conductance and blocks the action potential bursts of brainstem neurons, if they are triggered by prior hyperpolarization-induced activation of voltage sensitive low-threshold Ca^{2+} conductances /67,86/.

The Gonsalves-Gallager FL phenomenon; anticonvulsant action of FL and serotonin raphe system

The $BDZ/GABA_A$ receptors on somata of 5-HT neurons in the dorsal raphe nuclei develop tolerance or subsensitivity to BDZ/GABA agonists /46, 47,51/. Thus, one can conjecture that these neurons may loose their membrane potential and undergo phasic depolarization inactivation, rendering the antiepileptic BDZ therapy inefficient by eliminating inhibitory influences of the 5-HT system. The GABA subsensitivity of the raphe neurons /47,51/ and probably their depolarization inactivation, as well as tolerance of human subjects to BDZ's antiepileptic action, can promptly be reversed in a matter of minutes, and for a period of more than one week, by a single systemic administration of FL which fully restores the clinical efficacy of the antiepileptic BDZ treatment /131/. These observations are at least as difficult to interpret as those of the antiepileptic actions of FL discussed above. Nonetheless, they have a potential bearing on the

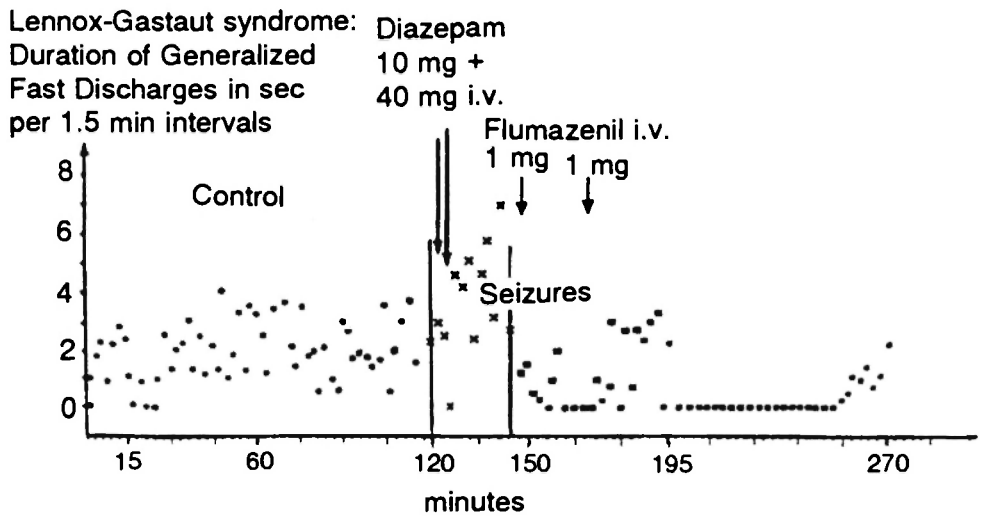


Fig. 11: Aggravating effect of diazepam contrasting with therapeutic effect of flumazenil (FL) on the occurrence of fast epileptic EEG discharges in a human subject suffering from the Lennox-Gastaut syndrome. Hexobarbital also had an aggravating effect, which was terminated by flumazenil (not shown). (Modified from Halasz /56/.)

GABAergic hypothesis of brain aging. As discussed above, in patients with advanced AD, the synthesis of GABA is elevated by 38-100% in the brainstem, thalamus and basal forebrain, relative to age-matched controls /124/. This condition, in theory, may totally block the antero- and retrograde transport in 5-HT axon terminals and varicosities through GABAergic depolarization. The somata of 5-HT neurons, deprived of retrograde flow of trophins, may not be able to retain normal GABA receptor function, leading to their "GABA subsensitivity" /47,51/. This, one can conjecture, may be reversed by FL's restoration of antero- and retrograde functions of axon terminals/varicosities. Also, one should recall that normal functions of 5-HT receptors on astrocytes are critical for both the formation of glycogen and glycogenolysis, and the release of lactate /34,60/, the main neuronal energy substrate /cf. 60/. When neurons become deficient in ATP and unable to control their membrane potential and ionic fluxes triggered by excitatory input, they may be prone to uncontrollable burst discharges, resulting in partial and generalized forms of epilepsy, such as the Lennox-Gastaut syndrome. Paradoxically, the latter can be controlled by i.v. injection of FL, but are triggered by BDZ/GABA agonists, such as diazepam and phenobarbital /56/. The most logical explanation of FL's antiepileptic action is disinhibition of BAAS neurons and particularly of its 5-HT neurons and axon terminals, because 5-HT, acting on 5-HT_{1A} receptors, causes an outward K^+ movement **and** blocks the low-threshold calcium currents which are known to be responsible for the burst firing mode of many brainstem and thalamic neurons /86/.

FL treatment of hepatic encephalopathy (HE)

The discussion of this topic is relevant to the BDZ/GABAergic hypothesis of brain aging and age-related loss of cognitive functions, because HE seems to represent a semi-acute, on the time scale of several months, increase in the tone of the BDZ/GABAergic system, leading to a loss of cognitive functions and coma.

The BDZ receptor agonist ligands 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 /cf. 11/. The source of the "endogenous" BDZ agonist ligands, such as diazepam, N-desmethyldiazepam and oxazepam, may be food stuffs and/or BDZ compounds synthesized *de novo* by intestinal flora /cf 11/. The levels of these BDZ ligands may increase with diet rich in milk, soy-

beans, 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 i.v. or oral administration of the BDZ receptor antagonist, FL /cf. 11/.

Although several successful treatments of HE with FL have been reported /cf 11/, the best documented and illustrative 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 porta-caval shunt /11/. 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 comatose again, remained chronically encephalopathic and had four further episodes of deep coma during the subsequent 3 months. Following reinstitution of FL treatment (25 mg of FL b.i.d.) and despite an unrestricted protein diet, she has been well for 14 months without any signs of encephalopathy, and she is able to lead a normal life.

The above case is interesting not only because FL was able to control the gross clinical signs of encephalopathy, but also because FL fully restored cortical event-related P300 evoked potentials. The amplitude of these endogenous potentials, e.g. normally generated after the attentive 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 only the passive process of impulse conduction to primary projection areas /cf. 93/. Two hours after the subject ingested 25 mg of FL, the amplitudes of the P300 potentials were comparable to those of healthy subjects and the subject's cognitive functions were fully restored /cf. 11/.

FL and subacute neurodegenerative processes

In humans, single i.v. administration of FL was shown to dramatically, though temporarily, ameliorate the comatose state and dysautonomia associated with degeneration of thalamic nuclei /cf. 2/. A single i.v. FL injection also dramatically improved resolution of cortical visual evoked potentials in patients suffering from a fatal spongiform encephalopathy, Creutzfeldt-Jacob disease /2/. These effects of FL are indicative of pathological increases in the tone of the BDZ/GABAergic system, which, in theory, could be a sufficient factor contributing to the neurodegenerative process. Whether or not chronic treatment of these diseases with FL would be able to retard their progress remains to be investigated.

Does FL prevent brain aging by antagonizing endogenous BDZ ligands and/or those ingested with food?

Food restriction has beneficial effects on longevity of humans and animals, including rats /cf. 99, 104/. Thus, it is tempting to conjecture that these beneficial effects 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 /cf. 11/. Thus, in the study of the anti-aging effects of FL /99/ in which there was no food restriction, the beneficial effects of chronic FL administration could have mimicked the effects of food restriction by "protecting" the BAAS and the cholinergic/peptidergic neurons from excessive levels of the known "endogenous" BDZ agonist ligands, diazepam, N-desmethyldiazepam and oxazepam /cf. 11/.

Repeated FL administration to healthy humans

In a cross-over double-blind design, Lavie *et al.* /77/ administered orally every 4 hours 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 working hypothesis was that, if FL has any effects on sleep, the subjects would be affected in their efforts to resist sleep when asked to lie down for 7 minutes in bed in a dark room, close their eyes and resist sleep. Six such 7 minute trials were spaced at 4 hr intervals; at the onset of each trial, the subject was given a FL tablet or placebo.

FL significantly decreased the subject's ability 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 than antagonist action on BDZ receptors /77/. This interpretation must, however, be challenged by the fact that, after each of six 7 minute sleep-resist trials, 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 /77/. 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 and coordination of motor reflexes /cf 61/.

The increased speed of motor/cognitive performance of the FL treated subjects is highly reminiscent of the 9-fold improvement of cognitive/motor skills observed in rats chronically treated with FL and challenged to resolve a swim-escape task /148/ (see below). The improved motor skills in healthy humans are probably related to disinhibition of the BAAS neurons and their preterminal axon varicosities, and resulting more efficient function of the cerebellum, as indexed by the previously discussed FL potentiation of cerebellar glucose utilization /125/ and energy metabolism.

Does chronic FL produce emotional imperturbability (ataraxia), i.e. a protection from environmental stress, resulting in superior cognitive/motor skills?

Such an action would have implication for brain aging, because there is a large body of evidence that emotional stress, resulting in destabilization of the autonomic system and massive serum corticosteroid increases, plays a major role in excitotoxic degeneration of large populations of neurons, particularly in the hippocampal formation /129/.

One can argue that chronic administration of FL should have stabilizing effects on the autonomic system, because it antagonizes both *in vitro* and *in vivo* actions of endogenous anxiogenic BDZ ligands /cf. 36,66/. Thus, FL may be expected to prevent large swings in emotional responses, a mechanism whose evolution was probably aimed at making our ancestors more sensitive to life-threatening environmental and social stimuli. However, in most current human affairs, emotional sensitivity and stress may be inappropriate and even excitotoxic to neurons through release of glucocorticoids (GCs) /129/, particularly if the metabolic functions of neurons have been compromised by age-related vascular pathology.

FL may be expected to improve cognitive and motor skills needed for adaptive behavior particularly in challenging circumstances that require their non-emotional analysis and skillful motor responses. Indeed, this may be the case, as shown by the following series of experiments:

1) Swim escape test. In this demanding test /148/, adult 3 month-old male rats chronically treated with FL (4 mg/kg/day in drinking water for 16 days) were challenged to escape from a water T-maze equipped with four ropes, two on each side of the T-arms, hanging from the overhead escape platform and touching the water surface, while only one rope was climbable, due to roughness of its surface and rope stability determined by anchoring it to the bottom of water tank. In this paradigm (Fig. 12), rats treated with FL needed only $1/9^{th}$ of the time for resolving the swim-escape task, relative to the age-matched controls /148/.

It appears that FL increases the capacity for associative motor learning mainly by disinhibition of the BAAS and cholinergic/peptidergic functions, actions that are consistent with the recent observation that *in vitro* associative long-term potentiation in piriform cortex requires a precisely timed block of $GABA_A$ receptors /cf. 53/.

2) Radial arm maze: non-appetitive, i.e. curiosity driven exploratory behavior and signs of emotional imperturbability (ataraxy) (Fig. 13). Mature 4 month-old male rats were partially food-deprived to motivate them to perform in the maze /102/. After 17 daily trials, guillotine gates were used to confine the animals for 10 sec to the center platform after each arm selection. This change in procedure failed to disturb the animals that had been chronically treated with FL, as indexed by unchanged "working memory" errors, while the performance of the control animals was significantly impaired (Fig. 13). The control animals showed significant increases in urination/defecation scores, indexing the animals' emotionality. The FL exposed rats, in addition to the food seeking

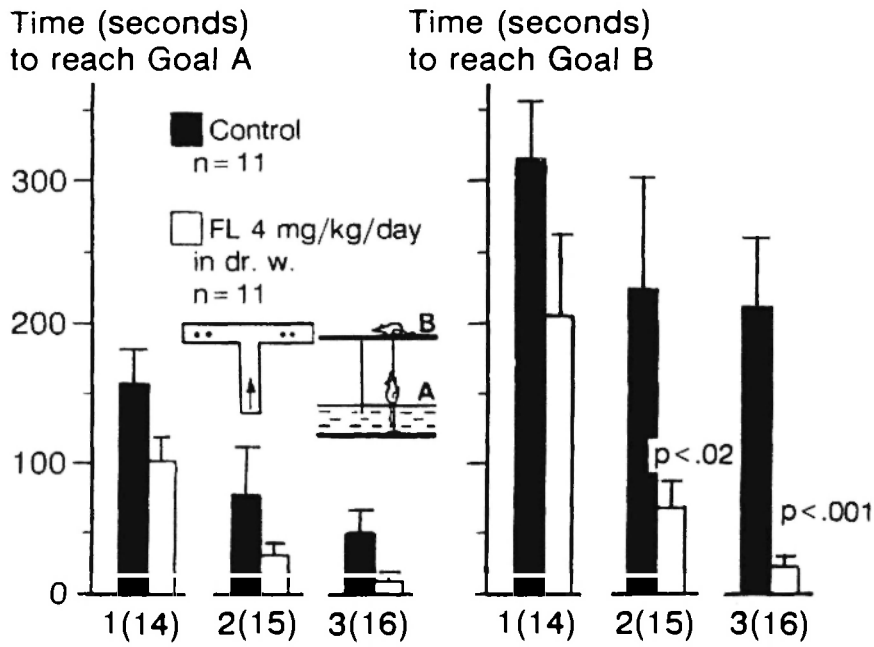


Fig. 12: Water T-maze escape paradigm (see inset in left panel). Mature 3 month-old male rats chronically treated with flumazenil (4 mg/kg/day in drinking water or the drug vehicle, 0.5% ethylene glycol in tap water). The animals were challenged to reach goal A and goal B on the overhead platform, using one of the 4 ropes (2 on each side of the T-maze); only one rope was climbable, for it was anchored to the bottom of the water tank. Note that there were no significant time differences (seconds) between the control and FL group in reaching goal A in trials 1-3 conducted at days 14, 15 and 16 of drug/vehicle treatment (left panel). However, as shown in right panel, in trial 3 (day 16 of drug treatment), the FL group needed less than $1/8^{th}$ time required by the control group ($df = 1,20$; $F = 10.0$; $p < 0.001$, ANOVA repeated measures). (Modified from Urbancic *et al.* /148/.)

performance, showed extraordinary curiosity aimed at objects located outside the maze by "rearing" on hindlegs with forepaws at the edge of the alley wall, lifting their bodies to gain full view of the room or climbing over the maze wall; they also showed unusual inventiveness and motor skills in lifting and/or climbing over the guillotine gates, behaviors never observed in the control group. It was obvious that this behavior was not motivated by fear, but by curiosity directed toward the environment outside the maze filled with objects of potential interest, because this behavior was not observed in alleys facing the darker and "dull" room corner. After FL withdrawal on day 17, the exploratory behavior remained unabated in 3 daily trials (a longer time period was not investigated), suggesting that chronic FL, as in the study of the REM sleep (see above), sets in motion complex brain metabolic processes that outlast the direct drug effects.

The autonomic system of FL-treated rats, as indexed by the defecation/urination scores relative to controls (Fig. 13), was significantly much more stable, not only following the introduction of guillotine gates, but also during most phases of the daily trials. In the FL group, these scores remained statistically unchanged, contrasting with the disoriented behavior of the control rats whose errors at days 15-20 significantly increased, and showed a 10-fold increase in defecation/urination scores. The validity of these observations is enhanced by the fact that across all 23 rats studied, there was a highly significant inverse correlation between the numbers of curiosity-driven exploratory episodes and the defecation/urination scores [$r(23) = -0.5$; $p < 0.003$].

The above exploratory behavior of rats treated with FL is reminiscent of healthy humans who, after a single 5 mg i.v. dose, reported that they experi-

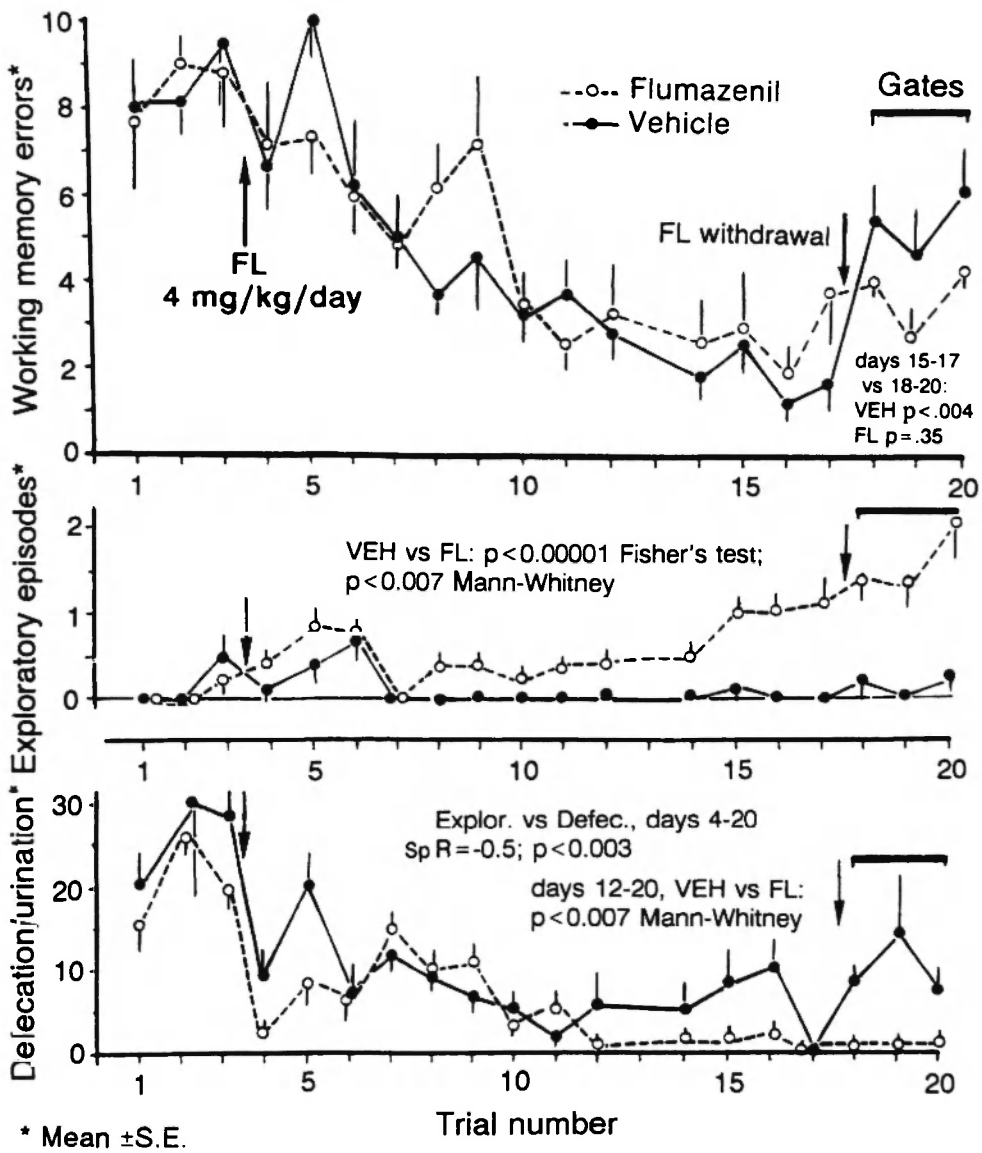


Fig. 13: Performance of mature 4 month-old male rats in the radial arm maze, prior to and during chronic administration of flumazenil, and during 3 days following drug/vehicle withdrawal, coinciding with the introduction of guillotine gates to keep the animal for 10 seconds in the center platform after each exploration of an alley. **Top:** numbers of "working memory" errors (ordinate) decreased equally in both groups ($p < 0.0001$ ANOVA repeated measures), and there were no group differences ($p = 0.17$). However, the introduction of gates and drug withdrawal revealed significant differences between the two groups: the control group, relative to the preceding three days, 15 to 17, made many more errors ($F(1,10)=13.84$, $p < 0.004$, ANOVA repeated measures), while the FL group was not significantly disturbed by this alteration of experimental procedure ($F(1,10) = 0.94$, $p = 0.35$). **Middle:** numbers of non-appetitively motivated, i.e. curiosity-driven, exploratory episodes (ordinate) increased over time in the FL group, relative to control ($F(1,21) = 12.92$, $p < 0.002$), and the difference between the two groups was largest after drug withdrawal/introduction of gates ($p < 0.001$). **Bottom:** Emotionality of rats in the control and FL group, as indexed by defecation/urination scores (ordinate), was not significantly different in trials 1 to 11, but in trials 12 to 20, the scores in the FL group were about 10 times smaller than in the control group ($p < 0.007$; Mann-Whitney). Even the introduction of gates coupled with drug withdrawal failed to influence the virtually scoreless FL group. Pairing these scores for days 4-20 with numbers of exploratory episodes, revealed a significant inverse correlation between the two measures ($R = -0.5$; $p < 0.003$; Spearman coefficient of rank correlation). Modified from Marczynski *et al.* /102/.

enced "pressure" to move and explore the environment /132/.

The distribution of the non-appetitively motivated exploratory behavior of individual rats is shown in Fig. 14.

3) Anxiolytic effect in elevated plus-maze (Fig. 15). Chronic FL administered to 4 month-old rats (4 mg/kg/day in drinking water) increased the time spent by the rats on open arms, particularly in response to minor changes in construction of the maze (Fig. 15). Here again, as in the radial maze, there was a significant inverse correlation between defecation scores and the time spent on open arms [$r(16) = -0.64$; $p < 0.007$].

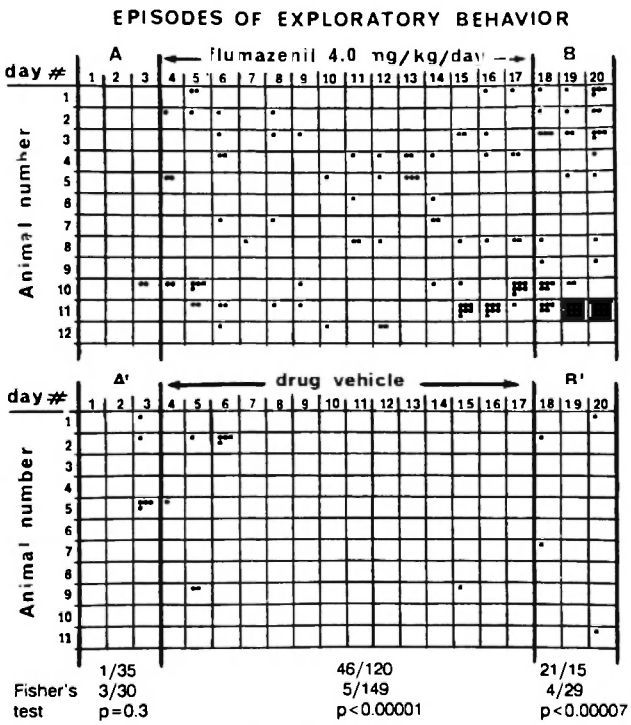


Fig. 14: Extension of Fig. 13: Occurrences of individual curiosity-driven exploratory episodes prior to (A), during flumazenil/vehicle treatment, and after drug/vehicle withdrawal (B). At the bottom, the Fisher's tests for the ratios between the number of trials during which the animals showed exploratory activity and the number of trials with no such behavior, revealed no significant group differences in phase A, and highly significant group differences during drug/vehicle treatment, and in phase B of drug/vehicle withdrawal. Based on data from Marczynski *et al.* /102/.

4) Social interaction test: anxiolytic/ataraxic effect (Fig. 16). These tests, with some modifications, were conducted as described by File /37/. FL was administered (4 mg/kg/day in drinking water) to 3 month-old male rats, which prior to tests were kept in separate cages. As shown in Fig. 16 left, on day 12 of FL treatment, the treated rats when paired showed a much faster habituation to the unfamiliar environment, as indexed by the time they spent in "friendly" social interactions, relative to untreated rats ($df=1.17$; $F=4.9$; $p<0.05$, ANOVA repeated measures). Next day, each individual animal was placed for 5 minutes in the testing arena to become more familiar with it. The following day, the untreated paired control rats, when placed in the now familiar arena, showed enhanced habituation, as indexed by faster than before increases in social interactions, and there was no difference between the FL and control groups at minutes 3 to 7 ($df=1,17$; $F=2.8$, $p=0.1$). However, the introduction (Fig. 16 right) of a novel inconsequential auditory stimulus (65 dB tone) disrupted the interactive behavior of the control rats, while the FL treated pairs ceased interacting only for about one minute. Thus, FL treatment significantly facilitated the animals' habituation to inconsequential environmental stimuli.

5) "Anticonflict" effect of prior chronic FL treatment, as ascertained in the punished drinking test conducted 3-10 days after drug withdrawal (Fig. 17). Vogel's test /147/ was carried out on adult 4 month-old male rats after treatment for 21 days with FL (4 mg/kg/day in drinking water). In the "conflict" tests, after 20 licks, a mild 0.35 mA current was delivered to the drinking spout. Each test was carried out after 44 h water deprivation. As shown in Fig. 17 left, the emotional state of the animals could be divided into "naive" and "shock experienced". On day 3 of drug withdrawal, the animals did not change their drinking habits, relative to pre-drug condition (-2 day). Following electric shock experience, the FL-exposed group was not significantly affected on day 6 and 10 of drug withdrawal. Conversely, in the control group, the shock experience significantly reduced unpunished drinking ($p < 0.003$; Wilcoxon matched pairs signed ranks test). On day 10, there was a significant difference between the groups

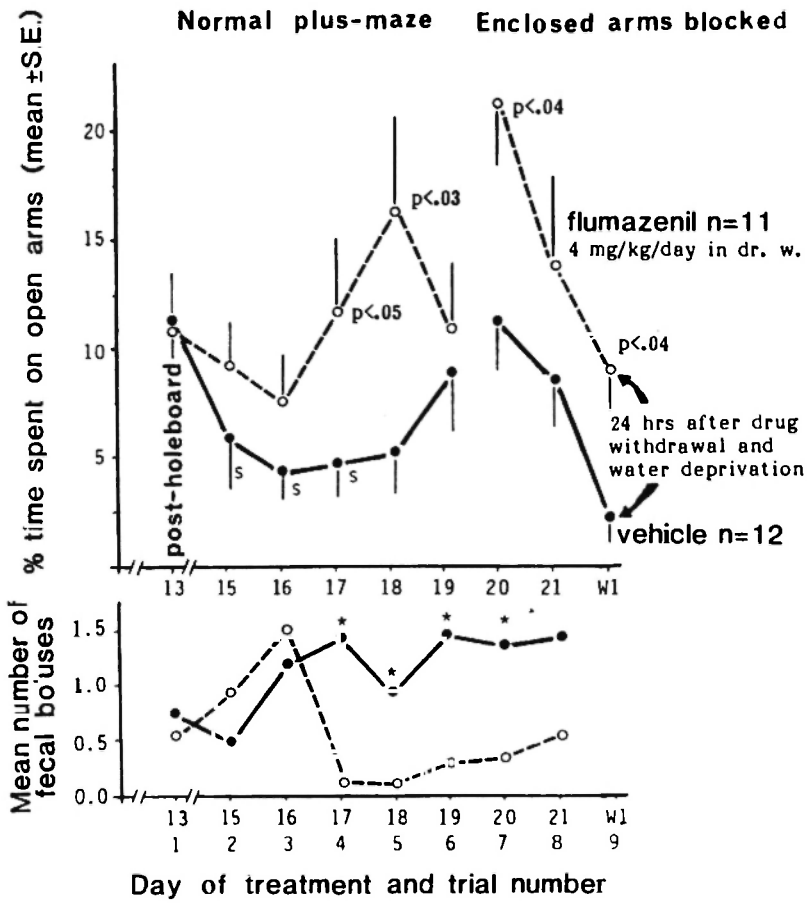


Fig. 15: **Top:** Enhanced exploratory behavior of rats during chronic 21-day treatment with flumazenil (4 mg/kg/day in drinking water), as measured by percent time spent on open arms of the elevated plus-maze (ordinate). Rats (4 months of age) were tested on day 13, and days 15 to 21 of treatment, and 24 hours after drug/vehicle withdrawal (W1; abscissa). Trial 1 (day 13 of treatment) was conducted immediately following a 5-min holeboard test which, to some extent, habituated the animals to handling and novel environment, and, therefore both groups showed comparable exploratory behavior. However, in subsequent trials 2, 3 and 4 (days 15 to 17 of treatment) the control group spent significantly less time on open arms, relative to trial 1 ($p < 0.02$; paired t-tests), while the FL group did not show a significant decline, and in trial 4 and 5 the differences between the groups became significant ($p < 0.5$ and $p < 0.03$; two-tailed t-tests). After the access to the enclosed arms was blocked (trial 7 through 9), the FL group spent more time on open arms than the control group ($p<0.04$). In the last trial, conducted 24 h after FL withdrawal and water deprivation, again the FL-exposed group spent more time in open arms ($p < 0.04$). **Bottom:** the numbers of fecal boluses (ordinate) left by the animals in the plus-maze after each of 1 to 8 trials (abscissa) were significantly higher ($p < 0.0001$; Fisher's exact test), relative to FL-treated rats. There was a significant inverse relationship ($r = -0.64$; $p = 0.007$) between the numbers of fecal boluses left by the animals after 8 trials and the percent time spent on open arms, thus validating the conclusion that FL stabilizes the animals' emotionality and the autonomic system function. (Modified from Urbancic *et al.* /147/.)

($p < 0.006$; two-tailed t-test). The punished drinking (right panel of Fig. 17) that followed 1 minute of unpunished drinking, also revealed a strong "anticonflict" effect of prior chronic treatment with FL, and on day 3 and 6 of drug withdrawal there were significant differences between the groups ($p < 0.04$ and $p < 0.006$, respectively; t-test).

Unique effect of single oral FL administration to healthy humans: combined enhancement of vigilance and habituation (Fig. 18)

To delineate differences between the electrophysiological and psychophysiological effects of diazepam and FL, Higgitt *et al.* /61/ studied the

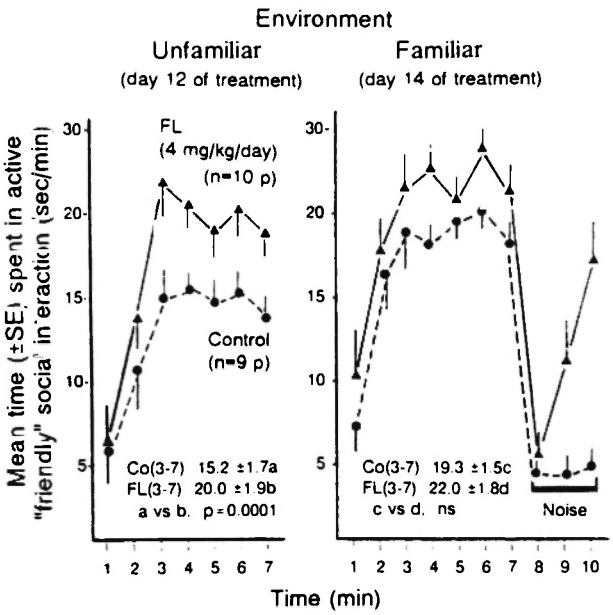


Fig. 16: FL enhancement of habituation of 4 month-old male rats to novel, but inconsequential environmental stimuli. During the test, paired rats occupied the same 50 cm x 50 cm testing platform, but were normally kept in separate standard cages. In unfamiliar environment (left panel) on day 12 of drug treatment (4 mg/kg/day in drinking water), the social interactions of control animals were significantly suppressed, relative to the FL treated rats (minutes 3 through 7; df = 1,17; F = 6.1; p < 0.03; ANOVA repeated measures). At day 14 of treatment, and in familiar environment (right panel), there were no significant group differences at minutes 3 to 7 (df = 1,17; F = 2.8; p = 0.1). The introduction of a novel continuous inconsequential 65 dB tone at minutes 8 through 10, caused the FL group to "freeze" behaviorally for one minute, but shortly thereafter the rats resumed their "friendly" interactions. Conversely, the control rats could not habituate to noise and remained inactive for 3 minutes (longer time periods were not investigated); the difference between the two groups at minutes 8 through 10 was highly significant (df = 1,17; F = 10.1; p = 0.005) (Urbancic, Gadek and Marczynski, unpublished observations.)

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

(i) Cortical potentials evoked by inconsequential stimuli (series of randomly spaced 32 clicks) which did not require the subject's attention, declined over six trials 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;

(ii) 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;

(iii) 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;

(iv) 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;

(v) Despite the above stabilizing actions on the automic system and facilitation of habituation to inconsequential environmental stimuli, FL had no measurable depressant effect on visual input processing, as measured by the Critical Flicker Fusion Threshold.

The increases in the EEG alpha mean frequency, and decreases in slow wave activity /132,163/ observed by Higgitt *et al.* were also reported by other investigators /132,163/, and are consistent with the view that FL increases vigilance in humans, while stabilizing the functions of the autonomic system and promoting habituation, which constitute a unique pharmacologic profile not observed in other drugs.

Another observation made by Higgitt *et al.* /61/ is difficult to interpret, but may be important, because it indicates that FL, even in a single dose, may affect mood. The volunteers differentiated the

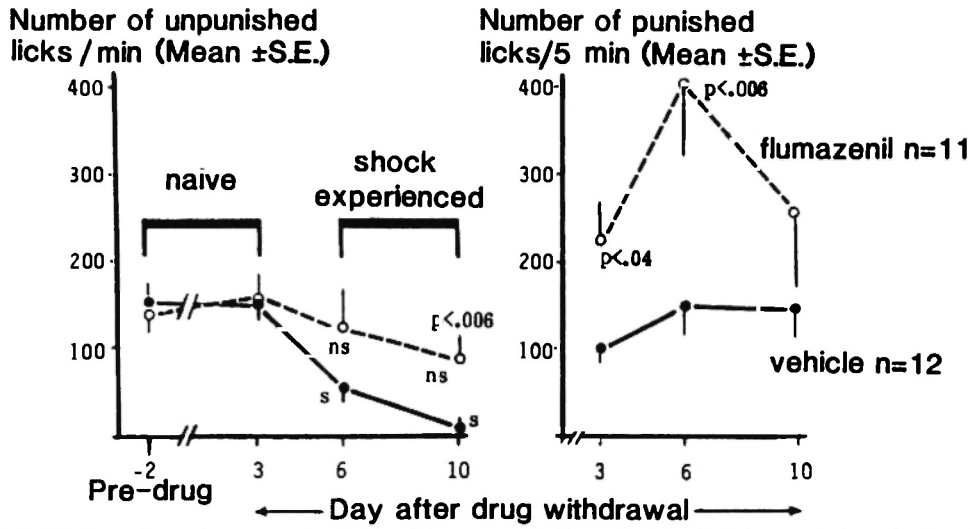


Fig. 17: "Anticonflict" effect of previous chronic 21 day FL treatment (4 mg/day in drinking water), as indexed by Vogel's test of unpunished and punished water licks counted in 44-h water deprived rats (left and right panel, respectively). In this procedure, 1 min of unpunished licking was followed by 5 min of punished licking (0.35 mA shock following each 20 licks), except for the pre-drug test (2 days prior to initiation of FL treatment). **Left panel:** In four trials of unpunished drinking, the animals' emotional condition could be divided into a shock-naive and a shock-experienced condition. In the control group, the shock experience significantly (s) inhibited unpunished drinking, relative to two naive state trials ($p < 0.003$; Wilcoxon test), while in the FL exposed group, on days 6 and 10 of FL withdrawal, the shock experience had no significant suppressing effect (ns, $p = 0.14$). On day 10 of drug withdrawal, there was a highly significant difference between two groups ($p < 0.006$; two-tailed t-test). **Right panel:** the punished drinking in the FL group, relative to controls, was significantly enhanced on days 3 and 6 after drug withdrawal ($p < 0.04$ and $p < 0.006$, respectively; t-test). (Modified from Urbancic *et al.* /147/.)

effect of FL from diazepam, since those treated with FL, when asked to self-rate their feelings 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 alteration could be related to the FL-induced feeling of 'pressure' to move and explore the environment, a feeling also reported by other investigators /132/ following a single 5 mg i.v. injection of FL to healthy volunteers. Parsimoniously, the feeling of discontent could be explained by the subject's three hour long confinement to the laboratory environment and the elaborate experimental protocol. The subjective 'pressure' to explore the environment seems to correspond to the surges of unusual exploratory behavior observed in adult rats chronically treated with FL /102/, as discussed above. The feeling of discontent caused by a single FL dose, contrasts with the elevated mood reported by patients suffering from epilepsy and chronically treated with FL tablets /134/.

Autonomic system stabilization and facilitation of habituation process as a potential basis for FL's nootropic 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 one 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 /69/. In contrast to FL, the BDZ agonists and the β-carboline "inverse" BDZ agonists /66, cf. 102/ and psychostimulants of the amphetamine group block habituation /6/. Moreover, the last two drug groups tend to distort cognitive processes and cause perseverative or even psychotic behavior /6/.

Intuitively, simultaneous enhancement of vigilance and habituation is often regarded as incompatible, mainly because vigilance is often wrongly conceptualized as a state of moderate arousal

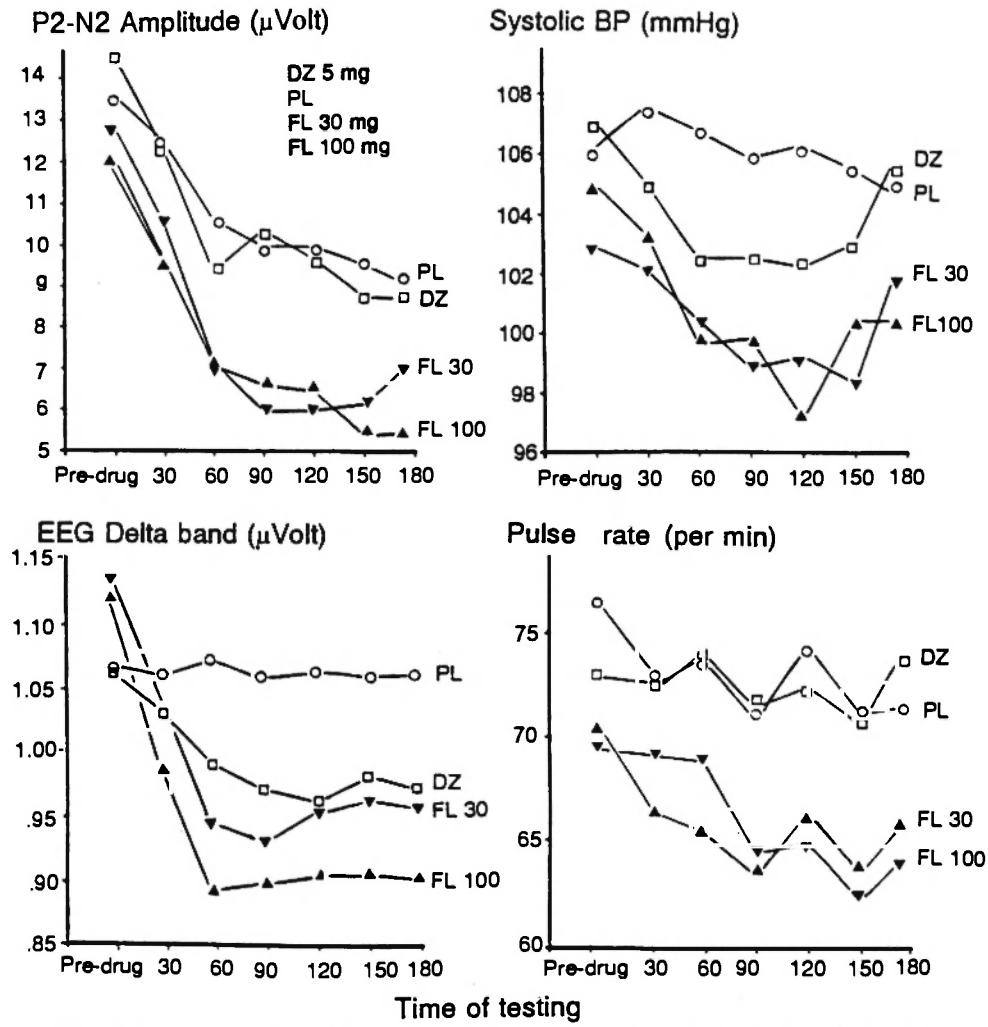


Fig. 18: Habituation-promoting and ataraxic actions of FL administered orally in a single 30 mg or 100 mg dose to healthy humans; responses were compared to placebo (PL) and diazepam (DZ; 5 mg p.o.) effects. Relative to PL or DZ, FL significantly ($p < 0.01$) facilitated subjects' habituation to repeated inconsequential and random auditory stimuli (clicks), as measured by declining electrocortical P2-N2 deflections. Note the lack of DZ effect which was comparable to that of placebo (PL). Also, note the FL-induced reduction of systolic blood pressure ($p < 0.02$), relative to PL or DZ ($p < 0.008$, $p < 0.02$, respectively), and the enhanced reduction in pulse rate ($p < 0.02$), relative to DZ and PL; these alterations occurred, despite the subjects displaying "activated" EEG patterns, as indexed by reductions in the EEG delta wave band ($p < 0.004$). These FL effects were coupled with: i) a significant reduction in fluctuation of galvanic skin conductance ($p < 0.001$); and ii) no changes in the flicker-fusion threshold (not shown), indexing unaltered vigilance and processing of sensory information. (Modified from Higgitt *et al.* /61/.)

which is thought to be antithetical to habituation /72/. However, the habituation process is known to be predominantly cholinergic in nature and is antagonized by scopolamine /18/, while psychostimulants of the amphetamine group block habituation by increasing arousal through preferential activation of the catecholaminergic system, which is known to cause severe and lasting endocrinologic responses similar to those of emotional stress /6/.

Although the habituation process is still poorly understood, it is apparent that this process is contingent upon analysis of the novel environment and subsequent feedback inhibition of input judged to be irrelevant /72/. The benefits of such a process are thought to be essential for cognitive functions and are brought about by an active process of suppressing focused attention and "freeing" the committed neurons for a subsequent cognitive task

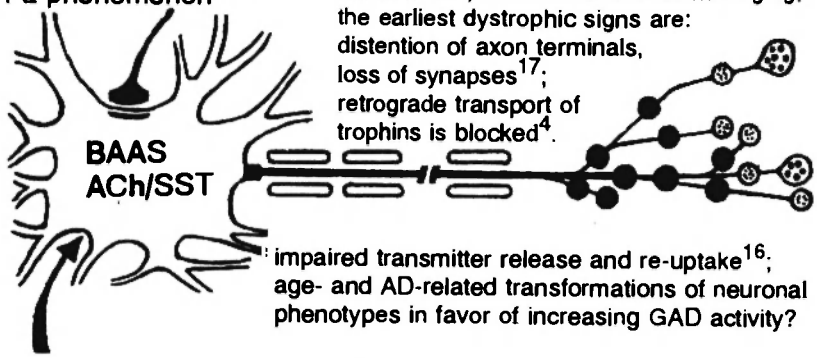
Extrasynaptic GABA deafferentation hypothesis of brain aging and Alzheimer's disease: apoptotic GAD activity, upregulation of receptors and their affinity[5-10,22]

Inward movement of Cl^- hyperpolarizing inhibition of all ascending systems, antagonized by bicuculline and flumazenil; e.g. medial septum ACh neurons[15].

Outward movement of Cl^- depolarizing inhibition of axon varicosities, antagonized by bicuculline & flumazenil; chronic PTZ antagonizes[7], while BDZ agonists promote brain aging[21].

pre- and extrasynaptic sites

Gonsalves-Gallager FL phenomenon[12,13]



chronic depolarizing inactivation of axonal varicosities[1]; in both AD and normal aging, the earliest dystrophic signs are: distention of axon terminals, loss of synapses[17]; retrograde transport of trophins is blocked[4].

impaired transmitter release and re-uptake[16]; age- and AD-related transformations of neuronal phenotypes in favor of increasing GAD activity?

metabotropic functions of aminergic axon varicosities↓[11] → deafferentation of cortex[2,17] → induction of βAPP mRNA[2], trophin accumulation[4] → dendritic sprouting[20], activation of kinases[22] → indiscriminate Tau phosphorylation[18]; depolarization of astrocytes[3] → lactate release↓ FL, but not the inverse agonists, prevents GABA/BDZ actions[3].

Energy deficit; decreased Na^+/K^+ATPase activity; inputs using EAA and AMPA receptors, become toxic by destabilizing Ca^{2+} homeostasis, where physiological concentration of $Ca^{2+}(i)=1/1000$ of $Ca^{2+}(e)$; this condition is aggravated by age- and/or stress-related high serum levels of GCs[14]. In hypometabolic foci with depolarized neurons, deficits of free ATP, ADP, P_i, and of monoamines (particularly dopamine),- disinhibit GAD[19], thereby further elevating the intra- and extracellular GABA[8,9,21].

1) Zhang & Jackson, Science, 259:1993; 2) Wallace & Haroutunian, Beh. Brain Res., 57:1993; 3) Backhus et al., Glia 2:1988; 4) Crutcher et al., J. Neurosci., 13:1993; Beal et al., TINS 16:1993; 6) Coyle & Putfarcken, Science, 262:1993; 7) Landfield et al., Science 214:1981; 8) Reinikainen et al., J. Neurol. Sci., 84:1988; 9) Myers & Komyskey, Brain Res., 343:1985; 10) Calderini et al. Neurobiol., Aging 2:1981; 11) Descaries et al., in: Avoli et al., Eds., Plenum Press, N.Y., 1988; 12) Gonsalves & Gallager, JPET, 244:1988; 13) Savic et al. Lancet, 337:1991; 14) Sapolsky, Beh. Brain Res., 57:1993; 15) Walsh et al., Brain Res. Bull. 31:1993; 16) Allard et al., Brain Res. 637:1994; 17) Masliah & Terry, Clin. Neurosci. 1:1993; 18) Matsuo et al., Neuron, 13:1994; 19) Martin & Rimvall, J. Neurochem., 60:1993; Lindefors, Prog. Neuropsychopharmacol. & Biol. Psychiat., 17:1993; 20) Ihara, Brain Res., 459:1988; 21) Moodley et al., Psychiat Res. 48:1993; 22) Smith & Sharp, J. Neurol. Transm. Gen. Sect., 97:1994; 23) Lindsay et al., TINS 17:1994.

Fig. 19: Synopsis of the BDZ/GABAergic deafferentation hypothesis of brain aging based on age and/or genetically determined excessive increase in the tone of the BDZ/GABAergic system, leading to a depolarizing block of the BAAS-peptidergic and ACh-somatostatin (SST) preterminal axon varicosities at the cortical and subcortical levels, resulting in functional and subsequently anatomic deafferentation of target systems. The metabolic consequences of the block of bidirectional axonal transport generate neurofibrillary tangles and cause induction of the βAPP-mRNA gene in the target systems. The excitatory amino acid (EAA) and/or excitatory cholinergic input to metabolically compromised neurons becomes toxic, mainly by destabilizing neuronal Ca^{2+} homeostasis. For further explanations, see text.

/93/. In cats, the dynamics of this processes, studied using behavioral, EEG and neuronal firing patterns, showed that, in a large population of cats, those that were unable to habituate to a novel environment had difficulties in acquiring a novel operant conditioned behavior, such as bar pressing for milk reward. This learning deficit was clearly related to the animal's reduced resourcefulness in exploring manipulanda and, as a result, these animals perseverated in stereotyped and ineffective motor exploratory modes /93/. These cats, compared to the resourceful ones, showed: (i) absent or reduced cortical P300-like potentials normally triggered by a food reinforced bar press; and (ii) unchanging spectra of neuronal firing patterns emitted by neurons in the thalamic sensory association nucleus (centrum medianum parafascicular complex), presumably reflecting the animal's tendency toward behavioral perseveration /93, and unpublished observations/.

CONCLUSION

The unique ability of FL to simultaneously promote vigilance and habituation may be contingent upon three mechanisms:

(i) Disinhibition of virtually all brainstem ascending neuronal systems; the advantage of manipulating the brain functions simply by attenuating the physiological GABAergic control of all ascending aminergic/peptidergic, cholinergic and glutamatergic systems involved in cognitive and, indirectly, motor functions, preserves the natural physiologic cooperativity among the ascending systems. Thus, a concerted crescendo may be expected to result in harmonious amplification of all physiologic brain functions and truly nootropic actions, contrary to most conventional pharmacologic manipulations of the ascending systems which tend to preferentially act on specific system components, often leading to untoward clinical effects;

(ii) By blocking access of endogenous anxiogenic ligands to BDZ receptors /36,66/, chronic FL stabilizes the functions of the autonomic system and emotional responses to challenging environmental and social stimuli;

iii) Owing to its ataraxic actions on emotional responses to challenging environmental stimuli, FL most likely reduces and stabilizes the release of glucocorticoids (GCs) which are known mediators of emotional stress and have been shown to cause excitotoxic neurodegeneration /129/. Such an action of FL may be expected to protect the subject from high levels of serum GCs, thereby protecting large populations of neurons from the excitotoxic effects of GCs /16,76,129/. These protective actions of FL may account for FL's retardation of age-related loss of cognitive functions and their neuropathologic correlates /129/.

Fig. 19 provides a synopsis of the BDZ/GABAergic hypothesis of brain aging; indirectly, the diagram also summarizes the arguments for nootropic actions of FL resulting from concerted disinhibition of most brainstem ascending systems involved in physiologic brain functions.

ACKNOWLEDGMENTS

The above studies were supported by BRSG grant #49121-R awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health and a grant AOSR 87-0364 from U.S. Air Force to T.J.M. We thank Dr. J.F. Zachary and Donna Epps, Department of Veterinary Pathology, University of Illinois, Urbana-Champaign, for making histologic preparations and Dr. J.R. Unnerstall, Department of Anatomy and Cell Biology, for helpful discussions. We also thank Dr. Peter Sorter (Hoffmann-LaRoche, Inc. Nutley, N.J.) for a generous supply of flumazenil, and Dr. N. Khoobyarian, Department of Microbiology, for letting us use the Olympus microscope and camera.

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Note added in proof

Since the presence of extrasynaptic GABA is essential for the GABAergic deafferentation hypothesis of brain aging, it is important to mention that recent *in situ* estimates of extracellular GABA, measured in rat brain microdialysates, indicate that about 50% of GABA derives from non-neural sources. [Smith S.E., Sharp T. An investigation of the origin of extracellular GABA in rat nucleus accumbens measured *in vivo* by microdialysis. J Neural Transm Gen Sect 1994; 97: 161-171].