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Section II: *Receptors in the C.N.S.: effects of endogenous and exogenous substances*

Molecular mechanism of aethimizol action

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Summary. – *The action of aethimizol on cAMP system and on steps of the metabolic reactions in which regulation of cAMP takes part, were investigated. Aethimizol inhibited the activity of cAMP phosphodiesterase, its potency being about half of that of theophilline. The drug increased markedly the rate of formation and the level of cAMP.*

Riassunto. – *E' stata studiata l'influenza dell'etimizolo sulle reazioni metaboliche che coinvolgono l'AMP ciclico. L'etimizolo inibisce l'attività della fosfodiesterasi dell'AMP ciclico; la sua potenza è circa metà di quella della teofillina. Il farmaco produce un aumento dei livelli di AMP ciclico a livello del sistema nervoso centrale.*

Introduction.

Aethimizol, a methylxantine derivative, is endowed with several pharmacological effects [1, 2]. It stimulates the respiratory centre and is used as a respiratory analeptic. It stimulates also the hypophysis–adrenal system, rising the level of corticosteroids in blood, and it is used in bronchial asthma and rheumatic arthritis. Since it improves also memory and learning, it may be supposed that this action is connected with its influence on brain metabolism.

The activity of methylxantines depends on their inhibitory action on cyclic nucleotide phosphodiesterase, leading to an increase of cAMP level in tissues. In this connection the action of aethimizol on cAMP system and on steps of the metabolic reactions in which regulation of cAMP takes part, were investigated.

Methods.

The investigations were carried out in male rats (weight 200–220 g). Aethimizol was injected intraperitoneally in doses of 10 and 20 mg/kg. The rats were decapited from 30 min to 3 hours after drug injection depending on the investigated processes and the brain was dissected out. The activity of cyclic AMP phosphodiesterase [3] was studied in homogenates of rat brain in experiments *in vitro*. Aethimizol was added in concentration of 0.01 M. The activity of adenyl cyclase [4] was measured in brain tissue 30 min after aethimizol injection (20 mg/kg), together with the content of cAMP, determined by enzymatic methods [5]. The level of calcium ions, by means of atom-absorption spectrophotometry, and the glucose content by the method of Slein [6] were also measured. The membrane permeability of brain synaptosomes was investigated by the rate of their swelling in 0.4 M glycerol [7] with addition of aethimizol *in vitro* in concentration of 10 mg/ml. In order to investigate the influence of aethimizol on transcription, histone/DNA ratio in nuclear fraction of brain was estimated 3 hours after aethimizol injection (20 mg/kg). The activity of RNA-polimerase reaction [8] and ribonuclease activity [9] was determined one hour after drug injection (10 mg/kg). The nuclear fraction of brain cells was isolated by centrifugation in 2.2 M sucrose at 34,000 $\times$ g. Histone proteins were extracted from nuclei with 0.25 N HCl. Their content was estimated by Lowry's method, DNA content was determined by Barton's method [10].

Results and discussion.

Aethimizol inhibited (30 %) the activity of cyclic AMP phosphodiesterase, its potency being about half of that of theophilline. The drug increased markedly the rate of cyclic AMP formation and the levels of cAMP (Tab. 1).

As calcium is the co-factor of the cyclic nucleotides regulating influence, the content of calcium ions in rat brain was investigated. Aethimizol increased Ca^{++} concentration (Tab. 1). Probably this effect is connected with the action of aethimizol on the adenylcyclase system and permeability of synaptic membranes. In fact aethimizol increases also membrane permeability of rat brain synaptosomes. It is known that the compounds raising cAMP content in tissue activate energy processes. Taking into account this fact, aethimizol, as well methylxantines, might be considered « energy » drugs [11]. Indeed, it was established that the content of brain glucose is increased under aethimizol influence (Tab. I). Moreover, it was shown in previous investigations the intensification of glycogenolysis, glycolysis, oxidative processes in brain tissue and the increase of synthesis of high-energy phosphates [12].

Table 1. – *Action of aethimizol on the rat brain metabolism* ($M \pm m$; $n = 5$)

	Control	Aethimizol
cAMP phosphodiesterase activity (mED/mg Protein)	32.8±1.6	22.1±1.2*
Adenyl cyclase activity (imp./mg protein/min) ..	178.0±23.6	602.0±96.0*
Cyclic AMP content (nM/g)	1.1±0.2	2.4±0.2*
Calcium ions concentration (μM/g)	14.2±0.9	18.4±1.5*
Permeability of synaptosome membranes (E_{520}/min).....	52.0±5.0	65.0±6.0*
Glucose content (μM/g)	1.59±0.04	2.25±0.12*
Histone/DNA ratio	1.22	0.94
RNA polymerase reaction activity (imp./mg DNA/min)	2837±353	6137±588*
Ribonuclease activity (E_{260}/mg DNA)	19.0±1.0	11.6±0.8*

(*) $P < 0.05$.

The action of cAMP on cellular metabolism is probably realized through an allosteric regulation of some enzymatic system. We have therefore investigated the influence of aethimizol on transcriptional activity, finding a decrease of the histone to DNA ratio in the nuclear preparation of brain tissue. Aethimizol increased ^{14}C–uridine incorporation in isolated cell nuclei of brain tissue. This effect may be connected both with activation of RNA synthesis and with the decrease of the rate of its enzymatic decay. After aethimizol treatment, ribonuclease activity in nuclear fraction of brain cells was found diminished.

In summary, it may be concluded that the molecular mechanism of aethimizol activity is connected with its ability to induce cAMP accumulation, therefore relying aethimizol action with that of the methylxantines. However, aethimizol, unlike methylxantines, is less active on cyclic AMP phosphodiesterase and induce cAMP accumulation in brain tissue by activation of the adenyl cyclase system. This, allegedly, promotes rapid and massive cAMP formation, triggering in turn a series of enzymatic reaction directed to activation of metabolic processes and to increase of functional cell activity.

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Repeated electroconvulsive shock prevents apomorphine-induced EEG synchronization

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Summary. – *Repeated (one shock daily for 8 days) but not single ECS suppresses the behavioural and EEG effects of a small dose of apomorphine (25 μg.kg^{-1}, s.c.) in rats. In control animals, this dose of apomorphine produces hypomotility and increases the time of synchronized EEG, during the 45 min after treatment. These effects were absent when apomorphine was given 3 days after the last of 8 ECS. The results suggest that repeated ECS results in diminished sensitivity in DA receptors that mediate sedation and sleep in rats.*

Riassunto. – *Il trattamento con ripetuti (ECS) (uno al giorno per otto giorni), ma non con un singolo ECS, antagonizza gli effetti comportamentali e le modificazioni EEG indotte da una piccola dose di apomorfina (25 μg.kg^{-1}) nel ratto. Negli animali di controllo tale dose di apomorfina inibisce l'attività motoria ed aumenta la percentuale dei tempi di sincronizzazione EEG durante i 45 minuti successivi alla somministrazione. I risultati indicano che ripetuti ECS producono una diminuita sensibilità dei recettori dopaminergici che mediano la sedazione e il sonno nel ratto.*

Introduction.

Minute doses of apomorphine decrease motor activity in rodents [1, 2]. This response reflects a true sedative effect since it is associated with a marked increase in the amount of EEG synchronization [3]. The effect of subcutaneous injection of apomorphine lasts less than one hour, due to the short half-life of the drug, but a persistent behavioural and EEG effect may be obtained with the continuous infusion of apomorphine [3]. Moreover, apomorphine-induced sedation and EEG changes are prevented by dopamine (DA) receptor blockers such as haloperidol, pimozide and (-)sulpiride, suggesting the existence of DA receptors mediating sedation in the rat CNS [1, 2, 3].

Chronic treatment with tricyclic antidepressants or mianserine prevents the sedative effect of low doses of apomorphine in rats [4, 5]. This antagonism is only observed after chronic treatment and persists long after treatment withdrawal, the time-course of this effect resembling that of the clinical effect of these antidepressants.

The aim of the present study was to clarify whether the ability to antagonize the sedative and hypnotic effects of low doses of apomorphine is a general property of antidepressant treatments, being also exerted by electroconvulsive shock (ECS), the most effective treatment for endogenous depression. Since hypomotility in rats, especially after ECS, may coexist with EEG arousal, the influence of ECS on apomorphine-induced synchronization was studied in order to evaluate a true sedative effect. The present results show that repeated, but not single, ECS eliminates both the behavioural and EEG effects of small doses of apomorphine.

Materials and Methods.

Male Sprague-Dawley CDR rats (Charles River, Como, Italy), weighing 250-300 g, were used. Under chloral hydrate anesthesia (400 mg · kg^{-1}), three stainless steel micro-screws were implanted over the visual, sensorimotor and frontal cortex. Hippocampal activity was recorded by a stainless steel wire (diameter 0.30 mm) insulated to the tip and placed in the left dorsal hippocampus [6, 7]. For EMG, two small stainless steel needles were inserted acutely, prior to recording, in the dorsal neck muscles, as previously described [3]. After surgery, the rats, allowed one week for recovery, were housed one per cage at 24 °C, humidity 50-60 %, with reversed light-dark cycles (light on from 10 p.m. to 10 a.m.) and with standard laboratory food and water *ad libitum*.

One single shock (150 volts; 50 Hz: for 1 sec) was given daily through ear-electrode alligators for 1 or 8 days, at 9 a.m. Controls were handled in the same manner, but no current was passed. The recordings were made during the dark phase, under red light, starting at 1 p.m. Each animal was recorded only once, for 45 min, starting 5 min after apomorphine injection. This schedule was chosen because the apomorphine effect is most evident in naive rats placed in an unfamiliar environment, during the dark phase of the cycle, when spontaneous motor activity is maximal. Apomorphine (25 μg · kg^{-1}), freshly dissolved in H_2O containing 0.2 mg · ml^{-1} of ascorbic acid, or solvent was injected subcutaneously through a long Silastic tube (Dow-Corning), so as not to disturb the animals. In each experiment, 4 animals were studied simultaneously: they were placed in individual

recording cages, within an electrically shielded sound-proofed room and observed through a one-way glass window.

EEG and EMG were recorded using a Grass-poligraph (Grass, Massachussetts, U.S.A.). Qualitative and quantitative EEG effects were analyzed by visual scoring of 10 sec epoch records and by the use of a frequency analyzer (Ecos, Sardinia).

The animals' state, as either awake, synchronized or slow or desynchronized or REM sleep was classified according to the standard criteria [6, 7].

An animal was considered sedated when the following conditions were concomitantly present: *a*) absence of body movements; *b*) presence in both the cortical and hippocampal leads of high voltage ($> 100\ \mu V$) slow frequency waves intermingled with spindles at 9–13 Hz and *c*) marked reduction of EMG activity. During these periods, the eyes may be either open or closed.

The statistical significance of the results was evaluated by the two-tailed Student's t-test.

Results.

After 15 min of control recording, apomorphine ($25\ \mu g \cdot kg^{-1}$) was injected in control rats and in those treated once or 8 times with ECS, three days after the last treatment. EEG recording was continued for 45 min, starting 5 min after apomorphine treatment. The recording was prolonged no further due to the short half life of the drug.

As previously reported [3], the administration of a low dose of apomorphine caused behavioural sedation and increased the amount of EEG synchronization (EEG sedation) from 12 to 50 % of the total recording time in control rats (Tab. 1). The effect of apomorphine in rats treated with a single ECS was quite similar to that observed in controls. On the contrary, the administration of apomorphine to rats repeatedly treated with ECS failed to elicit behavioural and EEG sedation. The EEG pattern of ECS-treated animals receiving no apomorphine was characterized by a slight increase in EEG desynchronization with respect to control rats.

Table 1. – *Antagonism by repeated ECS of the EEG-Synchronizing effect of apomorphine* ($25\ \mu g \cdot kg^{-1}$).

ECS Pretreatment	Slow waves as % of total recording time (45 min after treatment)			
	Solvent	No. of recordings	Apomorphine	No. of recordings
Sham	12.72±2.54	6	51.32±3.68*	6
Single	11.68±3.20	6	48.76±3.76*	8
Repeated ..	8.75±1.84	8	10.32±4.06**	10

Rats received ECS or sham ECS as described in Materials and Methods. Apomorphine or the solvent were given 3 days after the last ECS. Each value is the mean ± S.E. obtained from the reported number of recordings. Each animal was recorded only once.

(*) $P < 0.001$, with respect to solvent-treated rats.

(**) $P < 0.001$, with respect to sham or single ECS-treated rats receiving apomorphine.

Discussion.

The present results show that repeated ECS prevents the sedative and EEG effects of small doses of apomorphine. Changes in the response to apomorphine were observed after repeated treatments and were present long after treatment withdrawal. Since the effect of apomorphine is due to stimulation of DA receptors [1–3], the time course of ECS-induced changes suggests that DA receptors subserving sedation have become insensitive to the transmitter. However, the identity of such receptors is not clear. They might be identifiable with DA autoreceptors, whose stimulation results in decreased dopaminergic firing and DA synthesis [8, 9[, or a special kind of postsynaptic ones [10] having the same high sensitivity to DA agonists.

Experiments in progress have shown that the bilateral lesion of the nigrostriatal dopaminergic system, induced with the intranigral injection of 6-OHDA, which produced a fall in striatal DA content of over 95 %, failed to prevent the sedative effect of apomorphine, injected when the animals had recovered from the neurological deficits produced by the lesion. These results would apparently rule out the inhibition of the nigrostriatal DA system in the mechanism of the sedative effect of apomorphine. However, further experiments carried out with these 6-OHDA lesioned animals showed that inhibition of DA synthesis with *d*-methyl-*p*-tyrosine also resulted in a long-lasting sedative effect.

A possible interpretation of these results is that residual DA fibres after 6-OHDA lesion are sufficient to maintain a normal state of arousal and that the inhibition of dopaminergic transmission in these fibres results in a sedative response. Thus, these results support the idea that sedation is due to inhibition of dopaminergic firing in the nigrostriatal dopaminergic system.

An alternative interpretation of our data might be that ECS induces supersensitivity in postsynaptic excitatory DA receptors in the striatum or limbic areas [11, 12]. Accordingly, the sedative effect of apomorphine might be masked by the stimulation of such receptors. Indeed, such supersensitivity of postsynaptic DA receptors has been observed after withdrawal of chronic neuroleptic treatment [13]. However, unlike after neuroleptics, no changes in DA-sensitive adenylate cyclase [11] or dopaminergic binding in the striatum [14], two biochemical markers of DA receptor supersensitivity, have been observed after repeated ECS. Moreover, the effect of neuroleptics differs from that of ECS, in that these drugs potentiate not only the stimulant effect of apomorphine but also its sedative effect and inhibition of DA synthesis. After chronic haloperidol withdrawal, a dose of apomorphine as low as $6.2\ \mu g \cdot kg^{-1}$, which is ineffective in normal animals, decreased both motor activity and DA synthesis [4]. Such potentiation was not observed with chronic ECS.

The ability to antagonize the sedative effect of small doses of apomorphine appears to be a general characteristic of chronic antidepressant treatments, shared by tricyclic antidepressants [5], atypical antidepressants, such as mianserine [5], MAO inhibitors [4] and REM sleep deprivation [15].

Thus, also the present results suggest that the development of subsensitivity of DA autoreceptors mediates the antidepressant effect of antidepressants. Moreover, they indicate that apomorphine-induced sedation may be a useful model of mental depression.

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Possible involvement of prolactin in sulpiride-induced changes in nigral and striatal GAD activity

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Summary. – *In light of the different neuropharmacological spectrum of classical neuroleptics and benzamide derivatives we have investigated the comparative effects of haloperidol and sulpiride on the activity of the GABA-synthesizing enzyme GAD in rat corpus striatum and substantia nigra. Results obtained show that acute or chronic injection of sulpiride results in an increase of nigral and striatal GAD activity while no significant changes is observed following haloperidol administration. Since hypophysectomy completely prevents sulpiride-induced changes in nigral and striatal GAD activity, the possibility has been suggested that an anterior pituitary factor may be involved in the central effects of the benzamide derivative. In this regard, a specific role of prolactin, the synthesis and release of which is strongly stimulated by sulpiride, is suggested by the evidence that an increase in nigral and striatal GAD activity is observed in conditions of hyperprolactinemia induced by anterior pituitary transplantation under the kidney capsule.*

Riassunto. – *Sono stati analizzati gli effetti comparativi dell'aloperidolo e della sulpiride, sull'attività dell'enzima di sintesi del GABA, GAD, nel corpo striato e nella substantia nigra.*

Dai risultati ottenuti si evince che il trattamento acuto o cronico con sulpiride induce un incremento dell'attività GAD nella substantia nigra e nel corpo striato, mentre nessuna modificazione significativa si osserva dopo somministrazione di aloperidolo. Che un fattore adenoipofisario possa mediare l'effetto centrale del sulpiride si desume dall'evidenza che l'ipofisectomia previene l'incremento di GAD indotto dal derivato benzamidico. L'ipotesi di uno specifico coinvolgimento della prolattina deriva dall'evidenza che un incremento dell'attività GAD nigrale e striatale si osserva in condizioni di iperprolattinemia indotta da impianto di ipofisi sotto la capsula del rene.

Introduction.

The antidopaminergic activity of the benzamide derivative sulpiride appears markedly different from that of classical neuroleptics such as haloperidol. In fact, unlike haloperidol, sulpiride shows little efficacy in blocking amphetamine-induced hyperactivity and apomorphine-induced stereotypies and in causing catalepsy [1]. Moreover, it is well known that the parkinsonian syndrome and the tardive dyskinesia observed during haloperidol treatment and after its discontinuation rarely occur in sulpiride-treated patients [2].

The different neuropharmacological spectra of sulpiride and classical neuroleptics might be related to the fact that sulpiride would act preferentially on dopamine (DA) receptors not linked to adenylate cyclase (D2 receptors according to Spano *et al.* [3], and, in contrast, haloperidol would inhibit DA receptors both those linked (D1) and not linked to (D2) adenylate cyclase.

Few data are reported concerning the role of neurotransmitters other than DA in the neuropharmacological effects of haloperidol and sulpiride. Several

Table 1. – *Effect of acute and chronic (2.5-5 mg/kg, twice daily for 21 days) haloperidol or sulpiride i.p.; treatment (2 mg/kg each injection) on nigral (SN) and striatal (CS) GAD activity in male rats.*

	GAD ACTIVITY (umol $^{14}CO_2$/100 mg prot./60 min)	
	SN	CS
Acute treatment:		
Saline	41.64±1.42	19.90±2.71
Haloperidol	37.41±4.34	18.57±3.13
Sulpiride	60.28±2.12*	37.36±3.17*
Chronic treatment:		
Saline	43.19±2.61	18.88±3.04
Haloperidol	46.22±4.25	15.41±2.64
Sulpiride	62.81±3.16*	39.55±1.68*

GAD activity was assayed using the conditions described by Beaven et al. (14), slighty modified by Nisticò et al., (15). Values are meant of 8 animals for each group.

(*) $p < 0.01$ if compared to saline injected animals.

lines of evidence suggest that striato–nigral GABA ergic pathway plays an important role in the expression of DA–related striatal function [4].

In this paper the results are reparted of a study on the effects of acute and chronic haloperidol and sulpiride treatment on the activity of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD, EC 4.1.1.15).

No significant changes in nigral or striatal GAD activity were observed in both acute and chronic haloperidol–treated groups. These results are in accordance with previous data by Lloyd and Hornykiewicz [5]. Conversely, a sharp increase in GAD activity was induced by acute or chronic sulpiride treatment (Tab. 1).

Although a direct action of sulpiride on GABA neurons cannot be ruled out, it is reasonable to speculate that GAD activity changes may reflect a primitive action of the benzamide derivative on DA receptors. It has been suggested, in fact, that nigro–striatal DA ergic fibers could control striato–nigral GABAergic system by contacting the dendrites of striatal cholinergic interneurons [6]. Thus, the DA receptors agonist apomorphine may increase striato–nigral GABA turnover via removal of an inhibitory influence on the GABA cell bodies and dendrites [7]. In addition it has been recently proposed that DA can modulate the activity of cortico–striatal glutamatergic fibers [3]. This pathways is considered to play an excitatory role on striato–nigral GABAergic neurons [8]. Several lines of evidence suggest that, in corpus striatum (CS), D1 receptors mediate the DA–induced inhibition of cholinergic interneurons whilst D2 receptors would be preferentially located on cortico–striatal glutamatergic fibers.

The increase in GAD activity observed in sulpiride–treated animals could be explained through a potentiation of the GABAergic transmission by removal of the D2 mediated inhibition of cortico–striatal glutamatergic fibers. Still, the increase in GAD activity might also reflect the stimulation of nigro–striatal DA transmission due to the sulpiride–induced block of DA inhibitory autoreceptors. This hypothesis might be supported by the observation that sulpiride is able to counteract the hypomotility induced by low doses of apomorphine, effect allegedly associated with the stimulation of DA autoreceptors [9].

Since haloperidol fails to increase nigral and striatal GAD activity, it is conceivable that the blockade of D1 receptors may counteract the simultaneous D2 dependent effects on GAD activity. However, a major question concerning the neurochemical events related to sulpiride peripheral injection raises from the fact that the drug poorly crosses the blood–brain barrier [10]. Therefore, while exploring the possible activity of the benzamide derivative on GABAergic system, the possibility was considered of an indirect mediation by other events occurring outside the blood–brain barrier. In order to evaluate the involvement of prolactin (PRL), an anterior pituitary hormone whose synthesis and release are strongly stimulated by sulpiride [11–13], GAD activity was also assayed in sulpiride–injected hypophysectomized animals or in animals in which hyperprolactinemia had been induced by other approaches.

Hypophysectomy completely prevents the effects of sulpiride on GAD activity (Fig. 1), suggesting the involvement of an adenopituitary factor in the sulpiride –induced GAD activity changes in CS and substantia nigra (SN). In view of the above reported sulpiride effects on PRL synthesis and release, it is tempting to speculate on the possibility that PRL

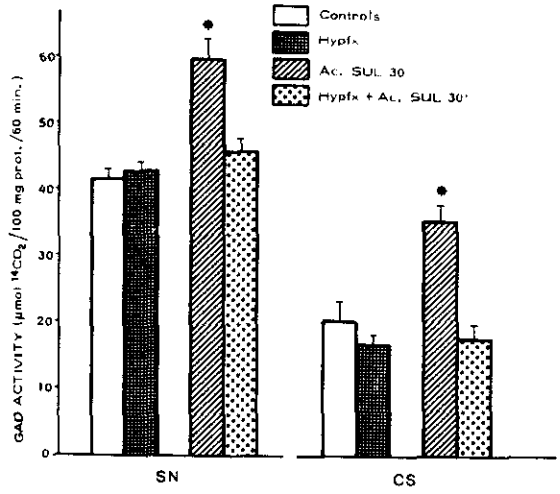

FIG. 1. – Effects of sulpiride injection (2 mg/kg. i.p. 30 min before sacrifice) on nigral (SN) and striatal (CS) GAD activity in intact and hypophysectomized (hypfx) male rats. Values are means ± S.E. of 8 animals for each group. (*) $p < 0.01$ if compared to saline–injected intact animals.

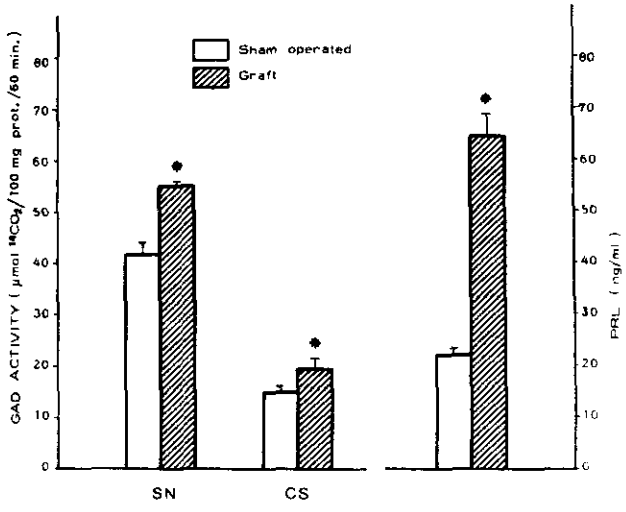

FIG. 2. – Effect of adenopituitary transplantation (Graft) on nigral (SN) and striatal (CS) GAD activity and plasma prolactin (PRL) levels in male rats. Values are means ± S.E. of 8 animals for each group. (*) $p < 0.01$ if compared to sham operated animals.

may be responsible for this effect. In support to this hypothesis, it is of interest to note that an increase in GAD activity is observed following adenopituitary homograft (Fig. 2), a condition of hyperprolactinemia independent from sulpiride injection. However, it must be also considered the possibility that other events possibly linked to hypophysectomy such as changes in the permeability of blood–brain barrier or in pharmacokinetics of sulpiride may interfere with the effects of the benzamide derivative on striato–nigral GABAergic system.

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Effect of acute and chronic ethanol exposure on the rat brain opiate receptor function

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Summary. – *A reduction of both maximal density and dissociation constant for the low-affinity component of methionine-enkephalin receptor binding sites was found in rats after chronic ethanol treatment. These changes disappeared within 48 h after the last day of treatment. On the other hand, acute ethanol administration (4 g/kg) elicited the disappearance of the high affinity component.* In vitro *ethanol significantly alters the binding of « opiate » peptides. The ultrafiltrate of the supernatant obtained from brain membrane fraction of rats chronically treated with alcohol was able to inhibit both maximal density and dissociation constant of the low-affinity component of opiate receptor binding.*

Riassunto. – *In ratti trattati cronicamente con etanolo è stata ritrovata una diminuzione del numero e della costante di dissociazione della componente a bassa affinità dei siti recettoriali della met-enkefalina. Un ritorno alla norma si ha dopo due giorni dalla fine del trattamento. Il trattamento acuto con etanolo (4 g/kg) provoca invece la scomparsa della componente ad alta affinità.* In vitro *l'etanolo influenza il legame dei peptidi oppioidi. L'ultrafiltrato del surnatante ottenuto dalle frazioni di membrana del cervello di ratti trattati cronicamente con etanolo inibisce il numero e la costante di dissociazione della componente a bassa affinità del legame del recettore oppiaceo.*

Introduction.

The question whether or not opiate systems are involved in development of tolerance to and physical dependence on ethanol begins to attract considerable attention [1]. For that reasons the demonstration of opiate-like effects by certain neuroamine-derived tetrahydroisoquinolines has a particular interest. It has been demonstrated [2, 3] that acetaldehyde may condense with norepinephrine, epinephrine, dopamine or serotonin to produce an intermediate Schiff base, which, in turn, undergoes a spontaneous molecular reorganization to form tetrahydroisoquinoline or tetrahydropapaveroline derivatives. The molecular structures of these compounds are similar to some opiate alcaloids with high addictive potency; thus, it has been suggested that addictive properties of ethanol may be related, in part, to the condensation of acetaldehyde during ethanol metabolism. Recent studies have also shown that chronic alcohol and chronic morphine exposure induces identical changes in endorphin and enkephalin turnover [4, 5].

In the present paper data are presented suggesting that opiate systems are involved in the development of dependence on ethanol, through a study of the rat brain opiate receptor function after acute and chronic ethanol exposure.

Methods.

A total of 50 Wistar male rats, weighing approximately 80–100 g was divided into 2 groups of 25 animals each. One group (thereafter called the « alcohol group ») was treated *per os* with 30 % (v/v) ethanol solution in a dose of 4 ml/kg body wt per day for one month. Then the alcohol group was fed the same standard solid pellet diet and separately a 30 % (v/v) ethanol solution in Richter drinking tubes for the following months. For the determination of ethanol consumption in rats of alcohol group the animals were housed individually in cages with two 100 ml graduated Richter drinking tubes fitted to the front wall of the cage. Alcohol intake of animals was measured by offering a free choice between water and 30 % v/v ethanol solution.

All results were reported as the mean $\pm$ S.E.M. of obtained values and comparisons between groups were made by the Student t-test. A P value of *less than* 0.05 was taken to indicate statistical significance.

Male Wistar rats (350–400 g) were killed by decapitation and the brains rapidly removed and placed in ice-cold 50 mM Tris-HCl buffer, pH 7.7 at 25 °C. The sections of midbrain plus hypothalamus were isolated and homogenized in 45 volumes of the Tris-HCl buffer. The homogenates were then centrifuged at 4 °C for 20 min at 30200 × g. The pellets were resuspended in 60 ml of Tris-HCl buffer, incubated for 40 min at 37 °C and centrifuged at 4 °C for 20 min at 30200 × g. Supernatants were discarded (unless otherwise indicated) or filtered through a Millipore PSAC filters (nominal cut-off 10,000 M. W.) and

filtrates were used for binding studies. The final membrane pellets were suspended in 50 mM Tris–HCl buffer, pH 7.7 (25 °C) and utilized for binding assays.

Binding experiments were performed as previously described [6] by using the filtration method of Snyder et al. [7]. The assay was carried out at 25 °C in a standard incubation mixture (final volume 1 ml of Tris–HCl buffer) containing 0.6–0.9 mg of protein of tissue suspension, containing 3 H–methionine–enkephalin (36 Ci/mmol; Amersham, England) or ^{3}H–D ALA^2–met–enkephalinamide (27 Ci/mmol; Amersham, England), in presence of bacitracin 2–7 Units (53, 500 U/g Sigma, USA).

After 40 min of incubation the reaction was terminated by rapidly filtering under vacuum through GF/B glass filters (Whatman, England), then the filters were washed three times with 2.5 ml of ice–cold Tris–HCl buffer. The filters were placed in 1 ml Protosol and counted with 10 ml standard toluene counting solution. All assays were performed in duplicate and the variability of the duplicate was usually less than 10 % of the mean. Opiate–specific binding was defined as the difference between the total binding and the binding that occurred in the presence of 2 mM methionine enkephalin.

In order to evaluate the presence into the supernatant of endogenous opiate–like material, the supernatant resulting from the last centrifugation of membranes, before the assay, was filtered through a Millipore PSAC filters (nominal cut–off 10,000 M. W.) and the filtrate was added in the incubation mixture before (40 min at 37 °) adding the ^{3}H–met–enkephalin.

The protein concentration was determined by the method of Lowry et al. [8].

Results.

During the 6 hours of administration of nalorphine (10 mg/kg ip × 3 times) a more than 3–fold increase of the rate of ethanol consumption in alcohol treated animals was observed (Fig. 1). However, from 3 till 18 hours after the last nalorphine injection we found a decrease of alcohol consumption rate.

Table 1. – *Characteristics of ^{3}H–met–enkephalin binding to opiate receptor sites in midbrain and hypothalamus of rats exposed to chronic ethanol treatment and withdrawal.*

TREATMENT	High affinity binding sites		Low affinity binding sites	
	K_D (nM)	B_{max} (fmol/mg prot)	K_D (nM)	B_{max} (fmol/mg prot)
Control	1.4±0.2	37±4	19±3	280±30
Chronic ethanol exposure:				
1 h post–withdrawal	1.0±0.2	44±4	11±2*	170±25*
48 h post–withdrawal	1.5±0.3	41±5	17±3	295±30

Rat brain tissue membrane fractions were prepared and assayed as described in « Methods ». Results are expressed as means ± S.E.M. for 6 rat brain samples in each group. (*) $P < 0.05$ compared to control rats.

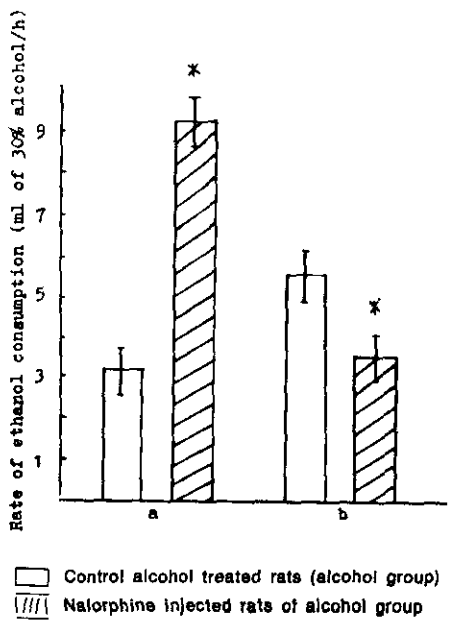

Fig. 1. – Effect of nalorphine on the rate of ethanol consumption ethanol treated rats.

(*a*) During nalorphine treatment (10 mg/kg × 3 times between 12 h and 18 h p.m.)

(*b*) After nalorphine injections (between 18 h p.m. and 12 h a.m. of the next day).

Chronic ethanol (daily dose of 4 g/kg) was administered as a 30 % w/v drinking solution during 11 months. Bar denoted S.E.M. (*) $P < 0.05$.

As showed in Table 1 no statistically changes were observed in the dissociation constant (K_D) and maximal number of high affinity binding sites (B_{max}) in midbrain and hypothalamus of rats following chronic ethanol treatment and withdrawal (1 h and 48 h post–withdrawal) as compared to the pair–fed controls. On the other hand, the value of K_D and B_{max} of low affinity binding sites for ^{3}H methionine enkephalin were significantly lower in hypothalamus and midbrain of alcohol treated rats (1 h post– withdrawal) than the pair–fed controls. Return to normal occurred 48 h after the end of treatment, when alcohol group and pair–fed control group gave similar binding characteristics.

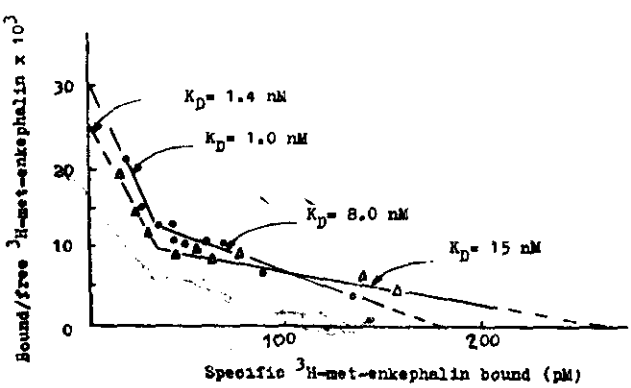

Fig. 2. – Scatchard analysis of ^{3}H–met–enkephalin binding in control rat brain tissue membrane fraction preincubated with supernatant of membrane fraction of rats, exposed to chronic ethanol treatment. Rat brain tissue membrane fraction were prepared, preincubated and assayed as described in « Methods ». The experiment was repeated 3 times. △ = Control; ○ = in presence of purified supernatant (40 min at 37 °C).

In order to clarify whether or not this effect can be ascribed to a direct effect of ethanol, we studied the direct influence of ethanol and/or acetaldehyde on opiate binding to its receptors sites in rat brain membranes. The exposure of membrane fractions to ethanol or acetaldehyde in concentration of 10 and 50 mM, respectively, did not cause a change in ^{3}H–D ALA binding activity, whereas at concentration higher than 10 or 50 mM, ethanol and acetaldehyde, respectively, caused an inhibition of the binding of ^{3}H–D ALA (Tab. 2). Similarly, the simultaneous exposure of the rat brain membranes to 2 mM ethanol and 0.02 mM acetaldehyde concentration has no effect on ^{3}H–D ALA binding. Moreover, after preincubation (40 min at 37°C) of membrane fractions with ethanol and/or acetaldehyde and then removing both substances by washing by centrifugation the membranes do not exhibited changes in ^{3}H–D ALA binding (Tab. 2).

In order to evaluate whether this effect in mediated by endogenous opiate receptor ligands we studied the inhibitory activity of the partially purified supernatant (see « Methods ») from rats chronically treated with alcohol on the binding of ^{3}H–methionine–enkephalin. The preincubation with the supernatant resulted in a decrease of the K_D (53 % of control) and B_{max} (69 % of control) for the low affinity component of ^{3}H–met–enkephalin binding (Fig. 2). Thus, changes of binding characteristics of low affinity opiate receptor binding sites observed after prolonged alcohol administration may be accounted for by formation of endogenous substances which alter ^{3}H–met–enkephalin binding in animal brain membranes during long–term alcohol administration.

Table 2. – *The effect of ethanol and/or acetaldehyde effects on ^{3}H–D ALA binding to rat hypothalamus plus midbrain tissue.*

TREATMENT	^{3}H–D ALA binding (% of control binding)	
	added directly in the assay (M ± m)	after previous incubation (a) (M ± m)
Control	100±9	100±10
1.7 M ethanol	7±3	102±12
0.4 M acetaldehyde	15±4	97±10
0.5 M ethanol plus 5 mM acetaldehyde	53±8*	110±14

Rat brain tissue membrane fractions were prepared and assayed as described in Methods. The binding was determined after preincubation of membrane fractions (40 min at 37 °C) with 50 mM Tris–HCl, pH 7.7 (Control), or ethanol and/or acetaldehyde before adding ^{3}H–D ALA (1.5 nM). The results are the means ± S.E.M. of 4 experiments for each group.

(*a*) Membrane fractions were incubated (40 min at 37 °C) with 50 mM Tris–DCl (Control), or ethanol and or acetaldehyde then centrifuged at 4 °C for 15 min at 30200 × g. The supernatants were discarded and pellets were resuspended in 60 ml of 50 mM Tris–HCl, pH 7.7 and centrifuged at 30200 × g for 15 min. Then pellets were resuspended and centrifuged at the same conditions once more before ^{3}H–D ALA binding experiment.

(*) $P < 0.001$ compared with the ^{3}H–D ALA binding of control, according to the Student's t–test.

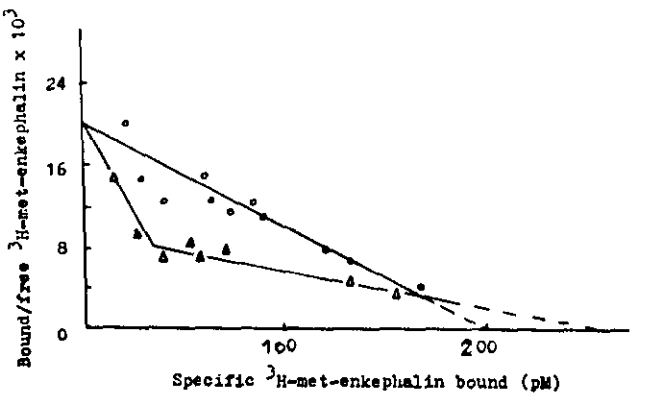

FIG. 3. – Scatchard analysis of ^{3}H–met–enkephalin binding in hypothalamus and midbrain of rats 1 hom o after acute ethanol (4 g/Kg × os) treatment. Rat brain tissue membrane fractions were prepared and assayed as described in « Methods ». The experiment was repeated twice. △ = Control; O = acute ethanol.

The disappearance of the high affinity component for ^{3}H–met–enkephalin binding has been observed in the midbrain and hypothalamus of rats at 1 h after an acute (4 ml/kg) dose of ethanol (Fig. 3). A monophasic Scatchard plot was noted after administration of a single dose of ethanol with a K_D of 7.2 nM and B_{max} of binding sites of 195 fmol/mg protein.

DISCUSSION.

The observation of discrete high and low affinity binding sites for ^{3}H–met–enkephalin in control and alcohol–treated animals was in good agreement with the postulate about the existence of two types of opiate receptors, one with high affinity for opiate alcaloids (named by Kosterlitz in analogy with the morphine or μ–receptor of Martin) and the other with high affinity for enkephalins (named δ). The enkephalinamides, β–endorphin and the opiate etorphine seem to bind equally well to both types of opiate receptors [9]. Therefore, the type of opiate receptor binding sites with high affinity for met–enkephalin revealed by us in hypothalamus and midbrain of control and alcohol group is δ–type of receptors, whereas the type of opiate receptor binding sites with low affinity for met–enkephalin is a μ–type of opiate receptors.

The differences in binding patterns, revealed in control and alcohol groups may be due to:

a) conformational or/and structural changes in opiate receptors of ethanol treated animals;

b) the modification in brain content of endogenous opioid ligands in alcohol treated animals;

c) direct effect of ethanol and/or acetaldehyde on membrane lipids or receptor proteins.

The study of direct effects of ethanol or/and acetaldehyde on enkephalin binding to opiate receptors showed that the reduction of opiate receptor binding activity by high concentrations of ethanol and/or acetaldehyde was a reversible process, and thus such reduction was not the cause of the changes of binding pattern for ^{3}H–met–enkephalin in rats after acute and chronic alcohol administration as observed in

our experiments. On the other hand the influence of preincubation with supernatant of rat brain membrane fraction of alcohol group on the enkephalin binding indicated that the observed changes of enkephalin binding after chronic alcohol treatment were responsible for the differences in endogenous opioid composition in brain tissues of control and alcohol treated animals.

The changes of binding of enkephalin with brain opiate receptors after chronic alcohol administration may be indicative of the participation of opiate systems in the mechanism of development of tolerance to and physical dependence on ethanol. In a study of Hoffman and coworkers [10] were also observed changes in opiate binding sites in striatal tissue of mice following chronic alcohol administration and withdrawal (24 h post-withdrawal).

At present it appears difficult to compare the disappearance of the high affinity component for ^{3}H-met-enkephalin binding in animals after acute alcohol consumption as compared to chronic ethanol treatment when the low affinity is affected. We might speculate that acute exposure to alcohol induces an increase of endogenous opiate-like material which affects the high affinity component of the ^{3}H-met-enkephalin binding. This increase might be reduced during the development of the tolerance after chronic exposure to this drug. These results are in a good agreement with the data of Schulz and coworkers [11] about more highly pronounced change of endorphin level in brain and pituitary after acute ethanol administration as compared to chronic ethanol one. However, in order to clarify the physiopatological involvement of the high affinity component during the acute treatment and the low affinity component during the development of tolerance, further study are required on the functional relationship between the two affinity components of opiate receptors.

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Hippocampal glucocorticoid binding: serotoninergic regulation and drug effects; relevance to behavioral-endocrine activities and depression

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Summary. – *The hippocampal glucocorticoid receptor serves as an integrative connection between endocrine activity of the hypothalamo–pituitary–adrenal axis and the associated behavioral responses, both governed by this limbic structures.*

Serotoninergic input to the hippocampus primarily modulates corticosterone binding in this area, indipendently of but cooperatively with the level of adrenocortical secretion. The modulatory role is essentially inhibitory.

In consideration that depressive illness can be associated with perturbation of the adrenocortical activity it is proposed that a disregulation in the limbic system–hypothalamus–pituitary–adrenal circuit is a salient feature of depression; the eventual beneficial effect of antidepressant drugs interfering with serotoninergic innervation of the hippocampus may be mediated through reparative influences on the behavior–endocrine integrated function of this circuit.

Riassunto. – *Il recettore glucocorticoideo ippocampale controlla, integrandole, l'attività endocrina dell'asse ipotalamo–ipofisi–surrene e le concomitanti risposte comportamentali.*

L'innervazione serotoninergica dell'ippocampo modula inibitoriamente la capacità di binding *del recettore glucocorticoideo, cooperando in tal senso con il livello di secrezione adrenocorticale.*

Poiché lo stato depressivo puó essere accompagnato da alterazioni dell'attività adrenocorticale, si ipotizza che una disregolazione nel circuito sistema limbico–ipotalamo–ipofisi–surrene sia componente caratteristica della depressione; l'effetto benefico operato da farmaci antidepressivi interferenti con l'innervazione serotoninergica ippocampale potrebbe fondarsi sulla normalizzazione della funzione integrativa di questo circuito.

Introduction.

The functional relevance of brain glucocorticoid receptor [1] in laboratory animals is now well established. In particular, the hippocampal glucocorticoid receptor serves as an integrative connection between endocrine activity of the hypothalamo–pituitary adrenal axis and associated behavioral responses [2–4], both governed by this limbic structure. On one hand, pituitary adrenal response to stress, namely early ACTH and late glucocorticoid secretion increase, is modulated by inhibitory and facilitatory influences from the limbic system [4–6[. On the other hand, the endocrine response modulates behavioral output from the hippocampus via a negative feedback on its glucocorticoid receptor, the binding capacity of which is reduced both by ACTH and glucocorticoid hormone [7, 8].

On the endocrine side of this integrative process, an example is given by the circadian rhythm in glucocorticoid binding capacity in the hippocampus [7]. This capacity can be measured in mice and rats a few hours after adrenalectomy, a time necessary to full desaturation of the receptor of the endogenous hormonal ligand. Figure 1 shows that the maximal value in *in vivo* specific ^{3}H–corticosterone uptake in the mouse' hippocampus was found at eight in the morning, time of adrenalectomy, or at noon, time of sacrifice, when blood corticosterone is lower, whereas minimal values were found in dark hours, when blood corticosterone is higher. Notice that the diurnal rhythm in pituitary glucocorticoid uptake is a mere reflection of blood corticosterone level. However, when uptake in the hippocampus was measured ten hours after adrenalectomy, maximal value was found at four in the morning, time of adrenalectomy, or two after noon, *i.e.* at the same time of the day as in four hour adrenalectomized mice. This indicates that the diurnal rhythm in binding capacity, aside the inverse regulation by hormonal feedback, is in part an autonomous property of the limbic structures.

On the behavioral side of the integrative process, an example is given by the role of the hippocampal glucocorticoid receptor in the physiology of extinction, namely when extinction of a learned behavior is a proper adaptive response [9]. In the adrenalectomized rat forced extinction of passive avoidance behavior cannot be obtained. At any rate, physiological behavior is specifically reinstated by exogenous corticosterone in proportion to the replenishment of glucocorticoid binding capacity in the hippocampus.

We have demonstrated that glucocorticoid binding capacity in the hippocampus is affected by monaminergic innervation [10, 11]. For instance, as shown in figure 2, neurotoxic lesion of brain serotoninergic pathways disrupted circadian rhythm in *in vivo* specific ^{3}H–corticosterone uptake in the rat's hyppocampus, in

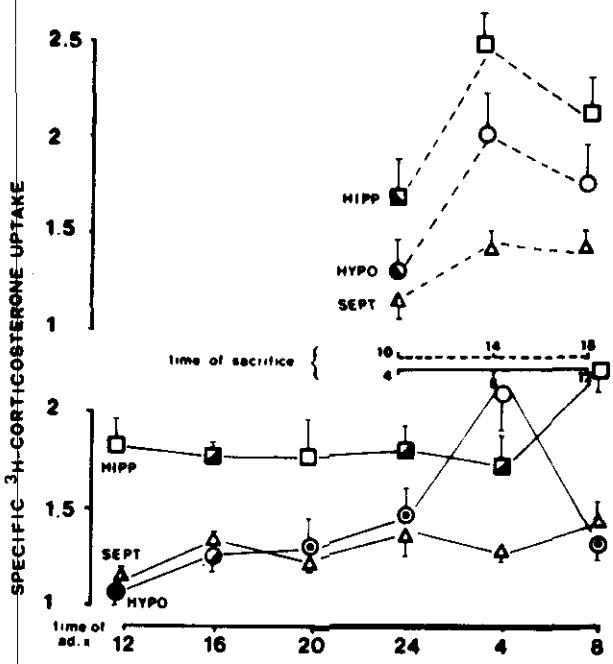

FIG. 1. – Circadian variations of brain specific (hippocampus: cortex, septum: cortex, hypophysis: blood) ^{3}H–corticosterone uptake after a same tracer dose in 4–hr (*continuous line*) and 10–hr (*dashed line*) adrenalectomized mice (N = 7–10). *Dotted, half filled* and *filled symbols*: p = 5, 1 and 0.1 %, respectively, versus peak value. *Heavy line*: dark period.

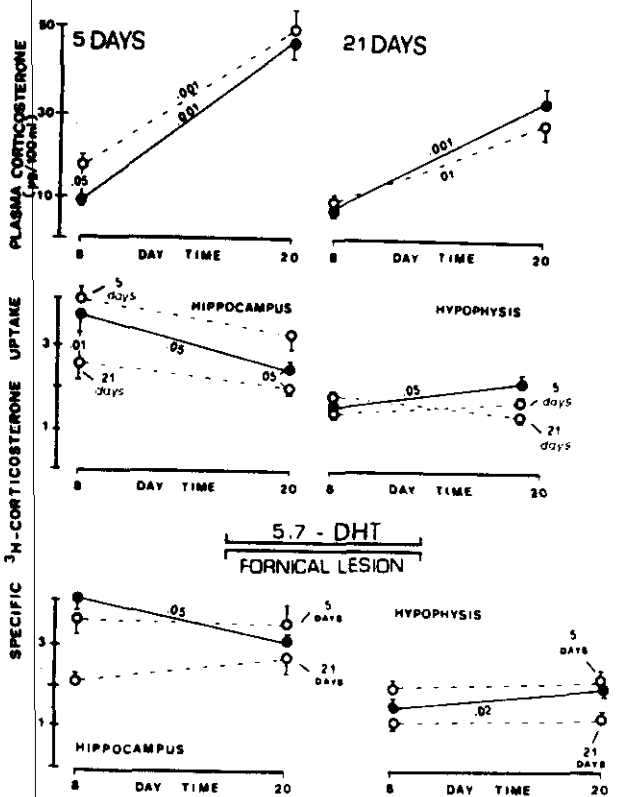

FIG. 2. – *Top and middle*: plasma concentrations of endogenous corticosterone and brain specific (hippocampus: cortex, hypophysis: blood) ^{3}H–corticosterone uptake of 12–hr adrenalectomized rats 5 and 21 days after 5,7–hidyroxytryptamine, 50 ug, in the third brain ventricle (N = 8). *Bottom*: uptake after fornical transection (N = 6–9). *Filled* and *open* circle: controls and treated.

presence of a dampened but not suppressed rhythm in blood corticosterone level. Again, this indicates that circadian variations in glucocorticoid binding capacity in the hippocampus are not a mere reflection of variations in corticosterone blood level.

As also shown in figure 2, even fornical transection produced a disruption in circadian rhythm of *in vivo* specific ^{3}H–corticosterone uptake in the rat's hippocampus. Evidently, this manipulation as well as

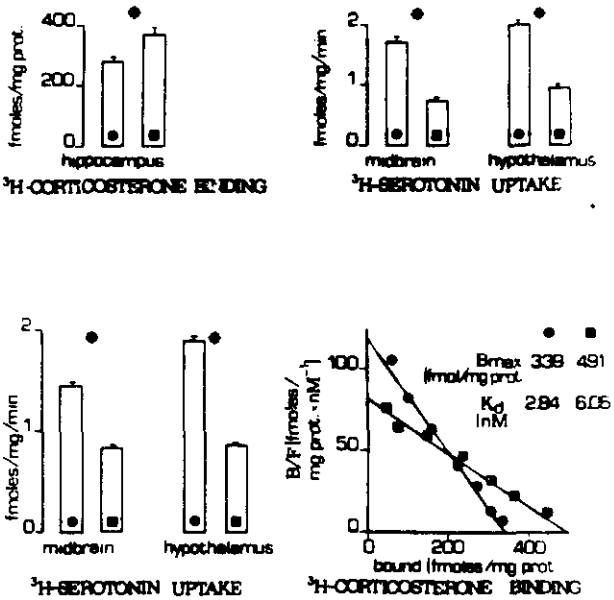

FIG. 3. – The relationship between serotoninergic innervation and cytosol (1.2 mg protein per ml) glucocorticoid binding in the 24–hr adrenalectomized rat's hippocampus. *Above*: individual maximal binding of ^{3}H–corticosterone 4 × 10 $^{-8}$M, and ^{3}H–serotonin 5 × 10 $^{-8}$M uptake (slices) in rats with an 8 day 5, 6 didydroxytryptamine (10 µg in 1 µl; 1 hour–pretreatment with desipramine 20 mg/kg i.p.) lesion of nucleus raphe dorsalis. ● = sham (7); ■ = lesion (6). *Below*: ^{3}H–serotonin uptake and Scatchard analysis of ^{3}H–corticosterone 0.06–4 × $^{-8}$M binding in lesioned (16) and sham (16) rats.

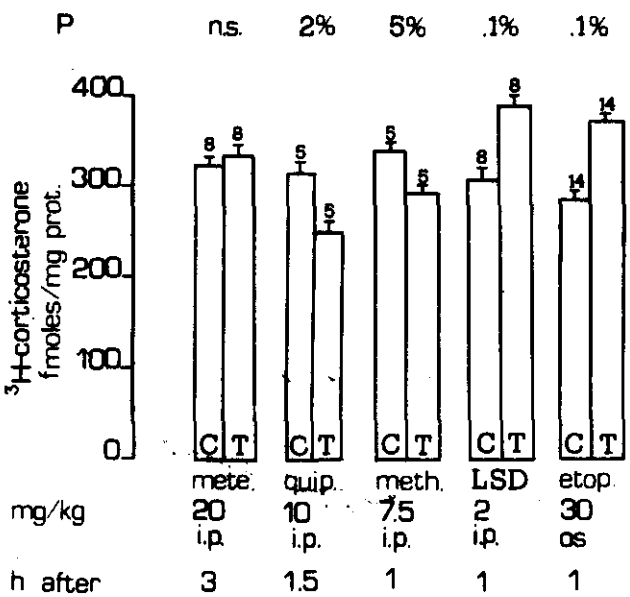

FIG. 4. – The effect of *in vivo* serotoninergic drugs on cytosol (1.2 mg protein per ml) individual maximal binding of ^{3}H–corticosterone (4 × 10^{-8}M) in the 24–hr adrenalectomized rat's hippocampus. *Plain area*: controls; *dotted area*: treated.

neurotoxic lesion interrupts a neuronal pathway essential to the manifestation of circadian activity in the hippocampus.

A positive correlation between serotonin content in the limbic system and plasma corticosterone rhythm has been found in the rat by other authors [12], and assumed to indicate a serotoninergic control of circadian periodicity of adrenocortical secretion in force of its suppression by inhibition of serotonin synthesis. On this basis we have investigated in more detail the role of serotoninergic input to the hippocampus in the regulation of its glucocorticoid binding capacity. As shown in figure 3, chemical lesion of the raphe dorsalis nucleus in the rat, able to produce a 50 per cent decrease in ^{3}H-serotonin uptake in the midbrain and hypothalamus, substantially increased maximal ^{3}H-corticosterone binding in the hippocampal cytosol. Scatchard analysis demonstrated that the latter was due to an increase in number of binding sites, accompanied by an apparent decrease in affinity. Since in the 8-day lesioned animal, no increase in serotoninergic receptor sensitivity is counteracting the effect of reduction in neuronal input [13], it would appear that serotoninergic innervation has an inhibitory role on the glucocorticoid binding capacity of the hippocampus and, possibly, on its functional expression with regard to behavioral and endocrine control exerted by the limbic structure.

In the light of these results, we have investigated the possible effect of serotoninergic drugs *in vivo* on glucocorticoid binding capacity in the hippocampus. As shown in figure 4, quipazine, a post-synaptic serotoninergic agonist, reduced ^{3}H-corticosterone binding. This effect was in agreement with the result of the pre-synaptic lesion of the serotoninergic system after 5,6-dihydroxytryptamine, indicating the inhibitory role of serotoninergic innervation on glucocorticoid binding capacity. Of the two post-synaptic serotoninergic blockers studied, metergoline was without effect, while methisergide produced a reduction in ^{3}H-corticosterone binding, this result is in contradistinction with the above postulated inhibitory role of serotoninergic innervation. Figure 5 shows the Scatchard analysis of hippocampal ^{3}H-corticosterone binding in rats treated with quipazine or methisergide: with both compounds a reduction in number of binding sites was evident, with no modification of affinity. LSD and etoperidone, two partial agonists of pre- and post-synaptic serotoninergic receptor, produced an increase in glucocorticoid binding (see fig. 4). This was agreement with the above postulated role. As shown in figure 6, the Scatchard analysis of hippocampal ^{3}H-corticosterone binding in rats treated with LSD demonstrate an increase in number of binding sites, possibly with a reduction in affinity.

The above measurements were carried out in previously adrenalectomized rats in order to have full desaturation of glucocorticoid receptors. In the intact rat the effect on hippocampal glucocorticoid binding of drugs acting on the serotoninergic system would have been more complex since some of these drugs, as shown in figure 7, were able to stimulate adrenal secretion, possibly through interference with monaminergic control of CRF release in the hypothalamus. Taking into account the hormonal negative feedback on glucocorticoid binding capacity in the hippocampus,

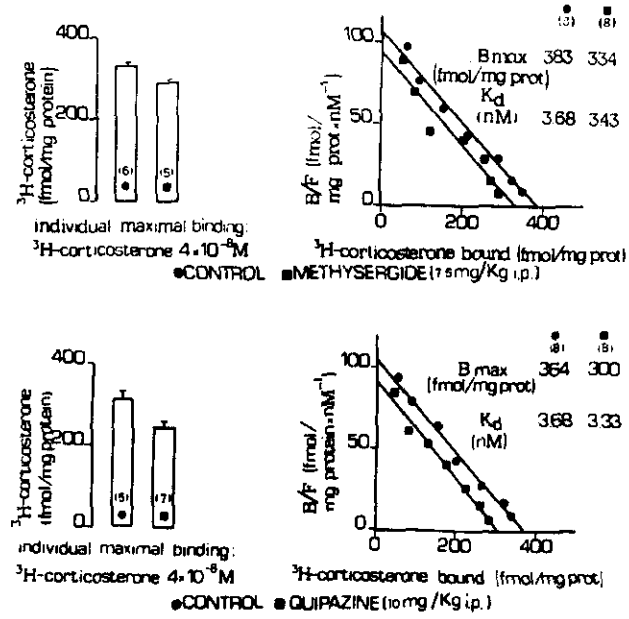

FIG. 5. – Individual maximal binding of ^{3}H-corticosterone and Scatchard analysis (0.06-4 × 10^{-8}M) of binding in the 24-hr adrenalectomized rat's hippocampus cytosol after methysergide or quipazine 1 hour before death.

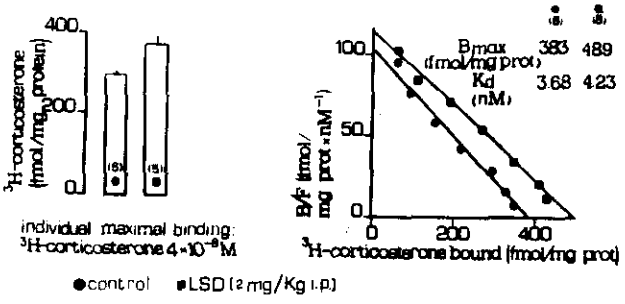

FIG. 6. – Individual maximal binding of ^{3}H-corticosterone and Scatchard analysis (0.06-4 × 10^{-8}M) of binding in the 24-hr adrenalectomized rat's hippocampus cytosol after LSD 1 hour before death.

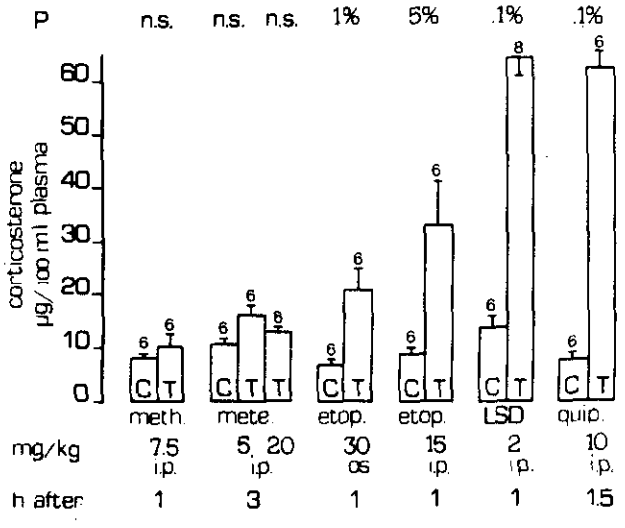

FIG. 7. – The effect of serotoninergic drugs on blood corticosterone level in the rat.

one should assume that in the intact rat the direct and indirect effects of quipazine on the binding would reinforce each other, whereas the two effects would tend to eliminate each other in the case of LSD and etoperidone.

On the basis of the above findings, it can be hypothesized that an alteration of the hippocampal function in the regulation of endocrine and behavioral activities might play a role in the depressive state. In a substantial portion of endogenous depressed patients, the pattern of hypothalamo–pituitary adrenal function is aberrant, with several peaks in diurnal cortisol plasma levels, flattening of circadian rhythm as a consequence of increased nocturnal activity, and a poor response in the dexamethazone suppression test [14, 15]. This abnormality is the neuroendocrinopathological counterpart of the behavioral disturbance. Both reflect a disfunction in a higher brain area [16], the limbic system [17], in which, as indicated by the previously outlined experimental evidence, programs for the circadian tonic inhibitory control of the hypothalamo–pituitary–adrenal activity, as well as for appropriate

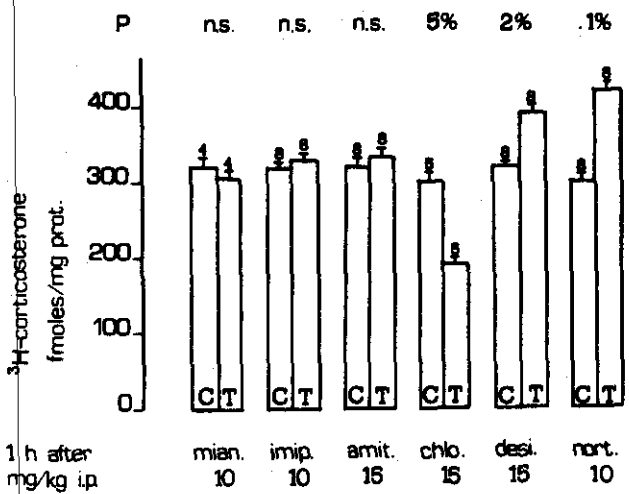

FIG. 8. – The effect of *in vivo* antidepressants on cytosol (1.2 mg protein per ml) individual maximal binding of ^{3}H–corticosterone (4×10^{-8}M) in the 24–hr adrenalectomized rat's hippocampus. *Plain area*: controls; *dotted area*: treated.

behavioral responses are engrammed and integrated. The basis for this intimate integration is given by glucocorticoid neuronal receptors. When tricyclic antidepressants produce recovery from depression, they also normalize adrenal activity [14]. Since these drugs interfere with monaminergic innervation, one may, ex-iuvantibus, prospect the depressive state accompanied by disturbances of the hypothalamo–pituitary–adrenal activity as a neuroendocrinopathy in which altered monaminergic activity is taking place in the hippocampus with ensuing alteration of glucocorticoid receptor function. So, it is of importance to know whether antidepressants modify glucocorticoid binding in the hippocampus. As shown in figure 8, among the antidepressants mianserine, imipramine and amitryptiline had no effect on ^{3}H–corticosterone binding in the hippocampus. Chlorimipramine reduced it, desipramine and nortryptiline increased it. These findings are apparently conflicting; one may attempt to explain the discrepancies among the various

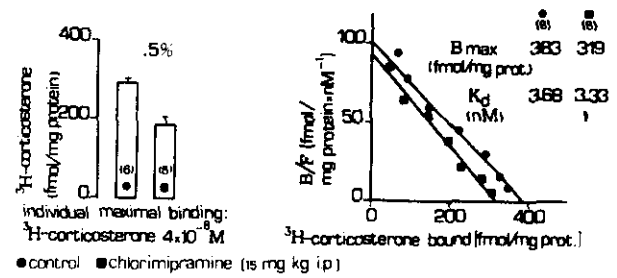

FIG. 9. – Individual maximal binding of ^{3}H–corticosterone and Scatchard analysis ($0.06–4 \times 10^{-8}$M) of binding in the 24–hr adrenalectomized rat's hippocampus cytosol after chlorimipramine 1 hour before death.

antidepressants considering that chlorimipramine is a rather selective blocker of serotonin re–uptake, whereas desipramine and nortryptiline prevalently block noradrenaline re–uptake. Assuming that increased post–synaptic serotoninergic activity inhibits, and increased post–synaptic noradrenergic activity facilitates glucocorticoid binding in the hippocampus, one could think that the lack of effect of imipramine and amitryptiline was due to a null balance between inhibitory and facilitatory influences, while in the case of mianserine it was due to its ascertained incapacity to block monamine re–uptake.

Figure 9 shows the Scatchard analysis of hippocampal ^{3}H–corticosterone binding in rats treated with chlorimipramine: a reduction in number of binding sites was evident, with no modification of affinity.

Figure 10 shows that all triciclic antidepressants investigated were able in large measure to activate adrenal secretion; it is possible that this effect, probably due to interference with monaminergic regulation of CRF release in the hypothalamus, offers a common denominator of the antidepressant activity, independently on the direct effect on hippocampal glucocorticoid binding capacity.

The data presented in this report on the physiological meaning, endocrine and behavioral, of the hippo-

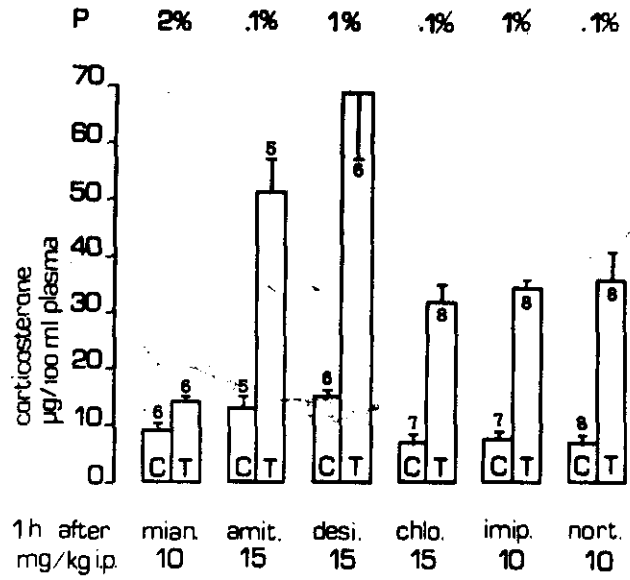

FIG. 10. – The effect of antidepressants on blood corticosterone level in the rat.

campal glucocorticoid receptor, on the role of serotoninergic innervation with regard to the regulation of this receptor, and on the effect of direct or indirect monaminergic drugs on glucocorticoid binding capacity may have a bearing on an antidepressant effect meant as a correction of a behavioral neuroendocrinopathy. At present these data cannot be interpreted in a unitary way. Incoherence may be only apparent, however, because of the imperfect knowledge of those many sites where the different drugs can act prevalently, simultaneously or sequentially, and of the inherent final balance; an incoherence which parallels the fact that through apparently antithetic different mechanisms of action, an antidepressant effect clinically relevant can be equally attained.

Acknowledgement.

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Genetic predisposition to epileptiform fits in rats: probable role of striatal mechanisms

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Summary. – *Some neurophysiological and neurochemical characteristic of rats of two lines: Wistar and Krushinsky–Molodkina (K–M), differing in their sensitivity to the convulsive effect of sound were studied. Wistar rats showed no reaction to the bell sound (exposed for 90 sec), K–M rats in 100 % of cases reacted to the sound developing an excitement fit proceeding into a clonic tonic convulsive fit. Wistar rats were more sensitive to the cataleptogenic effect of bulbocapnine than K–M animals.*

The animals of the two lines showed different kinetic properties of the striatal tyrosine–hydroxylase and also of the constants of ^{3}H–GABA uptake by cerebellum synaptosomes. No dissimilarities in the activity of glutamate–decarboxylase of homogenates of cortex or cerebellum, or difference in the level of GABA were found.

Riassunto. – *Sono stati studiati alcuni aspetti comportamentali e neurochimici di ratti Wistar e ratti Krushinski–Molodkina (K–M), che differiscono nella loro sensibilità alle convulsioni audiogene. I ratti Wistar sono più sensibili dei K–M agli effetti catatonizzanti della bulbocapnina. Le due linee differiscono anche nelle proprietà cinetiche della tirosina–idrossilasi striatale e nelle costanti di captazione del ^{3}H–GABA da parte dei sinaptosomi cerebellari. Non sono state ritrovate differenze nei livelli di GABA e nell'attività della glutamato–decarbossilasi di omogeneizzati di corteccia.*

Introduction.

Three decades ago it was shown that an increased tone of the thalamo–caudate inhibitory system was one of the factors which could stop or prevent convulsive epileptiform fits [1]. The susceptibility of brain motor cortex to the excitation spreading from an epileptogenic focus is known to be in large part related to the efficiency of the inhibitory input from the striatum which is involved in the regulation of locomotor activity and muscle tone [2, 3]. Recently, evidence has been gained in favour of the possibility of arresting convulsive seizures in animals or in men by direct electrical stimulation of the n. caudatus [2, 4, 5]. Dopaminergic nigro–striatal control is known [2, 3] to exert a modulating effect on the striatum: enhanced dopaminergic transmission inhibits striatal function whereas dopaminergic reduced input results in the activation of striatal inhibitory output. In the former case the increased locomotor activity (hyperkinesia) is observed, and in the latter the motility is slowed down (hypokinesia or catalepsy). Striatal hyperfunction clinically manifests itself as organic or drug-induced parkinsonism [3].

Our aim was a neurophysiological and neurochemical study of the striatum in two rat strains having different susceptibility to the epileptogenic effect of sound: Wistar rats were audiogenically insusceptible, while Krushinski–Molodkina rats manifested typical audiogenic convulsive seizures.

Methods.

65 Wistar and 69 Krushinski–Molodkina (K–M) rats weighing 180–220 g from the Animal Farm of M. V. Lomonosov State University (Moscow) were used. All the animals had been previously tested three times by stimulation with an electric bell of 105 decibels. Of the Wistar rats, only those not responding to the sound (audiogenically insusceptible) were selected, and of the K–M group, only those with a marked response to the sound, which manifested itself as sharp locomotor excitation followed by a typical epileptiform fit. Some animals (36) were implanted with steel electrodes in the cortical projection area of the forepaw; through them electric stimulation was applied, to estimate the following parameters: contralateral forepaw contraction threshold (CPCT), ipsilateral forepaw contraction threshold (IPCT) and Jacksonian seizure threshold (JST).

Threshold values were expressed in volts. Stimulation parameters were: 0.1 m·sec, 30–60 Herz, train duration 0.5 sec. Lowest thresholds were observed at 100–200 Herz.

Striatal hyperfunction was obtained with bulbocapnine, which is known to block nigrostriatal transmission, thus releasing the striatum from the inhibitory control of the nigra. Bulbocapnine was injected intraperitoneally at the dose of 40 mg·kg. The intensity of catalepsy was estimated using a 5–points scale. To

evaluate the functional state of the dopaminergic system, the activity of striatal tyrosine hydroxylase (TH) was studied.

Solubilized enzyme was prepared from brain homogenates of the animals of both strains according to Coyle and Axelrod [6].

The activity of the enzyme was determined using a direct spectrophotometric technique based on the measurement of the increase of DMPH² [7]. Synaptosomes were prepared using standard techniques, the kinetics of the uptake of ³H–GABA was studied using a « Millipore » filter system (pore size 0.45 μm). The radioactivity of the samples was measured by liquid scintillation spectrometry. The activity of glutamic acid decarboxylase (GAD, EC 4.1.1.15) was determined fluorimetrically [8]. The animals were decapitated, the brain was promptly removed in the cold, and separate structures were dissected according to commonly used techniques [9].

Results.

In audiogenically–insusceptible Wistar rats, bulbocapnine induced, within 10 min. after injection, an immobility state which was estimated as a 5–point score of catalepsy. The development of a cataleptic state in Wistar rats correlated with an increased contralateral forepaw contraction threshold (CPCT), which lasted 10–90 min, and pointed to a decreased excitability of the motor cortex. In K–M rats, bulbocapnine produced only a slight effect on cortical excitability which lasted less than 10 min and was less pronounced than that of audiogenically–insusceptible rats.

An increased intensity of stimulation resulted into a gradual dissemination of excitation from the local focus: the contraction of the contralateral forepaw was followed by that of the ipsilateral forepaw and then generalized Jacksonian seizures developed. The threshold of these convulsive events are shown in Fig. 1. The differences between the thresholds mentioned may serve as a quantitative index of the ability of excitation to irradiate along cortical and brain stem structures.

In audiogenically–insusceptible Wistar rats the spreading of excitation within motor cortex was sharply impaired by bulbocapnine: the threshold of ipsilateral forepaw contraction increased and so did the threshold of generalized Jacksonian seizures.

On other hand, in the K–M strain rats, bulbocapnine (10 min after injection) did not cause significant changes in the thresholds of forepaw contraction and of generalized Jacksonian seizures, (Fig. 1). After bulbocapnine, the type of movement caused by electrical stimulation of the motor cortex was found to be essentially different in K–M rats: local contraction of the forepaw almost inevitably led to hyperkinesias, clonic contractions of the forepaw, movements resembling athetoid hyperkinesia or even a kind of « torsion spasm »: turns of the head, neck and upper part of the body. Sometimes, such hyperkinesia occurred 10–12 sec after the stimulation was stopped. Audiogenic seizures manifestations after bulbocapnine were much more severe than in the control group, their tonic phase lasted longer, the rate of comas and the duration of postseizure excitation episodes were increased, death rate was higher. These data may be indicative of a lack of striatal inhibitory mechanisms in such animals.

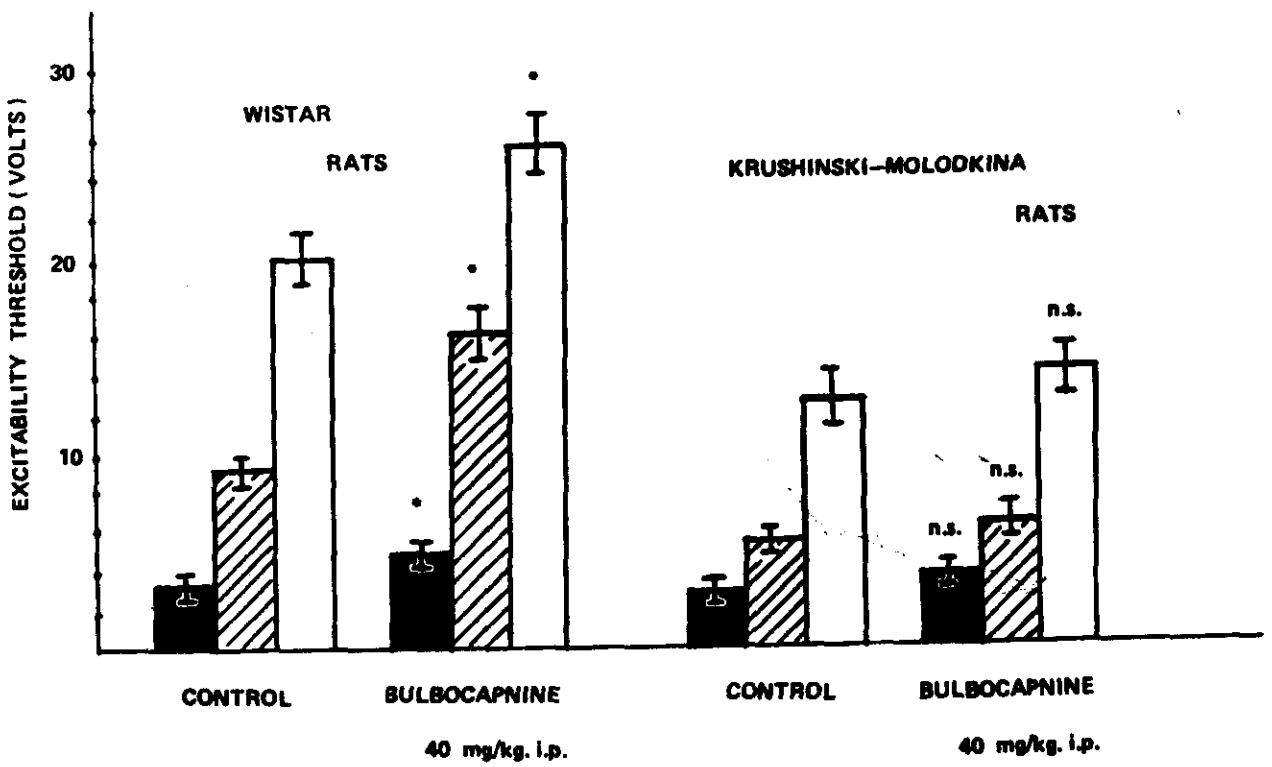

Fig. 1. – Effect of bulbocapnine on the forepaw contraction thresholds and Jacksonian seizure threshold in two rat strains. Black (contralateral forepaw), shaded (ipsilateral forepaw) and open (Jacksonian seizure) columns represent means ± SEM of thresholds measured in 8–10 rats. Asterisks indicate significant difference vs control group ($P < 0,05$)

Table 1. – *^{3}H-GABA uptake and GAD activity in the brain of two rat strains.*

ANIMALS	Region	Kinetic parameters of [^{3}H]-GABA uptake		GAD activity
		Km (μM)	Vmax $\left(\frac{\text{nmole GABA}}{\text{min} \times \text{mg protein}}\right)$	$\left(\frac{\mu\text{mole of GABA}}{\text{hour} \times 100\text{mg protein}}\right)$
Wistar (non sensitive to sound)	Brain Cortex	9.1 ±2.0	1.23 ±0.4	42.5 ±2.7
	Cerebellum	13.8 ±3.1	0.87 ±0.14	31.6 ±2.1
Krushinsky–Molodkina (sensitive to sound)	Brain Cortex	9.2 ±1.3	1.02 ±0.1	42.6 ±3.3
	Cerebellum	35.8 ±5.7	0.84 ±0.15	32.7 ±4.6

(*) p = 0.05.

It can also be seen in Fig. 1 that in K–M rats, the ratio between the threshold of generalized seizures, and that of contractions of ipsilateral and contralateral forepaws is substantially different from that observed in Wistar rats. In K–M rats with increased susceptibility to the sound, these thresholds were much lower.

The different susceptibility to the dopamine-blocking effect of bulbocapnine suggested that animals genetically predisposed to epileptiform fits may have altered DA biosynthesis in the striatum. To confirm this hypothesis, the kinetic characteristics of tyrosine hydroxylase prepared from the striatum of rats of both strains were studied. The greatest difference was found with respect to the affinity of the enzyme for the pterine cofactor of the reaction, $DMPH_4 \cdot K_M$ values for $DMPH_4$ were found to be 0.551 ● 0.054 mM in audiogenically-insusceptible Wistar rats, and 0.128 ± 0.021 mM in K–M rats. The corresponding values of maximum reaction rates (V_{max}) were 32.3 ± 5.1 μmole min^{-1} mg^{-1} protein in Wistar rats and 12.9 ± 2.6 in the animals from K–M group (Fig. 2).

The higher affinity of the enzyme for the hydroxylation cofactor $DMPH_4$ and the lower V_{max} value suggest the presence of genetically determined dissimilarities in the molecular structure of striatal TH in K–M rats as compared to Wistar rats.

To reveal the possible involvement of the GABA-ergic system in the origin of the increased susceptibility of rats to the convulsive effect of the sound, experiments were performed to compare the kinetics of ^{3}H-GABA uptake by synaptosomes obtained from the cerebral and cerebellar cortex of Wistar and K–M rats. Tab. 1 shows that the kinetic parameters of ^{3}H-GABA uptake by cerebral cortex synaptosomes were similar in the two strains of animals while for cerebellar synaptosomes the K_M value was much

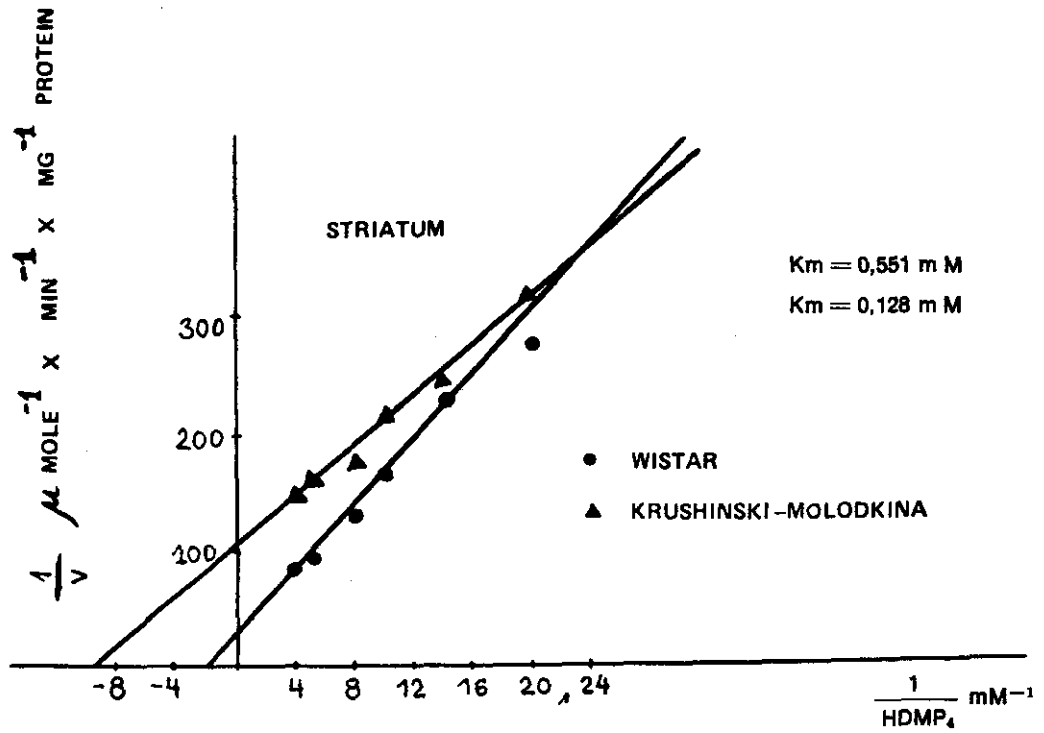

FIG. 2 – Kinetic parameters of striatal Tyrosine Hydroxylase in two rat strains: Wistar and Krushinski – Molodkina (Linewiver-Burk plot). Reciprocal units of TH activity are compared with reciprocal concentration of $DMPH_4$. Each point represents the mean value of 4–6 experiments.

higher in audiogenic sensitive rats indicating a lower affinity of the uptake system for GABA. No dissimilarities in the GAD activity between the two groups of animals have been found. The ratio between the enzyme activity in cerebral and cerebellar cortex was the same in both strains.

Discussion.

The relationship between the convulsive threshold, and in particular the audiogenic convulsions threshold, and the functional state of brain catecholamine systems has been repeatedly discussed in the literature [2, 10, 11], but the problem is still controversial. Our neurophysiological data show the existence of qualitative and quantitative dissimilarities in the effects of bulbocapnine on Wistar and K–M rats; these observations, as well as the neurochemical findings on the dissimilar properties of striatal TH kinetics in the two groups of animals suggest that a genetically determined failure of striatal inhibitory control may be at the basis of preposition to audiogenic fits.

This may be due to the excessive functioning of the nigro–striatal inhibitory mechanism of dopaminergic nature. Consistent with this view are the recent data on the increased level of DA and its metabolites in the striatum of K–M rats as compared to Wistar rats [11].

Our hypothesis is also in agreement with the finding that the increased DA level observed in the striatum in the first few days following electrolytic lesions of s.nigra in rats correlates with their increased sensitivity to penthylenetetrazol; the subsequent decrease of DA level results into a decrease of the sensitivity to this convulsive agent [12]. It is also known that L-DOPA and dopaminergic compounds are capable of potentiating convulsive manifestations in epileptic patients. On the other hand, there is clinical evidence for incompatibility between epilepsy and parkinsonism [13].

Our results may be also interpreted otherwise. The lack of direct measurements of DA turnover rate in the striatum does not allow to compare our data with the results of other workers [14] who showed, in particular, that during ontogenesis the period of decreased susceptibility to the sound in DBA–2 mice coincided with the time of maximal DA turnover rate. On the other hand, considering the complicated neurochemical organization of the striatum and its connections with other brain systems, it can be suggested that the failure of striatal function may be due to the involvement of other non–dopaminergic mechanisms, such as the GABAergic system.

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