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Section III: *Neurochemical and neuropharmacological aspects of pathological conditions of the brain*

Changes in kinetic properties of tyrosine hydroxylase due to dopamine effect on the external synaptosomal membrane

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Summary. – *In experiments on synaptosomes from rat brain hypothalamus the effect of the activation of the presynaptic membrane by dopamine on the kinetic properties of tyrosine hydroxylase (TH) isolated from those synaptosomes was studied.*

Our results indicate that the decrease of the rate of DOPA synthesis during the activation of presynaptic receptors by dopamine observed in in vivo *experiments may result from TH substrate inhibition which under these conditions is due to the changes of the properties of this enzyme. It is suggested that the regulatory influence of presynaptic receptors on TH is exerted via tyrosine-binding site of the enzyme molecule responsible for substrate inhibition.*

Riassunto. – *Sono stati studiati gli effetti dell'attivazione della membrana presinaptica sulle proprietà della tirosino-idrossilasi dei sinaptosomi dell'ipotalamo di ratto. Per l'attivazione sono stati impiegati la dopamina ed un suo polimero che non penetra nella membrana sinaptosomiale. I risultati ottenuti indicano che la diminuzione della sintesi di Dopa dovuta alla attivazione dei recettori presinaptici* in vivo *può dipendere dall'inibizione della tirosina idrossilasi. L'influenza regolatrice dei recettori presinaptici sulla tirosina idrossilasi potrebbe essere esercitata tramite il sito che lega la tirosina dell'enzima responsabile dell'inibizione del substrato.*

Introduction.

The regulation of tyrosine hydroxylase (TH) (E.C.1.14.3.2.) activity, the enzyme limiting catecholamine biosynthesis, involves many factors [1, 2]. Rapid changes of kinetic constants in physiological conditions are essential in TH regulation: the enzyme may be phosphorilated [3], and may change its aggregation state, with consequent changes in its kinetic properties [4]. Pre- and post-synaptic catecholamine receptors play a prominent role in the physiological regulation of TH activity in the brain. Presynaptic dopamine receptors [5, 6] control TH activity; TH inhibition by dopamine [7] resulting from the blockade of its release and reuptake from presynaptic cleft seems the most likely mechanism of TH reaction inhibition in this case.

Dopamine (DA) inhibitory effect on TH is competitive and directed to the pterine site of its molecule [4]. Therefore, our study on the TH physiological control by dopamine agonists deals with the kinetics of TH reaction in connection with the pterine cofactor. We have shown before that the antagonists of DA-receptors, neuroleptics, have a direct effect on TH as they prevent or eliminate the substrate inhibition of the enzyme [8]. We assume that TH substrate inhibition may play a certain role in the physiological mechanism of TH regulation alongside with the enzyme inhibition by the end-product, DA.

The idea that the mechanism of regulation of TH activity during the stimulation of presynaptic receptors could not be accounted for only by the dopamine-induced TH inhibition was set forth in a previous report [9]; the evidence for this concept was provided by experiments with isolated synaptosomes. It was shown that the inhibitory influence of extrasynaptosomal DA on synaptosomal TH activity, at a DA concentration lower than the K_m for uptake, was eliminated by fluphenazine. It was also shown that fluphenazine completely eliminated the inhibitory transmembrane effect on synaptosomal TH of the non-catecholamine DA analog, 7-hydroxy-*N*, *N*-di-n-propyl-2-aminotetraline, which, unlike DA, had no direct effect on TH.

To verify the suggestion that the inhibition of TH during the stimulation of presynaptic receptors is determined not only by end-product inhibition but also by other mechanisms, we carried out experiments on the kinetics of the TH reaction, using both the soluble and the membrane-bound enzyme prepared from synaptosomes pretreated with DA or its polymer derivative, in which DA is covalently linked to dextrane 70; this compound has the biological activity of natural DA but is unable to penetrate inside synaptosomes through the plasma membrane.

METHODS.

The experiments were carried out on synaptosomes isolated from rat hypothalamus according to [10]. The suspension of synaptosomes was divided into three parts and three types of samples were simultaneously incubated: 1) a control sample incubated in the buffer without additions, 2) a sample incubated with DA, 10^{-6}M, 3) a sample incubated with the polymer derivative of DA (PDA). The incubation was carried out for 30 min at 37 °C in a Krebs phosphate buffer with the following composition: 124 mM NaCl, 5 mM KCl, 20 mM Na_2HPO_4, 1,2 mM KH_2HPO_4, 1,3 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM glucose, pH 7.4. After the incubation the synaptosomes were isolated from the medium by centrifugation at 20000 g within 20 min and soluble and membrane-bound TH was isolated from them according to [4]. Protein was measured using the technique described in [12]. The concentration of DA(10^{-5} M) was chosen in accordance to Venter et al. [13] who showed that in order to obtain well-marked effects of immobilized catecholamines on receptors in cellular suspensions high concentrations (10^{-5}–10^{-4} M) have to be used. In our experiments the concentration of synaptosomal protein in the incubation medium was about 4 mg/ml. Hill coefficients were calculated according to [14].

RESULTS.

The curve expressing the relationships between the reaction rate and tyrosine concentration (for the membrane-bound enzyme in the control samples) had a marked sygmoid character.

Table 1. – *Alterations in kinetic properties of membrane-bound tyrosine hydroxylase with respect to tyrosine after incubation of synaptosomes with DA and PDA.*

SAMPLE	Hill Coefficient	Max. velocity of the reaction n mole $min^{-1} \cdot mg^{-1}$ protein	Half saturating concentration of tyrosine μM
Control	4,3±0,5	25,6±2,8	86,5±8,9
DA	1,5±0,2	28,2±3,0	30,0±2,7
PDA	1,3±0,1	38,3±0,4	18,0±2,0

The figures represent the mean of 7 experiments ± S.E.M.

Therefore, the kinetic parameters of the reaction were calculated using the Hill coefficient. Tab. 1 shows that the exposure of synaptosomes to dopamine or to its polymer derivative changed the kinetics of the reaction with respect to tyrosine. TH prepared from synaptosomes exposed to DA and PDA showed and increase in the maximum reaction rate, which in the case of exposure to PDA was higly significant ($p < 0.05$). Control and experimental samples differed markedly with respect to the half saturating concentration of tyrosine (S 0.5). The S 0.5 values were 3 or 5 times lower in the experimental samples previously exposed to DA or PDA respectively, as compared to controls. Thus DA and especially PDA caused a sharp decrease in the optimal tyrosine concentrations. Experimental samples differed from controls also with respect to the Hill coefficient which slightly exceeded 1 in the experimental samples and was about 3 times higher in controls.

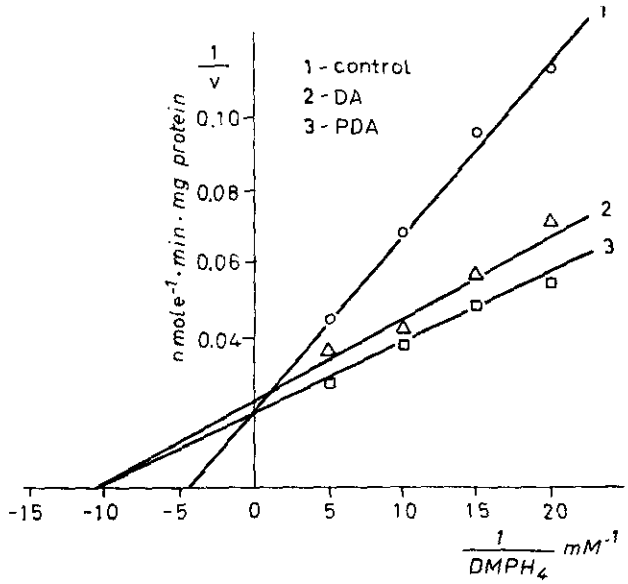

FIG. 1. – The transmembrane effect of DA and PDA on the membrane bound tyrosine hydroxylase activity as a function of $DMPH_4$ concentration. Buffer tris – maleate O, 1 M, pH 6,1. Tyrosine concentration is optimal for TH: in control sample 160 μM, in DA sample 60 μM, in PDA sample 30 μM.

In Fig. 1 the reaction rate is plotted against the concentration of $DMPH_4$. It can be seen that the contact of synaptosomes with DA and PDA somewhat decreased the Km of particulate TH for $DMPH_4$ and did not change the maximum reaction rate. Simi-

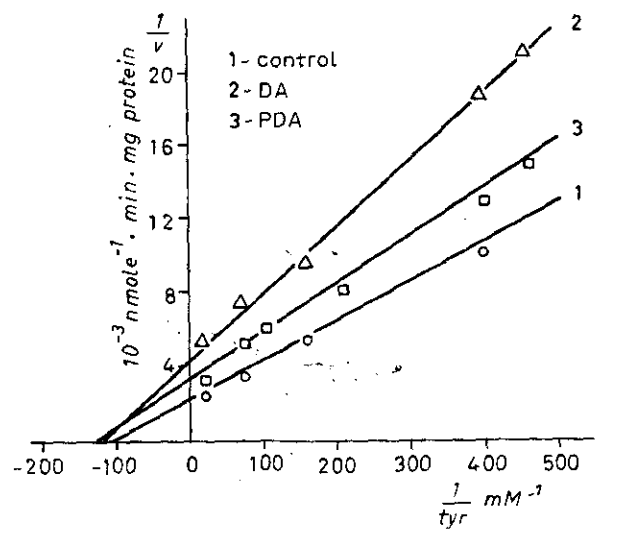

FIG. 2. – Kinetics of soluble synaptosomal tyrosine hydroxylase reaction with respect to tyrosine after incubation of synaptosomes with DA or PDA. Buffer: O, 1 M tris–maleate pH 6,1. $DMPH_4$ concentration 120 μM. Protein concentration 30 μg/ml.

lar results [9] were obtained with the soluble enzyme. Kinetic changes with respect to tyrosine were also observed for the soluble enzyme although they were less marked (Fig. 2). It can be seen that the incubation of synaptosomes with PDA and DA did not cause appreciable changes in the optimal tyrosine concentrations, but led to an increase of the maximal rates of the TH reaction.

The kinetic changes in the TH reaction during the activation of presynaptic receptors with dopamine and its polymer derivative proceed against a background of changes in the internal synaptosomal « milieu ». The permeability of the synaptosomal membrane increased, as confirmed by a 20 % increase in the protein concentration in the incubation medium in the samples incubated with DA and PDA (Tab. 2).

Table 2. – *The protein content in synaptosomal fractions and in the incubation medium after incubation of synaptosomes with DA and PDA.*

SAMPLE	Protein concentration		
	Supernatant after incubation	Membrane bound protein	Soluble tyrosine hydroxylase
Control	31,5±2,2	2998±207	80,0±12,8
DA	36,5±2,6	2440±109	41,6±2,6
PDA	39,6±2,4	2508±135	46,0±2,3

The figures represent the mean of 6 experiments ± S.E.M. The values of the columns 2 and 3 are expressed in micrograms of protein per milliliter. The values of column 2 represent the concentration of total protein in membrane-bound fraction.
Figures of column 3 represent the protein concentration of soluble fraction after its fractionation with ammonium sulfate and mainly consisted of tyrosine hydroxylase.

Protein concentration in the membrane-bound fraction was reduced by about 15 % respectively. Protein concentration of soluble TH decreased by 50 %.

DISCUSSION.

The data presented suggest that the stimulation of presynaptic receptors by dopamine agonists is associated with a decreased optimal tyrosine concentration for TH activity. This would mean that, in these conditions, TH substrate inhibition occurs at lower tyrosine concentrations. TH reaches a paradoxical state: decrease of optimal concentration of the substrate at unchanged or lowered K_m for the cofactor is usually considered as indicative of activation of the enzyme. On the other hand, physiological tyrosine concentrations [15] would already be inhibitory for the enzyme. Tyrosine concentration in the brain is fairly constant [15], and is maintained by the high rate of the phenylalanine hydroxylase reaction. However, increased enzyme affinity for tyrosine may result in the development of substrate inhibition at normal, physiological substrate concentrations. Thus, TH substrate inhibition might be controlled by enzymatic systems regulating TH conformation and activated by the stimulation of presynaptic receptors. Phosphorylation by protein kinase and *linkage* or *elimination* of peptide fragments by peptidases are known to be the most widespread and prompt ways to modify the enzyme molecule.

In conclusion, using DA covalently linked to the soluble polymer of 70000 molecular mass, having all DA active functional groups but not penetrating with sufficient speed inside synaptosomes, we could show that not all the dopamine effects are related to the direct action on TH (such as the enzyme inhibition by the end-product of a chain of biosynthetic reactions). Decreased optimal tyrosine concentration and increased maximum reaction rate with respect to tyrosine are related to the effect of dopamine on the external synaptosomal membrane. This effect would start a chain of reactions leading to TH modification. Kinetic changes of the reaction with respect to tyrosine under the influence of DA or PDA are qualitatively the same and therefore are not related to the mechanism of active transport of the transmitter into synaptosomes. Substrate inhibition of TH may be involved in mechanisms of inhibition of TH activity following the activation of presynaptic receptors.

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The release of GABA from the cerebral cortex: a biochemical approach to monitoring the activity of cortical GABA neurons

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Summary. – *The effects of inhibitors of GABA metabolism or uptake on GABA output from the cerebral cortex was studied by means of a collecting cup placed on the exposed cortex in urethane anaesthetized rats. GABA was identified and quantified by a mass-fragmentographic method.*

Ethanolamine-O-sulphate (EOS) $10^{-2}M$ applied directly on the cerebral cortex caused a long-lasting two fold increase in GABA output while DL 2,4 diaminobutyric acid (DABA) $5.10^{-3}M$ caused a seven fold increase and β-alanine was inactive.

The results indicate that glial uptake has little effect on GABA inactivation in the cerebral cortex. The inhibition of the neuronal uptake seems a more effective tool to increase GABA concentration in the synaptic cleft and consequently also GABA output, than the inhibition of GABA metabolism.

Riassunto. – *Alcuni inibitori della captazione gliale o neuronale e del metabolismo del GABA sono stati usati per studiare i meccanismi che determinano la cessazione dell'azione sinaptica di questo aminoacido. Il modello sperimentale utilizzato è stato il ratto in cui una coppetta era stata impiantata sulla corteccia parietale. Il GABA è stato misurato con tecniche mass-frammentografiche.*

I risultati ottenuti indicano che l'EOS ($10^{-2}M$), un inibitore della GABA-transaminasi, ove applicato sulla corteccia cerebrale, determina un raddoppio della quantità di GABA che diffonde nelle coppe corticali; il DABA, un inibitore della captazione neuronale di GABA, ne determina un aumento fino a circa il 700 %; mentre la β-alanina, un inibitore della captazione gliale dell'aminoacido è inattivo.

I risultati suggeriscono che, nella corteccia cerebrale del ratto, l'inibizione della captazione neuronale è più efficace dell'inibizione del metabolismo del GABA, nell'aumentare la concentrazione a livello sinaptico di questo neurotrasmettitore.

Introduction.

The activity of GABAergic neurons can be monitored « in vivo » by electrophysiological or biochemical techniques. Each technique has inherent advantages or limitations. The electrophysiological approach is usually very sensitive and offers the possibility of measuring rapid changes in the neuronal activity. However the anatomical complexity of the brain and the lack of adequate histochemical controls make it difficult for the electrophysiologist to ascertain the nature of the neurotransmitter released by a particular neuron. Moreover it is impossibile to assess by electrophysiological recording the global activity of the GABAergic neurons in a given brain area. The biochemical techniques usually provide information on the global function of a biochemically identified neuronal population. However they are not sensitive enough to monitor rapid changes in neuronal activity.

Among the biochemical techniques, turnover studies have been widely used in the last few years in order to monitor the rate of activity of cholinergic and dopaminergic neurons [1–3]. Several techniques have also been developed for the study of the turnover rate of GABAergic neurons [4]. However GABA is present not only in neurons but also in glial cells [5]. The compartmentalization between the two types of cells, its disposition and metabolism have not yet been completely clarified [6]. It is therefore difficult to calculate neuronal GABA turnover rate [4 – 7].

The measurement of the release of neurotransmitters from nerve endings has always been considered the most direct approach to the study of neuronal activity since the release is, within certain limits, proportional to the impulse flow [8, 9]. Therefore as an alternative approach to measuring « in vivo » the activity of cortical GABA- releasing neurons we attempted to investigate and quantify GABA outflow from the cerebral cortex.

Evidence of a neuronal origin of GABA released from the cortical surface.

Collecting cups were applied to the surface of the brain in adult male rats according to previously described techniques [10, 11]. Ringer solution was placed in the cups and was substituted every twenty minutes; the aminoacid content was measured by a sensitive and specific G. C.–M. S. method [10].

The amount of GABA released from the cortical surface was 55 ± 8 picomol. collection period and remained fairly constant for several hours [12]. The amount of glutamate found in the collecting cups was 1.25 ± 0.2 nmol/collection period. However, the blood contained 0.37 nmol/ml of GABA [12] and 150 nmol./ml of glutamate [13]. Therefore both the GABA and glutamate detected in collecting cups could have simply diffused from the blood and could therefore be unrelated to neuronal function.

In order to shed some light on the origin of these aminoacids, we measured the output of GABA and glutamate from the cortical surface while simultaneously recording the electrical activity from the same area.

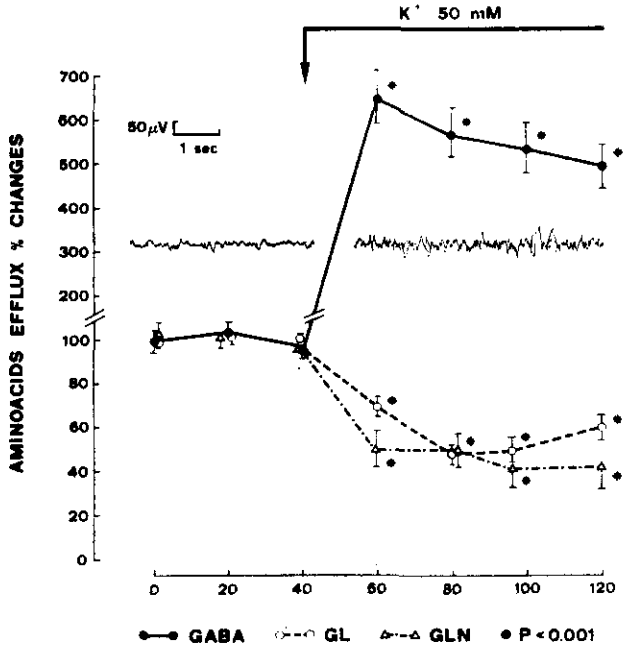

FIG. 1. – The effects of local application of 50 mM KCL on GABA, glutamate and glutamine output from the cerebral cortex and on the ECoG of urethane anaesthetized rats. (*) $P < 0.01$ (Data from [12]).

As shown in Fig. 1, the substitution of an equimolecular concentration of NaCl with KCl 50 mM in the Ringer solution filling the collecting cups was followed by a large increase in GABA output and a decrease in glutamate output. The peak increase in GABA output occurred in the first twenty minutes after changing the solution and was relatively long lasting. The modification in the aminoacid release pattern was accompanied by the appearance of an electrocorticogram (ECoG) characterized by high voltage low frequency waves. This electrocorticographic pattern suggests an enhanced cortical inhibition, as would be expected from an enhanced GABA output [14]. In order to ascertain whether the increase in GABA output was associated with an increase in neuronal activity, tetrodotoxin (TTX), which blocks the rapid Na^+ influx associated with depolarization [15], was added to the solution filling the collecting cups. TTX (3.10^{-5}M) completely prevented the increase in GABA output induced by the application on the cortical surface of a solution containing KCl 25 mM [10]. These results demonstrate a relationship between GABA output and neuronal activity and seem to indicate that GABA released from the cortical surface originates from neurons under the collecting cups.

Effects of inhibitors of GABA uptake and metabolism on GABA release.

In order to obtain further evidence that GABA detected in the collecting cups derives from the brain, attempts were made to modify GABA output without affecting blood GABA levels or cerebral blood flow. As shown in Table 1, the intracerebroventricular administration of 30 μg of aminooxyacetic acid (AOAA), an inhibitor of GABA metabolism [16], significantly increased the cortical level of GABA, doubled the amount released and caused a slight sedation without affecting blood GABA level.

A second attempt was made by adding ethanolamine–O–sulphate (EOS), a selective inhibitor of GABA metabolism [16, 17] to the Ringer solution filling the collecting cups. Figure 2 shows that the local application of 5.10^{-3} M and 10^{-2} M of EOS brings about a dose–dependent increase in GABA output. EOS is a relatively lipid–insoluble compound. In our experimental model it was applied on the external surface of the brain. This may explain the high concentrations which were necessary to obtain a pharmacological effect. Nevertheless, in spite of the large concentrations of inhibitor used, the increase in GABA output was only doubled.

This finding correlates well with the observation that a dose of 2000 mg./kg. i.p. of EOS only causes a 30 % increase in the convulsive threshold of several animal models [17], and indicates a relatively low potency of this drug. It also suggests that GABA-T plays a limited role in GABA inactivation.

Table 1. – *The effects of intracerebroventricular injections of AOAA.*

	%
Blood GABA content	110
Cortical GABA content	190
GABA release	200

30 μg of AOAA were injected 2 hours before. The control GABA release was 60 picomol./collection sample. Blood GABA content 0.37±0.04 nmol/ml; cortical GABA level: 21.40.5± nmol/mg protein.
Data from [7].

It has been demonstrated that a high affinity uptake of GABA occurs in presynaptic nerve terminals and in glial cells (see references in [18]). It has also been suggested that these uptake mechanisms play an important role in the removal of GABA from the synaptic cleft. The glial uptake is preferentially blocked by β–alanine, whereas *d*–diaminobutyric acid (DABA) interferes primarily with the neuronal or presynaptic uptake system [19].

As illustrated by Fig. 3, in our experiments the addition of DABA to the Ringer solution in the collecting cups was followed by a six-fold increase in GABA output. On the contrary, β-alanine at the same concentration was inactive. These results indicate that in the cerebral cortex the neuronal uptake is a powerful mechanism in removing GABA released from the nerve endings and reducing its concentrations at receptor level.

Discussion and conclusions.

From our findings it appears that GABA released from the cerebral cortex *in vivo* originates from the nervous tissue and that its release is related to neuronal activity in the upper cortical layers. Therefore, by measuring GABA output it is possible to monitor the functional activity of cortical GABA neurons.

Using this approach we showed that it is possible to increase GABA output and therefore presumably GABA concentration in the synaptic cleft by manipulating GABA metabolism and uptake. However, the inhibition of GABA-T by intracerebroventricular administration of AOAA or local application of EOS only brings about a two-fold increase in GABA release. On the contrary DABA, an inhibitor of neuronal GABA uptake, induced a six-fold increase in GABA output.

This observation confirms the hypothesis that GABA uptake is quantitatively more important than GABA-T as a mechanism for GABA inactivation. The finding that β-alanine was ineffective in our experimental model was unexpected and casts some doubt on the role of glial cells in modulating GABA transmission in the upper cortical layers. The inhibitors of the neuronal GABA uptake may therefore represent a class of compounds of future therapeutic interest in situation in which GABA neurotransmission is impaired.

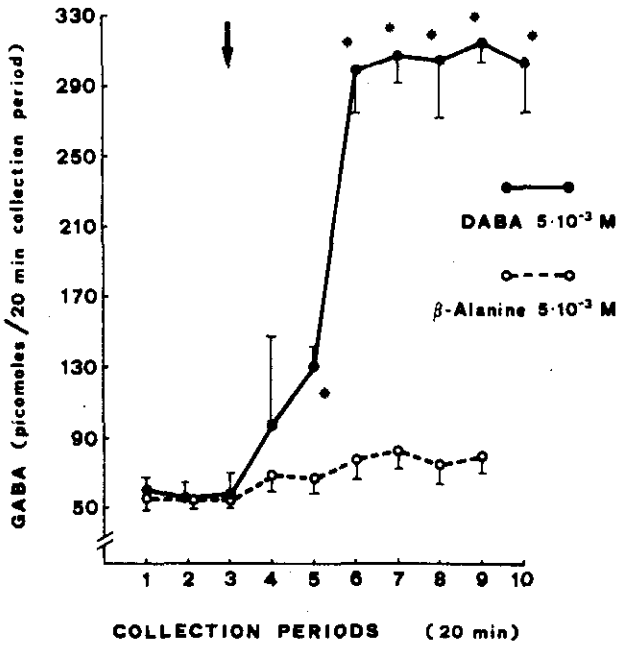

Fig. 3. – The effects of local application of β-alanine and of Dl-diaminobutyric acid (DABA) on the output of GABA from the rat cerebral cortex. (*) $P < 0.05$.

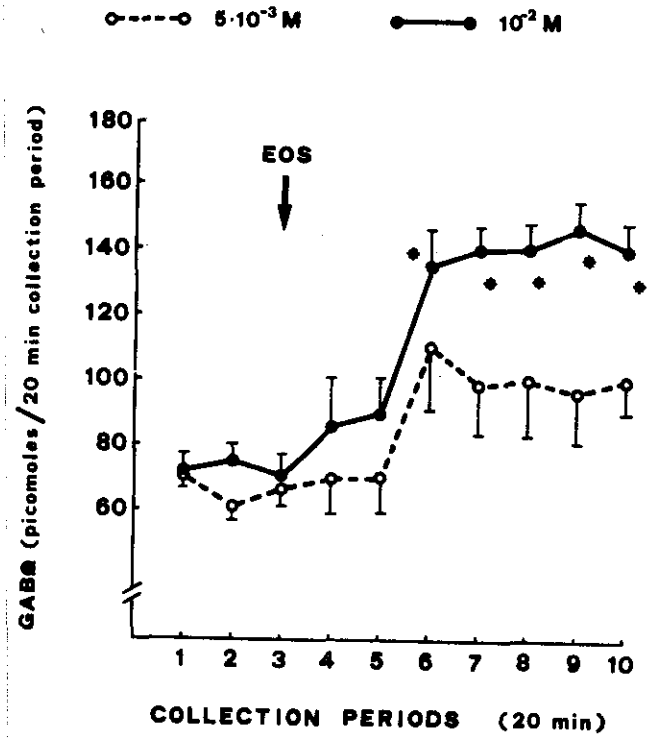

Fig. 2. – The effects of local application of ethanolamine o sulphate on the output of GABA from the rat cerebral cortex. (*) $P < 0.05$.

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A new class of endogenous peptide factors

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Summary. – *A purified brain extract or cerebro-spinal fluid (CSF) of animals with unilateral lesion of various parts of the motor system (motor cortex, cerebellum, descending motor tracts), introduced into the brain ventricular system of intact animals, induce a similar asymmetric functional state of the spinal centres.*

Biochemical analysis using column chromatography and enzymatic degradation has indicated that the active agent present in the brain extracts and in the CSF is a low-molecular peptide fraction with a molecular weight between 1000 and 2000.

At the later stages, corresponding behaviourally to the period of compensation of the damaged functions, in the CSF and in brain extracts of the donors a new factor appears, which is able to eliminate the postural asymmetry in lesioned animals at the early stages of the development of the pathological process. The reported results indicate the direct participation of specific endogenous factors in the pathogenesis and compensation of organic lesions of the brain.

Riassunto. – *Estratti cerebrali e liquido encefalorachidiano di animali con lesioni unilaterali del sistema motorio, introdotti nei ventricoli cerebrali di animali integri provocano un analogo deficit motorio a livello dei centri spinali. Ricerche chimiche hanno indicato che questi principi attivi sono dei peptidi con un peso molecolare variabile da 1000 a 2000. Quando l'animale si riprende dal deficit motorio, compare negli estratti di cervello e nel liquor un altro fattore che si è rivelato capace di eliminare le asimmetrie posturali in animali con analoghe lesioni del sistema nervoso centrale. I presenti risultati indicano un ruolo di fattori endogeni specifici nella patogenesi e nella compensazione di lesioni organiche del cervello.*

Introduction.

In 1929 Di Giorgio [1] observed that the postural asymmetry of the hind limbs developing in rats and in other animals after unilateral destruction of the anterior lobe of the cerebellum remains after section of the spinal cord at the thoracic level, if a certain period of time is elapsed between the appearance of the asymmetry and the spinal severing. Such a fixation of an asymmetrical functional state of the spinal centres represented an unusual phenomenon and was considered by some authors [2–4] as a form of spinal cord memory.

In our Department [5] it was demonstrated that purified brain extract or cerebro-spinal fluid (CSF) of animals with unilateral lesion of various parts of the motor system (motor cortex, cerebellum, descending motor tracts), introduced into the brain ventricular system of intact animals, induce a similar asymmetric functional state of the spinal centres, represented after thoracic spinalization by the asymmetry of posture and tone of the limbs. The development of asymmetry in recipients corresponded to the level and the side of lesion in the donor's central nervous system (Fig. 1).

Biochemical analysis using column chromatography and enzymatic degradation has indicated that the active agent present in the brain extracts and in the CSF was a low-molecular peptide fraction with a molecular weight between 1000 and 2000 [6].

Taking into consideration the data obtained, further research was undertaken in order to:

1) elucidate whether the participation of chemical factors in the reorganization of the functional state of the spinal cord can be applied also to more rostral centres;

2) to establish the species specificity of these factors;

3) to investigate more in detail the chemical identity of the liberated substances.

Methods.

The majority of the experiments were performed in adult male rats, but also some other laboratory species were employed. Two experimental models were used: unilateral extirpation of the motor cortex and Brown-Séquard hemisection of the spinal cord at Th_3–Th_4 level. The presence of chemical factors in the brain and/or CSF was tested by injection of the CSF or HCl-extracts of total brain of the operated animal-donor (10–50 μl) in the brain ventricles of intact recipient followed by spinalization.

To establish species specificity, dogs, cats and rabbits with unilateral central lesions were used as donors.

Intact rats, guinea-pigs and cats were used as recipients. In several cases the CSF of patients with unilateral brain lesions was injected subdurally to intact animals.

To investigate the role of chemical factors in compensatory processes, the CSF and brain extracts of

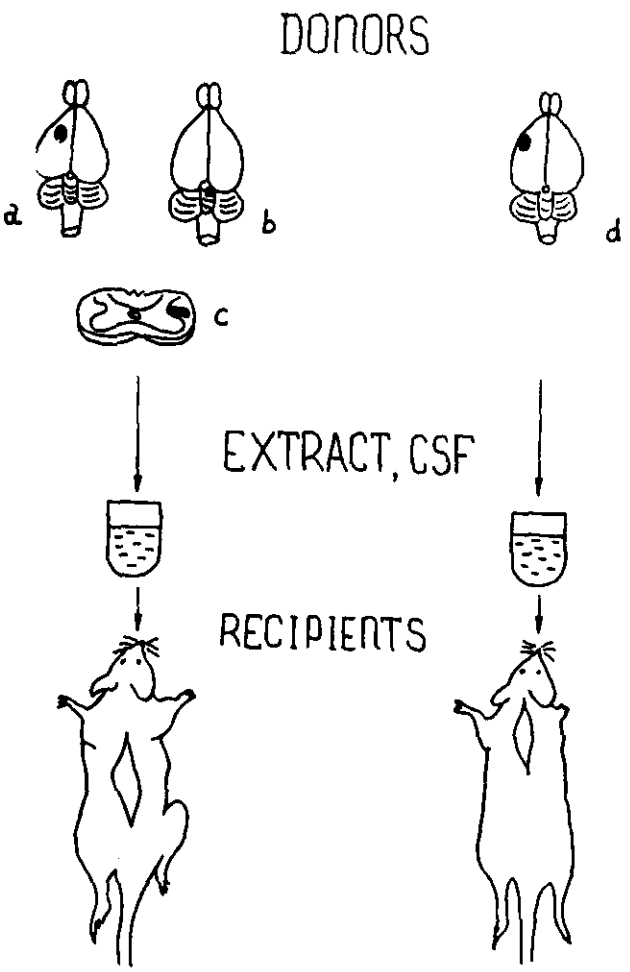

FIG. 1. – Specificity of action of the brain extracts and of the CSF upon the intact animals-recipients: (*a*) destruction of the left motor area controlling the movements of contralateral hind limb; (*b*) destruction of the right anterior lobe of cerebellum; (*c*) right side hemisection of the spinal cord at the Th_3Th_4 level. The brain extracts and the CSF of the animals with the above central lesions induce flexion of right hind limb in the intact animals-recipients after spinalization; (*d*) destruction of the left motor area controlling the movement of the contralateral fore limb. The extract and the CSF of an animal with such a lesion induce in the intact recipient the flexion of the right fore limb, which is clearly revealed after spinalization at cervical level.

operated animals which had already compensated their motor defect (3–4 weeks after operations) were used. Animals bearing analogues but recent (one week) brain lesions served as recipients in these experiments.

RESULTS AND DISCUSSION.

The experiments have shown that the brain extracts and the CSF of animals with unilateral brain lesions of different parts of the motor system (motor cortex, descending motor tracts) induced an asymmetrical functional state of the spinal centres in intact animals, represented by a postural asymmetry of the limbs after spinalization. The asymmetry induced in the recipients was dependent on the localization of the central lesion in the donor (Fig. 1): the brain extracts or the CSF of the donors with lesions of the cortical area controlling the hind limb (Fig. 1, *a*) or of the right anterior lobe of the cerebellum (Fig. 1, *b*), or with a right-side hemisection of the spinal cord (Fig. 1, *c*) always induced the flexion of the right hind limb. In case of contralateral lesions the active material induced a left side flexion of the hind limb. Moreover, when in the donor the motor area controlling the movement of the fore limb was removed, the flexion in the recipient occured only in the fore limb (Fig. 1, *d*).

These chemical factors inducing an asymmetrical functional state of the spinal cord centres have been named factors of postural asymmetry (FPA). Further experiments on different animal species and the use of CSF of patients with organic destruction of senso-motor cortex of traumatic and circulatory origin have revealed the species aspecificity of the FPA.

At the later stages, corresponding behaviourally to the period of compensation of the damaged functions, in the CSF and in brain extracts of the donors a new factor appears, which is able to eliminate the postural asymmetry in similarly operated animals at the early stages of the development of the pathological process [7]. It is worthwhile to point out that after boiling this FPA disappears from the extracts and CSF of compensated donors. These data indicate that the development of compensatory rearrangements in the CNS is, in turn, accompanied by the appearance of specific factors which can eliminate the asymmetrical state of spinal centers of operated animals. These factors are also of proteic nature and differ from FPA in being thermolabile and able to suppress the FPA activity *in vivo*.

Cats with traumatic lesions in cortical motor areas develop motor defects less severe than controls and compensate the damaged functions considerably faster when treated with the CSF of animals or humans with similar lesions of the CNS which have already compensated their motor defect.

The simptoms of organic lesions of the CNS were considered until now as a result of the damage of functions of the specialized brain tissue. The reported results indicate the direct participation of specific endogenous factors in the pathogenesis and compensation of organic lesions of the brain.

These factors might be similar to the inductors serving as markers of the neuronal chains in the course of ontogenetic development of the brain. These peptides exert a modulatory action upon the neurons of the motor system and induce changes in their functional connections. These changes are, in turn, the first step of intracentral rearrangements eventually leading to a compensatory effect.

The therapeutical effect observed in animal experiments is wortwhile of further investigations on the mechanism of action of the peptide factors, in order to lead to new treatments of organic diseases of brain, which until now do not have any effective form of treatment.

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Inhibition of nigral dopaminergic firing by N-n-propyl-norapomorphine: behavioural and biochemical correlates

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Summary. – *N-n-propyl-norapomorphine (NPA) is 10 to 20 times more potent than apomorphine in inhibiting the firing rate of nigral dopaminergic cells and striatal and limbic dopamine (DA) synthesis and also in producing hypomotility in rats. The intravenous ED_{50} of NPA to inhibit dopaminergic firing is 0.36 $\mu g \cdot kg^{-1}$, while that for apomorphine is 9.1 $\mu g \cdot kg^{-1}$. The threshold subcutaneous doses of NPA and apomorphine to significantly inhibit motor activity or DA synthesis are 1.25 and 25 $\mu g \cdot kg^{-1}$, respectively. The above effects of NPA, as those of apomorphine, are antagonized by haloperidol and, stereospecifically, by (–) sulpiride.*

Riassunto. – *La N-n-propil-norapomorfina (NPA) è da 10 a 20 volte più attiva dell'apomorfina nell'inibire l'attività delle cellule dopaminergiche nigrali, nel rallentare la sintesi della dopamina a livello dello striato e del sistema limbico e nell'inibire la motilità spontanea nel ratto. Questi effetti sono antagonizzati dall'aloperidolo e, stereospecificamente, dalla (–) sulpiride.*

It has been demonstrated that the behavioural stimulation produced by apomorphine is due to the activation of postsynaptic dopamine (DA) receptors in the central nervous system (CNS) [1, 2]. Moreover, it has been suggested that the sedative effect of low doses of apomorphine is mediated by the preferential stimulation of DA receptors located on the DA neuron itself, namely „autoreceptors" [1, 3–7]. In fact the stimulation of these receptors results in the inhibition of both DA synthesis and electrical activity of DA neurons [3, 8–10], supporting the idea that the sedative effect of apomorphine is the behavioural consequence of the inhibition of dopaminergic function.

Recently, N-n-propyl-norapomorphine (NPA) has been found far more potent than its homologue apomorphine in producing excitatory and stereotyped behaviour in different animal species [11–14]. With the aim of clarifying whether NPA were more potent than apomorphine in stimulating the autoreceptors, we studied the effects of low doses of NPA on the electrical activity of DA neurons in the rat substantia nigra, on motor activity and on DA synthesis in different brain areas. The present study shows that NPA is far more potent than apomorphine in inhibiting DA firing, decreasing DA synthesis and producing sedation.

Male Sprague-Dawley CD^{R} rats (Charles River, Como, Italy), weighing 230–250 g, were used. For the electrophysiological study, rats were anesthetized with 3.0 % halothane in air, tracheotomized, intubated, paralyzed with succinylcholine chloride (200 $mg \cdot kg^{-1}$, i.p.) and artificially ventilated. Additional injections of succinylcholine chloride were given as needed throughout the experiment. A long-acting local anesthetic (etidocaine HCl) was infiltrated in all incision sites and soft tissue pressure points, then the animals were mounted in a David Kopf stereotaxic apparatus. Recording glass " omega dot " electrodes, filled with a 2 % solution of Pontamine Sky Blue (PSB) in 5 M CH_3COONa (tips broken back to 1–2 μm, resistance 2–6 MΩ in vitro), were lowered into the substantia nigra (SN) pars compacta (PC): 1.8–2.0 mm anterior to the lambda suture, 1.4–1.9 mm lateral to the sagital suture and 7.1–7.8 mm ventral to the surface of the cortex with the skull flat (nose minus 2.3–2.5 mm). Extra-cellular action potentials from a single neuron were recorded and displayed using standard techniques [15–17].

In order to stimulate antidromically the nigro caudate pathway, electrical stimulations (30–90 V; 0.5–1.5 mA; 0.5–1.0 msec; monophasic square pulses) were delivered through a twisted pair of stainless steel 250 μm wire, insulated to the tip and placed in the medial forebrain bundle (6.6 mm anterior to the lambda; 2.0 mm lateral and 5.8 mm ventral) adjacent to the capsula interna.

Electrophysiological and anatomical identification of SN-DA neurons were performed as previously described by others [16, 18, 19] with some new characterizations of these cells. Briefly, the SN-DA cells were identified by their regular and bursting basal firing rate [16, 18], wide shape [16], low conduction velocity [16, 19], response to antidromic stimulation and collision test and design of computed Inter Spike Interval Histograms [20]. Finally, each recording site was marked by iontophoresis of PSB as cation (2–5 μA for a few min), followed by a standard histological preparation or a recently developed fluorescence histochemical method [21] (Fig. 1).

After electrophysiological identification of a SN-DA cell, its spontaneous activity was recorded for 5–10 min, then a drug solution or comparable volume (1 $ml \cdot kg^{-1}$) of the solvent was injected through a previously incannulated jugular vein. Body temper-

ature, expired CO_2 concentration, heart rate and frontal EEG were monitored throughout the experiment.

For motor activity studies, the animals were housed 4 per cage at 24°C, humidity 50–60 %, with a 12 h light–dark cycle and had standard laboratory food and water ad libitum. Motor activity was measured by placing the animals individually into motility cages (M/P 40 Fc Electronic Motility Meter, Motron Products, Stockholm, Sweden), as previously described [4]. *Motor activity was counted for 25 min starting* 5 min after the animals had been put into the motility cage, during the first 2 h of the dark phase of the cycle, when exploratory behaviour is maximal: a condition which is optimal to evidentiate the sedative ef*fect of DA agonists.*

DA synthesis was measured as DOPA accumulation following inhibition of DOPA decarboxylase by NSD 1015 [3]. The animals received NSD 1015, 100 mg. kg^{-1}, i.p., and were killed by decapitation 25 min later. *Caudate nucleus, nucleus accumbens* and frontal cortex were dissected on ice as previously described [22] and stored at — 30°C until analyzed. DOPA levels were determined by our radioenzymatic method based on the conversion of DOPA to labelled 3-O-methyl-DOPA by *catechol-O-methyl-transferase* in the presence of S-adenosyl-(^{3}H-methyl)-methionine (5–15 Ci/mmole, Amersham, England) as a methyl donor [23].

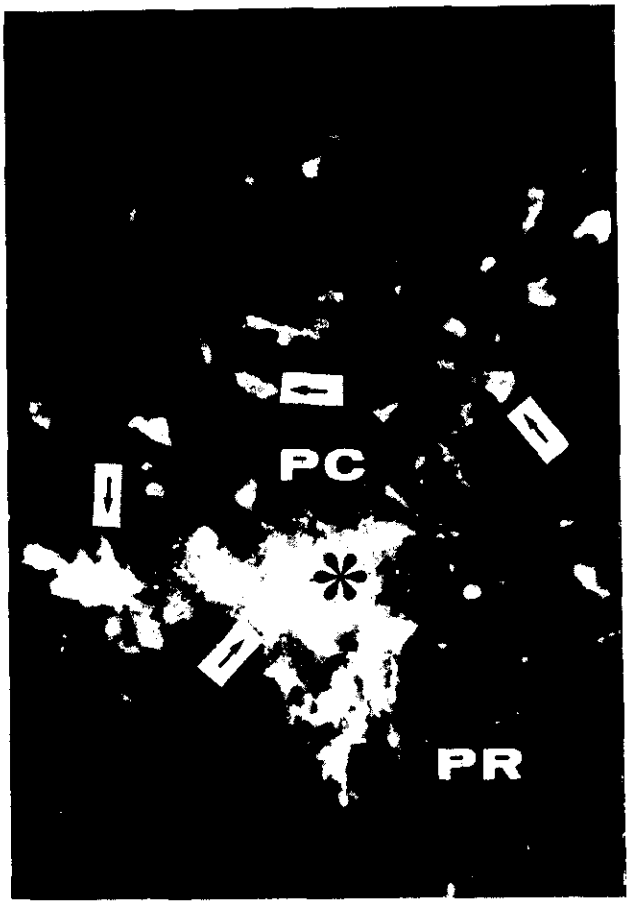

FIG. 1. – Fluorescence photomicrograph showing the « green-yellow » fluorescence of DA cells in the SN pars compacta (eg. as indicated by the arrows) and the « red » fluorescent PSB spot (black asterisk) confirming the recording site. The spot was produced by iontophoretically ejected PSB from the tip of the recording electrode (21).

(—) N-n-propyl-norapomorphine-HCl (Sterling Winthrop, Rensselaer, U.S.A.) and (—) apomorphine-HCl (Sigma, S. Louis, U.S.A.) were dissolved in saline containing 0.2 % ascorbic acid. (+) and (—) sulpiride were dissolved with a drop of acetic acid, diluted in saline

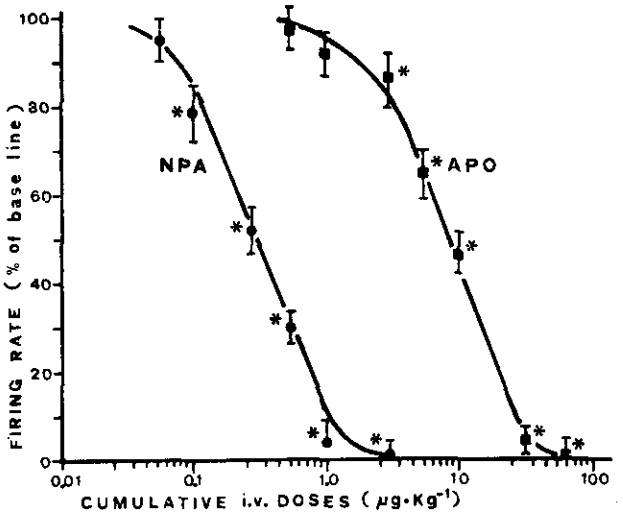

FIG. 2. – Log dose-response regression curves for inhibition of firing rate of DA neurons in substantia nigra pars compacta by apomorphine (APO) and N-n-propyl-norapomorphine (NPA), respectively. Each point represents the mean inhibition ± S.E. ($N = 5 \div 18$) obtained at a given cumulative dose. Apomorphine or NPA were administered in increasing doses through a previously incannulated jugular vein. Only one cell per rat was studied. (*) $P < 0.001$, with respect to baseline values (one tailed Student's t-test).

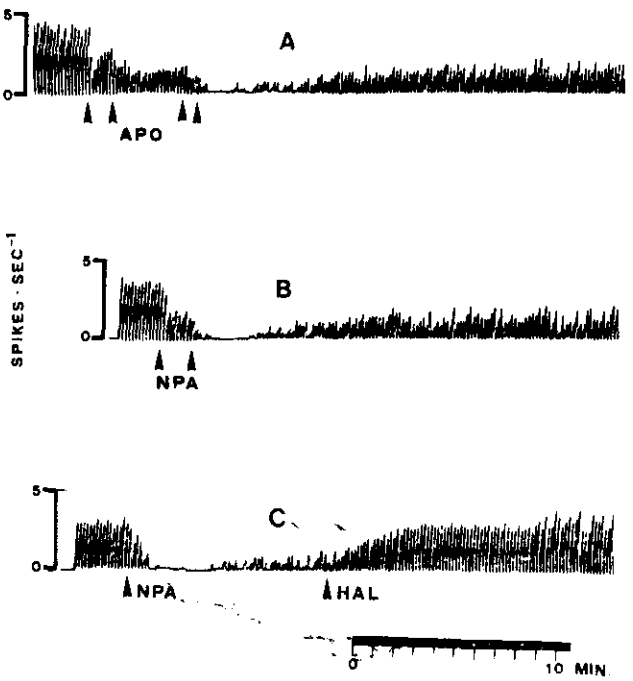

FIG. 3. – Effects of intravenous apomorphine (APO) and NPA on the firing rate of rat dopaminergic neurons: antagonism by haloperidol (HAL). *A, typical response to apomorphine.* Complete temporary interruption of activity was seen after the cumulative dose of 25, $\mu g \cdot kg^{-1}$ (5.0, 5.0, 5.0, 10.0, $\mu g \cdot kg^{-1}$, as indicated by the arrows). B, the same degree of inhibition was obtained with a cumulative dose of 1.0, $\mu g \cdot kg^{-1}$ (0.5, 0.5 $\mu g \cdot kg^{-1}$) of NPA. C, a single dose of 1.0, $\mu g \cdot kg^{-1}$ of NPA led to complete *inhibition within one min.* Haloperidol, given intravenously 9 min later, rapidly reversed the effect.

and the pH adjusted to about 4.5. Haloperidol (Serenase; Janssen, Belgium) was used in the commercially available solution.

Firing. Fig. 2 shows the dose-related inhibition of the bioelectrical activity of dopaminergic neurons in the substantia nigra pars compacta, induced by apomorphine and NPA.

In agreement with previous reports [8, 10], we found that the median effective dose that inhibited the firing rate of dopaminergic neurons by 50 % (ED_{50}) was 9.1 $\mu g \cdot kg^{-1}$ for apomorphine. The administration of 25 $\mu g \cdot kg^{-1}$ of the drug temporarily shut off 80 % of the DA cells tested (N=15). On the other hand, the ED_{50} of NPA was as low as 0.36 $\mu g \cdot kg^{-1}$ and the maximal dose used, 1.0 $\mu g \cdot kg^{-1}$, produced a transient complete inhibition in 95 % of the cells studied (N= 18). After injection of apomorphine (25 $\mu g \cdot kg^{-1}$) or NPA (1.0 $\mu g \cdot kg^{-1}$), recovery took place slowly with a 50–60 % return towards base-line occurring in 30 min (Fig. 3 A and B). Furthermore, the inhibition of nigral firing induced by these doses of apomorphine and NPA was readily reversed by 50 $\mu g \cdot kg^{-1}$ of haloperidol (Fig. 3C).

Motility. As expected from previous results [4, 6], apomorphine caused a biphasic effect. At the doses of 25–100 $\mu g \cdot kg^{-1}$, it significantly decreased motor activity and the animals appeared sedated. By contrast, the dose of 200 $\mu g \cdot kg^{-1}$ increased motor activity and elicited stereotyped behaviour characterized by repetitive sniffing and head and limb movements (Fig. 4). NPA was far more potent than apomorphine in decreasing motor activity in rats. A significant inhibition of spontaneous motor activity was produced by a dose as low as 1–10 $\mu g \cdot kg^{-1}$. Interestingly, after a dose of 10 $\mu g \cdot kg^{-1}$, although the animals appeared sedated, they showed repeated episodes of stereotyped gnawing movements. After a dose of 100 $\mu g \cdot kg^{-1}$, the animals no longer appeared sedated but exhibited only stereotyped behaviour, characterized by continuous sniffing, gnawing, biting and licking movements (Fig. 4).

DA Synthesis. As with apomorphine, a threshold dose of NPA to produce hypomotility also decreased DA synthesis in different brain areas. As shown in Tab. 1, a dose of NPA as low as 1.25 $\mu g \cdot kg^{-1}$ decreased DOPA accumulation by approximately 20, 30 and 32 % in the frontal cortex, nucleus accumbens and caudate nucleus, respectively. Maximal decrease in DA synthesis (40 %) was produced in all areas by a dose of 10 $\mu g \cdot kg^{-1}$.

As can be seen in Tab. 2, doses of haloperidol (50 $\mu g \cdot kg^{-1}$) and (—) -sulpiride (10 $mg \cdot kg^{-1}$) which, per se, do not modify motor activity, completely prevented the inhibitory effect of NPA on both motor activity and DA synthesis. The (+) enantiomer of sulpiride failed to prevent NPA effects.

The present investigation has shown that NPA has far greater potency than apomorphine in producing sedation, decreasing DA synthesis and inhibiting the firing rate of nigral DA neurons. All these effects

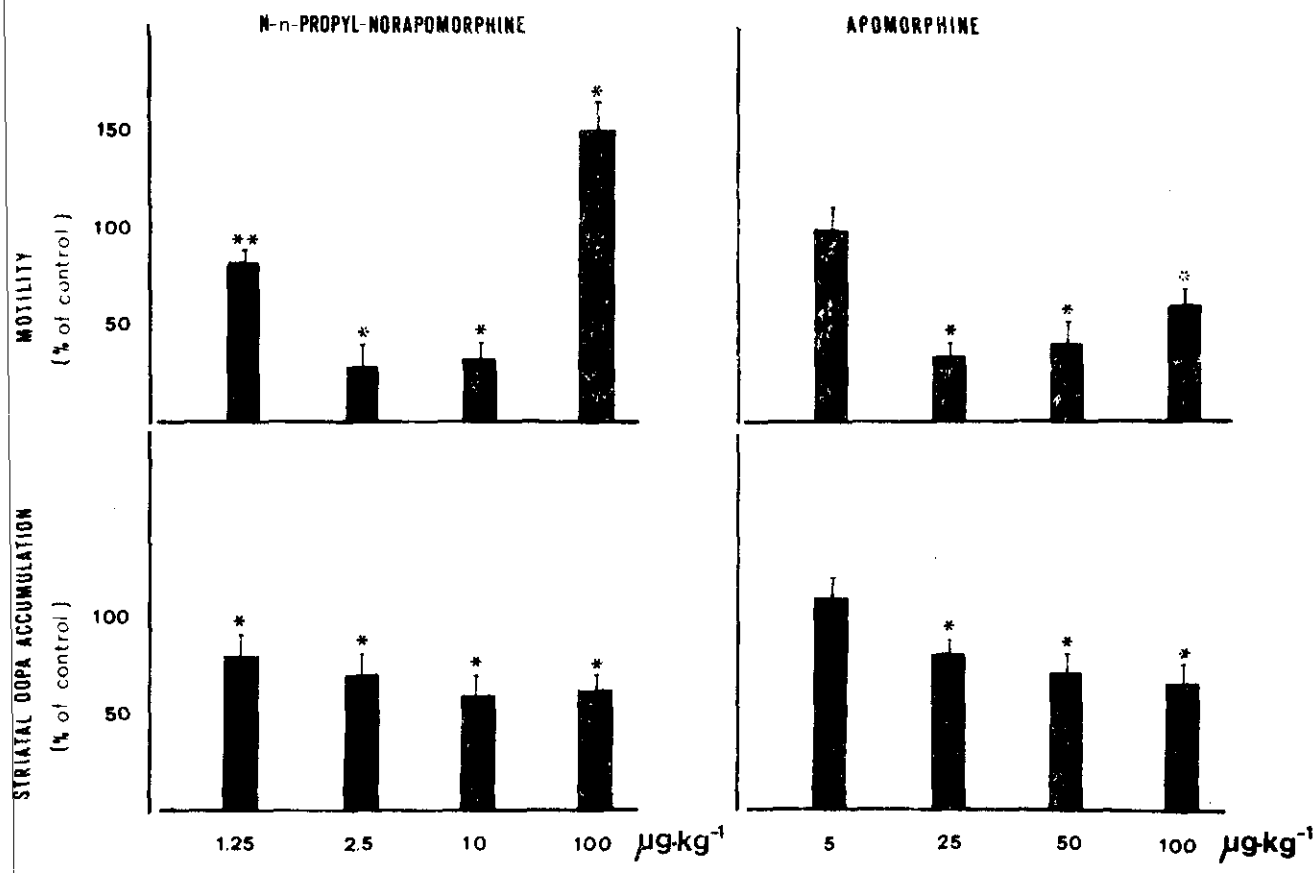

FIG. 4. – Comparison of the effects of NPA and apomorphine on motor activity and striatal DA synthesis. NPA and apomorphine were freshly dissolved in saline containing 0.2 % ascorbic acid and injected subcutaneously in the back of the neck. Motor activity and DA synthesis were measured as described in Materials and Methods. Values are expressed as percent of saline treated rats (controls). Each value is the mean ± S.E. of 10 animals. The total counts in 25 min were 834 ± 54 in saline-treated animals. Striatal DOPA levels are reported in Table 1. (*) $P < 0.001$; (**) $P < 0.05$, with respect to saline-treated rats.

are prevented by haloperidol and, stereospecifically, by (—) sulpiride, indicating that they are mediated by DA receptor stimulation. As with apomorphine, similar threshold doses of NPA are needed to elicit hypomotility and decrease DA synthesis and neuronal firing, suggesting that these responses are mediated by the stimulation of the same kind of DA receptors. However, NPA does not allow to distinguish whether these are the same DA receptors. Indeed, while there is convincing evidence that the inhibition of dopaminergic firing and DA synthesis is mediated by DA autoreceptor stimulation [4, 8], there is still some doubt that sedation is the behavioural consequence of the inhibition of dopaminergic activity [6, 24, 25]. On the contrary, the possibility remains that although DA receptors mediating sedation have the same sensitivity to apomorphine and NPA as DA autoreceptors, they may be postsynaptically located with respect to the DA neuron. If the sedative effect of apomorphine were a postsynaptic event, DA receptor mediated sedation would not reflect decreased dopaminergic function but the activation of dopaminergic transmission in a selected brain area.

Whatever the identity of such receptors may be, because of its extreme potency, NPA may have clinical usefullness. In fact, recent findings suggest that stimulants of DA autoreceptors might constitute a valid alternative the classical neuroleptic therapy in schizophrenia, Huntington's chorea, hyperkynetic disorders and tardive diskynesia [26–28]: all conditions in which increased dopaminergic activity is considered to play an important pathophysiological role.

Table 1. – *Inhibition of DA synthesis by NPA and apomorphine in different brain areas.*

TREATMENT $\mu g \cdot kg^{-1}$		DOPA accumulation ($ng \cdot g^{-1}$) in 25 min after NSD 1015		
		Caudate nucleus	Nucleus accumbens	Frontal cortex
Saline	.....	1378±62	1153±54	129±15
NPA	1.25.....	950±74*	807±58	103±18
	2.5	895±48*	780±32*	97±7*
	5.0	840±35*	730±36*	90±6*
	10.0	799±25*	691±24*	83±4*
APO	12.5	1347±58	1143±61	128±16
	25.0	1098±45*	927±50*	120±12
	50.0	971±40*	842±24*	94±6*
	100.0	851±32*	723±24*	91±5*

Apomorphine (APO) and NPA were administered subcutaneously, as described in Fig. 1, 5 min before NSD 1015 (100 mg. kg^{-1} i.p.). DOPA concentrations were determined radioenzymatically as described in Methods. Each value is the mean ± S.E. of 6 determinations. (*) $P < 0.001$, with respect to saline-treated rats.

Table 2. – *Antagonism by neuroleptics on NPA inhibition of motor activity and striatal DA synthesis.*

PRETREATMENT (mg·kg⁻¹) 30 min before NPA	NPA (2·5 μg·kg⁻¹) effect on	
	Motor activity (counts)	DA synthesis (DOPA accumulation)
	per cent of control	
None	30±2*	70±3*
Haloperidol (0.05)	95±4**	105±4**
(—) sulpiride (10)	98±7**	110±15**
(+) sulpiride (20)	31±3*	71±4*

Doses of neuroleptics were the highest which, per se, failed to significantly affect motor activity and DA synthesis. Each value is the mean ± S.E. of 6 experiments. (*) $P < 0.01$, with respect to values of untreated animals. (**) P 0.001, with respect to values of animals treated with NPA alone.

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Studies on a GABA receptor regulating glutamate release in the cerebellum

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Summary. – *GABA and muscimol at low concentrations (2–20 μM) potentiate the depolarization–induced release of ^{3}H–D–aspartic acid (a glutamate analogue sharing the same transport system) from cerebellar synaptosomal preparations. The effect is concentration–dependent, and is antagonized by bicuculline and picrotoxin. Moreover, the effect is linearly correlated with the logarithm of the K^+ concentration in the depolarizing medium. In similar experimental conditions GABA does not potentiate the release of acidic amino acids in other brain areas. The effect of GABA and muscimol is not present in immature animals (8 day old rats), at a developmental stage in which the parallel fibers have not yet formed in the cerebellum. GABA exerts its potentiating effect not only on the release of exogenous radioactive D–aspartate or glutamate, but also on that of glutamate previously synthesized from radioactive glutamine. The data suggest that GABA and muscimol exert their effect on glutamate release after interacting with GABAergic presynaptic receptors localized on the membrane of the glutamate releasing terminals. A preliminary study on the ionic requirements of the effect in question shows that the presence of Cl^- ions above a critical concentration is essential.*

Riassunto. – *GABA e muscimolo a basse concentrazioni (2–20 μM) potenziano la liberazione di acido ^{3}H–D–aspartico (un analogo dell'acido glutamico che utilizza lo stesso sistema di trasporto) indotta da depolarizzazione con 30 mM KCl in preparazioni sinaptosomali cerebellari. L'effetto è concentrazione–dipendente ed è prevenuto dagli antagonisti bicucullina e picrotossina. Inoltre è correlato linearmente con il logaritmo della concentrazione di K^+ nel mezzo di depolarizzazione. In condizioni analoghe il GABA non potenzia la liberazione di aminoacidi acidi in altre aree cerebrali. L'effetto del GABA e del muscimolo non è presente in animali immaturi (ratti di 8 giorni) in una fase di sviluppo in cui non si sono ancora formate le fibre parallele nel cervelletto. Oltre che sull'aminoacido esogeno preaccumulato, l'effetto del GABA può essere osservato anche sull'acido glutamico precedentemente sintetizzato usando come precursore radioattivo la glutamina. I dati suggeriscono che GABA e muscimolo esercitano la loro azione potenziante sulla liberazione di acido glutamico interagendo con recettori GABAergici presinaptici localizzati sulle terminazioni nervose che rilasciano acido glutamico. Uno studio preliminare sulla dipendenza ionica dell'effetto in questione mostra che la presenza di ione Cl^- al disopra di una certa concentrazione critica è essenziale perché l'effetto possa manifestarsi.*

The nature of the excitatory neurotransmitters in the cerebellum is still matter of debate, although there are good indications that the excitatory amino acid glutamate may act as a neurotransmitter in the terminals of the parallel fibers (originating from granule cells) and of the climbing fibers (originating from cells in the inferior olive) [1–6]. These putative „ glutamergic " terminals are localized in a milieu which is largely GABAergic. In fact, GABA (γ –aminobutyric acid) is utilized as an inhibitory neurotransmitter by Purkinje cells (which receive the putative „ glutamergic " innervation) and by many interneurons of the cerebellar cortex [7–9].

In the present investigation it will shown that GABA can influence the release of glutamate from the putative „ glutamergic " nerve endings, after interacting with GABA–presynaptic receptors, presumably localized in the glutamate–releasing terminals. Some characteristics of these receptors will be briefly described. Part of the results presented were published in previous reports [10, 11].

These studies were conducted on crude synaptosomal fractions prepared from adult rat cerebella, and superfused at 37°C on Millipore filters, in appropriate superfusion chambers [12, 13]. Other experimental details are given in refs. 10, 11.

Fig. 1 shows that low concentrations of GABA potentiated the depolarization–induced release of preaccumulated ^{3}H–D–aspartate from cerebellar synaptosomal preparations. ^{3}H–D–aspartate is a non–metabolized glutamate analogue which was used to label the glutamate „ reuptake pool " [14]. Panel A shows the concentration–dependence of the potentiating effect of GABA, when synaptosomes were depolarized with 30 mM KCl. The effect was evident at GABA concentrations as low as 2 μM, and was maximal at 20 μM; higher concentrations did not produce any further increase of ^{3}H–D–aspartate release. Panel B shows that the effect of 20 μM GABA on ^{3}H–D–aspartate depolarization–induced release was largely prevented by the GABA antagonist bicuculline. In the same panel it can be seen, more clearly than in panel A, that GABA had a modest stimulatory effect on ^{3}H–D–aspartate release even in the absence of depolarization, but this effect was not counteracted by GABA antagonists (see also panel C). The experiments of panel C

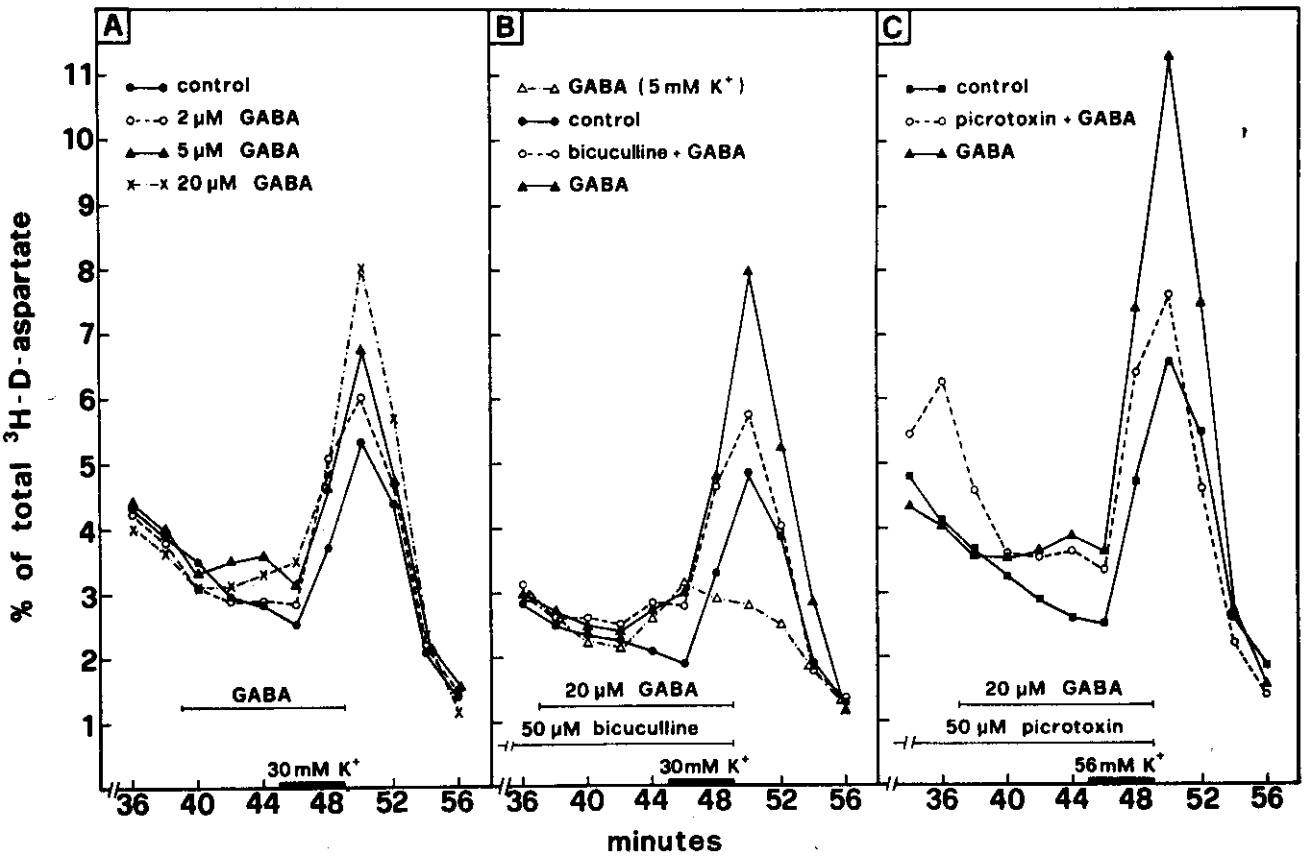

FIG. 1. – Effect of GABA on ^{3}H–D–aspartate release induced by high K^+depolarization from cerebellar synaptosomes. Synaptosomes (P_2 fractions) were superfused in 8–10 parallel superfusion chambers with the following procedure: standard medium (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4, and 20 mM N–2 hydroxyethylpiperazine N–2–ethanesulphonic acid (HEPES) at pH 7.35) for 5 minutes, then 15 minutes with a medium containing 6 nM ^{3}H–D–aspartate. After washing for 6 minutes, 2 min fractions were collected. The composition of the superfusion medium was changed as indicated in the panels. Picrotoxin and bicuculline were added at min 32. The ^{3}H–D–aspartate released in each fraction is given as a percentage of the total ^{3}H–D–aspartate recovered (total fractions from the time of collection (min 28) plus filter at the end of superfusion). Each curve is the average of 2–5 experiments, run in duplicate (taken from Levi and Gallo, ref. 11).

show that the GABA antagonist picrotoxin prevented the large effect elicited by GABA when synaptosomes were depolarized with 56 mM KCl.

The GABA agonist muscimol potentiated the K^+ –induced release of ^{3}H–D–aspartate in a way comparable to GABA, and its effect was equally reversed by picrotoxin (Fig. 2). Muscimol had hardly any effect on the spontaneous release of ^{3}H–D–aspartate. For this reason, in most of subsequent experiments, muscimol, rather than GABA, was selected as an agonist. Fig. 3 shows the relationship between the magnitude of the potentiating effect of muscimol on ^{3}H–D–aspartate release and the concentration of KCl in the depolarizing medium. The data point to a linear relationship between the stimulatory effect of muscimol and the logarithm of the concentration of KCl. An extrapolation of the curve to the value of 1 on the ordinate (lack of effect of muscimol) corresponds to a K^+ concentration of 7.8 mM which is probably too low to cause a substantial depolarization of synaptosomes, and is in fact close to the concentration of 5 mM present in our standard media.

It seemed interesting to investigate whether the release of ^{3}H–D–aspartate could be affected by GABA also in areas other than the cerebellum. Three areas (cerebral cortex, corpus striatum and hippocampus) were tested in conditions (presence of 20 µM GABA, depolarization with 30 mM KCl) giving clear-cut effects in the cerebellum. In these conditions, GABA did not exhibit any detectable effect on the release of ^{3}H–D–aspartate (Fig. 4).

The results presented so far concern the behaviour of exogenous ^{3}H–D–aspartate which was similar to that of exogenous ^{14}C–glutamate. Since acidic amino acids are known to be actively taken up by cerebellar glial cells [15–17] and glial fragments may well contaminate our synaptosomal preparations and take up ^{3}H–D–aspartate from the superfusion medium, it seemed necessary to ascertain whether GABA potentiated the K^+ –induced release of endogenous glutamate and of ^{14}C–glutamate previously synthesized from ^{14}C–glutamine. According to the current literature [18–20] the pool of newly synthesized glutamate should be mainly localized in nerve terminals, when radioactive glutamine is used as a precursor. In a set of experiments, cerebellar synaptosomes were superfused in the presence of ^{14}C–glutamine. The data on the release of endogenous glutamate and of ^{14}C–glutamate observed in these experiments are reported in Fig. 5. It can be seen that during depolarization with 30 mM KCl the release of ^{14}C–glutamate was greatly stimulated by GABA, while the overall release of endogenous glutamate was apparently unaffected. Picrotoxin largely prevented the potentiating effect of GABA on the K^+ –induced release of the newly synthesized amino acid, in the same way as it prevented the effect

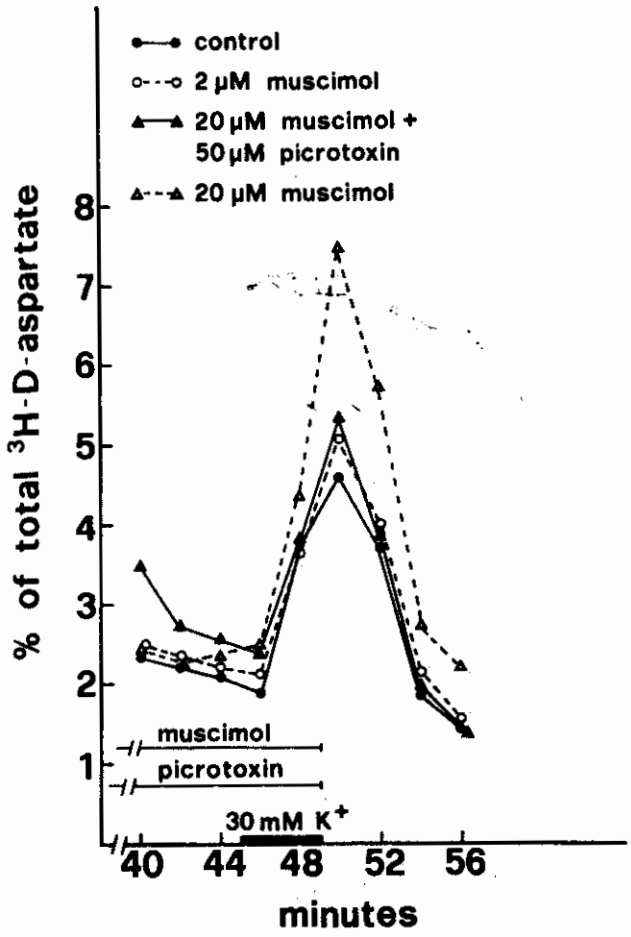

FIG. 2. – Effect of muscimol on the depolarization–induced release of ^{3}H-D-aspartate from cerebellar synaptosomes. The experimental procedure is described in the legend for Fig. 1. Each curve is the average of 2–3 experiments run in duplicate (taken from Levi and Gallo, ref. 11).

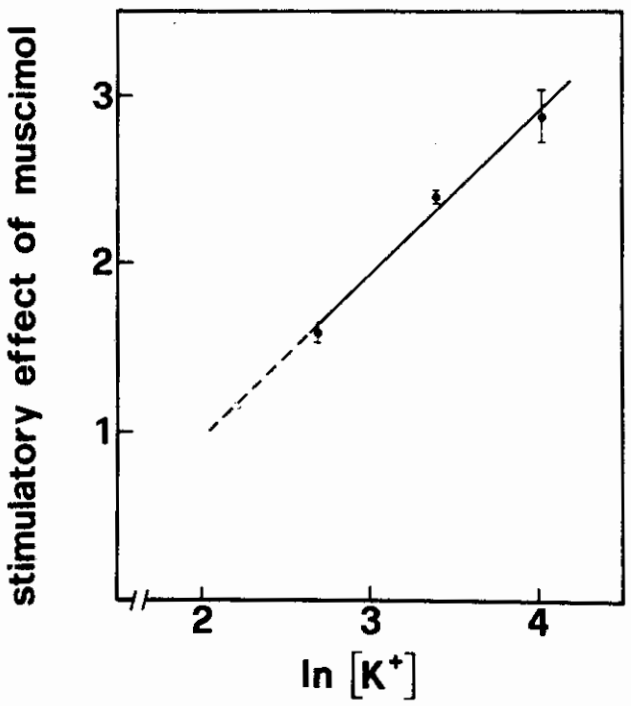

FIG. 3. – Relationship between the stimulatory effect of muscimol on ^{3}H-D-aspartate release and the KCl concentration in the depolarizing medium. The effect of muscimol was calculated as ratio between maximal KCl-evoked release of ^{3}H-D-aspartate in the presence and in the absence of the GABA agonist. Experimental procedure as in the legend for Fig. 1. Muscimol concentration: 20 μM. Concentration of KCl in the depolarizing medium: 15 mM, 30 mM or 56 mM. Each point is the average of 3 experiments run in triplicate ± S.E.M. (taken from Levi and Gallo, ref. 11).

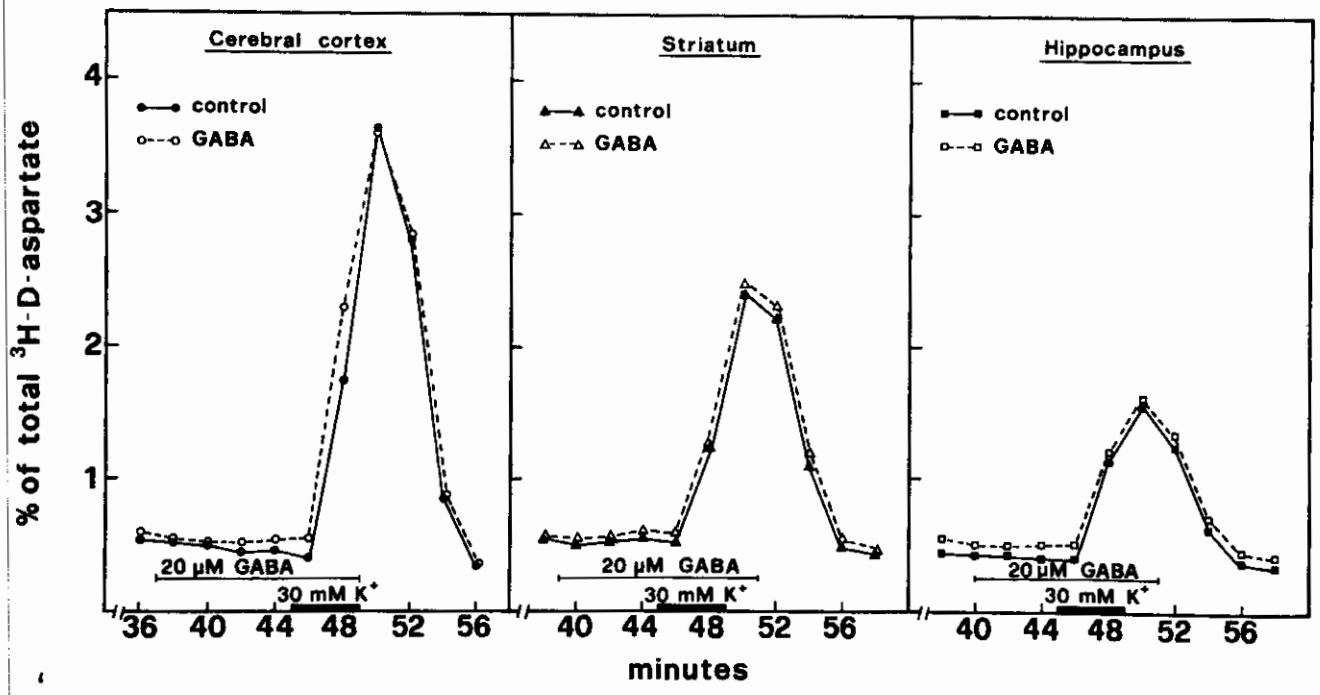

FIG. 4. – Lack of GABA effect on ^{3}H-D-aspartate release from cortical, striatal and hippocampal synaptosomes. Experimental procedure as in the legend for Fig. 1. Each curve is the average of 2 experiments (corpus striatum and hippocampus) or of 3 experiments (cerebral cortex) run in duplicate (taken from Levi and Gallo, ref. 11).

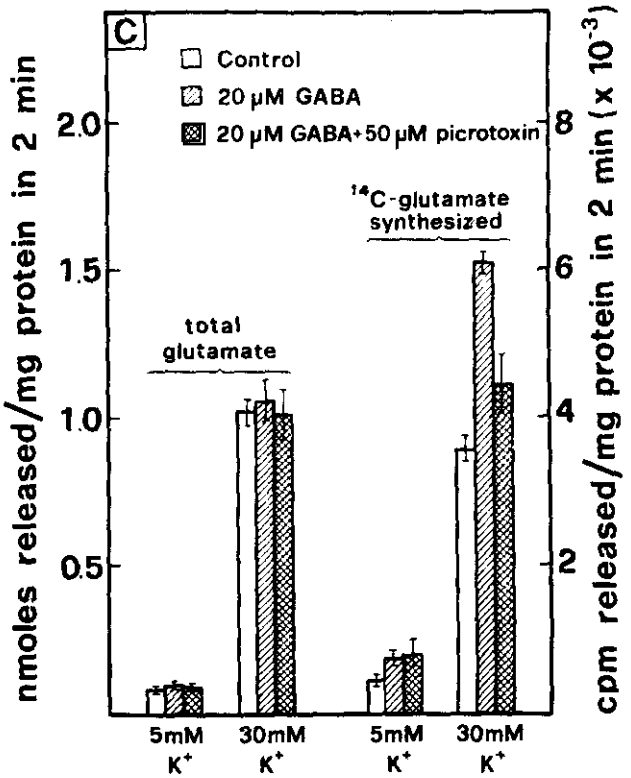

FIG. 5. - Effect of GABA on the depolarization-induced release of glutamate from cerebellar synaptosomes. Synaptosomes were superfused with a medium containing 12 μM ^{14}C-glutamine for 15 minutes. After washing, the superfusion was continued and fractions were collected every 2 minutes. The superfusion medium of two control superfusion chambers contained 30 mM KCl from min 45 to 49. In two other chambers, the medium contained, in addition, 20 μM GABA from min 39 to 49. In the last 2 chambers, the medium contained also 50 μM picrotoxin, from min 32 to 49. Endogenous glutamate and newly synthesized ^{14}C-glutamate were determined as previously described [11] (taken from Gallo, Levi, Raiteri and Coletti, ref. 12).

of GABA and muscimol on the evoked release of ^{3}H-D-aspartate.

The lack of effect on the release of total endogenous glutamate probably reflects the fact that, in the experimental conditions used, the „ reuptake pool " and the „ new synthesis pool ", which appear to be specifically affected by GABA, represent only a small fraction of total endogenous glutamate.

Fig. 6 shows a study on the age-dependence of the muscimol effect on ^{3}H-D-aspartate release. No effect could be detected in cerebella from 8-day old rats, while the effect was present in cerebella from 20-day old animals. This observation may offer a clue to the nature of the nerve terminals involved in the phenomena observed. In fact, in 8-day old cerebella the putative „ glutamergic " parallel fibers have not yet developed, while the other excitatory fibers (the climbing fibers) are already present and have synaptic contacts with the Purkinje cells [21].

In order to characterize more precisely the properties of the GABA receptor responsible for the activation of glutamate release, a series of studies were carried out on the ionic dependence of the biological effect observed. Table I summarizes the results of these studies. It is evident that the effect of muscimol was neither Ca^{2+} -dependent (line 2) nor Na^{+} -dependent (line 3 and 8). On the other hand, the effect was absent when the concentration of Cl^{-} in the medium was below a certain level (50–75 mM depending on the composition of the medium). It is interesting that, starting from a condition in which the effect was absent (line 6: 50 mM K^{+} 87 mM Na^{+}, 50 mM Cl^{-}) the effect could be restored by merely increasing the concentration of Cl^{-} (line 2 from bottom), or that of K^{+} (last line).

In conclusion, while in Fig. 3 it is shown that the magnitude of the effect of muscimol is related to the

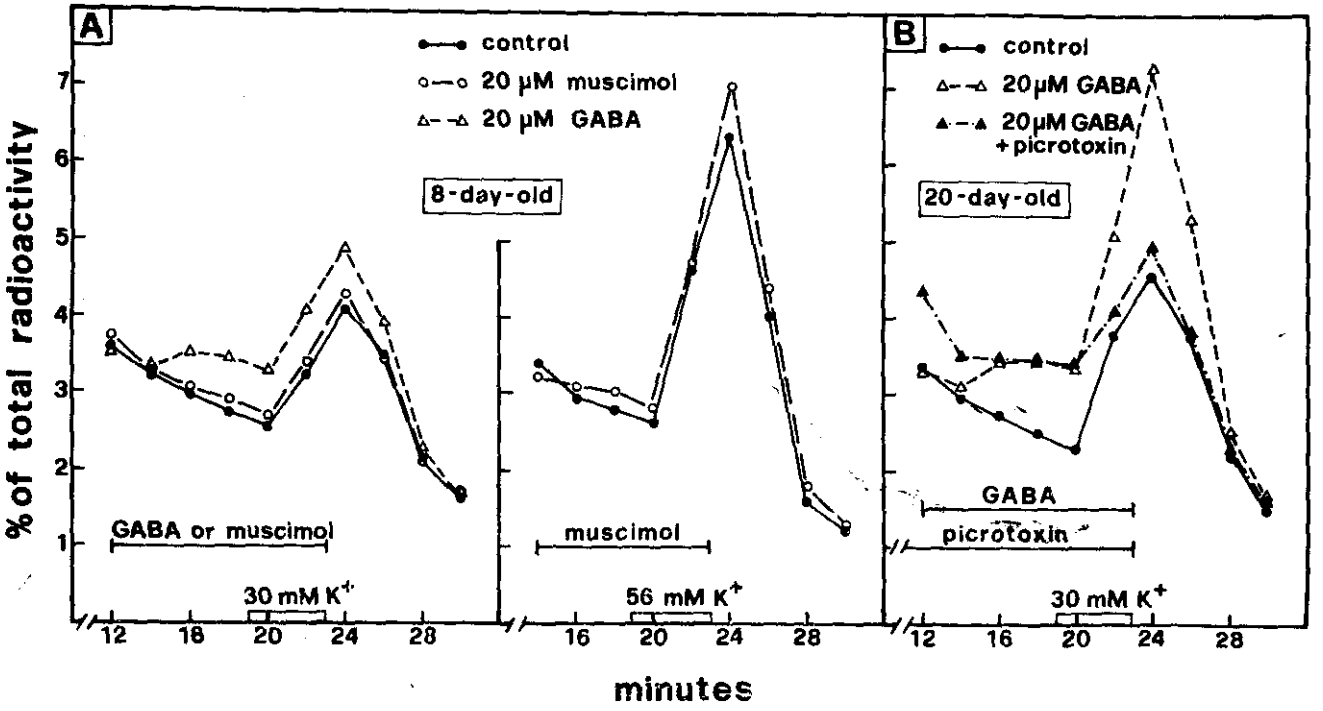

FIG. 6. - Effect of GABA on ^{3}H-D-aspartate release from synaptosomal preparations obtained from 8 day-old and 20 day-old rats. Experimental procedure as in the legend for Fig. 1. Each curve is the average of 2 experiments run in duplicate.

Table 1. – *Muscimol Effect on K+ -induced ^{3}H-D-aspartate Release in Different Ionic Conditions.*

Ionic concentrations				Effect of muscimol (20 μM)
K+	Ca++	Na+	Cl−	
(*a*) 15–56	2,7	(*a*) 120–79	133	present
(*a*) 30	—	(*a*) 113	133	present
(*a*) 130	2,7	—	133	present
(*a*) 30	—	(*b*) 113	—	absent
(*a*) 50	—	(*c*) 87	50	absent
(*a*) 70–100	—	(*c*) 67–37	70–100	present
(*a*) 70	2,7	(*d*) —	75	absent
(*a*) 100	2,7	(*d*) —	105	present
(*a*) 50	—	(*a c*) 87	80	present
(*a b*) 80	—	(*c*) 65	50	present

(*a*) Chloride; (*b*) Phosphate; (*c*) Isethionate; (*d*) Sucrose.

Effect of muscimol on ^{3}H-D-aspartate release induced by high K^+ in different ionic condition. Cerebellar synaptosomes (P_2 fractions) were labelled and superfused as described in the legend for Fig. 1 with media whose ionic composition is summarized in the table. The superscript letters a–b–c refer to the anion of the K^+, Na^+ and Ca^{2+} salts used. In a group of experiments (superscripts letter d) sucrose was used to replace NaCl. Cl^- was present as NaCl, KCl and $CaCl_2$. In the last column the word « present » means that muscimol elicited at least a 50 % increase on the depolarization – induced release of ^{3}H-D-aspartate. The word « absent » stands for no detectable effect of muscimol on ^{3}H-D- aspartate evoked release

extent of the depolarization, Table I shows that an adequate depolarization is a necessary but not sufficient condition to evoke the effect, which is not manifest unless a critical concentration of Cl^- is also present. It is interesting that, when the concentration of Cl^- is just below the critical level for a given depolarizing stimulus, an increase in the depolarizing stimulus can elicit the effect. It is thus possible that increasing depolarizations cause proportional increases in the affinity or in the availability of GABAergic receptors. It is not clear whether Cl^- ions are necessary for GABA binding to its receptors or to trigger the biological effect. Data reported in the literature on the binding of GABA to synaptic membrane preparations [22, 23] seem to exclude the first possibility; it seems possible that a Cl^- influx into the nerve terminals is promoted by the activation of presynaptic GABAergic receptors. The entry of the anion could be the triggering step of an unknown mechanism by which glutamate depolarization–induced release is increased by GABA.

The physiological implications of the present findings can be so far only matter of speculation. Since the most abundant nerve ending species presumably utilizing glutamate as a neurotransmitter in the cerebellar cortex is represented by the parallel fiber terminals [1, 2, 4, 5], it is reasonable to suggest that GABA exerts its stimulatory action on glutamate release at the level of these terminals. This suggestion is corroborated by the absence of effect at a developmental stage at which the parallel fibers have not yet developed.

Whatever may be the origin of the GABA released onto the parallel fiber terminals (Purkinje cell or Golgi cell dendrites, basket cell terminals or others), the modulatory action of GABA on glutamate release would ultimately serve the purpose of increasing the activity of the inhibitory output of the cerebellum, represented by the Purkinje cells.

Acknowledgements.

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Antibody to acetylcholine receptors increases the turnover of acetylcholine receptor: clinical relevance and mechanism of action

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Summary. – *The mechanism by which antibodies against nicotinic receptor (AChR) increase the degradation of AChR and the clinical relevance of this phenomenon has been studied. It has been observed that the serum fraction which increases AChR degradation is IgG and that this class is active also when separated from IgG3 subclass. The AChR degradation, both normal and antibody stimulated, is independent of the external Ca^{++}, is decreased by bacitracin, methylamine and ammonium chloride. Anti AChR antibodies increase the number of endocytosic vesicles indicating that they may also affect the internalization and degradation of other components of the plasma membrane. Anti AChR antibodies do not modify the rate of synthesis of AChR.*

The serum activity on AChR degradation has been also studied in several patients at different times and it has been found that it correlates strictly with the severity of myasthenic symptoms. In conclusion, it seems that anti AChR antibodies affect the function of neuromuscular junction by increasing the rate of degradation of AChR but not its rate of synthesis and probably by increasing the turnover of other membrane components which may be important for the morphology and activity of the neuromuscular junction. The clinical relevance of these findings are strenghened by the correlation in the patients between the serum activity on the AChR and the severity of the myasthenic symptoms.

Riassunto. – *Gli Autori hanno studiato il meccanismo con cui anticorpi antirecettore nicotinico aumentano la degradazione del recettore stesso e la rilevanza clinica di questo processo. Si è osservato che responsabile di questo effetto di aumentata degradazione è la componente gammaglobulinica del siero, che le IgG sono attive anche dopo che sono state private della componente IgG3. La degradazione del recettore, sia normale che dopo stimolazione da anticorpi, non è dipendente dal Ca^{++} extracellulare ed è antagonizzata dalla bacitracina, dalla metilamina e dal cloruro di ammonio. Gli anticorpi antirecettore aumentano il numero delle vescicole di pinocitosi e contribuiscono quindi alla degradazione non specifica della membrana cellulare. Gli anticorpi antirecettore non modificano acutamente la sintesi del recettore stesso.*

Studiando la attività del siero di diversi pazienti miastenici durante la evoluzione della malattia si è visto che vi è una buona correlazione tra attività del siero e severità dei sintomi miastenici. In base a questi dati gli autori concludono che gli anticorpi antirecettore possono essere responsabili della inefficienza della giunzione neuromuscolare, sia perchè diminuiscono il numero dei recettori sulla membrana postsinaptica, sia perché possono anche aumentare la degradazione di altre proteine di membrana. L'importanza di questi dati per la clinica è confermata anche dalla correlazione che è stata trovata nei miastenici tra attività del siero sul recettore e severità dei sintomi.

INTRODUCTION

In Myasthenia Gravis patients the main modification of the neuromuscular junction is a decreased number of acetylcholine receptors (AChR) in the postsynaptic infolding, which is probably the most relevant reason of the failure of the neuromuscular trasmission [1, 2].

Myasthenia Gravis (MG) is an autoimmune disease in which both a humoral and cellular immune response against the acetylcholine receptor has been clearly demonstrated [3–6], and it has been postulated that this autoimmune response may have a role in causing the myasthenic symptoms. This hypothesis was based on several data *a*) it is possible to induce an experimental model of MG in several animals immunizing them with Torpedo or electric Eel AChR, and the severity of the induced disease is related to the anti AChR antibodies [7]; *b*) the large majority of myasthenic patients have antibodies against AChR [6, 8]; *c*) anti AChR IgG from myasthenic patients injected in animals induce myasthenic symptoms [9]; *d*) the morphology of neuromuscular junction in MG patients and in the myasthenic animals is similar [10].

However it is not yet clear what is the mechanism by which antibodies can affect the activity of the neuromuscular junction. Recently it has been shown that anti AChR antibody may block the receptor with a curare like action [11] or increases the degradation of both junctional and extrajunctional AChR [12–14] and it has been suggested that such effects may explain the decreased number of AChR found in the neuromuscular junction of myasthenic patients.

In this paper it is presented:

a) evidence that the AChR antibodies increase in vitro the degradation of AChR, but not its synthesis;

b) an hypothesis on the mechanism by which degradation of AChR is carried out;

c) evidence obtained in a group of myasthenic patients that the activity of sera of MG patients in increasing the AChR degradation is correlated with the severity of the disease.

Materials and methods

Cells. The clonal muscle cell line BC3H-1 [15] was grown in Dulbecco modified Eagle's medium containing 10 % fetal calf serum (FCS) in a 6 % CO_2, 94 % air incubator in 35 mm Petri dishes at an initial density of 1.8×10^3 cells/cm^2. Confluency was obtained in about 5–7 days while maximum AChR densities were achieved by 10–12 days. Myogenic cells were obtained from posterior limb muscles of 3 days old Sprague Dawley rats according to the technique of Jaffe [16] and cultivated in a medium analogous to that used for the BC3H-1 cells.

Toxin. α – Bungarotoxin (α BT) was purified according to Borman [17] and labelled with ^{125}I using the Chloramine-T method [18].

Patients. Plasma or serum was obtained from patients suffering from moderate to severe generalized MG. The diagnosis was assessed by a clinical and electromyographic criteria and the clinical stage of the disease was classified according to Ossermann and Genkins [19] with slight modifications. 90 % of the patients received prednisone (25–100 mg) on alternate days and/or anticholinesterase drugs.

Assay of AChR degradation and synthesis. AChR degradation was measured both in BC3H-1 cells and myotubes with similar results, using the technique described by Kao and Drachman [13] and Devreotes and Fambrough [20].

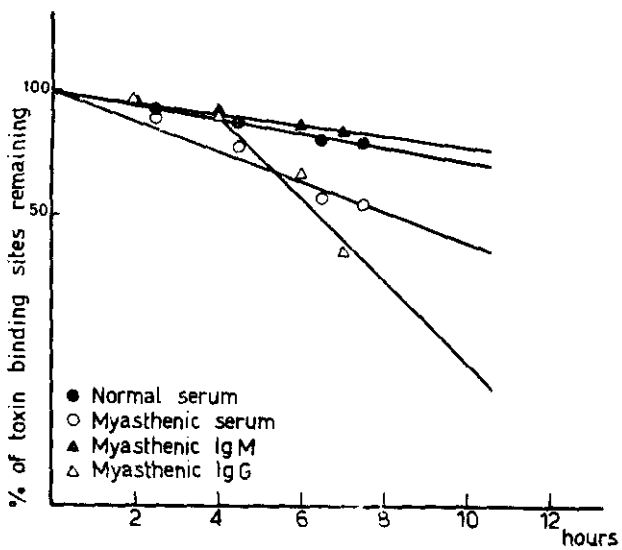

Fig. 1. – Effect of serum of a normal subject and of a MG patient and of antibody classes on the AChR degradation of BC3-H1 cells. Both serum and IgG from MG patient increase the AChR degradation while IgM does not affect it. The IgG used has been passed through a Sepharose-protein A-Column, so that they do not contain the IgG3 subclass.

Cells were incubated for 30 minutes at room temperature with 0.02 μM ^{125}I α-BT. Cultures were subsequently washed free of unbound toxin and returned to the incubator in growth medium containing, instead of FCS, 10 % of normal or myasthenic serum, since it was previously shown that this amount produces the maximum activity [21]. Medium was removed for measurement of ^{125}I at different times and fresh medium was returned to the dishes.

The rate of appearance of radioactivity in the medium is an indirect measurement of the rate of degradation of the AChR [13, 20]. All determination were perform ed in triplicate. The rate of synthesis of AChR was measured by incubating the cells for 30 minutes with 0.2 μM unlabelled α-BT. After 1, 2, 3, 4, 6 hours iodinated α-BT was then added at a concentration of 0.02 μM. The reaction was stopped by washing the cells three times with Earle and 2 % FCS. Cells were removed from the dish with a rubber policemen, and counted.

IgG Purification. IgG and IgM were purified by chromatography on DEAE-Affi-Gel blue (Biorad Laboratories, Richmond, California). IgG_3 subclass was isolated by affinity chromatography on a Protein-A sepharose column (Pharmacia, Uppsala).

Results.

A) *Class of antibody affecting AChR degradation.*

It was previously shown that sera of MG patients increase the rate of degradation of AChR and that this activity is proportional to the antiAChR antibody titer [21]. An example of this activity, using as a test BC3-H1 cells, is shown in fig. 1. In order to analyze which class of antibody is active in this process, both IgM and IgG were isolated; only IgG were found active on AChR degradation. Since MG patients have a high concentration of IgG_3 this subclass may be the active component of the antibody, therefore the IgG_3 component was separated from the IgG. The IgG without IgG_3 was found active in the same way as the parent IgG (Fig. 1), indicating that the IgG_3 subclass is not relevant for the serum activity on AChR degradation.

B) *Rate of synthesis and degradation of AChR.*

One of the more important question to ask is if the antibody which induces AChR degradation stimulates also its synthesis. For this purpose a pool of sera of several myasthenic patients which were very active in increasing the rate of degradation were selected and

Table 1. – *Rate of synthesis and degradation of AChR*

SERUM	Synthesis Rate (%/h)	Degradation Rate (%/h)	Half degradation time (hours)
Normal	3.7±0.6	3.1±0.06	16.2±0.3
Myasthenic	3.9±0.5	5.8±0.4	8.8±0.5*

(*) Significantly different from control ($P < 0.005$).

the effect of this pool was tested on the rate of AChR synthesis in BC_3H–1 cells. It was found that in presence of a normal serum the rate of degradation and the rate of synthesis of AChR are comparable, while in presence of myasthenic sera the rate of degradation is increased but the rate of synthesis is not modified even after 6 hours of culture (Tab. 1).

C) *Effect of drugs active on endocytosis on AChR degradation.*

It has been postulated that antibody clusters AChR on the surface of the cells and that this step triggers an increased endocytosis of plasmalemma, a subsequent fusion of the endocytotic vesicles with lysosomes and finally a degradation of AChR [8].

This hypothesis was tested using different drugs which affect endocytosis and lysosome functions. The BC_3H–1 cells were cultivated in a Ca^{++} free medium containing 1 mM EGTA and it was found that the rate of AChR degradation, both in normal conditions or after antibody stimulation, is not affected (Tab. 2), showing that this process is independent of the external calcium. Bacitracin and methylamine, drugs which interfere with the clustering process [22], decrease in a dose dependent manner the rate of degradation of AChR both in normal and after antibody stimulation (Tab. 2). However these drugs do not modify the rate of synthesis of AChR (Tab. 3) showing, also in this case, a dissociation between rate of synthesis and rate of degradation of AChR. The function of lysosomes was reduced by culturing the BC_3H–1 cells in medium containing NH_4Cl, and in this case the rate of degradation of AChR is apparently reduced (Tab. 2).

Table 2. – *Effects of Ca^{2+} and drugs interfering with endocytosis on AChR degradation.*

DRUGS mM	Normal Serum	Myasthenic Serum
	Half degradation time	
None	16.0	8.2
Ca^{2+} free medium + EGTA 1 mM	13.9	9.5
Bacytracin:		
0.5	20.6	16.9
5.0	34.0	44.3
Methylamine:		
0.1	33.2	21.0
1.0	27.9	50.0
NH_4Cl:		
10	24.1	15.9
20	33.7	—

Table 3. – *Effect of drugs active on endocytosis on AChR synthesis.*

DRUGS	%/hour
None	3.7
Bacytracin 1 mM	2.0*
Methylamine 0.1 mM	2.5*

(*) Not significantly different from control.

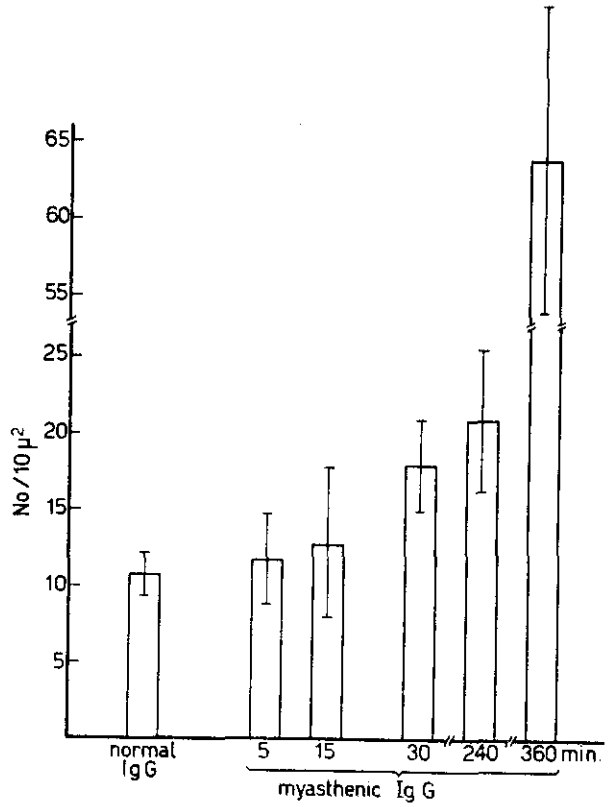

FIG. 2. – Number of pynocytotic vesicles on the surface of BC3H–1 cells. The number of pynocytotic vesicles have been counted in cells grown both in normal or myasthenic IgG at different time intervals. For each point the surface examined was 75 μ^2. After 30 minutes, myasthenic IgG increases the number of pynocytotic vesicles while the normal serum did not affect it at any time.

The cells were also prepared for electron microscope examination with the freeze fracture technique and the number of endocytocic vesicles were counted at different times after the exposure to the human sera. When the cells have been cultured in normal serum, the number of endocytotic figures does not change in time, but when the cells have been cultured in presence of sera of MG patients the number of endocytotic figures is increasing dramatically with time (Fig. 2), showing that the sera of MG patients stimulate endocytosis.

It has been thus shown that sera of MG patients increase degradation of AChR through a surface clustering of AChR, which leads to its increased internalization through endocytotic vesicles, step which is not calcium dependent, and finally to a destruction by lysosomes.

D) *Clinical relevance of AChR degradation.*

If antibodies can really increase the rate of AChR degradation, they may be then correlated with the decreased number of AChR receptors found in MG patients and should also be correlated with the severity of myasthenic symptoms. For these studies rat myoblasts, grown in vitro as described, were used as a tool.

A correlation has been previously described between the severity of the disease and the activity of the sera

Table 4. – *Correlations between clinical stage of 16 myasthenic patients and the activity of their sera.*

PATIENTS	Date of testing	Clinical stage (a)	AChR half life (hours)
1	13–1–1979	4	6,5
	6–4–1979	R F	11
2	11–12–1978	4	5,5
	8–1–1979	R F	8
3	21–12–1978	2b	6
	20–1–1979	R F	13.5
4	19–12–1978	2b	11,5
	11–5–1979	R F	19
5	30–1–1979	2b	7,75
	6–4–1979	R F	15,5
6	13–1–1979	4	13,5
	20–4–1979	R F	22,5
7	14–11–1978	4	17,5
	2–4–1979	4	16,5
8	31–8–1978	3	7,5
	18–1–1979	3	7,5
9	17–10–1978	2a	17,5
	14–4–1979	2a	18
10	15–8–1978	3	19
	13–1–1979	3	19
11	4–1–1979	3	9,5
	18–5–1979	4	10
12	16–5–1977	2b	6
	3–6–1977	2b	4,5
13	18–5–1979	R F	11
	19–8–1979	4	14
14	26–2–1979	3	8,5
	29–3–1979	2a	8,5
15	3–10–1977	2a	5
	3–11–1978	4	4
	27–3–1979	2b	4,5
16	8–11–1977	3	12
	6–1–1978	2b	14
	16–7–1978	plasmapheresis	—
	18–7–1978	2b	18,5
	25–7–1978	2b	21
	12–3–1979	4	10
	27–3–1979	4	9
	29–5–1979	4	9

(a) The severity of the disease was classified according to Osserman and Genkins (19) as follows: RF = Completely symptom-free or symptom-free with only minimal doses of anticholinesterase and/or corticosteroids drugs. — 1 = localized, nonprogressive form, usually ocular. — 2a = mild or moderate generalized form without bulbar involvement. — 2b = moderate or severe generalized form with bulbar manifestations. — 3 = Acute onset of generalized myasthenia with severe bulbar manifestations. — 4 = Late severe form, acute progressions from groups 1 and 2 with frequent respiratory crises.

The activity of the sera of these patients have been tested at different times and compares with the clinical state of the patients. The correlation between the two parameters is rather good except than in case 12 and 13.

of MG patients on the AChR degradation [21]. This correlation however is even more striking in the single patients. In the majority of cases, when the patients improve, the activity of their sera decreases, while when they become more severely ill the activity of their sera is increased (Tab. 4). The interesting fact is that the modifications of the serum activity follow the clinical evolution, whatever the « basal » activity. Out of the 12 cases with a positive correlation, two cases merit particular attention. In case n. 15 there is a constant strong acceleration of AChR turnover, and small variations of the AMd are closely parallel to wider fluctuations of the severity of the disease. In case n. 16, where correlation is also very close, the serum activity went to normal values for a short period of time after plasmapheresis as expected because of the induced reduction of all the antibodies' level.

Discussion.

In this paper it has been shown that anti AChR IgG affects AChR rate of degradation in vitro without affecting AChR synthesis. This fact is relevant since it may explain the decreased number of receptors found in the neuromuscular junction in MG patients.

The correlation between the severity of the disease and the activity of sera of MG patients on AChR degradation is a further support of the clinical significance of this phenomenon. However, the lack of effect of anti AChR antibodies on AChR synthesis has been measured in cells « in vitro » for a short period of time, thus in a situation completely different from the clinical one.

Furthermore the cells used for these studies are a continuous cell line derived from a mouse tumor which have some characteristics of the muscle cells, they contain contractile proteins and nicotinic AChR, but do not fuse and do not produce myotubes.

However the turnover rate of AChR is similar to that of rat normal myotubes [21] and human antiAChR antibodies cross-react to some extent with rat and mouse AChR [23]. With these limitations, BC3H-1 cells seem to be a reliable model for studying the effect of sera of MG patients on AChR turnover.

We have measured the rate of synthesis counting the new receptor molecules which have been exposed at the surface of the cells after their incubation with the serum to be tested. It is possible that for a short period of time the rate of insertion of new receptors does not depend on the rate of AChR synthesis, but on the availability of receptors present in a cytoplasmatic pool.

However it has been previously shown that the intracytoplasmatic pool of AChR is very small [24] and can account for the insertion of new receptor molecules at the cell surface for less than four hours if the protein synthesis is blocked [20]. In our case the rate of insertion of new AChR is the same even after 6 hours of culture.

Furthermore in preliminary experiments, in which the cells have been grown in presence of anti AChR antibody for three days, it was found that the total number of AChR exposed at the surface is dramatically decreased over the control cells. And this supports the hypothesis that the antibodies to AChR affect only the rate of degradation of AChR. Dissociation between rate of synthesis and rate of degradation of

AChR wasl also found when the rate of AChR degradation was decreased by methylamine and bacitracin; also in these experiments the rate of synthesis was not affected.

It seems that synthesis and degradation of AChR are not coupled and that it is possible to affect one process without affecting the other. It is possible that in humans, in which the situation is more complex and the time of esposition of the muscles to antibodies is very long, a different regulation of AChR turnover prevails. It has been found that AChR are internalized by a process which requires clustering of the surface molecules — since this process is decreased by the use of bacitracin and metylamine — and an increase of endocytosis. It seems that anti AChR antibodies act by stimulating the physiological process of AChR degradation since both normal and antibody-stimulated degradation is affected in the same way by drugs. Antibody-stimulated endocytosis may be not specific for the internalization of AChR and could affect other membrane molecules. In fact AChR molecules are a minimal part of the membrane protein, probably less than 0.1 %, and thus a selective retrieval of this class of molecules would have not increased the number of pynocitotic vesicles. Furthermore, in preliminary experiments it was found that the number of intramembrane particles of the plasmalemma are also decreased early after the medication with anti AChR antibodies, indicating that membranes are probably deprived of several protein. Furthermore, a large clustering of particles was not observed after antibodies exposure, indicating that the cluster of AChR which may trigger endocytosis is relatively small.

In conclusion, present data strongly suggest that the antibodies against nicotinic AChR impair the function of neuromuscular junction by increasing the AChR degradation and not AChR synthesis, and that in this process of AChR disposal by endocytosis other surface molecules may be affected. Probably, at the level of the postsynaptic membrane profound modifications are produced by anti AChR antibody, in agreement with the large morphological modification that has been found in the neuromuscular junctions of the MG patients.

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