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Changes in central GABAergic function following acute and repeated stress

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1 The function of γ-aminobutyric acid (GABA)ergic systems in response to acute and repeated stressful manipulations was evaluated in both the corpus striatum and frontal cerebral cortex of the rat.

2 In the corpus striatum the activity of the synthetic enzyme for GABA (glutamic acid decarboxylase, GAD) and the levels of GABA were reduced by acute immobilization stress (1 h). GABA turnover was reduced only by acute cold stress (3 h, 4°C).

3 In the frontal cerebral cortex no changes were observed after acute stressful manipulations, but repeated stress (0.5 h immobilization per day for 14 days) enhanced both GAD activity and GABA turnover, and reduced GABA levels.

4 In conclusion, it would appear that the GABAergic system in the corpus striatum of the rat is most sensitive to acute stress and that the system in the frontal cerebral cortex area is preferentially responsive to chronic stress. It is speculated that the cortical GABAergic system is responsible for adaptive responses to the adverse conditions prevailing during chronic stress.

Introduction

The neurochemical changes observed in response to the application of different types of environmental stress have been the subject of research for over two decades (Bliss & Zwanzinger, 1966; Blanc *et al.*, 1980; Kramarcy *et al.*, 1984; Tarizzo & Rubio, 1984). It is now clear that stressful manipulation induces metabolic and/or functional changes in several neurotransmitter pathways, both in the central and the peripheral nervous systems. Classical studies have described the effects of stress on central functions governed by aminergic mechanisms (Bliss *et al.*, 1968; Corrodi *et al.*, 1968; Irwin *et al.*, 1982; Saavedra *et al.*, 1982). More recently, however, evidence has accumulated that suggests a role for a novel neurotransmitter, γ-aminobutyric acid (GABA), in the pathophysiology of affective disorders (Gold *et al.*, 1980; Petty & Schlessler, 1981; Berretini & Post, 1982; Berretini *et al.*, 1982), in the development of certain types of behaviour (Matsuda *et al.*, 1984) and in the modulation of neuronal activity in the mammalian brain. There is little information concerning the possible participation of GABAergic systems in the neurochemistry of stress (Sherman & Gebhart, 1974; Green *et al.*, 1978; Yoneda *et al.*, 1983). It has been postulated that the effects of many antidepressants are mediated by changes in GABAergic function (Sherman & Petty, 1980). In view of the similar effects of antidepressants and exposure to chronic stress on both neurochemical and behavioural features (Zacharko *et al.*, 1983; 1984), it seems reasonable to suppose that GABAergic function might be affected during stress. Hence, the aim of this investigation was to study the function of the GABAergic system in defined areas of the rat brain following either acute or repeated stress.

The nature of the changes induced by stress appear to show a dependence on the type of stressor agent employed. For this reason, classical experimental methods have been adopted in this study and the stressful procedure in the acute treatments was either immobilization at room temperature or exposure to cold (4°C), with the time-course (1, 2 or 3 h) of each being evaluated. The repeated treatments involved only immobilization stress (0.5 h per day for 14 days). The activities of both GABA synthesizing and degrading enzymes were determined and measurements of endogenous GABA concentrations allowed the *in vivo* turnover of GABA to be estimated.

Methods

Male Wistar rats (180–250 g) were maintained in a 12-12 h light-dark cycle with free access to food and water. Stress application usually started at 10 h 00 min. For immobilization stress, each animal was placed

inside a narrow, acrylic cylindrical tube which was perforated for normal breathing and in which only restricted movements were possible. This treatment was given for 1, 2 or 3 h in the case of acute experiments or in sessions of 0.5 h for 14 consecutive days in the case of repeated treatments. For cold stress the animals were placed in separate cages in a cold room at 4°C (2 or 3 h). Control rats were left in their home cages.

At the end of the stress periods, or 1 day after the last stress session in the case of repeated treatments, the animals were killed by decapitation.

Enzymatic determinations

The brains obtained after decapitation were quickly removed and placed on ice. Frontal cerebral cortex and the corpus striatum (i.e. nuclei caudate and putamen) were dissected and homogenized in ice-cold distilled water 1:50 (w/v) to determine the activity of glutamate decarboxylase (GAD, E.C.4.1.1.15), or 1:10 (w/v) for 4-aminobutyrate-2oxo-glutarate aminotransferase (GABA-T, E.C. 2.6.1.19) determinations. GAD and GABA-T are the GABA synthesizing and degrading enzymes, respectively.

Glutamate decarboxylase assay

This was performed according to Albers & Brady (1959). Aliquots of 100 µl tissue homogenate were incubated in glass tubes for 60 min at 37°C in the presence of (concentrations in mmol l^{-1}): potassium phosphate buffer (pH:6.4) 50, 2-mercaptoethanol 10, substrate DL-[1-^{14}C]-glutamic acid (specific activity: 10 nCi µmol^{-1}) 4 (non-saturating concentration); D-penicillamine 10 nmol l^{-1} was added in the case of blanks.

The final volume in each tube was 0.5 ml. In order to trap the $^{14}CO_2$ formed, a plastic well was suspended from a rubber injection stopper containing folded paper soaked in 0.2 ml Protosol (New England Nuclear). The reaction was stopped by the injection of 0.5 ml 10% trichloroacetic acid and after 1 h of continuous shaking the plastic wells were removed, wiped with absorbent tissue, and placed in 5 ml of scintillation fluid.

In the case of repeated stress treatments the activity of GAD was also measured under saturating conditions. In these assays the cofactor pyridoxal-5'-phosphate was exogenously added (1 mmol l^{-1}) and the concentration of [1-^{14}C]-glutamic acid in the mixture was 100 mmol l^{-1} (specific activity: 5nCi µmol^{-1}).

GABA-T assay

The method described by Tunnicliff & Smith (1981) was employed with slight modifications. Aliquots of 10 µl of tissue homogenate were incubated for 30 min at 30°C in the presence of (concentrations in mmol l^{-1}): Tris (tris (hydroxymethyl) aminomethane) HCl buffer (pH 8.6) 50, pyridoxal-5'-phosphate 0.1, 2-mercaptoethanol 10, substrate γ-aminobutyric acid (GABA) 1 (non-saturating concentration), substrate 1-[^{14}C]-α-ketoglutaric acid (specific activity 0.44 µCi µmol^{-1}) 0.44 (non-saturating concentration) in a final volume of 100 µl. Blanks were made by replacing the homogenate with Tris buffer. The reaction was stopped with the addition of 100 µl of cold Tris buffer at 0°C. Following extraction of the remaining α-ketoglutarate, at room temperature with 0.5 ml tri-n-octylamine (0.2 mol l^{-1} in chloroform) and vigorous shaking for 20 s (Sterri & Fonnum, 1978), the radioactivity of 100 µl of the organic phase was determined by scintillation counting. In the case of repeated stress treatments, GABA-T activity was also measured in saturating concentrations of both substrates: GABA (5 mmol l^{-1}) and 1-[^{14}C]-α-ketoglutarate (2.2 mmol l^{-1}; specific activity 0.44 µCi µmol^{-1}).

GABA concentration

GABA was measured by the method of Lindgren *et al.* (1982). In order to prevent *postmortem* increase of GABA in the brain, the rats were injected with the GAD inhibitor 3-mercaptopropionic acid (dissolved in 0.01 N NaOH; 1 mmol kg^{-1}, i.p) 2.5 min before killing (Van der Heyden & Korf, 1978).

Extraction of GABA The tissues were weighed and homogenized in 5 ml ice-cold 0.4 N $HClO_4$ containing Na_2SO_3 (0.05%) and Na_2EDTA (0.1%). After centrifugation (10,000 *g*, 10 min, 4°C) the supernatants were adjusted to pH 3.0 using 5 N K_2CO_3 and centrifuged again (10,000 *g*, 10 min, 4°C). The supernatants were passed through a column (75 mm length, 4.0 mm diameter) containing the strong cation exchange resin Dowex 50 W × 4 (200–400 mesh), which had previously been washed with 20 ml 0.1 M phosphate buffer, pH 6.5 containing 0.1% Na_2EDTA. The column was then treated as follows: 10 ml water; 10 ml 0.02 M citrate buffer, pH 4; 0.4 ml 0.05 M citrate buffer, pH 5.2 (fraction for GABA assay); 4 ml 0.05 M citrate buffer, pH 5.2 (fraction for tissue blank). Recovery was calculated by the addition of a known amount of [^{3}H]-GABA to each sample at the beginning of the extraction procedure and by measuring the radioactivity in aliquots of the eluates obtained from the columns.

Spectrofluorimetric detection The reaction mixture contained: 0.05 M sodium citrate buffer, pH 5.2, 0.3 ml; aliquot of the eluate from the columns, 0.5 ml; 0.5 M sodium borate buffer pH 9.3, 0.4 ml; 2-mercap-

toethanol (solution, 1 μl ml^{-1} of 95% ethanol), 0.1 ml; distilled water, 0.1 ml.

The reaction was started by adding 30 μl *o*-phthalaldehyde (1 mg ml^{-1} of 95% ethanol).

Fluorescence was read within 30 min at 335/455 nm wavelength, and was linear from 0.5 to 4 μg of GABA per test tube.

Estimulation of GABA turnover in vivo

γ-Vinyl GABA (GVG) is an irreversible and specific inhibitor of GABA-T (Hammond & Wilder, 1985). Therefore, the turnover of GABA can be calculated from the difference between basal and accumulated GABA after GVG administration. For this purpose, rats were injected with GVG (10 mmol kg^{-1}, i.p.) 3 h before death. With the exception that the animals did not receive 3-mercaptopropionic acid before death GABA levels were determined as described above.

The following drugs were used: Protosol, New England Nuclear (NEN), γ-2, 3-[^{3}H]-γ-aminobutyric acid (specific activity 29.3 Ci mmol^{-1}) (NEN); α-1-[^{14}C]-ketoglutaric acid (sodium salt) (specific activity 53.6 Ci mol^{-1}) (NEN); *o*-phthalaldehyde (Sigma); tri-n-octylamine (Sigma); DL-([1-^{14}C])-glutamic acid (specific activity 50.3 mCi mmol^{-1}) (NEN); 3-mercaptopropionic acid (Sigma); γ-vinyl-γ aminobutyric acid (GVG) was a gift from Merrell Laboratories (Dr D.J. Wilkins); D-penicillamine (Sigma).

Statistical evaluations were performed according to Student's *t* test (Snedecor & Cochran, 1967).

Results

For tissues that had been subjected to acute stress treatments, the activities of the GABA-related enzymes were measured under assay conditions that would reveal changes in their apparent affinity (i.e.: non-saturation). Only after chronic stress treatments, when the period of treatment had been long enough also to promote changes in the total amount of enzyme, were the assays performed under saturation.

Effects of acute stress

Neither immobilization nor exposure to cold affected GABAergic systems in the frontal cerebral cortex (Table 1). As shown in Figure 1a, immobilization stress for 1 h reduced the activity of GAD in the corpus striatum by 27% ($P < 0.05$) and the GABA concentration by 36% ($P < 0.05$). These changes were not seen after prolonged periods of acute immobilization (2 or 3 h). Only a slight and transient inhibition of GABA-T (19%, $P < 0.05$, Figure 1a), was measured after immobilization for 2 h, conditions which did not affect striatal GABA turnover (Table 2).

Behavioural changes were observed during the first hour of immobilization. These consisted of struggling and signs of increased aggressiveness which later disappeared gradually.

Cold exposure (Figure 1b) for 1, 2 or 3 h did not modify the activity of the GABA-related enzymes, but after 3 h increased the endogenous content of GABA by 64% ($P < 0.05$). A tendency towards higher GABA levels was already observed after 2 h of cold exposure (40% increase, $P < 0.10$, NS, Figure 1b). Since no metabolic changes to explain the increase in GABA levels were detected, it seemed possible that there could have been a change in the *in vivo* turnover of the neurotransmitter. Hence, the accumulation of GABA following inhibition of its degradation was measured.

As shown in Table 2, 3 h of cold exposure reduced the GABA accumulated in the striatum by 56%

Table 1 Acute stress and GABAergic system in the frontal cerebral cortex

Treatment	*GAD activity* (μmol g^{-1} tissue h^{-1})	*GABA-T activity* (μmol g^{-1} tissue 30 min^{-1})	*GABA* (μmol g^{-1} tissue)
None	1.9 ± 0.3	8.9 ± 1.1	2.6 ± 0.3
Immobilization (1 h)	2.0 ± 0.3	8.5 ± 0.5	2.0 ± 0.1
None	2.3 ± 0.2	7.5 ± 0.6	2.1 ± 0.2
Immobilization (2 h)	1.8 ± 0.2	7.4 ± 1.0	1.8 ± 0.1
None	2.1 ± 0.2	8.1 ± 0.6	2.2 ± 0.2
Immobilization (3 h)	1.8 ± 0.2	8.3 ± 0.8	2.1 ± 0.2
None	3.8 ± 0.3	7.8 ± 0.6	2.4 ± 0.2
Cold (1 h)	3.0 ± 0.4	7.8 ± 1.0	2.3 ± 0.2
None	2.7 ± 0.2	8.0 ± 1.2	2.5 ± 0.3
Cold (2 h)	2.6 ± 0.3	7.1 ± 0.8	2.4 ± 0.1
None	3.0 ± 0.3	7.6 ± 0.8	2.6 ± 0.3
Cold (3 h)	2.9 ± 0.3	7.5 ± 0.6	2.5 ± 0.2

Results were obtained from at least 4 animals per group and are expressed as the mean ± s.e.mean.

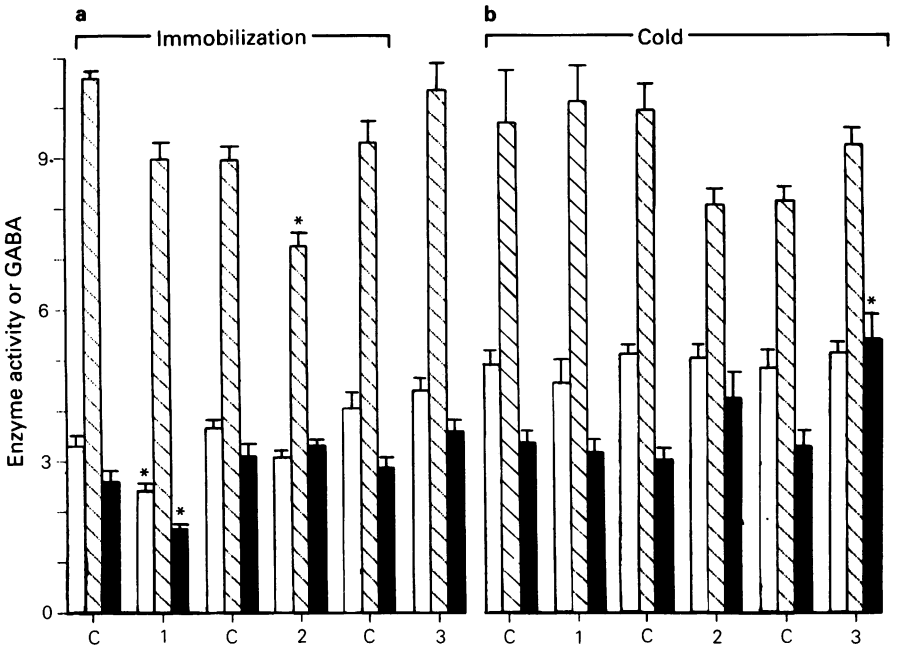

Figure 1 Effects of acute stress upon the striatal GABAergic system. Enzyme activity in μmol g^{-1} tissue h^{-1} for glutamic acid decarboxylase (open columns) and in μmol g^{-1} tissue 30 min^{-1} for 4-aminobutyrate-2-oxo-glutarate aminotransferase (hatched columns); endogenous GABA content (solid columns) in μmol g^{-1} tissue. C refers to the control group and 1, 2 and 3 refer to the groups exposed to stress for 1, 2 and 3 h, respectively. Results are expressed as the mean of 6 rats; vertical lines denote s.e.mean.
*$P < 0.05$ compared with respective control group.

Table 2 The effects of stress on GABA turnover *in vivo*

Treatment	*Endogenous GABA*	*GABA after GVG* (μmol g^{-1} tissue)	*Accumulated GABA*	*GABA turnover* (*k*, μmol g^{-1} tissue h^{-1})
Striatum				
None	3.1 ± 0.4	6.4 ± 0.8	3.3 ± 0.4	1.1 ± 0.1
Immobilization (2 h)	3.3 ± 0.2	4.9 ± 1.1	2.2 ± 0.9	0.7 ± 0.3
None	3.3 ± 0.5	6.8 ± 0.8	3.5 ± 0.4	1.2 ± 0.1
Cold-stress (3 h)	5.4 ± 1.3	7.3 ± 0.5	1.9 ± 0.2	0.6 ± 0.1*
Cortex				
None	2.6 ± 0.1	4.0 ± 0.1	1.4 ± 0.2	0.5 ± 0.0
Repeated stress (immobilization, 14 days)	2.3 ± 0.1	4.1 ± 0.1	1.8 ± 0.1	0.6 ± 0.0

Animals were injected with the GABA-T inhibitor, γ-vinyl GABA (GVG; 10 mmol kg^{-1}, i.p.) and divided into two groups: one group was exposed to cold stress (cold-stress 3 h) and the other immobilized for 2 h, 1 h post GVG injection. Repeatedly stressed animals (0.5 h immobilization per day for 14 days) were injected with GVG 1 day after the last stress session. In all cases the animals were killed 3 h after GVG injection and the accumulated GABA measured. Results are expressed as the mean ± s.e.mean of 4–6 animals per group.
*$P < 0.05$ compared with respective control group.

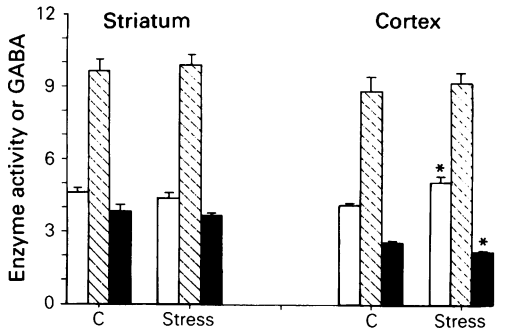

Figure 2 Effects of repeated immobilization stress upon GABAergic system. Enzyme activity in μmol g^{-1} tissue h^{-1} for glutamic acid decarboxylase (open columns) and in μmol g^{-1} tissue 30 min^{-1} for 4-aminobutyrate-2-oxo-glutarate aminotransferase (hatched columns); endogenous GABA content in μmol g^{-1} tissue (solid columns). Results are expressed as the mean of 6 animals per group; vertical lines denote s.e.mean. The animals were killed 1 day after the last stress session.
*$P < 0.05$ compared with respective control group C.

($P < 0.05$). This result indicates that GABA turnover *in vivo* was lowered in the 3 h cold-stressed animals (k: 0.6 μmol g^{-1} tissue h^{-1}) when compared with the untreated animals (k: 1.1 μmol g^{-1} tissue h^{-1}). Such an inhibition of GABA turnover would lead to the increase in endogenous levels of GABA seen after 3 h of cold exposure.

Effects of repeated stress

The response of the GABAergic systems to repeated immobilization stress was also area-dependent, though different from that observed after a single immobilization session.

The striatal GABAergic pathways were not affected by the repeated immobilization treatment (Figure 2). However, when measured at non-saturating conditions the activity of GAD in the cortex was increased by 24% ($P < 0.05$, Figure 2) and the endogenous level of GABA was reduced by 12% ($P < 0.05$, Figure 2).

Since the activity of GABA-T remained unaltered, the decrease in GABA levels and the enhancement of GAD activity could reflect the stimulation of GABA turnover. To test this hypothesis, the accumulation of cortical GABA after GVG administration was measured. A 29% increase ($P < 0.05$, Table 2) in the amount of GABA accumulated was observed in the repeatedly stressed group, thus revealing an enhanced turnover *in vivo* (k: 0.6 μmol g^{-1} tissue h^{-1} in stressed; k: 0.4 μmol g^{-1} tissue h^{-1} in untreated animals).

The maximal activity (i.e. under saturation) of the GABA enzymes in either the cortex or the corpus striatum was unaffected by repeated stress (Table 3).

Discussion

The present study has demonstrated changes in the metabolism and function of GABA in certain areas of the rat brain following either acute or repeated stress. Cortical GABAergic function was not affected by acute stress with only an initial decrease in GABA levels being detected after 1 h of immobilization and none following longer acute immobilization periods. This tendency was also observed by Sherman & Gebhart (1974) after acute restraint stress.

In contrast, the striatal pathways were sensitive to acute stress manipulations. The fact that, after 1 h of immobilization, striatal GABA content (and not only the activity of GAD) was reduced indicates that GAD inhibition had begun earlier. In addition, both GAD activity and GABA levels returned to control values between the first and second hours of stress. These results suggest that the first effect of stress is an interference with GABA function in the corpus striatum. The inhibition of GABA-T which follows after 2 h of stress would not influence GABA levels or GABA turnover.

The finding that the acutely immobilized animals displayed signs of hyperexcitability at a time when the striatal GABAergic system was apparently depressed is indicative of a link between GABAergic function

Table 3 Activity of the GABA enzymes after repeated stress

	Group	*GAD activity* (μmol g^{-1} tissue h^{-1})	*GABA-T activity* (μmol g^{-1} tissue 30 min)
Cortex	Control	40.9 ± 0.1	17.5 ± 0.2
	Stressed	43.8 ± 1.2	17.8 ± 1.4
Striatum	Control	47.3 ± 0.8	34.4 ± 0.4
	Stressed	56.3 ± 2.8	28.6 ± 2.8

Results are expressed as the mean ± s.e.mean of 6–8 animals per group.

and behaviour pattern. Furthermore, abnormalities in the striatal GABAergic system have been observed in the development of aggression.

Yoneda *et al.* (1983) found increased GABA levels in the striatum after 3 h of immobilization stress. This discrepancy with the present results might be at least partly accounted for by differences in the experimental conditions used to produce a stressful stimulus. Indeed, conflicting findings have been obtained when stress conditions were varied in studies of catecholaminergic systems. In the study of Yoneda *et al.*, animals were deprived of food for 16 h before being confined in metal restraining cages and kept in a water bath at 25°C for 3 h. In these experiments there was a suggestion of reduced striatal GABA turnover, although as in the current investigation, it did not attain statistical significance. This agreement, despite the differences in experimental design, would clearly indicate a tendency towards the inhibition of striatal GABA turnover by acute stress, regardless of the type of stressor used. GABA turnover in the corpus striatum was reduced when the animals were exposed to acute cold stress for 3 h. This may be a generalized effect of the low temperature since a reduction in the turnover of dopamine (Dunn & File, 1983) and noradrenaline (Stone, 1970) have also been induced by cold, but not by warm stress.

As no change occurred in the cortical GABAergic system, the results emphasize further that it is only the GABAergic fibres localized in the striatum that are involved in the acute response to stress. It has been demonstrated elsewhere that striatal GABAergic systems are the first to respond during acute stress (Yoneda *et al.*, 1983).

The functional consequences of the changes in the striatal GABAergic system in response to acute stress have yet to be recognized. However, it is important to note that anatomical studies have shown that the striatum is in a key position to serve as an integrating unit modulating functions in the cortex, thalamus and the limbic system (Scheel-Kruger & Magelund, 1981; Scheel-Kruger *et al.*, 1981; Scheel-Kruger, 1982).

It is interesting that the stress-induced enhancement of dopamine metabolism (Bliss *et al.*, 1968; Thierry *et al.*, 1978) is antagonized by diazepam (Fadda *et al.*, 1978; Lavielle *et al.*, 1978), a drug that is believed to act via GABAergic systems (Haefely *et al.*, 1975; McDonald & Barker, 1978; Waddington, 1978). Consequently, the effect on dopamine metabolism might be facilitated during the acute phase of stress due to a reduction in the tonic inhibitory influence of the GABAergic system.

Repeated immobilization for 14 days induced changes in the cortical, but not the striatal, GABAergic function. The increase in the activity of GAD under non-saturation and the absence of changes in GAD activity under saturation would indicate that repeated stress does not affect the amount of enzyme itself, but produces changes in its affinity which might be secondary to increased neuronal activity.

The enhancement of GABA release and turnover usually leads to GAD activation (Gold *et al.*, 1978; Otero Losada, 1985). In fact in the present study, cortical GABA turnover (estimated from the accumulation of GABA after GABA-T inhibition) was increased after repeated stress. These results indicate that cortical GABA-ergic neurones are involved in the response to this type of stress. The stimulation of cortical GABAergic function would have a protective role as one of the homeostatic mechanisms participating in adaptation to stress. Furthermore, repeated immobilization stress facilitates the response to chronic antidepressant treatments that are thought to involve an improvement of GABAergic neurotransmission (Sherman & Petty, 1980). According to the present findings, the synergism between the two treatments could be due to the stimulation of GABA function.

The enhancement of cortical GABAergic activity by repeated stress is also in agreement with two other observations. First, a reduction of the sympathetic peripheral function has been noted only in stress-adapted animals (Appelgren *et al.*, 1982) and GABA is known to reduce the neuronal discharge at sympathetic loci in the brain.

Secondly, cortisol levels rise during acute stress but are low after chronic stress due to feedback inhibition of its synthesis (Dallman & Jones, 1973; Young & Akil, 1985). Since cortisol enhances the affinity of the GABA receptor (Majewska *et al.*, 1985), the stimulation of GABAergic function would also act as a compensatory and adaptive mechanism to overcome the lack of positive hormonal influence upon this neuronal system (which is found in the early phases of stress), thus contributing to prevent the behavioural disturbances otherwise produced chronically by stressors.

Wielosz *et al.* (1985) found that repeated stress (but not acute) protected against seizures caused by GABA antagonists and it was suggested that this was due to stimulation of GABAergic transmission. The current findings are consistent with this hypothesis.

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