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Specific Effects of Punishment on Amino Acids Turnover in Discrete Rat Brain Regions

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MIYAUCHI, T., S. I. DWORKIN, C. CO AND J. E. SMITH. *Specific effects of punishment on amino acids turnover in discrete rat brain regions.* PHARMACOL BIOCHEM BEHAV **31**(3) 523–531, 1988.—Specific effects of punishment on the turnover rates of aspartate (Asp), glutamate (Glu) and gamma-aminobutyric acid (GABA) in 14 brain regions were investigated in rats exposed to punishment. Two yoked controls were also used in an attempt to separate the nonspecific effects of response rate, reinforcement density and direct effects of punisher (foot shock). Punished and unpunished littermate rats had similar response rates, and the reinforcement density was almost identical for both groups. A third group (yoked-shock rats) received food and shock independent of responding whenever these were given to the punished rats. When compared to the unpunished rats, the punishment increased the turnover rates of the three amino acids in all brain regions examined except GABA turnover in the caudate-putamen and preoptic-diagonal band. The majority of these changes by the punishment were similar to the effects of the yoked-shock (yoked-shock versus unpunished), although the magnitude of increase by the punishment was mostly larger than that by the yoked-shock. Six changes by the punishment (increase in the turnover rates of Asp in the thalamus, Glu in the hypothalamus and GABA in the cingulate cortex, entorhinal-subicular cortex, dentate gyrus and hypothalamus) appeared to be the specific effects of punishment since the yoked-shock did not affect these parameters. These results suggest that the punishment caused a hyperexcitation of the amino acidergic neurons in the limbic systems, particularly those in Papez's circuit.

Punishment Conflict Amino acid neurotransmitters Gamma-aminobutyric acid Anxiety Neurotransmitter turnover rates

GELLAR-SEIFTER type of conflict-punishment procedures (17) are useful animal behavioral techniques to investigate the effects and mechanisms of action of anxiolytic drugs. In this procedure, operant responding which is maintained by positively reinforcing stimuli (food, water, etc.) is suppressed (punished) by response-dependent presentation of noxious stimuli (electric foot shock, etc.), and anxiolytic drugs increase the rate of punished responding. A large body of pharmacological evidence exists implicating central gamma-aminobutyric acid (GABA) [reviewed by Sepinwall (38), Enna (14) and Sanger (36)] or serotonin [reviewed by Sepinwall (38) and Iversen (21)] in the antipunishment effect of anxiolytics in rats. In contrast to the numerous pharmacological studies using punishment procedures, however, there has been no report, to our knowledge, assessing the neurochemical consequence of punishment. This study was designated to detect punishment-specific changes in turnover rates of amino acid neurotransmitters as indexes of amino acidergic neuronal activities in discrete brain regions in rats.

Most punishment experiments utilize multiple schedule procedures in which punished (2–5 min duration) and unpunished (5–15 min duration) periods appear alternately during a session. However, these schedules are not suitable for the neurochemical procedure we are using to determine turnover rates (8, 24, 25, 39, 40). In this procedure, animals are killed 60 and 90 min after intravenous pulse injection of radiolabelled precursors of neurotransmitters, which requires animals being under a single component (punished or unpunished) during the pulse interval. Also, response rate and reinforcement density during the punished period are usually less than those during the unpunished period. The difference in motor activity (response rate) and food intake (reinforcement density) between the two components may obscure punishment-specific neurochemical effects.

In the present experiments, therefore, a behavioral procedure which was suitable for the neurochemical examination of punishment was developed. To separate the neurochemical effects of nonspecific factors described above, we used a behavioral procedure [yoked-box procedure (16)] which resulted in similar rates of responding and reinforcement density for punished and unpunished littermate rats. The yoking proce-

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dure for food reinforcement seems important for the neurochemical analysis because it has been reported that in rats performing lever pressing for water, catecholamine utilization in the causate nucleus, amygdala or brainstem changed in relation to the number of water reinforcement but not with response rate, response dependency or volume of water reinforcements (1, 13, 20). To separate the nonresponse contingent (e.g., sensory effects), electric foot shock and food was given to the third littermate (yoked-shock rats) independent of responding whenever these stimuli were delivered to the punished littermate. In this design, the specific neurochemical effects of punishment were determined by comparing the effects of punishment (punished compared with unpunished) with the nonspecific effects of shock (yoke-shock compared with unpunished) for individual compounds in each brain area.

METHOD

Behavioral Procedures

The thirteen triads of male F-344 littermates (3 months old at the time of the experiments) were food-deprived prior to beginning of training and were maintained approximately 80% of their free-feeding weights throughout. The animals were trained using a yoked-box procedure (16). The three littermates were run at the same time in three separate standard operant conditioning chambers located in ventilated, sound-attenuating enclosures in a room with white noise. Sessions were controlled and data collected and analyzed using Rockwell Aim 65 computers operating under MSC control, located in an adjacent room. One rat from each triad was randomly selected to respond on a random-ratio schedule. A food pellet was delivered to this rat after it completed a randomly determined number of responses (5—minimum, 100—maximum). For the second littermate, reinforcement was arranged according to a random interval schedule, directly yoked to the inter-reinforcement intervals produced by the first littermate, i.e., food was presented after the first response by this subject occurring after food was delivered to the first rat. The third littermate was placed on a response-independent food presentation schedule yoked to the first littermate, i.e., food pellet was presented to the third rat whenever the food was given to the first rat. Thus, the procedure resulted in equal inter-reinforcement intervals for the three subjects. Daily sessions terminated 90 min after the start or after 100 food presentations were delivered to the second littermate under the yoke-random interval schedule.

After rates of responding had stabilized (from the 29th session), a cojoint random ratio schedule (minimum—25, maximum—200) of electric foot shock (0.4 mA, 100 msec) was added to the ratio-food schedule for the first rat (punished rat). The same intensity and duration of shocks were given to the third rat independent of responding whenever the punished rat received the shock (yoked-shock rat). No shocks were delivered to the second rat under the yoked-random interval schedule (unpunished rat). Once responding under this condition was stabilized (after 39th session), all rats were implanted with chronic IV catheters into the jugular vein under pentobarbital (50 mg/kg, IP) anesthesia for pulse labelling of neurotransmitters (23,40). At the beginning of the 54th session, eight triads of the animals received IV injection of 0.2 mCi D-[U-^{14}C]-glucose (I.C.N., 210 mCi/mmol) in 50 μl of saline through the jugular catheters. The three rats in each litter were sacrificed at 60 or 90 min after the pulse labelling by immersion of the whole body into liquid nitrogen.

Five pairs of punished and unpunished rats that were not pulse labelled were used to examine the behavioral effects of pentobarbital. Sodium pentobarbital was administered IP 30 min before the start of sessions with doses sequenced in a random series with a minimum of seven days between determinations. Each dose (3.0–17.0 mg/kg) was evaluated at least twice in each subject. Doses are presented in terms of salt. Drug was administered to the punished and unpunished rats on different days.

Neurochemical Procedures

The heads were stored at −70°C and later warmed to −20°C in a cryostat, the brains dissected into 14 discrete regions. The individual tissue samples were pulverized in liquid nitrogen in a stainless steel mortar and stored at −70°C until extraction and assay.

A portion of each tissue sample was used to determine content of aspartate, glutamate and gamma-aminobutyric acid (GABA) by a modification of the method reported by Jones and Gilligan (22) using HPLC and fluorometer. In brief, the amino acids were extracted from the pulverized tissue samples with 200 μl of methanol and the tissue pellets saved for protein determination (26). To each tissue extract, 10 μl of 3 mM homoserine was added as an internal standard. Methanol extracts were dried at 37°C under dry N_2. Dried samples were dissolved in 20 μl of methanol, and 10 μl of the solution was mixed with 30 μl of OPT reagent (22). A portion (20 μl) of the mixture was injected into HPLC systems (Gilson) controlled by Apple IIc computer and utilizing a C_{18} reversed phase column coupled with a fluorometer. The HPLC mobile phase was 0.1 M sodium acetate buffer (pH 6.2) containing 0.1 μM EDTA and methanol in concentrations that changed linearly from 15 to 70%. The column temperature was 30°C and flow rate 1.8 ml/min. The levels of amino acids were calculated by comparing peak areas of each compound with standards. The radioactivity incorporated into the amino acids was measured by collecting each peak eluent from HPLC in a counting vials by means of a fraction collector (Gilson Model 202) and a controller (Gilson, 201-202), adding 10 ml of Aquasol-2 (New England Nuclear), and counting the samples in a Searle Isocap Model 6872 liquid scintillation counter.

Turnover Rate Calculation Procedures

Turnover rates were determined with the assumption that radiolabel from each neurotransmitter was disappearing from a single open pool since there is no acceptable method for determining CNS intraneuronal compartmentation in vivo. Thus, turnover = K × content where the apparent fractional rate constant (K) was a slope of the regression line derived from natural logarithmic plot of specific radioactivities (dpm/nmol) obtained at the two pulse times on the linear portion of the radioactivity decay curve for each neurotransmitter (8, 24, 25, 39, 40). The S.D. of K value was also calculated by regression analysis. It has been reported that the decline in specific activities for glutamate, aspartate, GABA and glycine was log-linear over a time course of from 40 to 150 minutes postinjection of radiolabelled glucose (39). Although specific activities at each of two pulse times were derived from only four rats, the variability in these values were small and the linearity of all of the regression lines was statistically significant ($r^2 > 0.68$, $p < 0.05$). The S.D. of slopes of the regression lines (K) ranged from 5.3 to 50.1 percent.

TABLE 1

MEAN RESPONSE RATES, REINFORCEMENT DENSITY AND SHOCK FREQUENCY OF THE THREE GROUPS BEFORE AND AFTER THE INTRODUCTION OF SHOCK CONTINGENCY

	Before Shock*		Shock†		
Group	Response/min	Reinforcement/min	Response/min	Reinforcement/min	Shock/min
Punished	86.3 ± 9.6	1.7 ± 0.20	10.9 ± 9.1	0.2 ± 0.14	0.09 ± 0.08
Unpunished	19.9 ± 4.5	1.7 ± 0.20	9.2 ± 3.4	0.2 ± 0.20	—
Yoked-shock	0.3 ± 0.3	1.7 ± 0.20	1.1 ± 1.1	0.2 ± 0.14	0.09 ± 0.08

Values are mean ± S.D. of 8 rats.
*Mean values during the last five days before the introduction of shock contingency.
†Mean values on the day of pulse labeling.

The turnover rate is expressed as nmol mg protein^{-1}·hr^{-1}. These turnover rates are assumed to be representative of the utilization of the respective neurotransmitters.

Behavioral and content values were compared by ANOVA followed by multiple comparison (46) and turnover rate values were compared by parallel-line assay.

RESULTS

Behavioral Observations

The random ratio schedule of food presentation initially engendered a high, constant rate of responding, while responding maintained by the yoked-random interval contingency occurred at a much lower rate. Extremely low rates of responding were elicited by the noncontingent presentation of food. The addition of the shock contingency to the random ratio schedule resulted in similar and stable rates of punished un unpunished responding. Reinforcement density for the three groups was identical both before and after the introduction of shock schedule (Table 1). Using another group of punished and unpunished rats, dose-effect curve for pentobarbital which has been reported to selectively increase punished responding (28) was examined (Fig. 1). Pentobarbital increased the punished responding and the peak dose of 5.6 mg/kg produced over an eight-fold increase in responding. Unpunished responding was not affected at doses that increased punished responding. The largest dose inhibited both punished and unpunished responding.

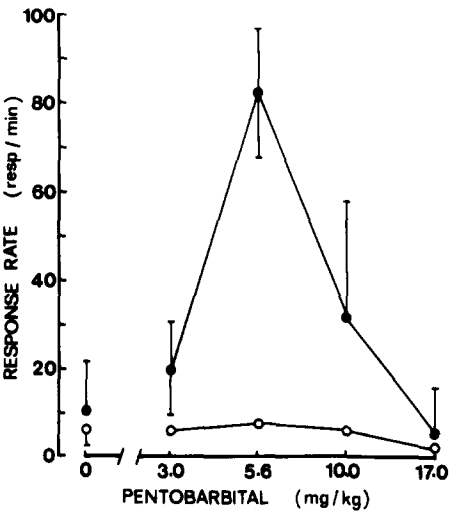

FIG. 1. Effects of pentobarbital on the response rates of punished (closed circle) and unpunished (open circle) rats. Each point represents mean and S.D. (vertical bars) of at least 10 observations.

Content of Amino Acids

As shown in Table 2, there was no significant difference between groups in the content values for Asp, Glu and GABA in any of the brain regions assayed. The absence of changes in content supports the hypothesis that neuronal systems are capable of maintaining sufficient neurotransmitter stores to meet functional needs within normal physiological limits. Such an interpretation would indicate that the effects of the behavioral manipulation in this study did not exceed these normal functional limits.

Turnover Rates of Amino Acids

In contrast to the content values, numerous changes in the turnover rates of the amino acids occurred as a result of either the punishment (the punished rats compared to the unpunished rats) or the response-independent shock (the yoked-shock rats compared to the unpunished rats), and all of the changes were increases (Figs. 2–4). Out of 42 combinations of the amino acids and the regions, only two showed ineffectiveness of the two behavioral manipulations, i.e., GABA in the caudate putamen and preoptic diagonal band. Asp and Glu turnover was significantly increased by the punishment in all of the 14 brain regions (44–106 and 60–161%, respectively), whereas the yoked-shock increased Asp turnover in 10 regions (31–114%) and Glu turnover in 12 regions (42–90%). GABA turnover was significantly increased by the punishment in 12 regions (19–195%) and by the yoked-shock in 8 regions (31–168%). Fifteen of the 40 changes observed in the punished rats were identical in the yoked-shock rats: increase in Asp turnover in the frontal cortex, pyriform cortex, entorhinal subicular cortex, nucleus accumbens, septum, caudate-putamen, amygdala and dentate gyrus, increase in Glu turnover in the frontal cortex, pyriform cortex, septum and caudate putamen and increase in GABA turnover in the frontal cortex, pyriform cortex and hippocampus. The remaining 25 elevated turnover rates in the punished rats were significantly different from those in the yoked-shock rats. However, the majority (19/25) of these changes in the punished rats were qualitatively similar to the effects of yoked-

TABLE 2

CONTENT OF AMINO ACIDS IN 14 BRAIN AREAS OF PUNISHED, UNPUNISHED AND YOKED-SHOCKED GROUPS

Brain Area	Group*	Amino Acids Content (nmol/mg protein) Asp	Glu	GABA
Frontal cortex	P	20.4 ± 9.1	113.3 ± 50.1	15.6 ± 6.3
	U	17.3 ± 8.7	84.8 ± 40.3	12.1 ± 6.0
	Y	19.8 ± 7.2	112.9 ± 46.2	15.8 ± 6.5
Pyriform cortex	P	25.6 ± 9.2	128.6 ± 50.5	30.7 ± 10.9
	U	18.5 ± 6.7	85.3 ± 32.2	21.3 ± 8.2
	Y	21.6 ± 8.5	105.5 ± 45.9	24.6 ± 10.8
Motor-somatosensory cortex	P	23.2 ± 1.4	103.4 ± 5.9	17.3 ± 2.0
	U	25.0 ± 1.8	106.5 ± 5.6	19.0 ± 1.5
	Y	24.2 ± 1.5	105.1 ± 7.0	18.8 ± 1.7
Cingulate cortex	P	27.6 ± 3.4	144.0 ± 9.6	20.4 ± 3.1
	U	24.8 ± 1.6	134.4 ± 8.8	20.9 ± 3.3
	Y	26.0 ± 2.6	140.4 ± 8.7	21.3 ± 2.3
Nucleus accumbens	P	29.8 ± 4.7	149.7 ± 19.3	44.3 ± 7.4
	U	30.1 ± 2.3	141.5 ± 7.5	44.7 ± 3.8
	Y	30.1 ± 7.4	149.1 ± 23.3	46.9 ± 9.5
Septum	P	25.0 ± 2.5	118.8 ± 9.7	61.3 ± 3.9
	U	28.6 ± 5.2	132.3 ± 22.8	68.7 ± 12.3
	Y	27.1 ± 7.6	130.1 ± 28.0	63.1 ± 11.0
Caudate-putamen	P	16.6 ± 2.3	103.2 ± 10.7	34.4 ± 5.4
	U	16.0 ± 2.4	97.4 ± 7.0	34.8 ± 5.1
	Y	17.1 ± 2.4	104.5 ± 9.8	32.0 ± 10.4
Preoptic-diagonal band	P	29.4 ± 5.1	99.8 ± 9.1	118.5 ± 23.9
	U	32.3 ± 10.8	104.0 ± 22.6	114.0 ± 32.0
	Y	26.2 ± 5.0	94.4 ± 9.3	109.7 ± 21.8
Amygdala	P	32.5 ± 10.9	153.6 ± 41.9	50.6 ± 12.3
	U	32.1 ± 5.2	153.6 ± 21.7	48.1 ± 6.4
	Y	31.7 ± 5.5	146.5 ± 17.3	43.2 ± 7.2
Hippocampus	P	15.9 ± 1.7	103.7 ± 7.3	21.2 ± 3.1
	U	17.3 ± 3.1	106.8 ± 12.0	24.0 ± 1.7
	Y	15.2 ± 1.7	101.3 ± 7.4	22.7 ± 1.7
Dentate gyrus	P	20.3 ± 2.0	129.3 ± 10.5	37.6 ± 4.0
	U	20.5 ± 4.9	122.0 ± 20.6	40.1 ± 7.8
	Y	20.8 ± 4.5	130.5 ± 16.3	40.5 ± 5.3
Hypothalamus	P	22.2 ± 5.4	99.2 ± 19.5	78.4 ± 13.0
	U	22.1 ± 2.4	100.2 ± 5.6	83.3 ± 7.9
	Y	24.3 ± 6.7	109.2 ± 21.2	86.6 ± 14.6
Thalamus	P	21.5 ± 3.1	96.3 ± 10.2	28.5 ± 5.0
	U	21.1 ± 3.1	93.7 ± 8.2	27.5 ± 3.4
	Y	20.2 ± 1.2	91.4 ± 6.6	25.2 ± 3.9

Values are mean ± S.D. of 8 rats.
*P=punished; U=unpunished; Y=yoked-shock.

shock since the yoked-shock also increased the turnover rates significantly, but with different magnitudes. Mostly (16/19), the changes caused by the yoke-shock were weaker than those by the punishment. These were: increase in Asp turnover in the motor-somatosensory cortex, cingulate cortex, preoptic diagonal band, hippocampus and hypothalamus, increase in Glu turnover in the regions except the frontal cortex, pyriform cortex, septum, caudate putamen and hypothalamus, increase in GABA turnover in the motor-somatosensory cortex, nucleus accumbens, septum, amygdala and thalamus. The remaining six changes, mostly in GABA turnover, appeared to be specific effects of the punishment since yoked-shock did not affect the turnover rates. These were: increase in Asp turnover in the thalamus,

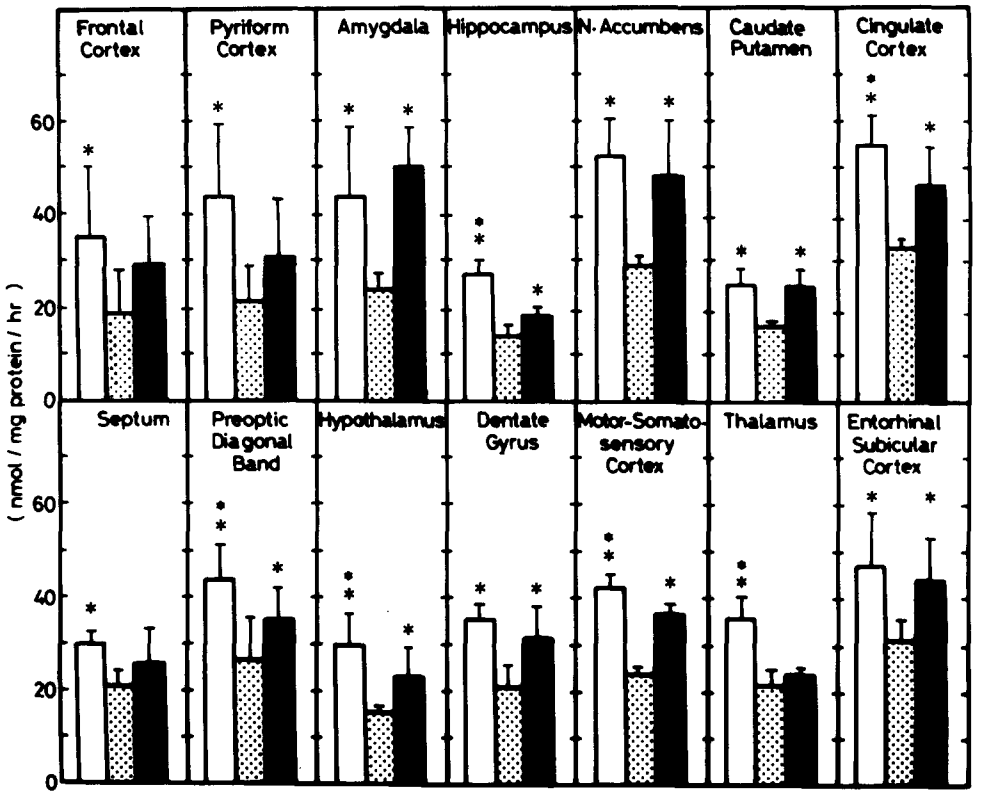

FIG. 2. Turnover rates of aspartate in 14 brain areas of punished (clear bars), unpunished (stippled bars) and yoked-shock (dark bars) groups. Data represent mean and S.D. calculated from 8 rats. *Significant difference ($p<0.05$) from the unpunished group. ★Significant difference ($p<0.05$) from the yoked-shocked group.

increase in Glu turnover in the hypothalamus, increase in GABA turnover in the cingulate cortex, entorhinal-subicular cortex, dentate gyrus and hypothalamus.

DISCUSSION

Behavioral Baseline

Using a yoked-box procedure, two groups of rats which had similar rates of responding and almost identical reinforcement density were obtained. Different effects of pentobarbital which has been reported to selectively increase punished responding (28) on the response rates for these two groups verified pharmacologically that the responding for one group under the random ratio schedule was punished and that for the other under the yoked-random interval schedule was unpunished.

Assessment of Amino Acid Neurotransmitter Utilization

The assumption that radiolabel from each neurotransmitter was disappearing from a single open pool is simplified since it disregards potential compartmentation of the neurotransmitters. For biogenic amine transmitters, the disappearance of radiolabel from an open compartment has been used to evaluate rates of utilization (23). Unfortunately, similar interpretation of amino acid neurotransmitter utilization is complicated by several factors. Glucose preferentially labels neuronal pools of amino acids which are readily releasable in the presence of depolarizing stimuli (11, 12, 31, 43, 44), although it does not distinguish intraneuronal compartmentation. This may not be a problem in the forebrain, where a majority of synapses are thought to release amino acids such as Glu, Asp and GABA, strongly suggesting that most of the amino acid pool(s) are dedicated to neurotransmitter function. Therefore, when large pool(s) of Glu, Asp and GABA are labelled from glucose, the contribution of these amino acids to nontransmitter functions (metabolic or synthetic) are probably small and can be ignored without resulting in significant error. The 60 and 90 minute pulse length were selected because they were on the linear portion of the decay in radioactivity curves (39), and they ensure that the animal is no way stressed nor artificially stimulated during ongoing behavior in the punishment paradigm. However, one may be concerned as to whether neurotransmitter utilization is being monitored at these extended time points. During short pulse intervals after glucose administration, Bertilsson *et al.* (4) observed a high flux of label from glucose into Glu and GABA, and using the method of Neff *et al.* (33), calculated turnover rates for the first 7 minutes postinjection, finding a trend toward decreasing fractional rate constants for GABA. This was attributed to the progressive recycling of radiolabel through the amino acids which is likely occurring in the present study, but does not reduce the usefulness of the observations. Even if recycling of radiolabel is occurring, the flux through the amino acids is a useful measure for deriving an apparent rate constant since any up- or

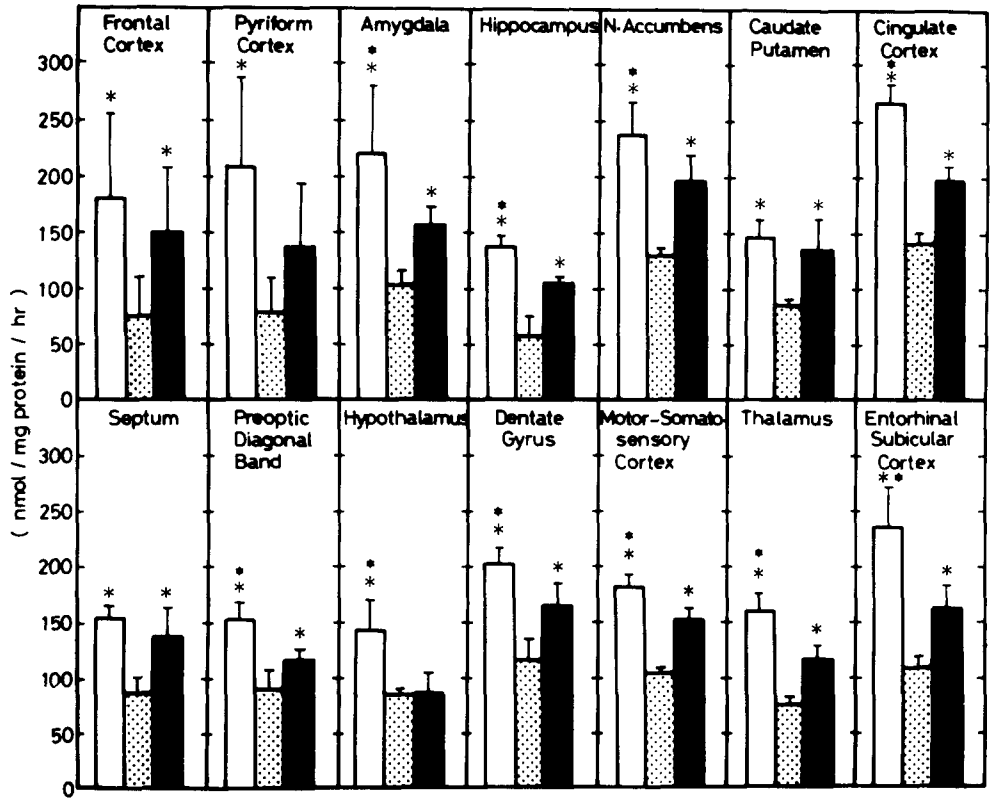

FIG. 3. Turnover rates of glutamate in 14 brain areas of punished (clear bars), unpunished (stippled bars) and yoked-shock (dark bars) groups. Data represent mean and S.D. calculated from 8 rats. *Significant difference ($p<0.05$) from the unpunished group. ★Significant difference ($p<0.05$) from the yoked-shock group.

down-regulation of neuronal activity would merely accelerate or diminish the contribution of recycling, and thus not effect the interpretation. Even considering these shortcomings, the turnover of amino acid neurotransmitters can be monitored in discrete brain regions after behavioral and/or pharmacological pertubation, with reasonable confidence that increased turnover rates represent elevated neuronal activity.

Punishment or yoked-shock caused increases in amino acid turnover rates in multiple brain regions. These relationships are summarized in Table 3. The 42 comparisons could be divided into 4 categories.

Effects not Associated Specifically With Punishment

(A) No differences among values for the three groups. This ineffectiveness of the behavioral procedures was seen only in GABA turnover rates in the caudate-putamen and preoptic diagonal band. The present results showing increases in turnover rates of the amino acids in widespread brain areas by the punishment and yoked-shock, which indicate a general stimulating effect of these behavioral manipulations on CNS neurons, are consistent with reports of an enhanced glucose utilization in extensive brain regions after stress (32).

(B) Same magnitude of increase in the turnover rates by the punishment and yoked-shock. In four out of 15 changes of this type, effects of the yoked-shock were statistically not significant (Asp in the frontal cortex and septum and Glu in the pyriform cortex), but the trend of increase was obvious in these cases. These types of changes were seen mostly in Asp turnover. It is most likely that these changes were due to direct or nonspecific effect of shock itself and/or interaction of food presentation and nonspecific effects of shock. It has been shown that the presentation of stimulus which was previously coupled with response-independent shock suppressed operant responding (conditioned suppression) (15,30). Therefore, one might speculate that, although the yoked-shock group was not trained for operant responding in this study, response-independent shock and punishment could cause similar neurochemical changes. Although the possibility cannot be ruled out, it seems unlikely since shock intensity (0.4 mA) and duration (100 msec) were considerably less than those used in the conditioned suppression (1–2 mA, 500 msec), and extreme emotional responses usually seen in the conditioned suppression such as bracing or freezing were not observed in the yoked-shock rats. Frequent approach to the food cup in the test box was observed in these rats throughout the session even just after receiving the shock.

(C) Different magnitude of increase in the turnover rates by the punishment and the yoked-shock. These types of changes were seen mostly in Glu turnover. The difference between the punishment and yoked-shock might have been due to specific up- or down-regulation [most (16/19) of the changes were up-regulation] of shock effect by the punishment. However, it is also possible that the differences resulted from the response dependency of the reinforcement, response

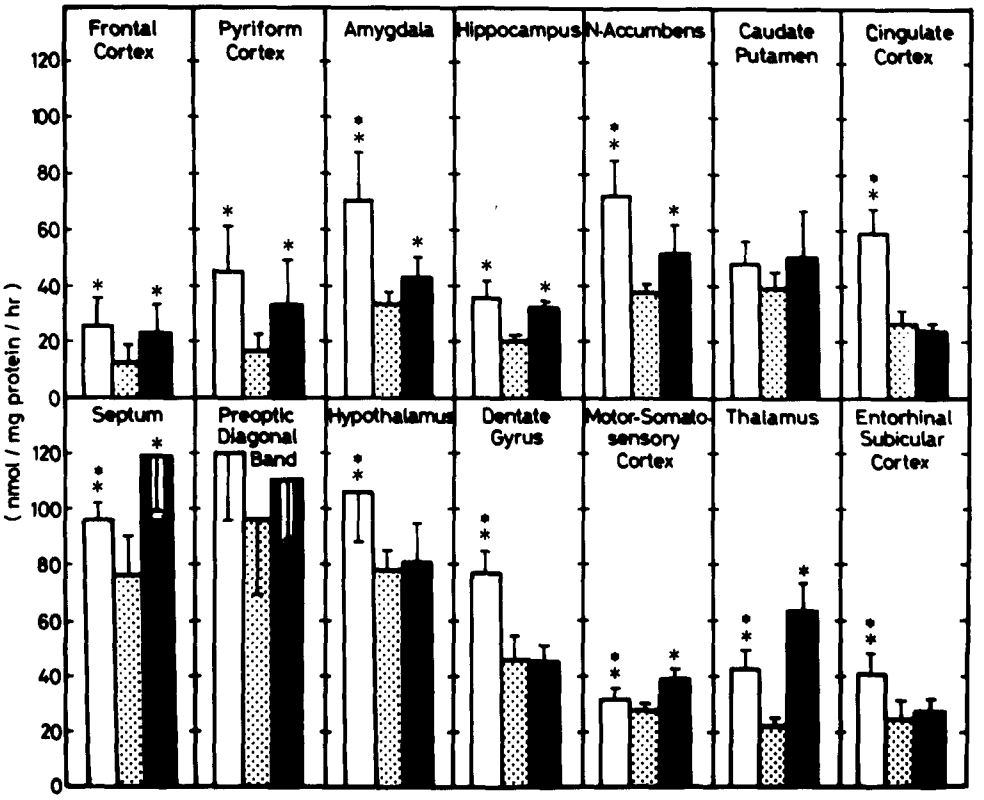

FIG. 4. Turnover rates of GABA in 14 brain areas of punished (clear bars), unpunished (stippled bars) and yoked-shock (dark bars) groups. Data represent mean and S.D. calculated from 8 rats. *Significant difference ($p<0.05$) from the unpunished group. ★Significant difference ($p<0.05$) from the yoked-shock group.

rates and/or interaction of these factors with shock. Therefore, more data are needed to determine whether the punishment specific effects were involved in this type of change.

Effects That can be Attributed Specifically to Punishment

(D) Significant increase in the turnover rates by the punishment but no effect by the yoked-shock. This type of change is most likely to result from specific effect of the punishment. These were: increases in turnover rates of Asp in the thalamus, Glu in the hypothalamus and GABA in the cingulate cortex, entorhinal-subicular cortex, dentate gyrus and hypothalamus. To be noted, the punishment specific changes were observed 1) only in the limbic structures forming Papez's circuit (hippocampal formation → hypothalamus → anterior thalamus → cingulate cortex → entorhinal cortex → hippocampal formation) (27) and 2) predominantly in GABA turnover. Since GABA neurons in these structures are mostly intrinsic interneurons which mediate feedback or feed forward inhibition [reviewed by Haefely and Polc (19)], the increase in GABA turnover suggests that the punishment specifically activated the limbic systems including Papez's circuit. Therefore, in addition to the punishment specific increase in Asp and Glu in the thalamus and hypothalamus, respectively, larger increase in Asp and Glu in the rest of the areas of Papez's circuit in the punished rats than in the yoked-shock rats may also be a specific enhancement of the shock effect by punishment. The specific activation of the neuronal activity in the limbic system by punishment may be related to the emotional states induced by the punishment, possibly anxiety and/or conflict.

GABAergic Systems and Punishment

It has been shown that the antipunishment action of benzodiazepines was potentiated and mimicked by the intraventricular or intracerebral injection of the GABA receptor agonist muscimol (7,37), while it was inhibited by drugs which reduce the responsiveness of GABA receptors (bicuculline, a GABA receptor antagonist and picrotoxin, a chloride channel inhibitor) (5, 37, 41, 45). On the other hand, inhibitors of GABA function (benzodiazepine receptor inverse agonists, GABA synthesis inhibitor, GABA antagonist, chloride channel inhibitor) have been reported to enhance the punishment-induced behavioral inhibition (9, 35, 42). These pharmacological data suggest that central GABAergic systems are inhibitory on the behavioral suppressing effect of punishment. This study showed that GABA turnover was selectively increased by the punishment and the turnover rates of excitatory Asp and Glu were elevated by the punishment much higher than by the yoked-shock. These results suggest that the inhibitory GABAergic interneurons were activated as a result of the pronounced excitation of the neurons in the limbic systems by punishment. The antipunishment effect of benzodiazepines may be a result of a further enhancement of the compensatory GABA-mediated inhibitory process. Alternately, it is also

TABLE 3

RELATIONSHIPS BETWEEN PUNISHMENT OR YOKED-SHOCK AND THE TURNOVER OF AMINO ACIDS

Brain Area	Asp Turnover				Glu Turnover				GABA Turnover			
	P*	Y†	P/Y‡	Category§	P*	Y†	P/Y‡	Category§	P*	Y†	P/Y‡	Category§
Frontal cortex	↑	—	—	B	↑	↑	—	B	↑	↑	—	B
Pyriform cortex	↑	—	—	B	↑	—	—	B	↑	↑	—	B
Motor-somatosensory cortex	↑	↑	↑	C	↑	↑	↑	C	↑	↑	↓	C
Cingulate cortex	↑	↑	↑	C	↑	↑	↑	C	↑	—	↑	D
Etorhinal-subicular cortex	↑	↑	—	B	↑	↑	↑	C	↑	—	↑	D
Nucleus accumbens	↑	↑	—	B	↑	↑	↑	C	↑	↑	↑	C
Septum	↑	—	—	B	↑	↑	—	B	↑	↑	↓	C
Caudate-putamen	↑	↑	—	B	↓	↓	—	B	—	—	—	A
Preoptic-diagonal band	↑	↑	↑	C	↑	↑	↑	C	—	—	—	A
Amygdala	↑	↑	—	B	↑	↑	↑	C	↑	↑	↑	C
Hippocampus	↑	↑	↑	C	↑	↑	↑	C	↑	↑	—	B
Dentate gyrus	↑	↑	—	B	↑	↑	↑	C	↑	—	↑	D
Hypothalamus	↑	↑	↑	C	↑	—	↑	D	↑	—	↑	D
Thalamus	↑	—	↑	D	↑	↑	↑	C	↑	↑	↓	C

*Punished versus unpunished groups.
†Yoked-shock versus unpunished groups.
‡Punished versus yoked-shock groups.
§Categories of relationships: A, no effect of the behavioral procedures; B, nonspecific effects of shock; C, nonspecific effects of shock or specific up- or down-regulation of shock effects by the punishment (see the Discussion section for details); D, specific effects of the punishment.
↑=Increased turnover; ↓=decreased turnover; —=no change.

possible that the increased GABA turnover was a response to impaired GABAergic transmission at the receptor site. It has been shown that GABA and benzodiazepine receptor agonists reduced GABA turnover rate, whereas GABA antagonists and benzodiazepine inverse agonists produced opposite effects (2, 3, 29) presumably via autoreceptors. On the other hand, the presence of endogenous peptides which share with β-carboline-like benzodiazepine receptor inverse agonists receptor binding properties and electrophysiological and behavioral effects has been reported (6, 10, 18). Thus, it can be speculated that punishment released endogenous substances which bind to benzodiazepine-β-carboline binding sites and inhibited GABAergic transmission at GABA receptor level, which in turn activated presynaptic GABA neuronal activity. Reduced GABAergic transmission was thereby lead to hyperexcitation of the neurons in these areas. The selective increases in GABA turnover by the punishment in the limbic areas could be explained by this hypothesis.

In conclusion, the results suggest that a hyperexcitation of the amino acidergic neurons in the limbic system, particularly those in Papez's circuit, is a specific effect of punishment. Although the present results do not address the mechanisms responsible for these changes, it is possible that this effect is mediated by endogenous ligands for benzodiazepine-β-carboline binding sites.

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