Pharmacology Biochemistry & Behavior, Vol. 16, pp. 329–340, 1982. Printed in the U.S.A.

Amino Acid Neurotransmitter Utilization in Discrete Rat Brain Regions Is Correlated with Conditioned Emotional Response

JOHN D. LANE, MICHAEL P. SANDS, MARK E. FREEMAN, D. R. CHEREK AND JAMES E. SMITH

Psychiatry Research Unit, Departments of Psychiatry, Physiology and Pharmacology
Louisiana State University Medical Center, Shreveport, LA 71130

Received 14 May 1981

LANE, J. D., M. P. SANDS, M. E. FREEMAN, D. R. CHEREK AND J. E. SMITH. *Amino acid neurotransmitter utilization in discrete rat brain regions is correlated with conditioned emotional response.* PHARMAC. BIOCHEM. BEHAV. 16(2) 329–340, 1982.—The content and utilization of amino acid neurotransmitters were evaluated in discrete brain areas of rats exposed to a conditioned emotional response (CER) procedure and in control groups which received either equivalent yoked shock history (shock only) or compound stimulus presentation (tone only). On test day, CER animals suppressed responding and exhibited anxious behavior after presentation of the CS, while shock only and tone only control groups, or CER animals which received an acute dose of diazepam prior to testing, did not suppress. Few changes were observed in the content of amino acids, suggesting that the behavioral manipulations were acting within normal physiological limits. On the other hand, numerous changes were observed in the utilization (turnover, metabolism) of the amino acid neurotransmitters. The effects of a history of shock presentation (shock only versus tone only) were persistent long after the conditioning sessions were terminated, and resulted in decreased turnover of the amino acids in many areas. CER conditioning-emotion (CER versus shock only) produced an increase in the turnover of aspartate and glutamate in many structures, while changes in GABA turnover were generally limited to decreases in limbic areas. If CER represents an animal model of anxiety, these observations may suggest roles for neurons which utilize amino acids in mediating or responding to emotional components of the paradigm.

Conditioned emotional response Shock history Stress Anxiety Amino acid neurotransmitters
Gamma-aminobutyric acid Neurotransmitter utilization

ONE reason that humans consume and abuse minor tranquilizers is to cope with anxiety-producing situations. These drugs may act by affecting the individual's perception of environmental conditioning stimuli that evoke anxious behavior and/or by modifying the individual's response to aversive situations. Understanding the neurobiological basis of anxiety produced by stress, and the interaction of the anxiolytic agents with these processes, may be beneficial in focusing the specificity of these agents. This research was designed to investigate neurochemical changes correlated with conditioned emotional response (CER; conditioned suppression). Since CER was first described by Estes and Skinner [15], it has received attention as a behavioral prototype of anxiety, because the warning signal for an unavoidable aversive event in the CER paradigm may be analogous to many everyday anxiety-producing situations for humans.

Although numerous studies have been conducted to characterize various parameters (e.g., [21,22]) and to assess the effects of drugs on the CER paradigm, particularly benzodiazepines (see review by Millenson and Leslie [32]), very few neurochemical studies have been reported [19, 26, 48]. Fortunately, there have been several neurochemical studies of analogous stressful paradigms (e.g., [2, 7, 17, 38]) that suggest a detailed neurochemical characterization of CER and its conditioning history would be beneficial. Furthermore, the discovery of benzodiazepine receptors in the central nervous system (CNS) and the potential interaction of these receptors with gamma-aminobutyric acid (GABA) [8, 17, 23, 29, 47], suggest that neurons which utilize amino acid neurotransmitters may play an important role in emotion or its alleviation by anxiolytics. Therefore, the content and utilization of amino acid neurotransmitters in the brains of rats subjected to the CER paradigm and its conditioning components were investigated.

METHOD

Behavioral Procedures

The CER conditioning-training was designed to accommodate the neurochemical procedures (refer to Fig. 1). Triads of male F-344 littermates (90–120 days old) were

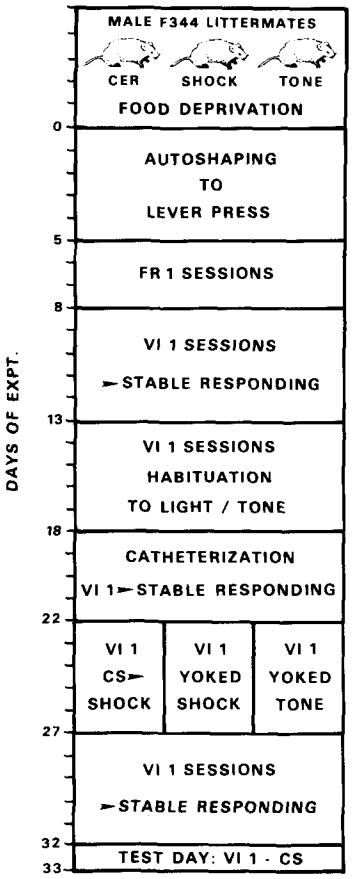

FIG. 1. Diagram of the conditioning of the CER, shock only and tone only littermates. Only during the five-day conditioning sessions (days 23–27) did the protocols differ.

shaped to lever press on a variable interval one minute (VI 1) schedule of food reinforcement in specially designed operant conditioning chamber-liquid nitrogen dipping apparatuses [39]. This VI 1 schedule made use of limited hold, i.e., a response must occur within 10 seconds of the variable interval's termination to be reinforced. Daily one-hour sessions of bar pressing on this schedule were carried out until three consecutive days of stable response rates occurred. After three consecutive sessions, when subject's response rates did not vary more than 100 responses per hour, habituation to a visual and auditory stimulus combination was initiated. A flashing light and a tone were presented during the middle 15 minutes of each session. The disruptive effects of this novel stimulus were extinguished within five sessions of habituation (defined by three consecutive sessions of continuous non-interrupted responding during this stimulus presentation).

Upon completion of the third session of successful habituation as required above, the triad of rats was surgically implanted with chronic intravenous jugular catheters [25, 39, 40, 41]. The catheter exited through a brass enclosure (approximately 15×15×10 mm, weighing 1 gram) that was affixed to a plastic plate mounted above the scapulae of each animal with teflon screws. The catheter was then filled with heparinized normal saline and sealed with cyclohexanone. The catheter was placed in the enclosure, which was wired shut to prevent access by the rat. The radiolabelled precursors were administered via this catheter on test day without disrupting behavior.

When recovery of continuous VI 1 responding in morning training sessions had occurred, afternoon sessions of respondent conditioning or control situations were initiated (Fig. 1). These made use of the same chambers customarily used by the respective rats for food responding, except that levers were removed. Three situations were employed: In one, the offset of the above described combination of stimuli was simultaneous with a 1 mA alternating current, 60 hertz, 500 millisecond shock to the feet (CER condition). In another, the subject received only the compound auditory and visual stimuli (designated as the tone only condition). In a third, the subject received only the shock (shock only condition). These three conditions allow the comparison of the classical conditioning and emotional phenomena (CER versus shock only) and the shock history (shock only versus tone only). The presentation and duration of CS were variable to prevent temporal discrimination. Of five one-hour sessions, the first made use of a VI 2.5 minute schedule, resulting in 24 events per hour, 12 of which were shocks. The second through fifth sessions employed a VI 5-minute schedule, producing 6 shocks per session. The range of the former was 1 to 4 minutes between events, and the latter 2 to 8 minutes. On the fifth day of respondent conditioning, after the session was completed, 80 μl 5% (w/v) sodium thiopental was injected via the catheter to ensure its viability, i.e., immediate anesthesia indicated a functional catheter. Daily VI 1 minute food maintained responding was continued after CER conditioning was terminated. When continuous, stable VI 1 responding without pause was predictable, animals were injected with the precursor to the amino acid neurotransmitters (0.2 mCi D-[U-^{14}C]-glucose, I.C.N., Spec. Act. 210 mCi/mmol) in 100 μl of saline through the jugular catheter. The animals were sacrificed at 60 and 90 minutes after injection by dipping into liquid nitrogen. The precursor was injected 15 or 45 minutes prior to the daily VI 1 minute food maintained session. After 30 minutes exposure to the VI 1 schedule, the conditioned stimulus combination (CS) was presented to all three animals and, after 15 minutes of continuous exposure, the rats were totally frozen as described [39].

Additional groups of animals were conditioned on the CER paradigm exclusively using the procedures described above. On test day, these animals received various acute doses (2.5–50 mg/kg) of diazepam or vehicle 30 minutes before the beginning of the VI 1 schedule. The diazepam was the generous gift of Dr. W. E. Scott of Hoffman-LaRoche, Inc. (Nutley, NJ). Thirty minutes into the VI 1 schedule, the CS was presented for 15 minutes as before to evaluate the effects of anxiolytics on this paradigm.

Neurochemical Procedures

The heads were stored at −70°C and later warmed to −20°C in a cryostat, the brains removed, coronally sectioned at 500–1000 μm intervals, and dissected into twenty-one discrete regions. The individual tissue samples were pulverized in liquid N_2 in a stainless steel mortar and stored at −70°C until extraction and assay.

The amino acids were extracted from a small portion of the tissue powder and assayed for content and specific radioactivity with an isotopic modification of a previously reported procedure [16]. A 3 to 10 mg portion of the tissue

TABLE 1

RESPONDING OF CONDITIONED EMOTIONAL RESPONSE, SHOCK ONLY AND TONE ONLY ANIMALS BEFORE AND DURING THE PRESENTATION OF THE CONDITIONED STIMULUS (CS)

Group	Total Responses for 30 min	Res/Min (Before CS)	Total Responses During 15 min CS	Res/Min (During CS)	RPM (during) / RPM (before)
CER	270.3 ± 94.3	9.1 ± 3.1	1.2 ± 1.5*	0.08 ± 0.01	0.01 ± 0.02
Shock Only	290.2 ± 103.8	9.7 ± 3.4	136.7 ± 59.4	9.2 ± 3.9	0.98 ± 0.33
Tone Only	345.9 ± 104.5	11.5 ± 3.5	147.4 ± 73.7	10.6 ± 4.5	0.92 ± 0.31

Values represent Mean ± SD, n=19–21 per group. RPM=responses per minute. Refer to behavioral paradigm described in the Method.

*A majority of the CER animals were totally suppressed during presentation of the CS.

was extracted with 7% trichloracetic acid, the trichloracetic acid removed with four 1 ml ether washes and the samples taken to dryness in a vacuum desiccator centrifuge. Protein was determined by the method of Lowry *et al.* [26]. Internal standards for the amino acids were added to brain extracts and processed in parallel. The dried samples were reconstituted in 0.1 M borate buffer, pH 10.0, added to a solution of ^{3}H-dinitrofluorobenzene (NEN, Spec. Act. 12 Ci/mmol) in benzene and incubated at 60°C for 30 minutes. The samples were acidified with 5 N HCl and the dinitrofluorobenzene and dinitrophenol removed with four 1 ml heptane/bromobenzene (v/v:80/20) washes. The ^{3}H-dinitrophenyl-amino acid derivatives were collected by two ether extractions which were combined and taken to dryness in a vacuum desiccator centrifuge. The dried extracts were redissolved in 1 N HCl and the two ether extractions repeated and dried as before. The ^{3}H-dinitrophenyl derivatives were separated by two dimensional thin-layer chromatography on 20×20 cm, 500 μm thick silica gel-G (E. Merck) glass plates (Analtech). The plates were first developed in a solvent system of ether/methanol/7 N NH_4OH (v/v:100/20/8) rotated 90° and developed in a solvent system of ether/glacial acetic acid/H_2O (v/v:100/10/10). The individual ^{3}H-dinitrophenyl-amino acid spots were scraped into counting vials, eluted with 1 ml of 0.01 M $NaHCO_3$, 15 ml of Aquasol-2 was added and the radioactivity in each sample determined by liquid scintillation spectrometry. Content (nmol/mg protein) was determined from the internal standards and specific radioactivity (dpm/nmol) calculated for each sample.

Since there is no acceptable method for determining CNS intraneuronal compartmentation in vivo, turnover rates were determined based on the assumption that radiolabel from each neurotransmitter was disappearing from a single open pool. Thus, $\text{Turnover}_A = K \times \text{content}_A$ where the apparent fractional rate constant $(K) = \ln 2/t_{1/2}$ and $t_{1/2}$ was obtained from a semilogarithmic plot of the specific radioactivities (dpm/nmol) obtained at the two pulse times on the linear portion of the decay in radioactivity curve for each neurotransmitter. The turnover rate is expressed as nmol mg protein^{-1} hr^{-1} and is the product of the rate constant (hr^{-1}) and the content values (nmol mg protein^{-1}). These turnover rates are assumed to be representative of the utilization or an index of metabolism of the respective neurotransmitters [25, 40–42, 56].

Values for content and turnover were compared by student's *t*-test for individual compounds in each brain area for both the conditioning-emotion (CER versus shock only) and shock history (shock only versus tone only). Since the numbers of observations were large (N=13), most differences were significant at the $p \leq 0.01$ level.

RESULTS

CER as a Behavioral Model of Anxiety

The classically conditioned littermates exhibited essentially total suppression of food reinforced responding during presentation of the CS. These CER conditioned animals would retreat to a corner of their operant chamber and exhibit behavior generally accepted as indicative of conditioned fear (anxiety), e.g., bracing, freezing, shaking, urination, defecation, etc. The shock only and tone only control littermates did not attend to the CS (Fig. 2) and had very similar response rates on the VI 1 schedule before and during the CS presentation (Table 1). The habituation portion of the initial training and conditioning procedures (Fig. 1) probably contributed to the response ratios approaching unity.

This CER paradigm was sensitive to the actions of anxiolytics. Low doses of diazepam were effective in reversing the conditioned suppression (with a return to normal VI 1 responding no different than pre-drug behavior (Fig. 3)), while injection of CER animals with vehicle yielded total suppression as observed with the initial studies (Fig. 2). There was a modest but consistent dose response relationship when comparing doses of 5–15 mg/kg with response ratios. Higher doses of drug produced increased VI 1 responding (appetite stimulation?), then ataxia and eventually stupor. The anxiolytic effects of low doses of diazepam support the use of the CER paradigm as an animal model of anxiety.

Content of Amino Acids

The content of aspartate, glutamate, glycine and GABA were measured in twenty discrete regions of animals subjected to CER, shock only and tone only (Fig. 4). There was substantial variation in the levels of the respective amino acids when comparing one with another in an individual brain region or the differential distribution of individual amino acids in various brain regions. Of 240 comparisons of means, only five statistically significant ($p<0.05$) changes in content were observed: glutamate in the pre-optic-diagonal band region (−23%—CER versus tone only); glutamate in the amygdala (−26%—CER versus shock only; and −41%—CER versus tone only); glutamate in the lateral hypothalamus (−28%—CER versus tone only); and GABA in the substantia nigra (−26%—CER versus tone only). A

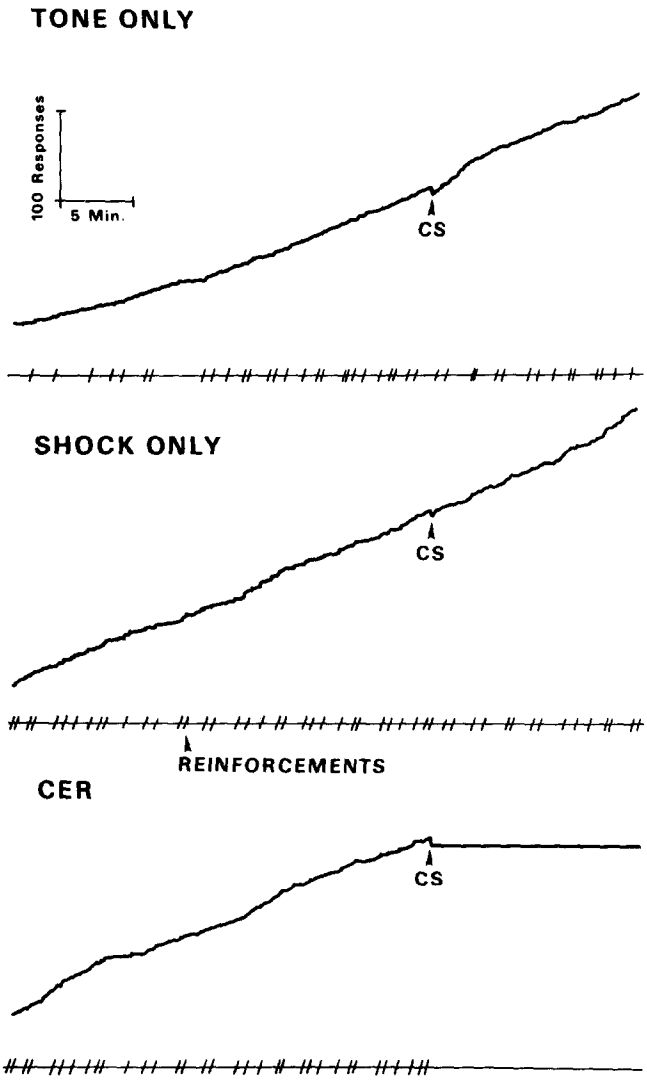

FIG. 2. Cumulative records for VI 1 food reinforced behavior for CER, shock only and tone only littermates. Reinforcements are indicated as deflections of the pen below each record. Thirty minutes into the session, all three littermates were presented with a 15-minute light/tone stimulus, although only the CER animal attended to it as a CS. The CER animal also exhibited bracing, shaking, freezing, urination, defecation, etc.

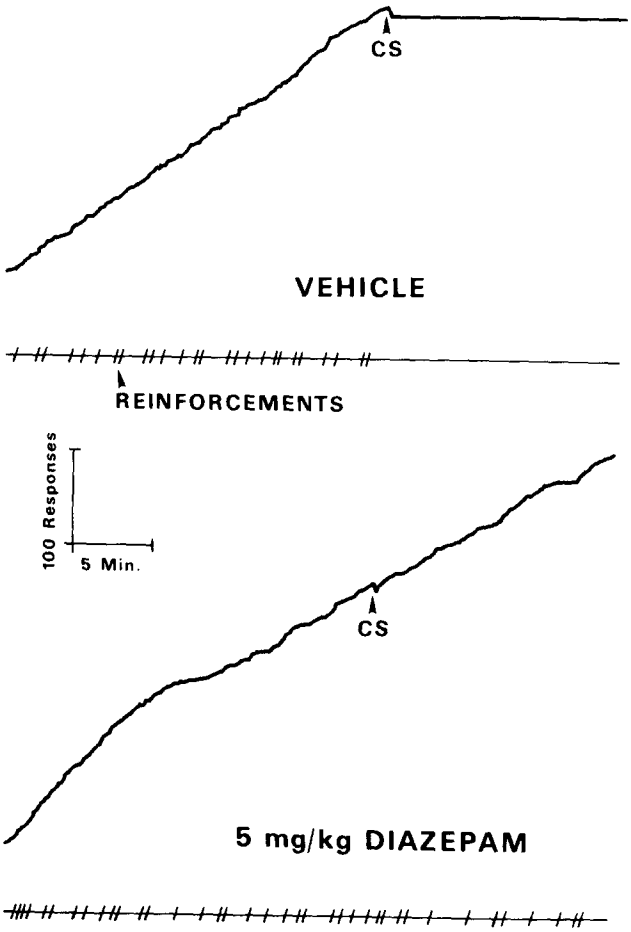

FIG. 3. Cumulative records for two CER conditioned littermates who received a single dose of 5 mg/kg diazepam or vehicle intraperitoneally 30 minutes prior to the VI 1 session. The acute dose of benzodiazepine reversed the suppression, while the vehicle had no effect on CER. There was a consistent dose-response relationship for acute intraperitoneal administration of diazepam: a dose of 2.5 mg/kg produced a response ratio (responses per minute during CS to before CS) of 0.47; higher doses up to 15 mg/kg yielded ratios approaching unity; doses above 15 mg/kg produced ataxia, and eventually stupor.

direct comparison of the CER and tone only groups was not considered in this investigation. These changes are assumed to be serendipitous, although analysis of these changes in conjunction with turnover measurements may later prove valuable. The overall absence of changes in content suggest that the behavioral manipulation is within normal physiological limits which utilize functional stores of each neurotransmitter.

Utilization of Glycine

The turnover of glycine in the forebrain was consistently lower than the other amino acids and changes in glycine related to the paradigm were very few—data not shown. Significant changes ($p<0.05$) in turnover of glycine with respect to conditioning (CER versus shock only) were limited to decreases of 62% in the motor-somatosensory cortex and 88% in the globus pallidus; and an increase of 800% in the caudate-putamen. Significant changes in turnover of glycine with respect to shock history (shock only versus tone only) were limited to decreases of 63% in the motor-somatosensory cortex, 68% in the nucleus accumbens and 97% in the caudate-putamen; and an increase of 36% in the globus pallidus. These few changes support the premise that glycine is primarily a neurotransmitter in the spinal cord [1] with limited evidence for its function in the forebrain [1,24].

Utilization of Aspartate, Glutamate and GABA

The turnover of these amino acids yielded rate constants of between 5 and 189 percent-per hour (Fig. 5). Two major comparisons of the turnover of aspartate, glutamate and GABA were conducted: an analysis of the conditioning component as a measure of emotionality (CER versus shock only, animals with equivalent shock history); and an analysis of shock history (shock only versus tone only). The entire conditioning and training protocol was routinely 33 days, and

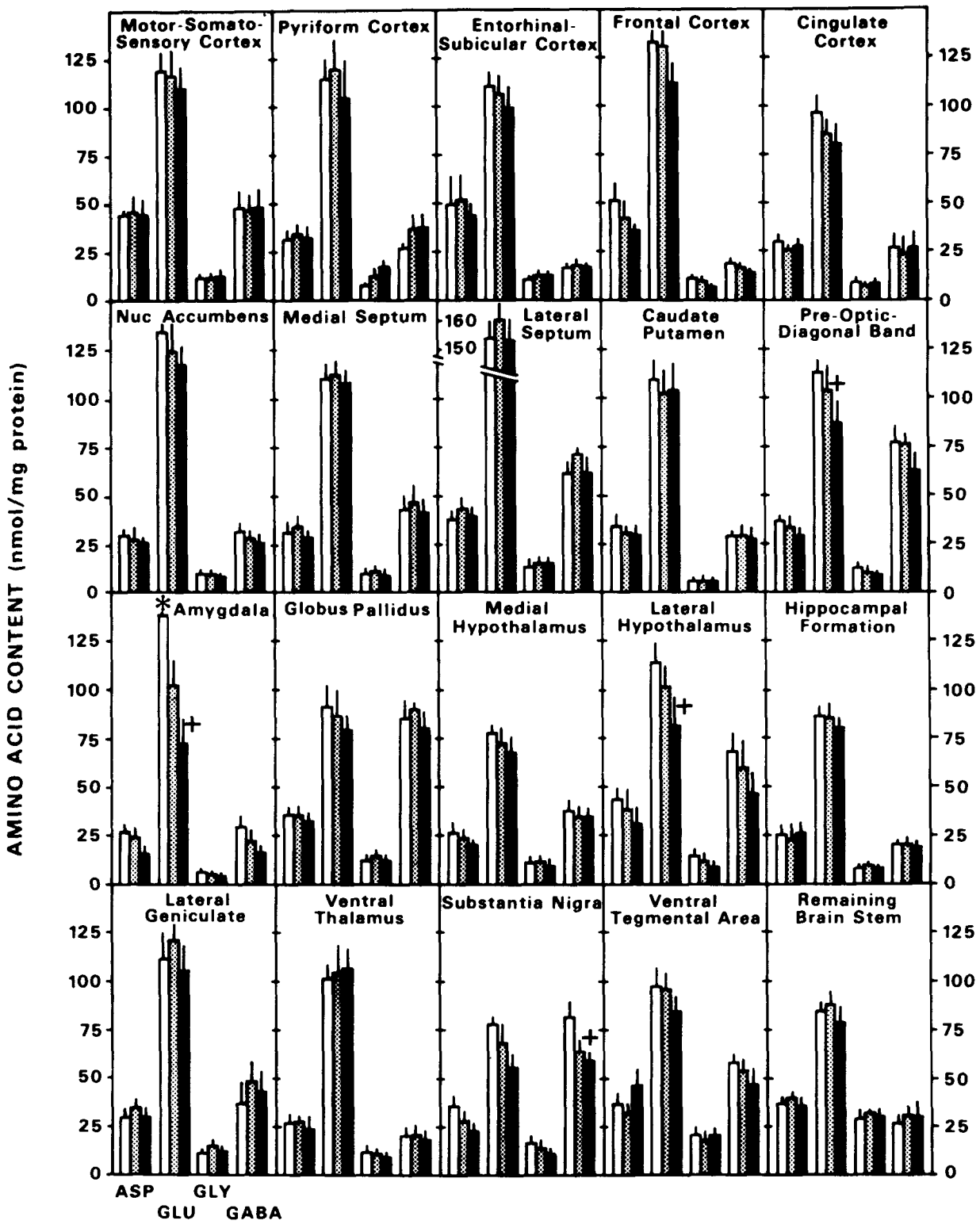

FIG. 4. The content of aspartate, glutamate, glycine and GABA in twenty discrete regions of the brains of rats receiving CER (clear), shock only (stippled) or tone only (dark) conditioning/training. Each block illustrates the groups of the three conditions for the four amino acids—left-to-right Asp, Glu, Gly and GABA—refer to lateral geniculate area in lower left corner. Data represent the Mean±S.D., N=13 observations per bar. (*) indicates a statistically significant ($p<0.05$) change between CER and shock only groups; (+) indicates a change between CER and tone only groups.

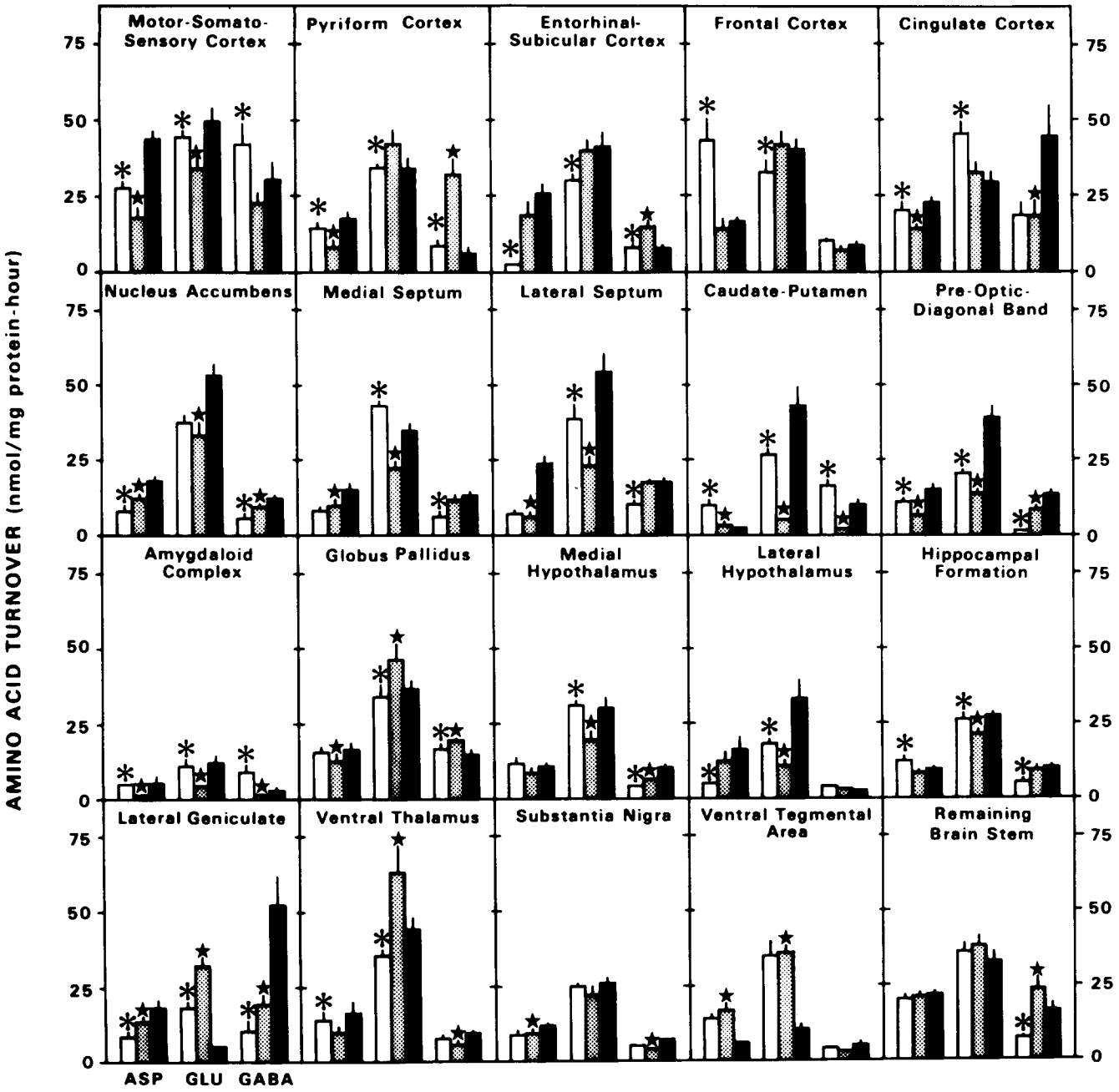

FIG. 5. The rates of utilization (turnover) of aspartate, glutamate and GABA in twenty discrete regions of the brains of rats receiving CER (clear), shock only (stippled) or tone only (dark) conditioning/training. Data represent Mean±S.D., N=13 observations per bar. (*) indicates a statistically significant ($p<0.01$) change between CER and shock only groups; (★) indicates a change between shock only and tone only groups.

only during days 23 to 27 (the conditioning sessions) did the protocols for the three animals vary (Fig. 1). This design was used to minimize effects of artifacts such as presentation of a novel stimulus on test day (to the tone only animal) or recent presentation of footshock (VI 1 schedules had stabilized and returned to normal rates of responding); and to maximize the probability of detecting changes resulting from the subtle behavioral manipulations.

The persistent effects of the aversive environment. The shock only animals were subjected to five sessions of inescapable, unavoidable footshock (36 total shocks—refer to Method and Fig. 1) in the same respective chambers where these animals customarily performed on a daily VI 1 schedule for food reinforcement. By test day 33, VI 1 performance had stabilized, but numerous statistically significant ($p<0.01$) changes in the turnover of aspartate, glutamate and GABA were observed with respect to shock history (shock versus tone animals). The utilization (turnover)

of aspartate decreased 50% in the pyriform, 59% in the motor-somatosensory, and 26% in the cingulate cortices, 26% in the nucleus accumbens, 39% in the medial septum, 74% in the lateral septum, 52% in the pre-optic diagonal band region, 76% in the amygdala, 22% in the globus pallidus, 29% in the lateral geniculate region (dorsal thalamus), 47% in the ventral thalamus and 28% in the substantia nigra; and increased 103% in the caudate-putamen and 54% in the ventral tegmental area.

The utilization (turnover) of glutamate decreased 31% in the motor-somatosensory cortex, 36% in the nucleus accumbens, 37% in the medial septum, 58% in the lateral septum, 88% in the caudate-putamen, 64% in the preoptic-diagonal band region, 54% in the amygdala, 33% in the medial hypothalamus, 67% in the lateral hypothalamus and 20% in the hippocampus; and increased 20% in the globus pallidus, 475% in the lateral geniculate area, 53% in the ventral thalamus and 273% in the ventral tegmental area.

The utilization (turnover) of GABA decreased 58% in the cingulate cortex, 25% in the nucleus accumbens, 86% in the caudate-putamen, 38% in the preoptic-diagonal band region, 56% in the amygdala, 36% in the medial hypothalamus, 63% in the lateral geniculate area, 40% in the substantia nigra and 47% in the ventral tegmental area; and increased 461% in the pyriform cortex, 103% in the entorhinal-subicular cortex, 39% in the globus pallidus and 41% in the brain stem.

Of sixty potential changes in turnover, there were 32 decreases, 10 increases and 18 showed no change; and although the changes were widespread, the utilization of amino acid neurotransmitters varied in an orderly manner to this aversive environment. The lingering association of the operant chamber with previously administered unsignaled, unavoidable, inescapable footshock resulted in many changes in the utilization rates of neurotransmitters in two groups with essentially identical VI 1 food-reinforced performance. There may be an emotional component of this behavior which is not revealed by the VI 1 performance (Fig. 2), e.g., the operant chamber may represent a pseudo-CS to the shock only animals.

The neurochemistry of the conditioning component. The comparison of the CER versus shock only animals yielded two forms of behavioral information related to the neurochemistry—the emotionality of the paradigm and a classical conditioning component. For this presentation, these will be considered together, although the behavior could also be evaluated in terms of a classical conditioning response to an aversive unconditioned stimulus producing the anxiety. Many significant ($p<0.01$) changes were observed.

The utilization (turnover) of aspartate decreased 86% in the entorhinal-subicular cortex, 28% in the nucleus accumbens, 60% in the lateral hypothalamus and 37% in the lateral geniculate area; and increased 221% in the frontal cortex, 80% in the pyriform cortex, 53% in the motor-somatosensory cortex, 50% in the cingulate cortex, 197% in the caudate-putamen, 58% in the preoptic-diagonal band region, 304% in the amygdala, 45% in the hippocampus and 54% in the ventral thalamus.

The utilization (turnover) of glutamate decreased 23% in the frontal cortex, 18% in the pyriform cortex, 25% in the entorhinal-subicular cortex, 24% in the globus pallidus, 41% in the lateral geniculate area and 47% in the ventral thalamus; and increased 24% in the motor-somatosensory cortex, 32% in the cingulate cortex, 94% in the medial septum, 70% in the lateral septum, 404% in the caudate-putamen, 49% in the preoptic-diagonal band, 112% in the amygdala, 52% in both areas of hypothalamus and 22% in the hippocampus.

The utilization (turnover) of GABA decreased 73% in the pyriform cortex, 40% in the entorhinal-subicular cortex, 39% in the nucleus accumbens, 44% in the medial septum, 39% in the lateral septum, 89% in the preoptic-diagonal band region, 14% in the globus pallidus, 51% in the medial hypothalamus, 45% in the hippocampus, 44% in the lateral geniculate area and 68% in the brain stem; and increased 83% in the motor-somatosensory cortex, 366% in the caudate-putamen and 280% in the amygdala.

Of sixty potential changes, there were 22 increases, 21 decreases and 17 that showed no change. The changes in utilization of aspartate and glutamate were primarily increases and the changes in utilization of GABA were increases in some motor-related structures and decreases in most limbic structures. Some of these observations may reflect differences in motor activity in the two groups. The shock only animals maintained normal VI 1 responding, while the CER animals were suppressed and engaged in other behaviors. The persistent effects of the aversive environment were generally reflected by a down regualtion of excitatory and inhibitory neurotransmitter function, while classical conditioning/anxiety produced a more varied and individual neurotransmitter response.

Both shock history (shock only versus tone only groups) and conditioning-emotion (CER versus shock only groups) had profound effects on the turnover of Asp, Glu and GABA in multiple brain areas. These relationships are summarized in Table 2. The 57 comparisons (the septum was considered whole) could be divided into five categories: no effects attributed to CER or shock, a single effect attributed to either CER or shock exclusively, a compensatory up/down relationship, or an enhancement by CER of the effect attributed to shock. Only six brain areas had no response to CER or shock related to amino acid turnover. CER enhanced the effects attributed to shock in seven areas, of which the four changes in GABA turnover were further reductions. Twelve responses were attributed exclusively to CER and ten responses were attributed exclusively to shock history. The remaining changes (21 of 57) were compensatory, i.e., a down-regulation related to shock was overcome by the effect of CER, or vice versa. Of these observations, a majority of the changes were decreases in the turnover of Asp and Glu attributed to shock, which were reversed by CER.

DISCUSSION

Assessment of Amino Acid Neurotransmitter Utilization

Amino acids probably act as neurotransmitters at a majority of all synapses in the CNS [6,24], therefore an analysis of an animal model of anxiety was initiated to evaluate the role of amino acid neurotransmitters in this paradigm. Changes in content of the amino acids were few (Fig. 4), suggesting that shock history and classical conditioning manipulated small physiologically functional pools of amino acid neurotransmitters whose fluctuations were not detectable. Therefore a further evaluation of amino acid turnover was undertaken (Fig. 5). This was based on previously published approaches for evaluation of biogenic amines [24, 35, 40–42, 56].

Interpretation of biogenic amine turnover presented in most reports is straight forward, albeit simplified, since it disregards potential compartmentation of the neurotransmitters. In most instances where biogenic amines appear to

TABLE 2

RELATIONSHIPS BETWEEN CONDITIONING-EMOTION OR SHOCK HISTORY AND THE UTILIZATION OF AMINO ACIDS

	Asp Turnover			Glu Turnover			GABA Turnover		
Brain Area	CER*	Shock†	Category‡	CER*	Shock†	Category‡	CER*	Shock†	Category‡
Frontal Cortex	↑	–	A	↓	–	A	–	–	–
Pyriform Cortex	↑	↓	C	↓	–	A	↓	↑	C
Motor-Somatosensory Cortex	↑	↓	C	↑	↓	C	↑	–	A
Entorhinal-Subicular Cortex	↓	–	A	↓	–	A	↓	↑	C
Cingulate Cortex	↑	↓	C	↑	–	A	–	↓	B
Nucleus Accumbens	↓	↓	D	–	↓	B	↓	↓	D
Septum	–	↓	B	↑	↓	C	↓	–	A
Caudate-Putamen	↑	↑	D	↑	↓	C	↑	↓	C
Preoptic-Diagonal Band	↑	↓	C	↑	↓	C	↓	↓	D
Amygdala	↑	↓	C	↑	↓	C	↑	↓	C
Globus Pallidus	–	↓	B	↓	↑	C	↓	↑	C
Medial Hypothalamus	–	–	–	↑	↓	C	↓	↓	D
Lateral Hypothalamus	↓	–	A	↑	↓	C	–	–	–
Hippocampus	↑	–	A	↑	↓	C	↓	–	A
Lateral Geniculate	↓	↓	D	↓	↑	C	↓	↓	D
Ventral Thalamus	↑	–	A	↓	↑	C	–	↓	B
Substantia Nigra	–	↓	B	–	–	–	–	↓	B
Ventral Tegmental Area	–	↑	B	–	↑	B	–	↓	B
Brain Stem	–	–	–	–	–	–	↓	↑	C

↑=increased turnover; ↓=decreased turnover; –=no change.

*CER versus shock only groups.

† Shock only versus tone only groups.

‡ Categories of relationships: A=related to CER exclusively; B=related to shock history exclusively; C=compensatory up- or down-regulation when comparing conditioning-emotion versus shock history; D=conditioning-emotion enhances the effects of shock history.

serve important functions, the disappearance of radiolabel from one open compartment is used to evaluate rates of turnover [25, 40, 56]. The apparent fractional rate constants obtained with this assumption generally range from 20 to 100 percent-per hour, and are consistent with many previous reports. Unfortunately, similar handling and interpretation of amino acid utilization is complicated by several factors, which must be addressed by four assumptions: (a) It has been reasonably well established that the carbon flux (from the precursor glucose) labels neuronal stores of amino acids, specifically pool(s) which are readily releasable in the presence of depolarizing stimuli [10, 11, 33, 51]. This labelling procedure would be preferentially labelling neuronal pools of amino acids, although it does not distinguish intraneuronal compartmentation. This may be a moot point in the forebrain, where a majority of all synapses release amino acids such as Glu, Asp, Gly and GABA, strongly suggesting that a majority of the amino acid pool(s) are dedicated to neurotransmitter function (this argument is well illustrated by the eleven-fold differential distribution of Gly in the dorsal root and the ventral gray of the spinal cord, where Gly functions as an inhibitory transmitter [1]). Therefore, when large pool(s) of Asp, Glu, Gly and GABA are labelled from glucose, the contributions of these amino acids to ancillary metabolic or synthetic functions are probably small and can be ignored in computation without resulting in significant error. (b) This interpretation does not rule out the possibility that observations of increased or decreased turnover represent a generalized increase or decrease in neuronal activity. If this is the case, those groups of neurons which utilize the neurotransmitter would be identified, because each group would reflect the dominant content of the respective amino acids. (c) The times 60 and 90 minutes post-injection were selected because they represent the linear portion(s) of the decay curves in radioactivity (Fig. 6), because they can be coordinated with the administration of monoamine precursors, and because they insure that the animal is in no way stressed nor artificially stimulated during ongoing behavior in the CER paradigm. Use of these extended time points raises a question as to whether neurotransmitter utilization is being monitored. Alternative methods have been proposed by other investigators. During very short time points after ^{13}C-glucose administration, a very high flux of label from glucose into Glu and GABA was observed [5], and turnover rates were calculated on the first seven minutes post-injection [35]. There was a trend toward decreasing fractional rate constants and this was attributed to the progressive recycling of radiolabel through the amino acids. This is very likely occurring in the present study, but does not necessarily reduce the usefulness of the observations. Even with recycling of radiolabel, the flux through the amino acids is a useful measure for deriving an apparent rate constant. Any up- or down-regulation of neuronal acitivty would merely accelerate or diminish the contributions of recycling,

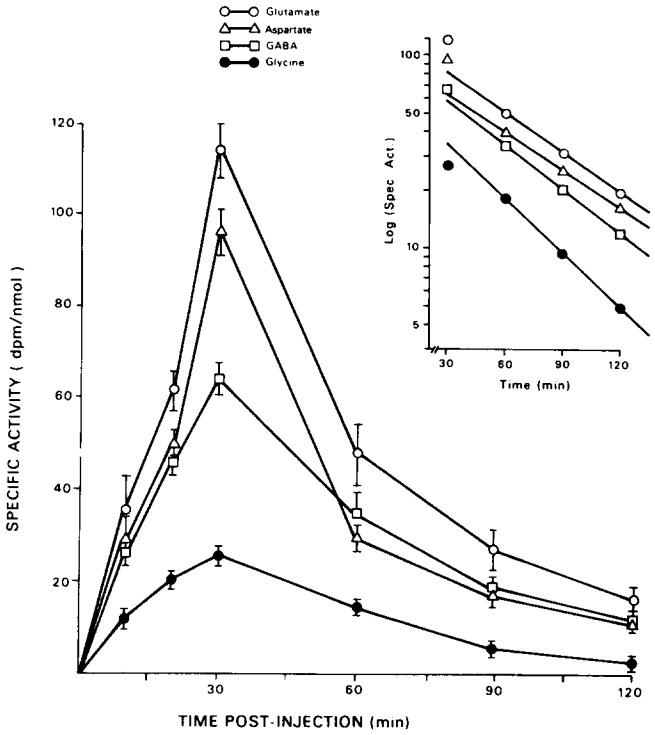

FIG. 6. The time course for flux of ^{14}C-radiolabel from an intravenous pulse of 0.2 mCi D-[U-^{14}C]-glucose through the putative amino acid neurotransmitters in rat cerebral cortex. The large plot shows the temporal specific activities (Mean±S.D., N=5 for each time point) and the smaller plot shows the mean values plotted in a logarithmic fashion. The decline in specific acitivities is log-linear over a time course of from 40 to 150 minutes post-injection.

and thus not effect the interpretation. (d) Despite the latter three concerns, these comparisons are always conducted within a stringent protocol, and therefore, observed differences likely reflect bonafide changes in turnover with respect to manipulation (CER versus shock only; shock only versus tone only). By weighing these constraints, the utilization of putative amino acid neurotransmitters can be monitored in discrete CNS regions after behavioral and/or pharmacological manipulation with reasonable confidence. Generally this approach is predicated on increased turnover representing increased neuronal activity irrespective of the inherent excitatory or inhibitory character of the neurotransmitter.

Origin and Termination of Neuronal Pathways Which Utilize Amino Acids

A growing number of pathways and intrinsic neurons have been identified, characterized, and demonstrated to utilize amino acid neurotransmitters. There now exists evidence to support the following: Many areas of the cerebral cortex utilize GABA in intrinsic neurons, such as the aspinous stellate cells in the visual cortex [6, 14, 36]. The frontal cortex sends glutamatergic efferents to other frontal and pyriform cortical areas, the thalamus and the caudate-putamen [12, 14, 30]. The entorhinal perforant pathway sends glutamatergic efferents through the subiculum to the hippocampus [14, 45, 55]. The nucleus accumbens utilizes GABA, aspartate and glutamate in intrinsic neurons [54] and sends GABAergic efferents to the globus pallidus and ventral tegmental area [53]. The dorsolateral septum utilizes GABA in intrinsic neurons, which are regulated by glutamatergic input from CA3 pyramidal cells of the hippocampus [28]. The caudate-putamen utilizes GABA and glutamate in intrinsic neurons [13] and sends GABAergic efferents to the globus pallidus [13] and substantia nigra [13,17]. The globus pallidus sends GABAergic efferents to the substantia nigra [13]. The hippocampus utilizes aspartate and glutamate in intrinsic neurons (glutamate in mossy fibers) [34,45] and GABA in the intrinsic basket cells [45] and sends glutamatergic and aspartergic efferents to the septum and hypothalamus [28,46]. Glutamate is the putative transmitter of Shaffer's collaterals which innervate the septum [14,28]. The hippocampus sends glutamatergic efferents to the subiculum [45,46], lateral septum [14,45], nucleus accumbens [14, 24, 45], and mammillary bodies of the hypothalamus [14,45]. In addition, the interhippocampal commisural fibers utilize glutamate and aspartate [14, 34, 44, 45]. GABAergic efferents innervate the habenula via the stria medullaris [14]. The substantia nigra utilizes glutamate, GABA and perhaps glycine in intrinsic neurons [14, 15, 17]. GABAergic efferents also innervate the cerebello-vestibular and cuneate nuclei systems [17]. Since amino acids are neurotransmitters at many synapses in the CNS [1, 6, 14, 15, 17, 23, 45], it seems reasonable to presume that glutamate, aspartate and GABA function in these aforementioned and additional structures to mediate or respond to behaviors. In addition, these pathways are intimately related to neuromodulatory pathways which utilize acetylcholine [19], biogenic monoamines (discussed elsewhere [26]) and peptides. With these characterized neuronal interconnections in mind, the observations concerning utlization of aspartate, glutamate and GABA (Fig. 5) can be considered further.

The consequences of shock history. The comparison of the shock only groups with the tone only groups revealed the persistence of the effects of an aversive environment (although footshock was previously delivered in the same chamber, these conditioning sessions had been terminated at least five days before test day). A history of footshock yielded a decreased utilization of aspartate, glutamate and GABA in many instances (Fig. 5—30 of 40 changes were decreases). Since detailed behavioral and neurobiological studies (effects of lesions, etc.) of shock history have not been completed to date in the context of this investigation, not all of the changes with respect to brain areas can be assessed or explained at this time. However, many changes are consistent with the proposed neurotransmitter function of the amino acids. These observations demonstrate the importance of amino acid connections between the frontal cortex, nucleus accumbens, septum, striatum and hippocampal formation in responding to the aversive environment (primarily in the direction of down-regualtion). There are probably anxiety-provoking components to this behavior (e.g., chamber as a pseudo-CS) which cannot be assessed by VI 1 performance in the paradigm. However, these many changes, in groups of animals with essentially identical VI 1 performance, should be given credence in the design of future experiments.

The components of conditioning and emotion. Since several studies of classical conditioning and CER have been reported, this comparison can be restricted to the areas of interest: the hippocampus [3, 49, 50], septum [4,18], caudate [20], frontal cortex [31,37], amygdala [31, 43, 49, 50],

mesencephalic reticular formation [37, 49, 50], lateral geniculate nuclei [37, 49, 50], hypothalamus [49,50] and raphé nuclei [48]. Lesions of, or intracranial drug injections into, these areas have been demonstrated to disrupt CER or classical conditioning; and extracellular recordings in these areas are demonstrated to correlate with CS presentation in analogous conditioning paradigms. Twenty-seven of 41 observed changes (Fig. 5) involved these structures when comparing the CER group and the shock only group. Unlike the former comparison of shock history, a majority of these changes in the utilization of the excitatory transmitters, aspartate and glutamate, were increases. The changes in GABA utilization were predominantly decreases in limbic structures. Again, not all changes can be explained, but several changes are consistent with the proposed neurotransmitter function of the amino acids. These include: Asp in intrinsic interneurons of the frontal-pyriform cortex, nucleus accumbens, caudate-putamen and hippocampus; Glu in intrinsic interneurons of the frontal-pyriform cortex; two Glu pathways, originating in the frontal cortex, which terminate in the caudate-putamen and ventral thalamus; two Glu pathways, originating in the hippocampus, which terminate in the septum and entorhinal-subicular cortex; a Glu pathway, originating in the entorhinal-subicular cortex, which terminates in the hippocampus; GABA in intrinsic interneurons of the frontal-pyriform and entorhinal-subicular cortices, nucleus accumbens, septum, caudate-putamen, and hippocampus; a GABA pathway, originating in the nucleus accumbens, which terminates in the globus pallidus; and a GABA pathway, originating in the caudate-putamen, which terminates in the globus pallidus.

Acetylcholine [19], monoamines (discussed elsewhere [26]) and peptides are utilized as neuromodulators in the aforementioned CNS areas, and are likely important in these behaviors. Changes in aspartate and GABA utilization in the amygdala, ventral thalamus, hypothalamus, lateral geniculate and brain stem (all areas implicated in CER or classical conditioning—see above) may represent the actions of intrinsic neurons. The changes in glutamate utilization in the amygdala, hypothalamus and lateral geniculate may be explained by heretofore unidentified efferents which impinge on these areas. Changes in the utilization of all three amino acids in the other cortical regions and the preoptic-diagonal band region suggest that additional CNS areas may play important roles in mediating or responding to the behavioral paradigm. These observations demonstrate the importance of the frontal cortex, nucleus accumbens, septum, striatum, amygdala and hippocampal formation in conditioning and emotion.

The Relationship Between Anxiety, Anxiolytics and Neurotransmitters

The CER paradigm is sensitive to the acute administration of diazepam (Fig. 3). Benzodiazepines are known to effect the CNS turnover of acetylcholine, catecholamines, serotonin and GABA in behaviorally naive animals [8, 17, 29, 47, 48]; benzodiazepines facilitate GABAergic transmission [8, 17, 47]; and benzodiazepines and GABA agonists have similar effects on CER and analogous behavioral paradigms [7, 17, 23, 48, 50, 52]. It seems reasonable that an animal model of anxiety (CER) and its attenuation by anxiolytics would be responsive to or mediated by classical neurotransmitters such as aspartate, glutamate and GABA. The administration of muscimol and diazepam reduce the turnover of GABA in the caudate-nucleus and nucleus accumbens, but not in the globus pallidus [8, 17, 29]; therefore, one action of diazepam on the CER paradigm could be to reduce the elevated turnover of GABA in the caudate-nucleus. The actions of anxiolytics in other CNS areas likely involve additional neurotransmitters. [8, 17, 47, 48].

The actions of benzodiazepines are not limited to their anxiolytic properties, i.e., they act as muscle relaxants, anticonvulsants, etc. There is also growing evidence that more than one benzodiazepine receptor exists [23,47]. Therefore, it may be difficult to determine whether, by assessing neurotransmitter utilization in discrete brain regions of behaviorally naive animals, benzodiazepines are acting as anxiolytics or in other ways. The key to studying the differential effects of these agents may rely on their neurochemical action in the appropriate animal model. Accordingly, the analysis of the neurochemical effects of acute diazepam administration in reversing conditioned suppression (CER) and attenuating emotional/anxious behavior is currently being assessed in this laboratory, and should provide information about whether anxiolytics act by reversing the neurochemistry attributed to anxiety, or act through novel neuronal pathways to bring about their response.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. James D. Altazan for excellent assistance and Ms. Shirley Hickox for preparation of the manuscript. This research was supported by N.I.M.H. Grant MH-31835 (to J. D. L.).

REFERENCES

1. Aprison, M. H. Glycine as a neurotransmitter. In: *Psychopharmacology: A Generation of Progress*, edited by M. A. Lipton, A. DiMascio and K. F. Killam. New York: Raven Press, 1978, pp. 333–346.
2. Barchas, J. D. and D. X. Freedman. Brain amines: response to physiological stress. *Biochem. Pharmac.* **12:** 1232–1235, 1963.
3. Berger, T. W. and R. F. Thompson. Neuronal plasticity in the limbic system during classical conditioning of the rabbit nictitating membrane response. I. the hippocampus. *Brain Res.* **145:** 323–346, 1978.
4. Berry, S. D. and R. F. Thompson. Medial septal lesions retard classical conditioning of the nictitating membrane response in rabbits. *Science* **205:** 209–210, 1979.
5. Bertilsson, L., C. C. Mao and E. Costa. Application of principles of steady-state kinetics to the estimation of γ-aminobutyric acid turnover rate in nuclei of rat brain. *J. Pharmac. exp. Ther.* **200:** 277–284, 1977.
6. Bloom, F. E. and L. L. Iversen. Localizing ^{3}H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. *Nature, Lond.* **229:** 628–630, 1971.
7. Cananzi, A. R., E. Costa and A. Guidotti. Potentiation by intraventricular muscimol of the anticonflict effect of benzodiazepines. *Brain Res.* **196:** 447–453, 1980.
8. Costa, E. and A. Guidotti. Molecular mechanisms in the receptor action of benzodiazepines. *A. Rev. Pharmac. Toxicol.* **19:** 531–545, 1979.

9. Crawford, I. L. and J. D. Connor. Localization and release of glutamic acid in relation to the hippocampal mossy fiber pathway. *Nature, Lond.* **244:** 442–443, 1973.
10. Cremer, J. E. Amino acid metabolism in rat brain studied with ^{14}C-labelled glucose. *J. Neurochem.* **11:** 165–185, 1964.
11. DeBelleroche, J. S. and H. F. Bradford. On the site of origin of transmitter amino acids released by depolarization of nerve terminals in vitro. *J. Neurochem.* **29:** 335–343, 1977.
12. Divac, I., F. Fonnun and J. Storm-Mathisen. High affinity uptake of glutamate in terminals of corticostriatal axons. *Nature, Lond.* **266:** 377–378, 1977.
13. Dray, A. The striatum and substantia nigra: a commentary on their relationships. *Neuroscience* **4:** 1407–1440, 1979.
14. Emson, P. C. and O. Lindvall. Distribution of putative neurotransmitters in the neocortex. *Neuroscience* **4:** 1–30, 1979.
15. Estes, W. K. and B. F. Skinner. Some quantitative properties of anxiety. *J. exp. Psychol.* **29:** 390–400, 1941.
16. Freeman, M. E., C. Co, T. R. Mote, J. D. Lane and J. E. Smith. Determination of content and specific activity of amino acids in central nervous system tissue utilizing tritium and carbon-14 dual labelling. *Analyt. Biochem.* **106:** 191–194, 1980.
17. Haefely, W. E. Behavioral and neuropharmacological aspects of drugs used in anxiety and related states. In: *Psychopharmacology: A Generation of Progress,* edited by M. A. Lipton, A. DiMascio and K. F. Killam. New York: Raven Press, 1978, pp. 1359–1374.
18. Harvey, J. A., C. E. Lints, L. W. Jacobson and H. F. Hunt. Effects of lesions in the septal area on conditioned fear and discriminated instrumental punishment in the albino rat. *J. comp. physiol. Psychol.* **59:** 37–48, 1965.
19. Hingtgen, J. N., J. E. Smith, P. A. Shea, M. H. Aprison and T. M. Gaff. Cholinergic changes during conditioned suppression in rats. *Science* **193:** 332–334, 1976.
20. Hirasuna, N., S. A. Deadwyler and E. J. Wyers. Disruption of the conditioned emotional response by caudate nucleus stimulation. *Brain Res. Bull.* **2:** 173–179, 1977.
21. Hunt, H. and J. Brady. Some effects of electro-convulsive shock on a conditioned emotional response ("anxiety"). *J. comp. physiol. Psychol.* **44:** 88–98, 1951.
22. Kamin, L. and R. Schwab. Effects of conditioned stimulus intensity on the conditioned emotional response. *J. comp. physiol. Psychol.* **56:** 502–506, 1963.
23. Klepner, C. A., A. S. Lippa, D. I. Benson, M. C. Sano and B. Beer. Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharmac. Biochem. Behav.* **11:** 457–462, 1979.
24. Krnjevic, K. Chemcial nature of synaptic transmission in vertebrates. *Physiol. Rev.* **54:** 418–540, 1974.
25. Lane, J. D., C. Co and J. E. Smith. Determination of simultaneous turnover of serotonin, dopamine and norepinephrine in the telencephalon of unrestrained behaving rats. *Life Sci.* **21:** 1101–1108, 1977.
26. Lane, J. D., M. P. Sands, C. Co, D. R. Cherek and J. E. Smith. Biogenic monoamine turnover in discrete rat brain regions is correlated with conditioned emotional response and its conditioning history. *Brain Res.,* in press.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193:** 265–275, 1951.
28. Malte-Sorenssen, D., K. K. Skrede and F. Fonnum. Release of d-[^{3}H]-aspartate from the dorsolateral septum after electrical stimulation of the fimbria in vitro. *Neuroscience* **4:** 1255–1263, 1980.
29. Mao, C. C., E. Marco, A. Revuelta, L. Bertilsson and E. Costa. The turnover rate of γ-aminobutyric acid in the nuclei of telencephalon: implications in the pharmacology of antipsychotics and of a minor tranquilizer. *Biol. Psychiat.* **12:** 359–371, 1977.
30. McGeer, P. L., E. G. McGeer, U. Scherer and K. Singh. A glutamatergic cortico-striatal path? *Brain Res.* **128:** 369–373, 1977.
31. McIntyre, D. C. Effects of focal vs generalized kindled convulsions from anterior neocortex or amygdala on CER acquisition in rats. *Physiol. Behav.* **23:** 855–859, 1979.
32. Millensen, J. R. and J. Leslie. The conditioned emotional response (CER) as a baseline for the study of anti-anxiety drugs. *Neuropharmacology* **13:** 1–9, 1974.
33. Minchin, M. C. W. The release of amino acids synthesized from various compartmented precursors in rat spinal cord slices. *Expl. Brain Res.* **29:** 515–526, 1977.
34. Nadler, J. V., W. F. White, K. W. Vaca, B. W. Perry and C. W. Cotman. Biochemical correlates of transmission mediated by glutamate and aspartate. *J. Neurochem.* **31:** 147–155, 1978.
35. Neff, N. H., P. F. Spano, A. Groppetti, C. Wong and E. Costa. A simple procedure for calculating the synthesis rate of norepinephrine, dopamine and serotonin in rat brain. *J. Pharmac. exp. Ther.* **176:** 701–710, 1971.
36. Ribak, C. E. Aspinous and sparsely-spinous stellate neurons contain glutamic acid decarboxylase in the visual cortex of rats. *Neurocytology* **7:** 461–478, 1978.
37. Schwartzbaum, J. S. Interrelationship among multiunit activity of the midbrain reticular formation and lateral geniculate nucleus, thalamocortical arousal and behavior in rats. *J. comp. physiol. Psychol.* **89:** 131–157, 1975.
38. Smith, J. E., J. D. Lane, P. A. Shea, J. N. Hingtgen, W. J. McBride and M. H. Aprison. Levels of eight putative neurotransmitters in the telencephalon of rats receiving shock avoidance training. *Fedn Proc.* **34:** 292, 1975.
39. Smith, J. E., W. R. Leckrone and C. Co. Combination operant conditioning-liquid nitrogen immersion chamber for studying neurotransmitter systems and behavior. *Pharmac. Biochem. Behav.* **7:** 167–173, 1977.
40. Smith, J. E., C. Co and J. D. Lane. Turnover rates of serotonin, norepinephrine and dopamine concurrently measured in seven rat brain regions. *Prog. Neuropsychopharmac.* **2:** 359–367, 1978.
41. Smith, J. E., C. Co, M. E. Freeman, M. P. Sands and J. D. Lane. Neurotransmitter turnover in rat striatum is correlated with morphine self-administration. *Nature, Lond.* **287:** 152–154, 1980.
42. Smith, J. E., C. Co, M. E. Freeman and J. D. Lane. Brain neurotransmitter turnover correlated with morphine-seeking behavior of rats. *Pharmac. Biochem. Behav.,* in press, 1982.
43. Spevack, A. A., C. T. Campbell and L. Drake. Effect of amygdalectomy on habituation and CER in rats. *Physiol. Behav.* **15:** 199–207, 1975.
44. Storm-Mathisen, J. Glutamic acid and excitatory nerve endings: reduction of glutamic acid uptake after axotomy. *Brain Res.* **120:** 379–386, 1977.
45. Storm-Mathisen, J. Localization of transmitter candidates in the brain: the hippocampal formation as a model. *Prog. Neurobiol.* **8:** 119–181, 1977.
46. Storm-Mathisen, J. and M. Waxenopsahl. Aspartate and/or glutamate may be transmitters in hippocampal efferents to septum and hypothalamus. *Neurosci. Lett.* **9:** 65–70, 1978.
47. Tallman, J. F., S. M. Paul, P. Skolnick and D. W. Gallager. Receptor for the age of anxiety: pharmacology of the benzodiazepines. *Science* **207:** 274–281, 1980.
48. Thiebot, M. -H., A. Jobert and P. Soubrie. Conditioned suppression of behavior: its reversal by intra raphé microinjection of chlordiazepoxide and GABA. *Neurosci. Lett.* **16:** 213–217, 1980.
49. Umemoto, M., Y. Marai, M. Kodama and R. Kido. Neuronal discharge patterns in conditioned emotional response. *Brain Res.* **24:** 347–351, 1970.
50. Umemoto, M. and M. E. Olds. Effects of chlordiazepoxide, diazepam and chlorpromazine on conditioned emotional behavior and conditioned neuronal activity in limbic, hypothalamic and geniculate regions, *Neuropharmacology* **14:** 413–425, 1975.

51. Voaden, J. J. and B. Morjara. The synthesis of neuroactive amino acids from radioactive glucose and glutamine in the rat retina: Effects of light stimulation. *J. Neurochem.* **35:** 95–99, 1980.
52. Waddington, J. L. Behavioral evidence for GABAergic activity of the benzodiazepine flurazepam. *Eur. J. Pharmac.* **51:** 417–422, 1978.
53. Waddington, J. L. and A. J. Cross. Neurochemical changes following kainic acid lesions of the nucleus accumbens: implications for a GABAergic accumbal-ventral tegmental pathway. *Life Sci.* **22:** 1011–1014, 1978.
54. Walaas, I. and F. Fonnum. The effects of surgical and chemical lesions on neurotransmitter candidates in the nucleus accumbens of the rat. *Neuroscience* **4:** 209–216, 1977.
55. White, W. F., J. V. Nadler, A. Hamberger, C. W. Cotman and J. T. Cummins. Glutamate as transmitter of hippocampal perforant path. *Nature, Lond.* **270:** 356–357, 1977.
56. Zilversmit, D. B. The design and analysis of isotope experiments. *Am. J. Med.* **29:** 832–848, 1960.