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ALTERATIONS IN BRAIN 5-HYDROXY-TRYPTAMINE METABOLISM DURING THE 'WITHDRAWAL' PHASE AFTER CHRONIC TREATMENT WITH DIAZEPAM AND BROMAZEPAM

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1 Daily administration of diazepam or bromazepam (10 mg/kg) for 22 days significantly increased the activity of mid-brain tryptophan hydroxylase by 36% and 39%, respectively. The concentration of tryptophan was also enhanced in the mid-brain region of rats subjected to benzodiazepine treatment.

2 Chronic therapy with either of the two anti-anxiety agents enhanced the endogenous levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in cerebral cortex, hypothalamus, pons-medulla, mid-brain and striatum.

3 Whereas diazepam treatment decreased (13%) the activity of monoamine oxidase in mid-brain, bromazepam failed to exert any effect, suggesting that the observed elevation in 5-hydroxy-indoleacetic acid levels is not associated with enhanced deamination of 5-hydroxytryptamine.

4 Discontinuation of treatment for 48 h significantly decreased the activity of mid-brain tryptophan hydroxylase to levels that were significantly lower than those seen for benzodiazepine-treated and normal rats. The concentrations of mid-brain tryptophan and 5-hydroxytryptamine were also reduced in various brain regions examined.

5 Withdrawal from diazepam or bromazepam therapy further augmented the levels of brain 5-hydroxyindoleacetic acid.

6 The results demonstrate that the depressant effects on behaviour of these agents are accompanied by increased metabolism of 5-hydroxytryptamine in the brain. Withdrawal from these minor tranquillizers, on the other hand, reduces the synthesis of this indoleamine.

Introduction

Considerable attention has recently been focussed on the role of 5-hydroxytryptamine (5-HT) in certain behavioural states (Sheard, 1969; Redmond, Mass., Kling, Graham & Keirmenzian, 1971; Boelkins, 1973). Indeed, evidence supports the notion that changes in this important indoleamine are involved in sleep processes (Jouvet, 1969) and anxiety states (Wise, Berger & Stein, 1970). In addition, brain 5-HT has been implicated in the control of seizures in animals (Jenner, Chadwick, Reynolds & Marsden, 1975). Benzodiazepines represent a group of compounds that are extensively used as sedatives, hypnotics, central muscle relaxants, antiepileptic, pre-anaesthetic and antianxiety agents. However, the beneficial effects of benzodiazepines in anxiety states are temporary and continuous administration of the drugs over a prolonged period may be required. Furthermore, psychophysiological studies have shown that upon cessation of therapy, there is a clinically observable return of previous anxiety levels (Rastogi, Lapierre & Singhal, 1976c; Lapierre, unpublished data). Of greater interest is the finding that in certain cases, the post-drug anxiety was even greater than the pre-treatment level which would seem to be in line with the 'rebound' phenomenon noted by Borland & Nicholson (1975) following discontinuation of nitrazepam treatment.

Despite the profound influence of benzodiazepines on the central nervous system, no unified concept is yet available that could account for various pharmacological effects of these drugs by a specific action on a given neuronal system. Recently, chronic administration of diazepam or bromazepam was found to decrease spontaneous locomotor activity and the turnover of noradrenaline and dopamine. These changes were accompanied by decreased activity of tyrosine hydroxylase and increased levels of the catecholamines in hypothalamus, pons-medulla, mid-brain and striatum (Rastogi, Lapierre & Singhal, 1976b). The levels of striatal homovanillic acid (HVA), the chief metabolite of dopamine, were significantly decreased in rats treated chronically with either diazepam or bromazepam. Catecholaminergic projections in the brain as well as the 5-

hydroxytryptaminergic system are diffuse systems linking together many cerebral structures. Since available evidence indicates involvement of 5-HT in behavioural manifestations of anxiety as well as sleep processes (Jouvet, 1969; Stein, Wise & Belluzi, 1975), the present study was undertaken to examine the effects of chronic treatment with diazepam and bromazepam on the activity of tryptophan hydroxylase (TPH) as well as the levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in certain discrete brain regions. In order to elucidate the neurochemical basis of the 'rebound' phenomenon seen during the withdrawal phase after repeated exposure to benzodiazepines, the influence of 48 h discontinuation of treatment also was studied on the biosynthetic capacity of 5-hydroxytryptaminergic systems.

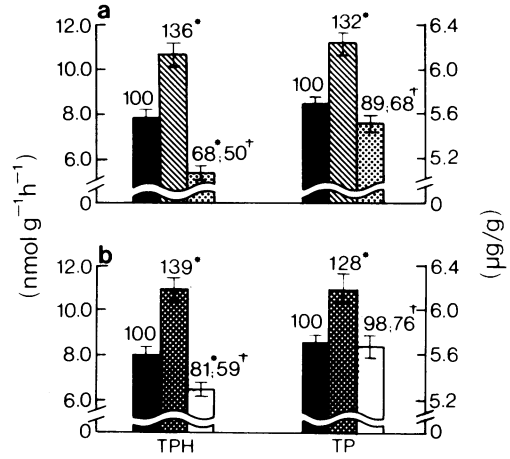

Figure 1 Effect of (a) chronic diazepam, (b) bromazepam and 48 h withdrawal on mid-brain tryptophan hydroxylase (TPH) and tryptophan (TP). Each column represents the mean of 6 rats in the group. Vertical lines show s.e means. Animals were injected daily with diazepam or bromazepam (10 mg/kg) subcutaneously for 22 days and killed 6 h after the last injection. Groups of animals treated with either drug for 20 days were subsequently withdrawn for 48 h ('withdrawn' rats). Numbers above columns express results as percentages, taking the values of control rats as 100%. Solid columns = control rats; hatched columns = diazepam-treated rats; stipplied columns = diazepam withdrawn rats; cross hatched columns = bromazepam-treated rats; open columns = bromazepam withdrawn rats.

* Statistically significant difference when compared with the values of normal control rats ($P < 0.05$).

† Statistically significant difference when compared with the corresponding values of diazepam- or bromazepam-treated rats ($P < 0.05$).

Methods

Albino male Sprague-Dawley rats (100–115 g) were housed in a group of 6 per cage and exposed to regular alternate cycles of 12 h light and darkness under constant environmental conditions (24°C, 60% relative humidity). The animals had access to Master Laboratory Chow and water *ad libitum*.

In the first series of experiments, animals were injected subcutaneously (10 mg kg^{-1} day^{-1}) with diazepam or bromazepam dissolved in propylene glycol, in a fixed volume of 0.2 ml/100 g for a period of 22 days. Control rats received an equal volume of propylene glycol and animals were killed 6 h after the last injection of the drug or the vehicle. In another series, groups of rats which had been exposed either to diazepam or bromazepam for 20 days were maintained without any treatment for an additional period of 48 h when the animals were killed for biochemical assays.

Sample preparation and biochemical assays

Rats were killed between 09 h 30 min and 12 h 30 min by the 'near-freezing' technique of Takahashi & Aprison (1964). Following decapitation, the brain was dissected into five different regions (cerebral cortex, pons-medulla, hypothalamus, mid-brain and striatum) according to the procedure of Glowinski & Iversen (1966). To determine the activity of TPH and monoamine oxidase (MAO), the mid-brain was homogenized in 20 volumes of 0.28 M sucrose. The assay for TPH was carried out under linear kinetic conditions according to the procedure of Peters, McGeer & McGeer (1968) with minor modifications as described earlier (Rastogi & Singhal, 1974). The concentration of tryptophan was determined in mid-brain according to the procedure of Hess & Udenfriend (1959). The activity of MAO was assayed according to the method of Wurtman & Axelrod (1963) as described in an earlier study (Rastogi, Lapierre & Singhal, 1976a). In order to determine the levels of 5-HT and 5-HIAA, the brain tissue was extracted and assayed according to the procedure of Curzon & Green (1970).

Data were analysed for significance of differences by Student's *t* test.

Results

Effects of chronic benzodiazepine treatment and subsequent withdrawal on mid-brain tryptophan hydroxylase activity and tryptophan levels

Data shown in Figure 1 demonstrate that repeated exposure of rats to diazepam and bromazepam for 22

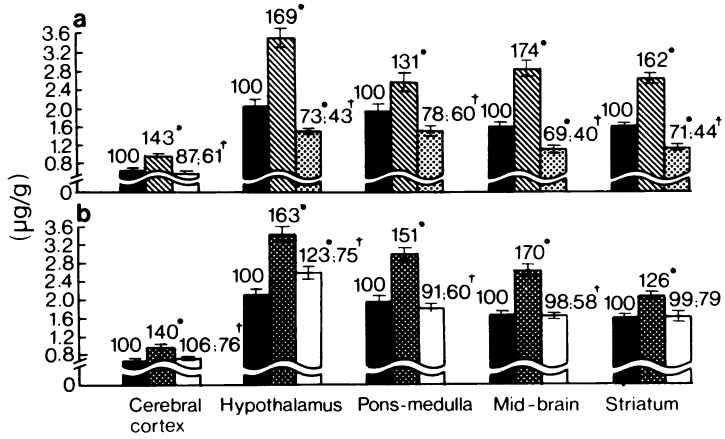

Figure 2 Effect of (a) chronic diazepam, (b) bromazepam and 48 h withdrawal on 5-hydroxytryptamine concentrations in certain discrete brain regions of rats. Each column represents the mean of 6 rats in the group. Vertical lines show s.e. means. Solid columns = control rats; hatched columns = diazepam-treated rats; stippled columns = diazepam withdrawn rats; cross hatched columns = bromazepam-treated rats; open columns = bromazepam withdrawn rats. For other details see legend to Figure 1.

days significantly elevated the activity of mid-brain TPH to 136% and 139% of the normal values, respectively. Since brain 5-HT synthesis may be controlled by the availability of dietary tryptophan, the concentration of this amino acid also was examined in control as well as in rats subjected to chronic benzodiazepine therapy. As seen in Figure 1, the levels of brain tryptophan were also significantly increased following treatment with either of these anxiolytic drugs. Upon cessation of therapy for 48 h, the activity of TPH was reduced to the values that were significantly lower than those seen for the corresponding 'treated' and normal controls. The tryptophan concentration in mid-brain of rats withdrawn from diazepam and bromazepam treatment also was significantly reduced by 32% and 24% respectively, the values were, however, statistically indistinguishable from those of normal animals.

Changes in 5-hydroxytryptamine concentrations of certain discrete brain regions

Administration of diazepam or bromazepam for 22 days increased the steady state levels of 5-HT in all regions of the brain examined (Figure 2). In the case of diazepam, the maximal rise was observed in mid-brain, hypothalamus and striatum. Discontinuation of diazepam treatment for 48 h significantly reduced 5-HT concentrations in all regions when compared with the values of the 'treated' group. In hypothalamus, mid-brain and striatum, 5-HT concentrations were significantly lower than those seen for even normal rats. Treatment with bromazepam considerably increased 5-HT concentrations in pons medulla also. Withdrawal from bromazepam lowered the 5-HT concentration to normal limits except in the hypothalamus where it was still significantly higher than the normal values.

Effects on 5-hydroxyindoleacetic acid concentrations

As with 5-HT, the concentration of 5-HIAA was significantly increased in virtually all regions of the brain studied in rats exposed chronically to diazepam or bromazepam (Figure 3). The rise in this brain indoleamine metabolite was more pronounced in animals receiving bromazepam. Withdrawal for 48 h following 20 days of benzodiazepine therapy further augmented the levels of 5-HIAA in several regions of the brain. Again, the magnitude of change was somewhat greater in rats exposed to bromazepam in all regions examined, except striatum.

Changes in mid-brain monoamine oxidase activity

In order to examine whether the observed rise in 5-HIAA was associated with an enhancement in the activity of the deaminating enzyme MAO, the influence of chronic treatment with diazepam or bromazepam as well as subsequent withdrawal was investigated on this metabolizing enzyme in mid-brain. Data in Table 1 show that treatment of rats with diazepam slightly (13%) but significantly reduced the activity of mid-brain MAO. Upon withdrawal from this minor tranquillizer, the level of this enzyme was

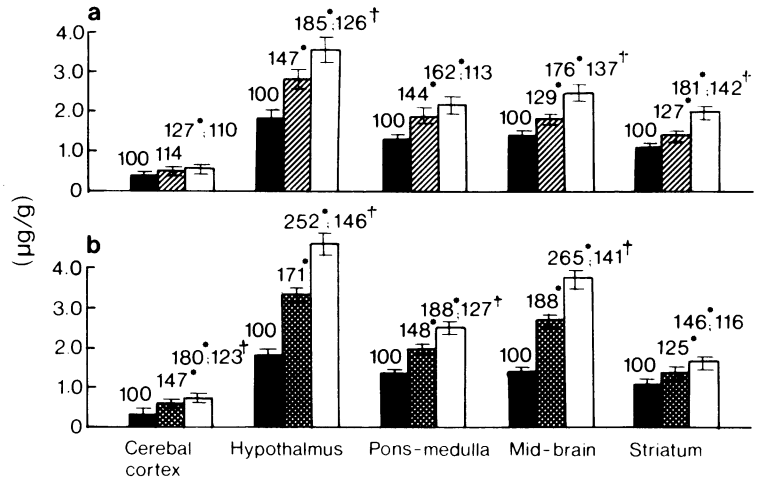

Figure 3 Influence of (a) chronic diazepam, (b) bromazepam and 48 h withdrawal on 5-hydroxyinoleacetic acid concentrations in certain discrete brain regions of rats. Each column represents the mean of 6 rats in the group. Vertical lines show s.e. means. Solid columns = control rats; hatched columns = diazepam-treated rats; stippled columns = diazepam withdrawn rats; cross hatched columns = bromazepam-treated rats; open columns = bromazepam wtihdrawn rats. For other details see legend to Figure 1.

significantly increased to 123% of the values seen for diazepam-treated rats. Data in Table 1 also demonstrate that neither bromazepam nor withdrawal from bromazepam treatment exerted any significant effect on the activity of mid-brain MAO.

Table 1 Effect of chronic diazepam, bromazepam and 48 h withdrawal on mid-brain monoamine oxidase (MAO)

Treatment	*MAO (nmol g^{-1} h^{-1})*	*% Change*
Control	116.72 ± 1.19	(100)
Diazepam	101.55 ± 2.10	(87*; 100)
Diazepam-withdrawn	124.89 ± 1.71	(107; 123†)
Bromazepam	113.96 ± 1.18	(98; 100)
Bromazepam-withdrawn	117.52 ± 1.02	(101; 103)

Each value represents the mean ± s.e. mean of 6 animals in the group. Rats were injected daily with diazepam or bromazepam (10 mg/kg) subcutaneously for 22 days and killed 6 h after the last injection. Groups of animals treated with either drug for 20 days were withdrawn for 48 h ('withdrawn' rats). Data in parentheses express results in percentages taking the values of control rats as 100%.

* Significantly different from normal control values at $P < 0.05$.

† Significantly different from values of diazepam-treated rats at $P < 0.05$.

Discussion

Several drugs acting on the central nervous system appear to interact specifically with the mechanisms that are involved in the maintenance of dynamic equilibrium of neurotransmitter substances. Recent studies have shown that acute treatment with diazepam and nitrazepam decreased noradrenaline and dopamine turnover in certain brain regions and antagonized the increased catecholamine turnover induced by electro-foot shock stress (Taylor & Laverty, 1969). Fennessy & Lee (1972) found no change in the endogenous levels of 5-HT and 5-HIAA in brains of mice 30 min after oral administration of diazepam (7 mg/kg). Wise, Berger & Stein (1972) demonstrated that daily treatment with oxazepam (20 mg/kg) for 6 days decreased 5-HT turnover in rat brain as evidenced by elevated levels of [^{14}C]-5-HT and [^{14}C]-5-HIAA in rats pretreated with [^{14}C]-5-HT by the intraventricular route. Furthermore, Lidbrink, Corrodi, Fuxe & Olson (1973) found decreased turnover of 5-HT in cortex of rats treated with a single injection (25 mg/kg) of chlordiazepoxide. This is in line with our recent work in which significantly decreased synthesis of 5-HT, as evidenced by lowered tryptophan and TPH activity, was found in mid-brain of rats killed 2 h after a single injection (10 mg/kg) of diazepam (unpublished data). This is not too intriguing since opposite effects on central monoamine turnover have been demonstrated following acute and chronic administration of several psychoactive agents (Javoy, Thierry & Kety, 1968; Schildkraut, Winokur,

Draskoczy & Hensle, 1971). Thus, any apparent discrepancy between our data and those reported earlier may be explained on the grounds of different doses and duration of treatment. It is probable that acute or subacute administration of oxazepam for 6 days (Wise *et al.*, 1972), by lowering the turnover of brain 5-HT, reduces the concentrations of this amine at the vicinity of corresponding receptor sites; this in turn, by feedback mechanisms may result in induction of TPH enzyme in mid-brain (a region rich in 5-hydroxytryptaminergic cell bodies) as has been documented after repeated administration of a number of psychoactive drugs by Knapp (1975). The increased TPH activity may thus not be in direct response to the effect of chronic diazepam treatment, but may reflect an adaptive change as a consequence of alterations in 5-HT neuronal activity resulting initially from decreased 5-HT turnover after short term treatment with these anxiolytic drugs.

Changes in brain tryptophan levels are relevant to 5-HT mediated functions because in the brain, tryptophan is known to modulate the synthesis of 5-HT (Tagliamonte, Tagliamonte, Perez-Cruet, Stern & Gessa, 1971; Hamon & Glowinski, 1974). Since tryptophan binds avidly to albumin in plasma (McMenamy & Oncley, 1958), the observed rise in brain tryptophan levels might be the result of displacement of tryptophan from the binding site in albumin molecule by benzodiazepines which are known to be bound to plasma protein (80–90%) (Goodman & Gilman, 1975). The elevated levels of endogenous tryptophan as well as the activity of TPH may underlie the enhanced synthesis of 5-HT in brains of rats exposed to diazepam or bromazepam. In addition, there might be several other mechanism(s) through which repeated exposure to benzodiazepines could increase 5-HT turnover e.g. acceleration of 5-HT release, blockade of 5-HT receptors or enhanced nervous activity in 5-hydroxytryptaminergic neurones. However, with the information available in the literature, the latter possibility seems to be favoured. Our data on the elevated brain 5-HT and 5-HIAA concentrations are consistent with the previous reports (Fernstrom, Shabshelowitz & Faller, 1974; Jenner *et al.*, 1975). The increased concentrations of 5-HIAA might be due to increased turnover of 5-HT and impeded egress of this metabolite from the brain. Chase, Katz & Kopin (1970) observed a reduced efflux of labelled [^{14}C]-5-HIAA from the brain after its intraventricular injection in rats pretreated with diazepam. The possibility also remains that observed elevation in 5-HIAA levels might be related to increased activity of the deaminating enzyme. However, our data showed that whereas diazepam treatment decreased MAO activity, administration of bromazepam exerted no effect on this metabolizing enzyme.

Behavioural states of excitation with increased locomotor activity have been observed after the administration of drugs that are known to enhance the activity of catecholaminergic neurones in the brain (Ernst, 1967; Carlsson, 1970). Drugs that reduced central catecholamine transmission produced sedation and hypoactivity. In contrast, a state of general excitement with increased locomotor activity was noted after pharmacological and neurological manipulations which selectively lowered brain 5-HT (e.g. inhibition of 5-HT synthesis by *p*-chlorophenylalanine: Sheard, 1969; Brody, 1970) and destroyed 5-hydroxytryptaminergic neurones by electrolytic lesions placed in the mid-brain raphe (Kostowski, Giacalone, Garattini & Valzelli, 1968; Neill, Grant & Grossman, 1972). Recently, it was shown that there is a decrease in brain catecholamine turnover (Rastogi *et al.*, 1976c) along with increased synthesis and turnover of 5-HT following chronic diazepam and bromazepam treatment. This may suggest that benzodiazepines manifest their central actions by acting antagonistically on the 5-hydroxytryptaminergic and catecholaminergic neurones.

In a separate set of experiments, it has been demonstrated that chronic administration of diazepam and bromazepam decreased spontaneous locomotor activity by 61% and 49%, respectively (Rastogi *et al.*, 1976b). However, a 48 h withdrawal from these drugs significantly elevated the locomotor activity to 328% and 253%, respectively taking the values for 'treated' groups as 100%. In fact, the rats withdrawn from these minor tranquillizers displayed significantly higher mobility when compared to untreated normal rats. This is in line with the 'rebound' phenomenon observed in patients following withdrawal of anti-anxiety agents (Borland & Nicholson, 1975; Lapierre, unpublished data). It may be speculated that the elevated levels of 5-HIAA in 'withdrawn' rats could partly be due to enhanced catabolism of 5-HT within the synaptic clefts whose synthesis is decreased, but its neuronal uptake is diminished by about 30% of the values seen in diazepam- or bromazepam-treated rats (unpublished data). Additionally, the enhanced activity of MAO during withdrawal may account for lowered 5-HT and increased 5-HIAA levels seen in rats withdrawn from diazepam therapy. Since the activity of MAO did not alter significantly in rats withdrawn from bromazepam, it is conceivable that elevated levels of 5-HIAA after bromazepam withdrawal may be independent of changes in MAO activity but may be related to a reduced active transport from the brain.

In conclusion, the present study implicates central 5-hydroxytryptaminergic neurones in mediating the depressant effects of benzodiazepines on behaviour as well as in the 'rebound' phenomenon during the 'withdrawal' phase from benzodiazepine treatment as noticed earlier (Rastogi *et al.*, 1976b). However, the question whether these neurones are affected directly

or are conveying the effects of another neuronal system remains open. Recent studies suggest that benzodiazepines may exert a primary action on γ-aminobutyric acid (GABA) containing neurones, some of which in turn may regulate transmission at monoaminergic synapses (Polc, Mohler & Haefely, 1974; Costa, Guidotti, Mao & Suria, 1975; Mao, Guidotti & Costa, 1975; Stein *et al.*, 1975). Padjen & Bloom (1975) suggested that acute injection of 1,4-benzodiazepines may enhance the release of GABA at 5-hydroxytryptaminergic nerve endings which by presynaptic inhibition results in a reduction of 5-HT release. However, in view of recent studies by Sherman & Gebhart (1974; 1976) demonstrating that pain induced by hot plate elevated GABA levels in the brain, it is not clear whether the increased GABA seen 1–2 h after acute administration of benzodiazepines is indeed in response to pain induced by injection or by the drug itself. Further work is necessary to gain additional insight into the effects of long-term exposure of benzodiazepines and withdrawal on GABA-containing neurones to correlate the observed changes in 5-HT metabolism with those in GABA neuronal systems. Although there is lack of evidence pertaining to the interrelationship with glycine and 5-HT metabolizing systems, the possibility remains that these anxiolytic drugs may modulate 5-HT metabolism by directly activating glycine receptors in the brain (Young, Zukin & Snyder, 1974).

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