15. D. H. Nugteren and E. Hazelhof. *Biochim. biophys. Acta* **326**, 448 (1973).
16. A. A. Abdel-Latif, J. P. Smith, S. Y. K. Yousufzai and R. K. Dover, in *International Symposium on Neural Membranes* (Eds. G. Y. Sun, A. Y. Sun, J. Y. Wu, N. Bazan and G. Porcellati). The Humana Press, Clifton, NJ, in press.
17. R. A. Akhtar and A. A. Abdel-Latif, *Gen. Pharmac.* **10**, 445 (1979).

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Effects of acute and chronic desmethylimipramine on levels of cyclic AMP *in vivo**

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Although the clinically relevant mechanism of action of tricyclic antidepressants including desmethylimipramine (DMI) is not certain, DMI clearly affects various aspects of noradrenergic synaptic function. DMI has been shown to decrease the reuptake of norepinephrine acutely [1, 2], and chronic DMI administration has been shown to desensitize alpha-adrenergic presynaptic receptors [3, 4], decrease firing rates of locus coeruleus neurons [5], increase 3-methoxy-4-hydroxyphenylglycol (MHPG) levels [6, 7], and decrease the sensitivity of beta-adrenergic receptors [8, 9]. Since the time required for therapeutic efficacy of DMI and the development of subsensitive beta-receptors share a similar time course, it has been suggested that the change in beta-receptor sensitivity is important in the antidepressant mechanism of action of DMI.

The beta-adrenergic receptor in the CNS appears to be linked to adenylate cyclase, and the pharmacological effects of beta-adrenergic stimulation by catecholamines can be assessed by measuring cyclic AMP response [10, 11]. *In vitro* studies have shown diminished cyclic AMP response to exogenous noradrenergic stimulation in tissue prepared from rats chronically treated with DMI [12–16]. Pineal cyclic AMP response to isoproterenol has been shown to be decreased *in vivo* following chronic administration of DMI [17].

Using a high-power microwave system [18, 19] to rapidly inactivate adenylate cyclase and phosphodiesterase, our laboratory has been investigating the cyclic AMP and cyclic GMP responses to various neurotransmitter agonists in discrete rat brain regions *in vivo* [20–22].

In this report, we describe the effects of a single acute dose of DMI on cyclic AMP levels in twenty-one brain areas as well as the effects of chronic DMI treatment on basal levels of cyclic AMP in rat brain regions. Chronically treated groups were also challenged with a beta-adrenergic agonist (isoproterenol) to assess beta-noradrenergic receptor sensitivity. DMI has been reported to block muscarinic receptors *in vitro* [23, 24], and chronic treatment might be expected to result in supersensitive muscarinic receptors. Since we have shown a significant cyclic AMP response *in vivo* to cholinergic agonists [22], DMI chronically treated rats were also challenged with a muscarinic cholinergic agonist (oxotremorine).

Methods

Animals. All experiments were performed with Wistar derived random bred male rats from the Walter Reed colony (300 ± 20 g).† The rats were housed in individual cages with food and water freely available. Lights were on from 6:00 a.m. to 6:00 p.m.

Drugs. Desmethylimipramine hydrochloride (USV Pharmaceutical Corp., Tuckahoe, NY), methylatropine nitrate, oxotremorine sesquifumarate, and DL-isoproterenol hydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved in saline immediately prior to use. Doses are expressed as the base.

Experiment 1: Effect of acute DMI on cyclic AMP. Rats were alternately injected with DMI (30 mg/kg) i.p. or an equivalent volume of saline. Thirty minutes following injection, at which time brain levels of DMI have been reported to be maximal [25], rats were killed by high power microwave irradiation.

Experiment 2: Effect of chronic DMI on cyclic AMP. Rats were injected daily with DMI (10 mg/kg) or saline for 6 weeks. Twenty hours after the last injection, groups of saline and DMI-pretreated rats were given an i.p. injection of saline, isoproterenol (10 mg/kg) or methylatropine nitrate (0.5 mg/kg). Saline and isoproterenol-treated rats were killed 10 min following injection. The methylatropine-pretreated (to prevent excess peripheral cholinergic stimulation) groups were injected with oxotremorine (2 mg/kg) 30 min after the methylatropine pretreatment and then killed 10 min later.

Sacrifice and tissue preparation. Animals were killed by a 5-sec exposure to high intensity microwave irradiation at 2450 MHz using 2.5 kW forward power [26–28]. Since brief immobilization is required during the sacrifice procedure, animals were placed in a plastic cylinder which was then inserted into the wave guide such that the longitudinal axis of the body was perpendicular to the microwave E field. After sacrifice by microwave irradiation, the rats were decapitated. The heads were briefly cooled on dry ice, the desired brain regions were dissected and weighed, and then they were sonicated in 50 mM sodium acetate buffer, pH 6.2. The sonicates were centrifuged at 12,000 *g* at 4° and the supernatant fractions were stored at −70° until assayed for cyclic AMP and cyclic GMP.

* This material has been reviewed by the Walter Reed Army Institute of Research, and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

† In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

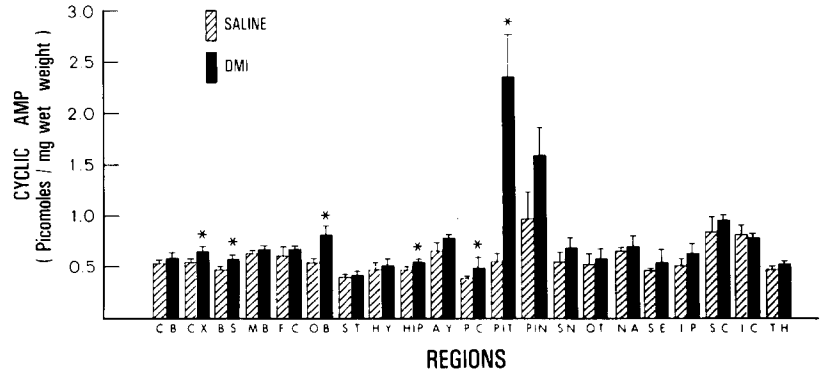

Fig. 1. Effects of acute injection of DMI (30 mg/kg, i.p.) on levels of cyclic AMP. Each value represents the mean ± S.E.M. of six animals. Asterisks indicate values significantly different from control ($P < 0.05$). Abbreviations: CB, cerebellum; CX, cortex; BS, brain stem; MB, midbrain; FC, frontal cortex; OB, olfactory bulb; ST, striatum; HY, hypothalamus; HIP, hippocampus; AY, amygdala; PC, pyriform cortex; PIT, pituitary; PIN, pineal; SN, substantia nigra; OT, olfactory tubercle; NA, nucleus accumbens; SE, septal region; IP, interpeduncular region; SC, superior colliculus; IC, inferior colliculus; and TH, thalamus.

Assay procedures. Cyclic AMP and cyclic GMP levels were determined by radioimmunoassay using antibodies developed and characterized in our laboratories [29, 30]. For measurement of the cyclic nucleotides in the smaller brain regions, a modification of the method described by Harper and Brooker [31] was employed.

Highly specific antisera were used at final dilutions of 1:400,000 for cyclic AMP and 1:20,000 for cyclic GMP. The cyclic AMP antiserum exhibited cross-reactivities for ATP and cyclic GMP of less than 0.00007 and 0.14% respectively. Standards and samples were assayed in triplicate. The data were analyzed by computer using a nonlinear 4 parameter logistic model weighted for nonuniformity of variance [32]. The sensitivity for cyclic AMP (minimal detectable amount) was 0.10 pmole/assay tube for the routine assay and 3 fmoles for the acetylated assay. The intra-assay coefficient of variation was 7% for cyclic AMP. Phosphodiesterase treatment of tissue extracts reduced cyclic AMP and cyclic GMP to undetectable levels representing a reduction greater than 95% for cyclic AMP and 80% for cyclic GMP in each region.

Table 1. Basal and isoproterenol-stimulated levels of cyclic AMP following chronic administration of DMI*

	Cyclic AMP (pmoles/mg wet weight)			
	Chronic saline treatment		Chronic DMI treatment	
	Acute challenge		Acute challenge	
Region	Saline	Isoproterenol	Saline	Isoproterenol
Cerebellum	0.428 ± 0.052	0.533 ± 0.073	0.349 ± 0.068	0.517 ± 0.066
Cortex	0.639 ± 0.040	0.766 ± 0.072	0.530 ± 0.068	0.820 ± 0.074
Brainstem	0.480 ± 0.152	0.472 ± 0.156	0.587 ± 0.198	0.530 ± 0.139
Midbrain	0.770 ± 0.103	0.635 ± 0.080	0.540 ± 0.078	0.650 ± 0.102
Frontal cortex	0.909 ± 0.119	1.091 ± 0.177	0.891 ± 0.132	0.929 ± 0.056
Olfactory bulb	0.761 ± 0.107	0.935 ± 0.085	0.780 ± 0.048	1.158 ± 0.311
Striatum	0.243 ± 0.032	0.328 ± 0.029	0.237 ± 0.033	0.338 ± 0.042
Hypothalamus	0.623 ± 0.043	0.845 ± 0.215	0.559 ± 0.044	1.012 ± 0.191
Hippocampus	0.526 ± 0.028	0.719 ± 0.058	0.493 ± 0.037	0.596 ± 0.056
Amygdala	0.942 ± 0.095	1.256 ± 0.194	0.973 ± 0.167	1.369 ± 0.144
Pyriform cortex	0.847 ± 0.053	1.137 ± 0.092	1.032 ± 0.128	1.078 ± 0.090
Pituitary	1.233 ± 0.276	2.097 ± 0.183	0.876 ± 0.047	1.610 ± 0.181
Pineal	1.575 ± 0.292	5.857 ± 1.932	1.346 ± 0.430	7.604 ± 2.264
S. nigra	0.188 ± 0.029	0.342 ± 0.054	0.158 ± 0.021	0.224 ± 0.050
Olf. tubercle	0.942 ± 0.047	1.177 ± 0.150	0.900 ± 0.078	1.087 ± 0.076
N. accumbens	0.492 ± 0.024	0.556 ± 0.043	0.469 ± 0.032	0.447 ± 0.074
Septal region	1.342 ± 0.241	1.913 ± 0.214	1.289 ± 0.123	1.493 ± 0.222
Interped. region	1.158 ± 0.128	1.646 ± 0.243	1.007 ± 0.057	1.588 ± 0.330
S. colliculus	0.430 ± 0.021	0.659 ± 0.029	0.423 ± 0.024	0.587 ± 0.082
I. colliculus	0.672 ± 0.046	0.883 ± 0.052	0.667 ± 0.048	0.881 ± 0.131
Thalamus	0.643 ± 0.037	0.771 ± 0.058	0.530 ± 0.058	0.662 ± 0.063

* Rats were injected (i.p.) daily with DMI (10 mg/kg) or saline for 6 weeks. Twenty hours after the last injection, groups of saline and DMI-pretreated rats were given an i.p. injection of saline or isoproterenol (10 mg/kg). Rats were killed 10 min later by microwave irradiation. Values represent the means ± S.E.M. for six rats in each group.

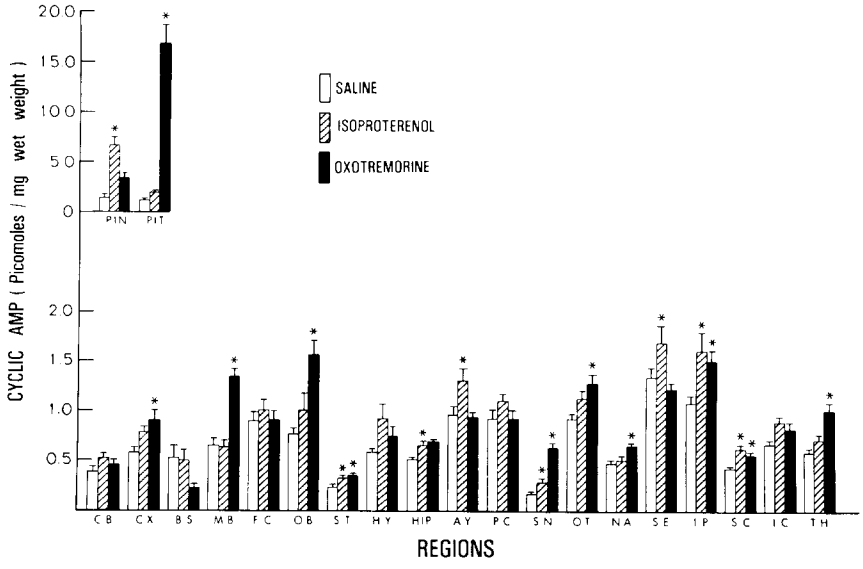

Fig. 2. Effects of isoproterenol (10 mg/kg) and oxotremorine (2 mg/kg) on levels of cyclic AMP. DMI and saline chronically pretreated groups were not different from each other, and data from these groups are pooled. Therefore, each value represents the mean ± S.E.M. of twelve animals. Asterisks indicate values significantly different from control ($P < 0.05$). Abbreviations are given in the legend of Fig. 1.

Results

Experiment 1. Cyclic AMP levels were elevated in twenty of the twenty-one brain regions examined following a single acute dose of DMI (30 mg/kg) as shown in Fig. 1. Two-way analysis of variance (ANOVA) showed a significant effect of treatment ($F = 33.06$; $df = 1$; $P < 0.001$) and brain region ($F = 11.03$; $df = 20$; $P < 0.001$) and a significant treatment X region interaction ($F = 6.39$; $df = 20$; $P < 0.001$). There were no significant effects of DMI treatment on levels of cyclic GMP (data not shown). The pineal and pituitary gland cyclic AMP levels rose most markedly following DMI. Both these regions lie outside the blood–brain barrier.

Experiment 2. Chronic pretreatment with DMI did not affect basal levels of cyclic AMP or cyclic AMP response to isoproterenol (Table 1) or oxotremorine (data not shown). Overall two-way ANOVA showed no effect of DMI pretreatment ($F = 0.30$; $df = 1$; $P > 0.5$). There was, however, a significant effect of drug challenge. Since DMI pretreatment had no effect on cyclic AMP response to drug challenge, saline and DMI-pretreated groups were combined and analyzed by one-way ANOVA for drug challenge effects region by region (Fig. 2). Post-hoc *t*-tests were performed on regions with significant F values (all regions except hypothalamus, inferior colliculus, frontal cortex, pyriform cortex, brainstem and cerebellum). Isoproterenol significantly increased levels of cyclic AMP in striatum, hippocampus, s. nigra, olfactory tubercle, amygdala, interpeduncular region, superior colliculus, septal region, and pineal. Oxotremorine significantly elevated cyclic AMP levels in pituitary, striatum, thalamus, olfactory bulb, s. nigra, midbrain, olfactory tubercle, interpeduncular region, superior colliculus, nucleus accumbens and cortex.

Discussion

DMI acutely increased levels of cyclic AMP throughout the rat brain. The magnitude and pattern of response were similar to that seen after isoproterenol (this report and Ref. 21). Cyclic AMP levels have also been shown to be elevated in mouse cortex 24 hr following amitriptyline [33]. The most likely explanation of this effect is that NE reuptake is acutely inhibited by tricyclic antidepressants, resulting in excess NE in the synapse and at the postsynaptic beta-NE receptor. The potentiation of beta-receptor stimulation leads to increased cyclic AMP levels.

Alternately, the increase in cyclic AMP seen after acute DMI treatment could be adenosine mediated. Sattin *et al.* [34] have shown that DMI-induced cyclic AMP increases *in vitro* can be inhibited by adenosine receptor blockers.

Although it was expected that cyclic AMP response to isoproterenol *in vivo* would be diminished in rats chronically administered DMI based on reports of subsensitive beta receptors tested *in vitro*, we did not find a subsensitive cyclic AMP response to isoproterenol *in vivo*. It is possible that changes in cyclic AMP response in DMI-treated rats would have been observed if a higher dose of isoproterenol had been used. However, since the experiments were conducted *in vivo*, the amount of isoproterenol given was selected to minimize peripheral effects of the drug, e.g. cardiovascular, that might indirectly affect the brain. Similarly, we did not find a supersensitive response to cholinergic stimulation although DMI has been shown to block muscarinic receptors *in vitro*.

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REFERENCES

1. A. Carlsson, H. Corrodi, R. Fuxe and T. Hokfelt, *Eur. J. Pharmac.* **5**, 367 (1969).
2. J. Glowinski and J. Axelrod, *Nature, Lond.* **204**, 1318 (1977).
3. B. A. McMillen, W. Warnack, D. C. German and P. A. Shore, *Eur. J. Pharmac.* **61**, 239 (1980).
4. F. T. Crews and C. B. Smith, *Science* **202**, 322 (1978).
5. T. H. Svensson and T. Usdin, *Science* **202**, 1089 (1978).
6. M. F. Sugrue, *Life Sci.* **26**, 423 (1980).
7. S. W. Tang, D. M. Helmeste and H. C. Stancer, *Naunyn-Schmiedeberg's Archs Pharmac.* **305**, 207 (1978).
8. S. P. Banerjee, L. S. Kung, S. J. Riggi and S. K. Chanda, *Nature, Lond.* **268**, 455 (1977).
9. D. A. Bergstom and K. J. Kellar, *J. Pharmac. exp. Ther.* **209**, 256 (1979).
10. L. L. Iversen, *J. Neurochem.* **29**, 5 (1977).
11. J. W. Kebabian, F.E. Bloom, A. L. Steiner and P. Greengard, *Science* **190**, 157 (1975).
12. J. Vetulani, R. J. Stawarz, J. V. Dingell and F. Sulser, *Naunyn-Schmiedeberg's Archs Pharmac.* **293**, 109 (1976).
13. J. Schultz, *Nature, Lond.* **261**, 417 (1976).
14. A. Frazer, M. E. Hess, J. Mendels, B. Gable, E. Kunkel and A. Bender, *J. Pharmac. exp. Ther.* **206**, 311 (1978).
15. B. B. Wolfe, T. K. Harden, J. R. Sporn and P. B. Molinoff, *J. Pharmac. exp. Ther.* **207**, 446 (1978).
16. J. A. Moyer, L. H. Greenberg, A. Frazer and B. Weiss, *Molec. Pharmac.* **19**, 187 (1981).
17. J. A. Moyer, L. H. Greenberg, A. Frazer, D. J. Brunswick, J. Mendels and B. Weiss, *Life. Sci.* **24**, 2237 (1979).
18. R. H. Lenox, P. V. Brown and J. L. Meyerhoff, *Tins* **2**, 106 (1979).
19. R. H. Lenox, J. L. Meyerhoff, O. P. Gandhi and H. L. Wray, *J. Cyclic Nucleotide Res.* **3**, 367 (1977).
20. G. J. Kant, J. L. Meyerhoff and R. H. Lenox, *Biochem. Pharmac.* **29**, 369 (1980).
21. G. J. Kant, V. E. Bates, R. H. Lenox and J. L. Meyerhoff, *Biochem. Pharmac.* **30**, 3377 (1981).
22. R. H. Lenox, G. J. Kant and J. L. Meyerhoff, *Life Sci.* **26**, 2201 (1980).
23. S. H. Snyder and H. I. Yamamura, *Archs gen. Psychiat.* **34**, 236 (1977).
24. M. Rehavi, S. Maayani and M. Sokolovsky, *Biochem. Pharmac.* **26**, 1559 (1977).
25. A. Biegon and D. Samuel, *Psychopharmacology* **65**, 259 (1979).
26. P. V. Brown, R. H. Lenox and J. L. Meyerhoff, *IEEE Trans. Biomed. Engng* **25**, 5 (1978).
27. R. H. Lenox, O. P. Gandhi, J. L. Meyerhoff and H. M. Grove, *IEEE Trans. Microwave Theory Tech.* **24**, 58 (1976).
28. J. L. Meyerhoff, R. H. Lenox, P. V. Brown and O. P. Gandhi, *Proc. IEEE* **68**, 155 (1980).
29. A. L. Steiner, A. W. Parker and D. M. Kipnis, *J. biol. Chem.* **247**, 1106 (1972).
30. R. H. Lenox, G. J. Kant, G. R. Sessions, L. L. Pennington, E. H. Mougey and J. L. Meyerhoff, *Neuroendocrinology* **30**, 300 (1980).
31. J. F. Harper and G. Brooker, *J. Cyclic Nucleotide Res.* **1**, 207 (1975).
32. D. Rodbard, R. H. Lenox, H. L. Wray and D. Ramseth, *Clin. Chem.* **22**, (3), 350 (1976).
33. G. C. Palmer, D. J. Jones, M. A. Medina and W. B. Stavinoha, *Neuropharmacology* **16**, 435 (1977).
34. A. Sattin, T. W. Stone and D. A. Taylor, *Life. Sci.* **23**, 2621 (1978).

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Metabolic activation of 2,4-xylidine and its mutagenic metabolite

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Xylidines (dimethylanilines) are metabolic products of certain food dyes, cosmetics and pharmaceuticals [1, 2], and their hepatotoxic effects in dogs, rats and mice [2, 3] have been reported. Among their isomers, 2,4-xylidine showed the most marked toxic effect on the liver of rats [4] and is known as a reductive product of the azo dye, Ponceau R, which is tumorigenic in rats and mice [5, 6]. Although the metabolism of 2,4-xylidine in rats has been studied earlier by Lindstrom [7], the relationship between the specific metabolite formation and the induction of toxicity has not been sufficiently established.

Recently, we and other workers found that 2,4-xylidine was mutagenic in *Salmonella typhimurium* TA100 after metabolic activation by polychlorinated biphenyl (PCB)-treated rat liver 9000 *g* supernatant fraction [8, *]. This finding suggests that 2,4-xylidine is converted to a mutagenic metabolite which is capable of inducing mutation without further activation. We isolated the metabolites of 2,4-xylidine by using high performance liquid chromatography (HPLC) and tested their mutagenicity with a bacterial mutation assay. It has been reported that the HPLC analysis is the most reliable method for separation of the metabolites that are thought to be present at low concentrations and are susceptible to air oxidation [9, 10]. Concomitantly, the bacterial mutation test provides a simple and very sensitive method for detecting such small amounts of metabolites [11]. In this paper, we report the structure of the mutagenic metabolite of 2,4-xylidine and discuss the relationship between the formation of mutagenic metabolite and the enzyme-mediated mutagenicity of 2,4-xylidine.

Materials and methods

Materials. 2,4-Xylidine was purchased from Wako Pure Chemicals, Osaka, Japan. 2,4-Xylidine was purified by distillation (b.p. 101°/20 mm Hg), and the purity was checked by HPLC with acetonitrile–water (3:7) as described in the legend to Fig. 1. Authentic samples of 2,4-dimethylphenylhydroxylamine and 2,4,2′,4′-tetra-

* K. Yoshikawa *et al.*, unpublished observations.