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EFFECTS OF DRUGS AFFECTING ENDOGENOUS AMINES OR CYCLIC NUCLEOTIDES ON ETHANOL WITHDRAWAL HEAD TWITCHES IN MICE

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1 Twenty-four hours after ethanol withdrawal, dependent mice exhibited frequent head twitching. Naive mice exhibited similar twitching 15 min after treatment with 5-hydroxytryptophan (5-HTP) or 6 h after α-methyl-*p*-tyrosine (AMPT). Ethanol lessened the incidence of head twitches induced by any of these treatments. 5-HTP and AMPT each increased the incidence of head twitches induced by withdrawal of ethanol from dependent mice.

2 Drugs that affect the amount or activity of endogenous amines or cyclic nucleotides modified the incidence of head twitches. Nearly all drugs acted in the same direction on twitching elicited by any of these three treatments.

3 The incidence was lessened by: (a) methysergide, methergoline, MA 1420, *p*-chlorophenylalanine and *p*-chloroamphetamine; (b) dopamine, noradrenaline, L-DOPA, amphetamine and apomorphine; (c) hyoscine and nicotine; and (d) adenosine triphosphate, dibutyryl cyclic adenosine-3′,5′-monophosphate (db cyclic AMP) and prostaglandins E_1 and E_2.

4 The incidence was increased by: (a) acetylcholine, carbachol and physostigmine; and (b) guanosine triphosphate, dibutyryl cyclic guanosine monophosphate (db cyclic GMP), theophylline and 3-isobutyl-1-methyl-xanthine.

5 These findings suggest that head twitching induced by these three treatments arises from a common biochemical mechanism, which may ultimately be a change in favour of cyclic GMP of the balance between this nucleotide and cyclic AMP within appropriate neurones. This imbalance appears to be elicited or increased by 5-hydroxytryptamine and acetylcholine and to be decreased by dopamine, noradrenaline and E prostaglandins.

6 Neither actinomycin D nor cycloheximide, given during the induction of ethanol dependence, altered the incidence of head twitches after ethanol withdrawal.

Introduction

One of the obstacles to exploring the mechanism of ethanol dependence in experimental animals is a scarcity of recognizable abstinence signs. In mice, however, we observed that head twitching was much more frequent than usual between about 18 and 36 h after withdrawal of ethanol, administered subchronically either by mouth or by inhalation (Hammond & Schneider, 1973). For reasons discussed below, we regarded this as an abstinence sign. To explore the biochemical mechanism of ethanol dependence, we tested certain drugs, given after ethanol withdrawal, on the incidence of head twitching.

Because the intraperitoneal administration of 5-hydroxytryptophan (5-HTP), or the intracerebroventricular administration of 5-hydroxytryptamine (5-HT) had been shown to elicit head twitching in mice (Corne, Pickering & Warner, 1963; Mawson & Whittington, 1970), we began by testing drugs interacting with 5-HT mechanisms. The study was then extended to drugs affecting other brain amines (Collier, Hammond & Schneider, 1974). Because of the interaction of some amines with cyclic nucleotides (Weiss & Costa, 1968; Ferrendelli, Steiner, McDougal & Kipnis, 1970; Wellman & Schwabe, 1973; Daly, 1975) and because another drug of dependence, morphine, interacts with cyclic nucleotides (Collier & Roy, 1974; Gullis, Traber & Hamprecht, 1975), we then studied the effects of some other drugs affecting cyclic nucleotide mechanisms on the incidence of head twitches after ethanol withdrawal. These studies are described in detail below; some of these findings have been briefly reported (Hammond & Schneider, 1973; Collier *et al.*, 1974; Hammond & Schneider, 1974; Collier, Hammond & Schneider, 1976).

Methods

Animal treatment and observation

Male albino T/O strain mice weighing 23 to 25 g at the start of the treatment were used. They were randomized into groups of five and coloured for identification. In the ethanol withdrawal experiments, mice received by intragastric tube a daily dose of ethanol (40% w/v, in distilled water) starting at 4 g/kg (i.e. 10 ml/kg) on the first day and increasing daily by 1 g/kg to 7 g/kg on the fourth day. Head twitches were counted 24 h after the last dose of ethanol. In experiments in which head twitches were elicited in naive mice, 5-HTP (400 mg/kg in 20 ml saline/kg) was given intraperitoneally and head twitches were counted 15–19 min later. α-Methyl-*p*-tyrosine (AMPT) (300 mg/kg suspended in 10% w/v gum acacia in distilled water) was given orally (10 ml/kg) and head twitches counted 6 h later.

Drugs tested for ability to modify the incidence of head twitches were given in a dose volume of 10 ml/kg by the oral, intraperitoneal or subcutaneous route. Intracerebroventricular injections (up to 50 µl in volume) were carried out by the method of Haley & McCormick (1957), modified by using 3 mm depth of injection and light ether anaesthesia. Most drugs were dissolved in 0.9% w/v NaCl solution (saline) or distilled water; MA 1420 was suspended in 10% w/v gum acacia in distilled water, and methergoline was dissolved in 1% w/v ascorbic acid in distilled water.

Individual head twitches were counted during periods of 4 min, after transferring the mice in groups of five to opaque plastic boxes (30 cm long × 12 cm high × 12 cm wide) fitted with a perforated Perspex lid. Observers did not know the treatment each mouse had received.

Design and analysis of experiments

In experiments on the induction of head twitching, mice received either inducing treatment (ethanol, 5-HTP or AMPT) or vehicle. In experiments on the modification by drugs of the incidence of head twitches, some mice received inducing treatment, and, of these, some received modifying drug and the rest vehicle; other mice received both vehicles. The effect of a modifying drug on induced head twitching was expressed numerically as the ratio of the mean twitch count in mice treated with modifying drug to that in mice receiving its vehicle (the incidence ratio).

Because values were not distributed normally, statistical significance was determined by the non-parametric Mann-Whitney 'U' test (Siegel, 1956). When only one dose is reported, this is a submaximally effective dose. The experimental design did not allow statistical comparison to be made between drugs.

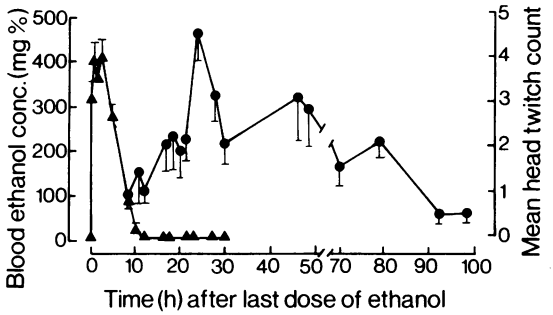

Figure 1 Blood ethanol levels and incidence of head twitches in mice that had received oral doses of ethanol, increasing from 4 to 7 g/kg, once daily for four days. (▲) Ethanol concentration; (●) mean head twitch count/4 min; vertical lines represent s.e. mean.

Ethanol concentration in brain homogenate or blood was determined by gas-liquid chromatography, as described by Hammond (1975a).

Drugs

The following drugs were used: acetylcholine hydrobromide, adenosine triphosphate (ATP), amphetamine sulphate, apomorphine hydrochloride, carbachol, dibutyryl cyclic-3′,5′-adenosine monophosphate (db cyclic AMP), dibutyryl cyclic-3′,5′-guanosine monophosphate (db cyclic GMP), L-DOPA, dopamine, ethanol (96% v/v), guanosine triphosphate (GTP), hyoscine hydrobromide, imidazole, 3-isobutyl-1-methylxanthine (IBMX), 6-acetamido-3-(3-(4-phenyl-1-piperazinyl)-propyl)-2,4-quinazolinedione maleate (MA 1420), methergoline, α-methyl-*p*-tyrosine, methysergide bimaleate, nicotine hydrogen tartrate, noradrenaline hydrochloride, *p*-chlorophenylalanine methylester hydrochloride (PCPA), *p*-chloroamphetamine hydrochloride (PCA), physostigmine salicylate, prostaglandins E_1, E_2 and $F_{2\alpha}$, theophylline. Doses are expressed in terms of active acid or base.

Results

Induction of head twitching

When ethanol was given once daily to mice on four successive days, its concentration in the blood reached a peak (400 mg %) 30–60 min after the last dose (Figure 1). The concentration then fell to < 3 mg % at 12 h after the last dose of ethanol. The concentration of ethanol in the brain closely paralleled that in blood.

As the blood ethanol concentration declined, head twitching increased to a peak, at which each mouse exhibited about 1 twitch/min, 24 h after the last dose.

Table 1 Modification by ethanol and other drugs of the incidence of head twitches induced by ethanol withdrawal, by 5-hydroxytryptophan (5-HTP) or by α-methyl-*p*-tyrosine (AMPT)

Modifying drug	*Route*	*Dose*	*Incidence ratio after induction with*		
			Ethanol	5-HTP	AMPT
Ethanol	oral	2000 mg	1.70	NT	NT
		4000 mg	0.33†	0.30†	0.25†
		6000 mg	0.11‡	NT	NT
Methergoline	oral	4 mg	0.10‡	0.15‡	0.00†
Noradrenaline	i.c.v.	5 µg	0.25†	0.40‡	0.25‡
db cyclic AMP	i.c.v.	100 µg	0.10†	0.35†	0.05†
db cyclic GMP	i.c.v.	50 µg	2.28†	3.20†	1.75*
Theophylline	oral	100 mg	2.60†	2.60‡	1.85†
Imidazole	oral	100 mg	1.15	0.20‡	1.10

Ethanol dependence was induced by oral doses of ethanol once daily for 4 days, increasing from 4 g/kg to 7 g/kg. Head twitches over a 4 min period were counted 24 h after the last dose of ethanol. 5-HTP (400 mg/kg) was given intraperitoneally (i.p.) 15 min before head twitch counting started. AMPT (300 mg/kg) was given orally 6 h before head twitch counting. Modifying drugs were given orally 1 h or i.c.v. 35 min before counting. Controls received the same inducing treatment as test animals, but only the vehicle of the modifying drug. The incidence ratio is the ratio of the head twitch count in mice receiving modifying drug to that in mice receiving vehicle of modifying drug.
NT, not tested; db cyclic AMP, dibutyryl cyclic AMP; db cyclic GMP, dibutyryl cyclic guanosine monophosphate. Oral doses in mg/kg; intracerebroventricular (i.c.v.) doses in µg/mouse.
* $P<0.05$; † $P<0.01$; ‡ $P<0.001$ (Mann-Whitney 'U' Test).

Table 2 Modification of the incidence of ethanol withdrawal head twitches by drugs affecting 5-hydroxytryptamine (5-HT) mechanisms

Effect sought	*Modifying drug*	*Route*	*Dose (mg base/kg)*	*Incidence ratio*
Inhibition of 5-HT biosynthesis	PCPA	i.p.	50	0.70
			200	0.63*
	PCA	i.p.	1.25	0.74†
			5	0.44†
			20	0.35†
Specific antagonism of 5-HT	Methysergide	s.c.	6	0.37‡
			24	0.13‡
	Methergoline	s.c.	0.4	0.44*
			4	0.02*
		oral	4	0.10‡
	MA 1420§	oral	25	0.71
			100	0.17‡
Increase of 5-HT	5-HTP	i.p.	400	3.36*

All mice received oral doses of ethanol, increasing from 4 to 7 g/kg, once daily for four days. *p*-Chlorophenylalanine (PCPA) and *p*-chloroamphetamine (PCA) were given intraperitoneally (i.p.) 1 h before each ethanol dose. Antagonists were given orally or subcutaneously (s.c.) 1 h, and 5-hydroxytryptophan (5-HTP) was given i.p. 15 min before counting started. Other details as Table 1.
§ Hong, 1973.

From this peak, head twitching slowly decreased during about 72 h to a normal level of about 0.75 twitch/min (Figure 1). Further treatment with ethanol suppressed the twitching in a dose-related way (Table 1). A comparable increase in head twitching may be observed when mice are withdrawn from continuous exposure for 8–14 days to 20 mg/l of ethanol in the air they breathe (Hammond, 1975b).

The head twitching in naive mice after treatment with 5-HTP or with AMPT was similar in appearance and incidence to that after ethanol withdrawal. Ethanol suppressed head twitches induced by 5-HTP or AMPT (Table 1), each of which in turn increased ethanol withdrawal twitching (Tables 2 and 3). Six of seven drugs tested modified head twitching induced by all three treatments in the same direction (Table 1). The seventh drug, imidazole, a weak stimulant of phosphodiesterase (Butcher & Sutherland, 1962) inhibited only the head twitching induced by 5-HTP.

Modification by drugs of withdrawal head twitching

Two inhibitors of protein biosynthesis were tested for their effect on the induction of ethanol withdrawal head twitching. Neither actinomycin D (50 μg/kg) nor cycloheximide (40 mg/kg), injected intraperitoneally 1 h before each daily dose of ethanol, significantly changed the incidence of head twitches, 24 h after ethanol withdrawal.

Drugs affecting 5-hydroxytryptamine mechanisms. With two exceptions, drugs tested for ability to modify the expression of withdrawal head twitching were given after the last dose of ethanol. When given during the induction of ethanol dependence, the inhibitors of 5-HT biosynthesis, PCPA and PCA, significantly reduced the incidence of head twitches after ethanol withdrawal, PCA being roughly 40 times more potent than PCPA (Table 2). Because, in this instance, the effect might have resulted from an inhibition of the induction of ethanol dependence, three specific antagonists of 5-HT were tested, by giving each in a single dose 23 h after the last dose of ethanol. All these antagonists reduced the incidence of withdrawal head twitching, whereas 5-HTP increased its incidence (Table 2).

Table 3 Modification of the incidence of ethanol withdrawal head twitches by drugs affecting catecholamine mechanisms

Effect sought	*Modifying drug*	*Route*	*Dose*	*Incidence ratio*
Increase of brain dopamine	L-DOPA	oral	2 × 50 mg	1.06
			2 × 200 mg	0.51†
			2 × 800 mg	0.64†
	Dopamine	i.c.v.	50 μg	0.31*
			150 μg	0.04‡
		i.v.	1000 μg	0.84
Stimulation of dopamine receptor	Apomorphine	oral	1 mg	0.47*
			5 mg	0.66*
Increase of brain noradrenaline	Noradrenaline	i.c.v.	2.5 μg	0.27†
			5 μg	0.25†
			10 μg	0.11‡
		i.v.	10 μg	1.23
	Amphetamine	s.c.	5 mg	0.58*
			10 mg	0.23‡
			20 mg	0.00‡
Inhibition of catecholamine biosynthesis	AMPT	oral	300 mg	2.00†

L-DOPA was given in two doses, one at 18 h and one at 1 h before counting head twitches. Dopamine and noradrenaline were given 35 min before counting. Other drugs were given 1 h before counting. Oral and subcutaneous (s.c.) doses are in mg/kg; intracerebroventricular (i.c.v.) and intravenous (i.v.) doses in μg/mouse. Other details as in Tables 1 and 2.

Drugs affecting catecholamine mechanisms. Dopamine or noradrenaline inhibited ethanol withdrawal head twitching. Whereas a single intracerebroventricular injection of either catecholamine was highly effective, intravenous injection was not (Table 3). L-DOPA was, however, effective when given in two oral doses after ethanol withdrawal. Other drugs that had sympathomimetic activity, such as apomorphine and amphetamine, also lessened withdrawal head twitching; whereas AMPT, which inhibits endogenous biosynthesis of dopamine and noradrenaline (Spector, Sjoerdsma & Udenfriend, 1965), increased it.

Drugs affecting acetylcholine mechanisms. By the intracerebroventricular route, acetylcholine or carbachol increased the incidence of ethanol withdrawal head twitching whereas nicotine reduced it (Table 4). By the intraperitoneal route, physostigmine increased head twitching and hyoscine lessened its incidence.

Cyclic nucleotides. By the intracerebroventricular route, db cyclic AMP lessened considerably the incidence of withdrawal head twitches, whereas db cyclic GMP increased their incidence (Table 5). After db cyclic AMP, mice were slightly sedated. ATP and GTP given intraperitoneally, affected the incidence of twitches in the same direction as did the dibutyryl salts of the corresponding cyclic nucleotides. The phosphodiesterase inhibitors, theophylline and IBMX, increased withdrawal head twitching; but imidazole was inactive (Table 5). Prostaglandins E_1 and E_2, which increase brain cyclic AMP, also lessened head twitching but prostaglandin $F_{2\alpha}$, which has little effect on brain cyclic AMP, was inactive.

If theophylline and IBMX acted by inhibiting phosphodiesterase, they would be expected to increase neuronal cyclic AMP, which probably inhibits head twitching, as well as cyclic GMP, which probably intensifies it. To investigate why theophylline and IBMX increased head twitching, we tested the effect of intracerebroventricular injections, at a dose of 50 μg/mouse, of mixtures of db cyclic AMP and db cyclic GMP, 20 min before intraperitoneal injection of 5-HTP (400 mg/kg). Compared with saline, a mixture of 25 μg db cyclic AMP and 25 μg db cyclic GMP increased the incidence ratio due to 5-HTP to 2.34 ($P < 0.05$). Even as little as 5 μg db cyclic GMP in the mixture increased this ratio to 1.69. Hence an increase of cyclic GMP would outweigh an increase of cyclic AMP in their effects on head twitching.

Discussion

Three findings argue that the large increase of head twitching that occurs after withdrawal of ethanol, given subchronically to mice, is an abstinence sign. First, increased head twitching was not seen at any time after a single intragastric dose of 4 g/kg ethanol; but it was seen after this dose had been repeated on three successive days. Second, after withdrawal of ethanol, head twitching increases as the blood and brain concentrations of ethanol fall and it reaches a peak when ethanol can no longer be detected. Third, increased head twitching is suppressed by giving a large dose of ethanol.

Several findings suggest that the increased head twitching elicited by ethanol withdrawal, 5-HTP and AMPT arises centrally. Thus, twitching elicited by these treatments was intensified by db cyclic GMP and inhibited by noradrenaline or db cyclic AMP, injected by the intracerebroventricular route (Table 1). Again, ethanol withdrawal head twitching, although

Table 4 Modification of the incidence of ethanol withdrawal head twitches by drugs affecting acetylcholine (ACh) mechanisms

Effect sought	*Modifying drug*	*Route*	*Dose*	*Incidence ratio*
Increase of brain ACh	Acetylcholine	i.c.v.	10 μg	1.56*
	Physostigmine	i.p.	0.02 mg	1.63*
Simulation of ACh	Carbachol	i.c.v.	1 μg	1.81*
	Nicotine	i.c.v.	0.125 μg	0.22
			0.5 μg	0.20*
			1 μg	0.00†
Specific antagonism of ACh	Hyoscine	i.p.	2 mg	0.33*

Acetylcholine, carbachol and nicotine were given 35 min and physostigmine and hyoscine 1 h before counting head twitches began. Intraperitoneal (i.p.) doses are in mg/kg; intracerebroventricular (i.c.v.) doses in μg/mouse. Other details as in Tables 1 and 2.

inhibited by intracerebroventricular dopamine or noradrenaline, was not inhibited by larger intravenous doses of either drug (Table 3). It was, moreover, intensified by intracerebroventricular acetylcholine or carbachol, but lessened by intracerebroventricular nicotine (Table 4). Yet again, 5-HT elicits head twitching when injected by the intracerebroventricular route (Mawson & Whittington, 1970).

Tables 2–5 give the effects sought by administering modifying drugs to mice dependent on ethanol. It is not contended that these are the only effects of the drugs given, but there is good evidence in the literature that they are the outstanding effects. The internal consistency of the results obtained with drugs modifying each mechanism in different ways supports the validity of this approach.

A seeming exception is nicotine, which lessens head twitching, whereas acetylcholine, physostigmine and carbachol increase it. This exception is, however, consistent with the findings that stimulation of nicotine receptors in rat adrenal medulla increases cyclic AMP formation (Guidotti & Costa, 1974) and that stimulation of muscarinic but not nicotinic receptors in chick cerebral hemispheres increases the level of cyclic GMP (Nahorski, Pratt & Rogers, 1976).

The main aim of this work was to elucidate the biochemical mechanism of ethanol dependence by studying the effect on an abstinence sign of drugs modifying brain amine and/or cyclic nucleotide mechanisms. The results are unexpectedly clear-cut. On the one hand, drugs that probably increase the level or activity of 5-HT, acetylcholine or cyclic GMP intensify head twitching, and drugs that probably decrease any of these inhibit head twitching. On the other hand, drugs that probably increase the level or activity of dopamine, noradrenaline or cyclic AMP inhibit head twitching and drugs that probably decrease any of these intensify it. Hence it may be supposed that head twitching arises from a change in favour of cyclic GMP of the balance between this nucleotide and cyclic AMP, resulting from a change in the balance of neurohumoral mediators, particularly against dopamine and/or noradrenaline.

There is evidence from the experiments of Carlsson and colleagues with AMPT (Carlsson, Engel & Svensson, 1972; Ahlenius, Carlsson, Engel, Svensson & Södersten, 1973; Engel, Strömbom, Svensson &

Table 5 Modification of the incidence of ethanol withdrawal head twitches by drugs related to cyclic nucleotides

Effect sought	*Modifying drug*	*Route*	*Dose*	*Incidence ratio*
Increase of brain cyclic AMP	db cyclic AMP	i.c.v.	50 µg	0.95
			100 µg	0.10†
	ATP	i.p.	50 mg	0.36†
Stimulation of adenylate cyclase	Prostaglandin E_1	s.c.	0.125 mg	0.38
			0.5 mg	0.21‡
			2 mg	0.03‡
	Prostaglandin E_2	s.c.	0.5 mg	0.41*
	Prostaglandin $F_{2\alpha}$	s.c.	2 mg	0.86
Increase of brain cyclic GMP	db cyclic GMP	i.c.v.	50 µg	2.28†
	GTP	i.p.	20 mg	2.07†
Inhibition of phosphodiesterase§	Theophylline	oral	100 mg	2.60†
	IBMX	oral	10 mg	2.11†
Stimulation of phosphodiesterase	Imidazole	oral	100 mg	1.15

Dibutyryl cyclic AMP (db cyclic AMP) and dibutyryl cyclic 3′,5′-guanosine monophosphate (db cyclic GMP) were given 35 min before counting head twitches began. ATP and guanosine triphosphate (GTP) were given 19 h before counting. The remaining drugs were given 1 h before counting. Other details as in Tables 1 and 2.
§ Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970.

Waldeck, 1974) that ethanol produces euphoria and other effects by stimulating appropriate brain neurones responding to catecholamines. There is also evidence from our experiments and from those of Goldstein (1973), Blum & Wallace (1974) and Griffiths, Littleton & Ortiz (1974) that abstinence effects are associated with a relative lowering of the amount or activity of catecholamines in the brain during ethanol withdrawal. The steps by which continued treatment with ethanol produces a state of dependence may be surmised on the basis of published evidence to be as follows. First, ethanol liberates catecholamines in the brain, thus stimulating neurones concerned with its pharmacological effects and increasing catecholamine turnover (Thadani & Truitt, 1973; Hunt & Majchrowicz, 1974; Pohorecky, 1974). Second, probably as a result of this effect, there is a slow increase of dopamine and noradrenaline concentrations in the brain during continued treatment with ethanol, followed by a rapid decline after its withdrawal (Griffiths *et al.*, 1974). Third, an increase in catecholamine concentration at the specific receptors causes a decrease in the number of these receptors, as observed in other situations (Mukherjee, Caron & Lefkowitz, 1975; Mickey, Tate & Lefkowitz, 1975; Kebabian, Zatz, Romero & Axelrod, 1975). Fourth, such a decrease in receptor numbers leads to subsensitivity towards catecholamines in the stimulation of neuronal cyclic AMP formation, as observed in slices of the cerebral cortex of the rat chronically treated with ethanol (French, Palmer, Narod, Reid & Ramey, 1975). Fifth, on withdrawal of ethanol, a shortage of catecholamines and/or a subsensitivity towards them produces the imbalance that leads to abstinence effects, as described above. This conjectural mechanism of dependence, based on the development of subsensitivity to a neurohumoral transmitter through a drug-induced change in receptor number, conforms with the hypothesis proposed some years ago (Collier, 1966). A comparable mechanism involving supersensitivity to 5-HT could be envisaged. The earlier steps in this mechanism would also give rise to tolerance.

The evidence of our experiments and that in the literature summarized above suggests that L-DOPA may be useful in the clinical management of alcoholism. It is possible, too, that specific antagonists of 5-HT might also be useful in this condition.

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