Increased release of striatal dopamine after long-term treatment with methadone in rats: inhibition by agents which increase central 5-hydroxytryptamine transmission

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A single dose of methadone increases striatal dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels (Wood et al 1980) with no effect on 3-methoxytyramine (3-MT) (Spampinato, unpublished results). Since DOPAC and 3-MT are considered indices respectively of intraneuronal and extraneuronal metabolism of dopamine (DA) (Kehr 1976; Ponzio et al 1981a), this agrees with other studies suggesting that a single dose of methadone, like morphine, increases the intraneuronal synthesis and metabolism of dopamine with no effect on DA release (Wood et al 1980). Recently, repeated treatment with methadone was found to cause intense stereotyped movements which were completely blocked by haloperidol (Cervo et al 1981), a blocker of dopamine receptors in the brain (Van Rossum 1966; Janssen 1967). Together with the fact that stereotyped behaviour is commonly associated with increased dopamine function (Andén et al 1967; Ernst 1969; Costall et al 1977) these studies suggested that repeated treatment with methadone increases dopamine release in the rat brain. This hypothesis was tested in the present study by measuring 3-MT levels in the striatum of animals which had received repeated treatment with methadone. Since in the study by Cervo et al (1981) m-chlorophenylpiperazine (CPP), a potent 5-hydroxytryptamine receptor agonist (Samanin et al 1979; Invernizzi et al 1981), and (+)-fenfluramine, a 5-HT releaser and uptake inhibitor (Garattini et al 1979) completely inhibited methadone-induced stereotypy, these drugs were included to see whether they inhibited the effect of methadone on 3-MT.

Materials and Methods

Male CD-COBS rats (Charles River, Italy), 200 g at the beginning of the experiments, were housed, three per cage, at a constant room temperature (22 ± 1 °C) and relative humidity (60%) with free access to food and water and a 12 h light-12 h dark cycle (7 a.m.–7 p.m.).

The animals received two daily subcutaneous injections (at 10 a.m. and 6 p.m.) of 5 mg kg⁻¹ methadone hydrochloride (Ist. Franco Tosi, S.p.A., Milan, Italy) for 10 days: 48 h after the last dose the animals were injected intraperitoneally with 2·5 mg kg⁻¹ m-chlorophenylpiperazine hydrochloride (Aldrich-Europe, Bel-

gium), 2·5 mg kg⁻¹ (+)-fenfluramine hydrochloride (Servier Laboratory, Neuilly-sur-Seine, France) or distilled water and 15 min later received subcutaneously 5 mg kg⁻¹ methadone or distilled water. At 30 and 60 min after methadone injection the animals were killed by microwave irradiation focussed on the head (1·3 kW at 2·45 GHz for 4·25 s) using a commercial microwave unit adapted by Medical Engineering Consultants (Lexington, Massachusetts). The rats were decapitated and the striatum was dissected according to Glowinski & Iversen (1966) and immediately frozen on dry ice. DOPAC and HVA were measured by high performance liquid chromatography (h.p.l.c.) with electrochemical detection according to Wightman et al (1977) with minor modifications (Invernizzi & Samanin 1981). 3-MT was measured by h.p.l.c. according to Ponzio et al (1981b). The data were statistically analysed by ANOVA 2 × 2 (Rocchetti & Recchia 1982). The F test for significant interaction was followed by Tukey's test to compare the various experimental groups.

Results

As shown in Table 1, 5 mg kg⁻¹ methadone significantly raised DOPAC and 3-MT concentrations respectively 60 and 30 min after injection. HVA levels were also significantly increased 60 min after methadone. DOPAC and HVA at 30 min and 3-MT at 60 min in methadone-treated rats were not significantly different from vehicle (data not shown). Rats treated with methadone showed intense stereotyped movements with peak effect 30 min after injection.

In a separate experiment a single dose of 5 mg kg⁻¹ methadone significantly raised DOPAC (controls 414 ± 38, methadone 687 ± 29 ng g⁻¹ ± s.e., P<0·01 Student's t-test) and HVA (controls 290 ± 18, methadone 568 ± 38 ng g⁻¹ ± s.e., P<0·01 Student's t-test) with no effect on 3-MT (controls 19 ± 1, methadone 20 ± 1 ng g⁻¹ ± s.e.). As in animals repeatedly treated with methadone, DOPAC and HVA were measured 60 min and 3-MT 30 min after methadone injection.

CPP 2·5 mg kg⁻¹ did not significantly modify dopamine metabolites; 2·5 mg kg⁻¹ (+)-fenfluramine had no effect on 3-MT but significantly raised the concentrations of DOPAC and HVA. Both drugs completely blocked the effect of methadone on 3-MT and significantly reduced the effect on DOPAC and HVA (F interaction P<0·01). Stereotyped movements were also

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Table 1. Effect of methadone alone or in combination with *m*-chlorophenylpiperazine (CPP) or (+)-fenfluramine on striatal concentrations of DOPAC, HVA and 3-MT in animals treated repeatedly with methadone for 10 days.

Treatment	Striatal concn. (ng g^{-1} ± s.e.)		
	DOPAC	HVA	3MT
Vehicle + vehicle	362 ± 13	241 ± 13	14 ± 1
Vehicle + methadone	747 ± 31*	653 ± 28*	23 ± 1*
CPP + Vehicle	357 ± 25	286 ± 18	13 ± 1
CPP + methadone	629 ± 15†	450 ± 40†	15 ± 1†
Vehicle + vehicle	395 ± 15	303 ± 10	16 ± 1
Vehicle + methadone	740 ± 15*	540 ± 13*	23 ± 1*
(+)-fenfluramine + vehicle	490 ± 16*	410 ± 9*	15 ± 1
(+)-fenfluramine + methadone	570 ± 17†	426 ± 11†	16 ± 1†

Each value is the mean of 6 determinations. 3-MT was measured 30 min and DOPAC and HVA 60 min after methadone injection. CPP, 2·5 mg kg^{-1} and (+)-fenfluramine, 2·5 mg kg^{-1}, were injected i.p. 15 min before methadone, 5 mg kg^{-1} s.c.
*$P<0.01$ compared with vehicle + vehicle.
†$P<0.01$ F interaction.

completely inhibited in rats which had received CPP or (+)-fenfluramine before methadone.

Discussion
Repeated treatment with methadone, unlike a single dose, raised striatal 3-MT, suggesting that dopamine release was increased in these animals. This agrees with previous findings showing dopamine-dependent stereotyped behaviour in animals repeatedly treated with methadone (Cervo et al 1981). The mechanism of the different effects of acute and repeated treatment with methadone on dopamine release is not clear. Opiate sites, which activate or reduce dopamine function, have been described in the rat brain (Iwamoto & Way 1977; Kuschinsky et al 1975; Celsen & Kuschinsky 1974). Since an increase is found in DOPAC and HVA (but not 3-MT) when impulse flow in dopamine neurons is blocked (Walters & Roth 1972; Kehr 1976), inhibitory opiate sites may be particularly involved in the effect of a single dose of methadone. This is also suggested by the fact that acute treatment with methadone completely blocks the stereotypy caused by (+)-amphetamine (Cervo et al 1981), an effect commonly attributed to dopamine release from nerve terminals (Andén et al 1967; Ernst 1969; Costall et al 1977). Tolerance to the inhibitory effect may reveal the excitatory component of opiate sites during repeated methadone treatment with the appearance of stereotyped movements and increased 3-MT.

It has been recently suggested that 5-HT is one mechanism by which methadone inhibits dopamine-containing neurons (Cervo et al 1981). In fact, *p*-chlorophenylalanine, an inhibitor of 5-HT synthesis (Koe & Weissman 1966), completely prevented the inhibitory effect of a single dose of methadone on amphetamine-induced stereotypy (Cervo et al 1981). On the basis of this and other findings it was suggested (Cervo et al 1981) that the increased dopamine function is revealed

when an adaptive reduction in 5-HT receptors develops in animals treated repeatedly with narcotics (Samanin et al 1980). 5-HT-sensitive sites have recently been described which inhibit dopamine release in nerve terminals in the striatum (Ennis & Cox 1982; Westfall & Tittermary 1982).

The fact that CPP and (+)-fenfluramine at doses reported to selectively activate brain 5-HT mechanisms (Samanin et al 1979; Invernizzi et al 1981; Garattini et al 1979), completely blocked the increase in 3-MT levels and stereotypy found during long-term treatment with methadone is compatible with the hypothesis that drugs increasing 5-HT transmission very effectively block dopamine-dependent behavioural activation in rats repeatedly treated with narcotics (Cervo et al 1981).

REFERENCES

Andén, N. E., Rubenson, A., Fuxe, K., Hökfelt, T. (1967) J. Pharm. Pharmacol. 19: 627–629

Celsen, B., Kuschinsky, K. (1974) Naunyn-Schmiedeberg's Arch. Pharmacol. 284: 159–165

Cervo, L., Romandini, S., Samanin, R. (1981) Life Sci. 29: 2585–2591

Costall, B., Naylor, R. J., Cannon, J. G. Lee, T. (1977) J. Pharm. Pharmacol. 29: 337–342

Ennis, C., Cox, B. (1982) Neuropharmacology 21: 41–44

Ernst, A. M. (1969) Acta Physiol. Pharmacol. Neerl. 15: 141–154

Garattini, S., Caccia, S., Mennini, T., Samanin, R., Consolo, S., Ladinsky, H. (1979) Curr. Med. Res. Opin. 6, suppl. 1: 15–27

Glowinski, J., Iversen, L. L. (1966) J. Neurochem. 13: 655–669

Invernizzi, R., Cotecchia, S., De Blasi, A., Mennini, T., Pataccini, R., Samanin, R. (1981) Neurochem. Int. 3: 239–244

Invernizzi, R., Samanin, R. (1981) Pharmacol. Res. Commun. 13: 511–516

Iwamoto, E. T., Way, E. L. (1977) J. Pharmacol. Exp. Ther. 203: 347–359

Janssen, P. A. J. (1967) Int. J. Neuropsychiatry 3: S10–S18

Kehr, W. (1976) Naunyn-Schmiedeberg's Arch. Pharmacol. 293: 209–215

Koe, B. K., Weissman, A. (1966) J. Pharmacol. Exp. Ther. 154: 499–516

Kuschinsky, K., Celsen, B., Huppertz, A. (1975) Int. J. Clin. Pharmacol. Biopharm. 12: 129–133

Ponzio, F., Achilli, G., Algeri, S. (1981b) J. Neurochem. 36: 1361–1367

Ponzio, F., Achilli, G., Perego, C., Algeri, S. (1981a) Neurosci. Lett. 27: 61–67

Rocchetti, M., Recchia, M. (1982) Comput. Programs Biomed. 14: 7–20

Samanin, R., Cervo, L., Rochat, C., Poggesi, E., Mennini, T. (1980) Life Sci. 27: 1141–1146

Samanin. R., Mennini, T., Ferraris, A., Bendotti, C., Borsini, F., Garattini, S. (1979) Naunyn-Schmiedeberg's Arch. Pharmacol. 308: 159–163

Van Rossum, J. M. (1966) Arch. Int. Pharmacodyn. Ther. 160: 492–494

Walters, J. R., Roth, R. H. (1972) Biochem. Pharmacol. 21: 2111–2121

Westfall, T. C., Tittermary, V. (1982) Neurosci. Lett. 28: 205–209

Wightman, R. M., Plotsky, P. M., Strope, E., Delcore, R. Jr., Adams, R. N. (1977) Brain Res. 131: 345–349

Wood, P. L., Stotland, M., Richard, J. W., Rackham, A. (1980) J. Pharmacol. Exp. Therm. 215: 697–703

Naloxone radioimmunoassay: an improved antiserum

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Naloxone is a potent opiate antagonist which has found clinical utility in the treatment of narcotic overdose. More recently the use of naloxone to identify the modulation of various systems by endogenous opiates (Sawynok et al 1979) has renewed interest in its pharmacokinetics in man and laboratory animals. Although a radioimmunoassay (RIA) procedure for naloxone was developed several years ago (Berkowitz et al 1975), both the antiserum and antigen are currently unavailable (Spector, personal communication). Since we required a sensitive and specific assay for naloxone in body fluids and tissues for various ongoing studies in our laboratory (Kreek et al 1983), we were prompted to prepare a new antiserum against the opiate antagonist.

The major determinants of antibody specificity in the naloxone molecule that were of interest to us were the D-ring nitrogen function and the C-3 hydroxyl group. Since the C-6 carbonyl group in naloxone has been shown to lend itself readily to modification (Hahn et al 1975), we decided to produce an antibody which was raised against an antigen containing a substituent at C-6. This antibody was expected to exhibit little binding to other narcotic structures which could be present in the tissues and body fluids to be studied.

In this manuscript we describe the preparation and characterization of a new antiserum to naloxone. The cross reaction of the antiserum with various narcotic substances is examined and discussed. The use of the antiserum for the assay of naloxone in human serum is also reported.

Antiserum preparation. Carboxymethylamine hemihydrochloride (0·39 mmol, Aldrich Chemical Co.) and 2 M potassium hydroxide (0·5 ml) were added to a solution of naloxone (0.26 mmol) in ethanol (10 ml). The reaction was heated under reflux for 2 h, after which time the pH of the cooled solution was adjusted to 7 with 10% hydrochloric acid in methanol. The solvent was removed in-vacuo and the residue was purified by preparative thin layer chromatography on silica gel (Analabs, North Haven, CT) using chloroform–methanol–ammonia (60:30:5) as the solvent

system. An infrared spectrum (KBr) of the isolated naloxone-6-(O-carboxymethyl) oxime derivative I (Fig. 1) showed that the C-6 ketone absorption was no longer present. The nuclear magnetic resonance spectrum (MeOH-d⁴) showed an absorption for the added methylene group in the C-6 oxime derivative. The bovine serum albumin (BSA) derivative of compound I was prepared by a modification of the mixed anhydride procedure (Wainer et al 1972).

Naloxone-6-(O-carboxymethyl) oxime (0·12 mmol) was dissolved in dioxane (5 ml) and isobutylchloroformate (0·12 mmol, Aldrich Chemical Co.) and tributylamine (0·12 mmole, Eastman) were added. The reaction mixture was stirred for 30 min at about 18 °C after which a solution of BSA (2·4 μmol) in water (20 ml) and dioxane (20 ml) at pH 8·5 was added. The mixture was stirred at 5 °C for 4 h, and then dialysed for 48 h with water changes every 12 h. The antigen was isolated after removal of the water by lyophilization.

Rabbits were injected once intramuscularly with naloxone-6-(O-carboxymethyl) oxime-BSA (NLX-ag) in complete Freund's adjuvant (100 μg/0·5 ml). Two weeks after the first injection the rabbits received
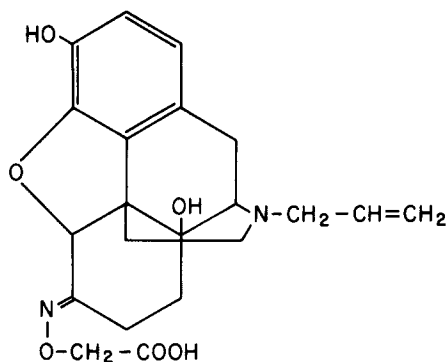
Naloxone - 6 - (O - Carboxymethyl) Oxime

FIG. 1. Structural formulae of the naloxone hapten.

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