

Research Report

Repeated administration of MDMA down-regulates preprocholecystokinin mRNA expression but not tyrosine hydroxylase mRNA expression in neurones of the rat substantia nigra

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Abstract

The effect of repeated administration of 3,4-methylenedioxymethamphetamine (MDMA) on the expression of tyrosine hydroxylase and preprocholecystokinin (CCK) messenger RNAs in substantia nigra was examined by in situ hybridisation histochemistry. Sections hybridised with ³⁵S-labelled oligonucleotides were subjected to computerised image analysis to determine the density of silver grains above positively labelled cells as an index of steady state mRNA levels. In the substantia nigra pars compacta, CCK mRNA levels were significantly reduced in drug-treated animals 24 h and at 2 weeks after the last dose of MDMA (10 mg/kg i.p., twice daily for 4 days). In the same animals, MDMA caused no change in the level of tyrosine hydroxylase mRNA in this brain region. The results show that MDMA can produce changes in dopamine neurones. Furthermore, since tyrosine hydroxylase and cholecystokinin are co-expressed in substantia nigra pars compacta, these results suggest that the expression of the tyrosine hydroxylase and CCK genes are regulated independently.

Key words: Preprocholecystokinin; In situ hybridization; Gene expression; Brain; Dopamine; MDMA

1. Introduction

The popular drug of abuse, 3,4-methylenedioxymethamphetamine (MDMA, or 'ecstasy') has been reported to be a potent neurotoxin of the serotonin system, causing long-term reductions in brain serotonin, tryptophan hydroxylase and serotonin uptake sites as well as swollen and broken serotonin neurones (reviewed by Rattray [32]). The mechanism of neurotoxicity is not known, although several groups have suggested that MDMA-induced loss of serotonin is dependent upon dopamine release [35,42]. Depletion of central dopamine stores or destruction of the substantia nigra prevent the 'neurotoxic' effects of a single

high dose of MDMA, and, conversely, L-DOPA potentiates MDMA-induced decrease in serotonin levels [36].

Although the primary acute effect of MDMA is to release serotonin in the CNS, acute MDMA administration has also been shown to affect dopamine systems. It has been shown that MDMA decreases the firing rate of dopamine neurones in the substantia nigra [18,22], although this result was not confirmed by another group [29]. Consistent with a decrease in the firing rate of nigrostriatal neurones, MDMA has been shown to increase the release of dopamine from nigrostriatal neurones in vivo and in vitro [15,26,27,44]. However, low doses of MDMA have been reported to decrease dopamine release [10]. It is likely that MDMA alters dopamine release by an indirect interaction with dopamine neurones through enhanced serotonin release, rather than by a direct interaction with dopamine neurones.

Although a single administration of MDMA alters dopamine release and turnover, repeated administration of MDMA under conditions which produce pro-

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found degeneration of serotonin neurones produces no major changes in the levels of dopamine or its metabolites, or the activity of tyrosine hydroxylase [2,16,42]. However, Johnson et al. [16] reported that, although dopamine levels were unaffected by repeated MDMA administration, the levels of neurotensin which co-exists with dopamine in nigrostriatal neurones was increased, suggesting that repeated MDMA administration is capable of altering dopamine-containing neurones.

In this study, we have investigated whether repeated MDMA administration produces changes to the dopamine system by examining the level of expression of tyrosine hydroxylase messenger RNA in the substantia nigra. In addition, we have examined the level of expression of the messenger RNA which encodes a neuropeptide, cholecystokinin which immunocytochemical and *in situ* hybridisation studies have indicated is co-localised with tyrosine hydroxylase mRNA and dopamine in dopamine neurones of the mesencephalon including the substantia nigra pars compacta (SNc) [34,37,38].

2. Materials and methods

Male Wistar rats (200–250 g) were injected twice daily for 4 days with ($\pm$)-methylenedioxymethamphetamine (10 mg/kg, *i.p.*) or saline vehicle. Twenty-four hours or 14 days after the last drug injection, animals were killed and their brains removed and rapidly frozen. Coronal brain sections (20 μ m) were cut onto gelatin-chrome alum-treated microscope slides. Sections were fixed in fresh 4% paraformaldehyde in PBS, pH 7.4 for 10 min, washed twice for 10 min in PBS, acetylated in 0.25% acetic anhydride/0.1 M triethanolamine/0.15 M NaCl for 15 min and then dehydrated and delipidated through a graded series of alcohols and chloroform. Sections were air dried and stored at -70°C until use.

Oligonucleotides complementary to the rat preprocholecystokinin (CCK) mRNA [6] (CCA GCC CAT GTA GTC CCG GTC ACT TAT CCT GTG GCT A) and rat tyrosine hydroxylase mRNA [19] (TGG TAG GTT TGA TCT TGG TAG GGC TGC ACA) were synthesised on an ABI oligonucleotide synthesiser and purified by polyacrylamide gel electrophoresis. Four picomoles of each oligonucleotide were radiolabelled by incubating with 30 pmol [^{35}S]dATP (NEN) and 60 units deoxynucleotidyl terminal transferase (Life Technologies) for 60 min at 31°C in the manufacturer's reaction buffer. Labelled probe was separated from unincorporated deoxynucleotides on a size exclusion column (NICK, Pharmacia), freeze-dried and resuspended to a final concentration of 2 nM in hybridisation buffer (50% formamide, 0.02% bovine serum albumin, 0.02%

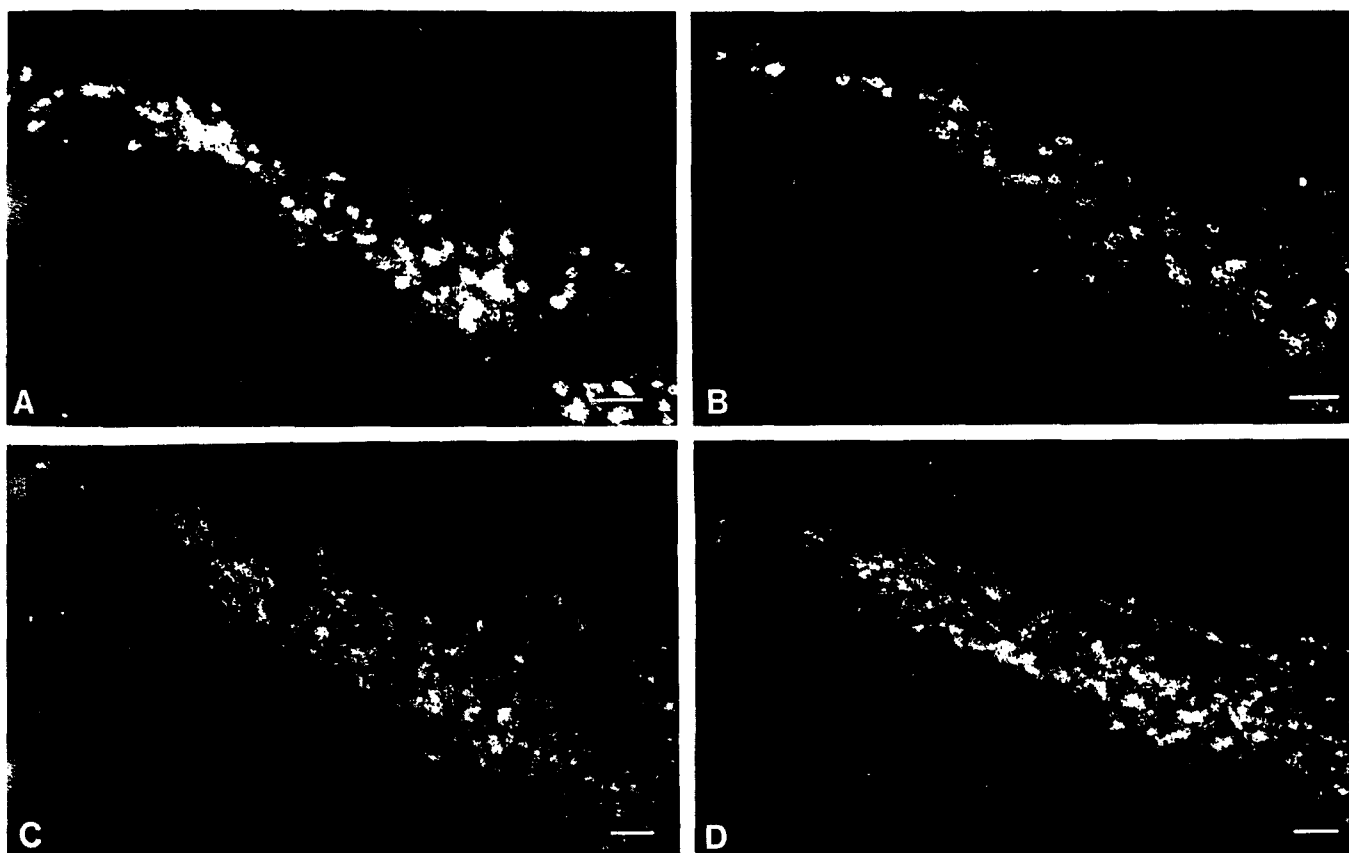


Fig. 1. Expression of CCK messenger RNA (A,B) and tyrosine hydroxylase messenger RNA (C,D) in the substantia nigra pars compacta of control and MDMA-treated sections processed for *in situ* hybridisation histochemistry. CCK mRNA expression was lower in MDMA-treated animals (B) compared to control animals (A). There was no change in tyrosine hydroxylase mRNA levels between control animals (C) and MDMA-treated animals (D). Bar = 100 μ m.

polyvinylpyrrolidone, 0.02% ficoll-400, 100 µg/ml polyadenylate, 100 µg/ml denatured herring sperm DNA, 4×SSC, 10% dextran sulphate, 10 mM dithiothreitol). 1×SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0. The specific activity of the CCK and tyrosine hydroxylase probes were 6900 Ci/mmol and 5000 Ci/mmol, respectively.

Prior to hybridisation, sections were dehydrated through a graded series of alcohols and air-dried. Ten µl of probe was applied to each section, the slides coverslipped and incubated overnight at 37°C in sealed humidified containers. Following hybridisation, the slides were washed in 1×SSC at room temperature for 20 min, 1×SSC for 1 h at 55°C and 1×SSC for 10 min at room temperature. Slides were dehydrated through a series of alcohols, air-dried and dipped in photographic emulsion (Ilford K5, 1:1 with water). Slides were exposed for 30 days. After developing, the sections were stained in toluidine blue, dehydrated and coverslipped for microscopic analysis.

The number of silver grains over neurones which expressed CCK mRNA or tyrosine hydroxylase mRNA were determined using a Leitz laborlux microscope with Sight Systems image analysis software [31]. The grains were visualised under epi-polarised illumination to minimise interference from the underlying tissue section and the images captured using a Hitachi CCTV camera [30]. The field detected area was calculated within a fixed size rectangular frame placed over positive neurones. The number of grains per µm² for each positive cell was calculated from a calibration graph. To construct the calibration graphs, field detected area readings were plotted against the manual grain counts over a series of neurones on a standard slide. Cells on the standard slide were measured at the beginning and end of each counting session. This standardisation procedure was performed to correct for fluctuations in the epi-polarised light source during counting sessions, and also served to correct for fused grains over heavily labelled cells.

3. Results

The tyrosine hydroxylase probe showed heavy labelling of dopamine and noradrenaline cell groups, but insignificant labelling elsewhere. On RNase-treated sections, no labelling was observed with the tyrosine hydroxylase probe (results not shown). The specificity of the CCK probe has been reported elsewhere [33].

In midbrain, both CCK and tyrosine hydroxylase mRNA was expressed in most neurones of the substantia nigra pars compacta (SNc) and ventral tegmental area, with a similar pattern to that reported by other workers. Most neurones in the SNc expressed both CCK and tyrosine hydroxylase mRNAs, suggesting that they are co-expressed in dopamine cells (Fig. 1). After MDMA administration, the grain density above cells hybridised with the CCK probe was noticeably reduced (compare Fig. 1A to Fig. 1B), whereas the labelling with the tyrosine hydroxylase probe was relatively unchanged (compare Fig. 1C to Fig. 1D).

Quantitative image analysis of silver grain densities above positive cells showed that repeated administration of MDMA produced a significant reduction in CCK mRNA expression in SNc. This effect was apparent both at 24 h and 2 weeks after the last drug dose (Table 1). However, there was no significant change in the levels of tyrosine hydroxylase mRNA levels (Table

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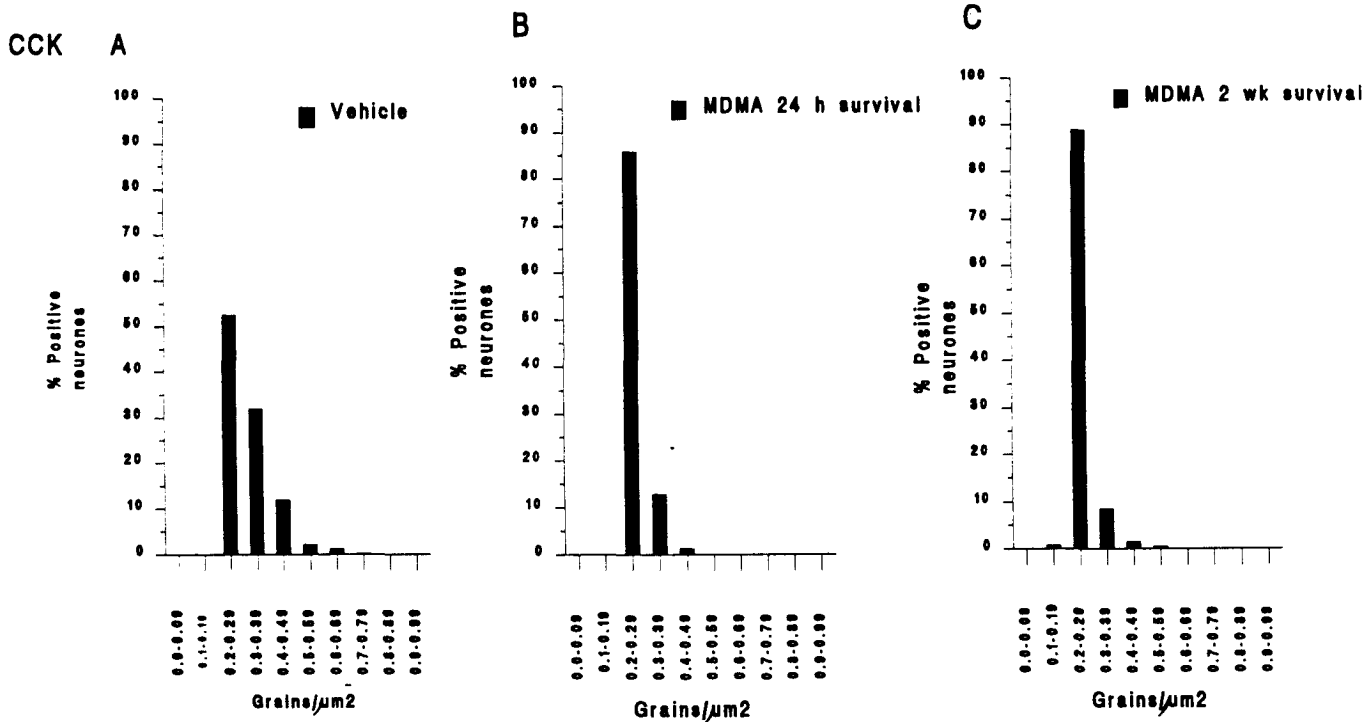


Fig. 2. Population distribution histograms showing the range of levels of CCK messenger RNA expression in substantia nigra expressed as grains/µm² for vehicle-treated animals (A) and animals treated with MDMA (10 mg/kg, twice daily for 4 days) and killed 24 h (B) or 14 days (C) after the last drug dose. Each histogram shows combined measurements of the expression levels of 318-485 neurones pooled from 6 animals per drug-treated group and 11 animals in the control groups.

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Table 1
Effect of chronic MDMA administration on CCK and tyrosine hydroxylase mRNA levels in rat substantia nigra pars compacta

	Gene expression (silver grains/ μm^2)	
	CCK mRNA	TH mRNA
24 hour survival		
Vehicle	0.240 $\pm$ 0.016 (5)	0.230 $\pm$ 0.025 (5)
MDMA	0.152 $\pm$ 0.011 (6) **	0.242 $\pm$ 0.023 (6)
2 week survival		
Vehicle	0.195 $\pm$ 0.010 (6)	0.205 $\pm$ 0.016 (6)
MDMA	0.153 $\pm$ 0.012 (6) *	0.228 $\pm$ 0.011 (6)

Rats were injected with ($\pm$)-MDMA (10 mg/kg, i.p.) or control vehicle twice daily for 4 days. Animals were killed 24 h or 2 weeks after the last injection of MDMA. Expression of the CCK and tyrosine hydroxylase (TH) genes were measured. For each animal, image analysis was carried out on 27–108 positive neurones taken from the same part of the substantia nigra pars compacta. Numbers show the steady-state level of gene expression (silver grains per μm^2 over positive neurones) $\pm$ S.E.M. with the numbers of animals used in brackets. ** $P < 0.005$, * $P < 0.05$ by Student's *t*-test.

1). Population distribution histograms suggest that there is a single population of cells expressing CCK and tyrosine hydroxylase mRNA (Figs. 2 and 3). After MDMA administration, the amount of CCK mRNA in positive neurones is markedly shifted, so that few cells remain that express high levels of this mRNA (Fig. 2). There is no evident shift of expression of tyrosine

hydroxylase mRNA after MDMA administration (Fig. 3).

4. Discussion

The results of this study indicates that repeated administration of MDMA produces decreased CCK mRNA levels in neurones of the substantia nigra, 24 h and 2 weeks after the last dose of the drug. Paradoxically, tyrosine hydroxylase mRNA levels were unaffected even though this messenger RNA is expressed in the same cells as CCK mRNA. The lack of effect of MDMA on tyrosine hydroxylase mRNA levels is consistent with reports from other groups which have shown that repeated MDMA administration does not cause changes in levels of dopamine or its metabolites [2,8,15,42]. Our results contrast with the effects of amphetamine, which is specific to the dopamine system and has been reported to increase both CCK and tyrosine hydroxylase mRNA levels in rat ventral tegmental area [14].

Since tyrosine hydroxylase mRNA and CCK mRNA are co-localised within dopamine neurones in substantia nigra pars compacta, but tyrosine hydroxylase messenger RNA levels do not change, our findings suggest that CCK mRNA and tyrosine hydroxylase mRNA are

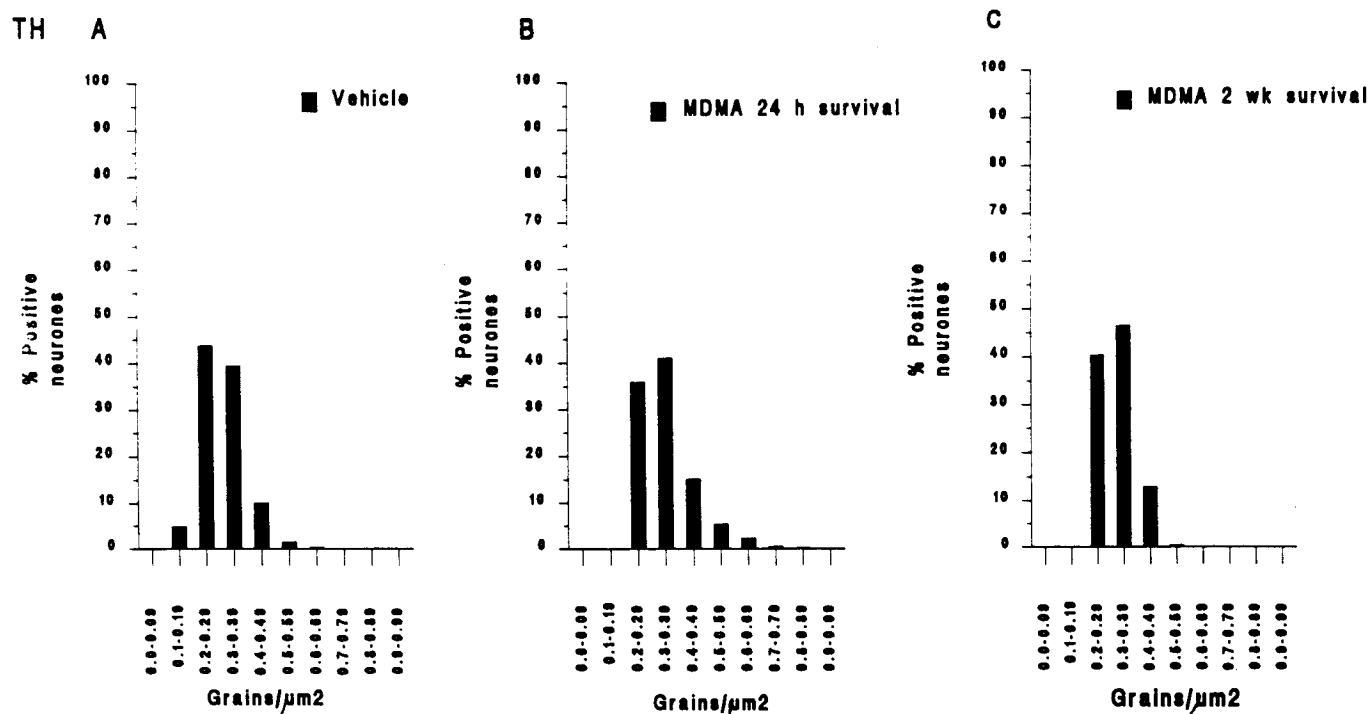


Fig. 3. Population distribution histograms showing the range of levels of tyrosine hydroxylase messenger RNA in neurones in substantia nigra pars compacta. Messenger RNA levels are expressed as grains/ μm^2 . A: vehicle-injected controls. B,C: mRNA expression in cells 24 h (B) or 2 weeks (C) after chronic MDMA administration. Each histogram shows combined measurements of the expression levels of 406–1083 neurones pooled from 6 animals per drug-treated group and 11 animals in the control groups.

regulated in different ways. Differential regulation of CCK mRNA and tyrosine hydroxylase mRNA has previously been reported in the ventral tegmental area of the marmoset: the neurotoxin MPTP has been shown to reduce the number of cells which express tyrosine hydroxylase mRNA with a less marked decrease in the number of cells that express CCK mRNA [40]. The same group has recently reported that in substantia nigra of rats, ageing decreases the expression of tyrosine hydroxylase mRNA more than CCK mRNA [39].

Our results suggest that CCK mRNA expression, but not tyrosine hydroxylase mRNA expression is controlled by serotonin innervation of the substantia nigra. The effects of repeated MDMA administration on CCK messenger RNA levels are consistent with the hypothesis that MDMA causes selective degeneration of serotonin neurones. Dopamine neurones of the substantia nigra receive extensive innervation from serotonin neurones [4], and are responsive to serotonin administration [7]. Quantitative autoradiographic studies have shown that repeated MDMA administration reduces the number of serotonin uptake sites in substantia nigra by 70% 18 h after the last drug administration [3]. The same authors reported that 14 days after the last dose of MDMA, the number of serotonin uptake sites in substantia nigra were still only 50% of controls [3]. Thus, the changes in CCK mRNA levels observed here correlate well with these observations, and suggest that the level of CCK mRNA is controlled by serotonin innervation of the substantia nigra.

The biochemical basis of this differential regulation of CCK and tyrosine hydroxylase gene expression after loss of serotonin innervation of substantia nigra is unclear. One explanation of our results is that CCK messenger RNA levels are maintained by tonic activation of serotonin receptors, whilst tyrosine hydroxylase messenger RNA levels are not. We note that dopamine neurones in the substantia nigra expresses high levels of the 5-HT₂ receptors, mainly of the 5-HT_{2C} subtype [1,24], but not 5-HT_{1A}, 5-HT_{1B} or 5-HT₃ receptors [11,17,25], and that receptors of the 5-HT₂ class use inositol trisphosphate (IP₃), but not cAMP as a secondary messenger. The expression of both CCK mRNA [13] and tyrosine hydroxylase mRNA [8] are known to be activated by cAMP, and both genes have AP-1 and AP-2 sites which are putative enhancer elements for transcription factors that are induced by IP₃. Whether these genes can be differentially activated by inositol trisphosphate-stimulated systems is not known. Alternatively, the lack of effect of MDMA on tyrosine hydroxylase messenger RNA levels may be a consequence of the reduction in CCK messenger RNA levels. A loss of CCK in nigrostriatal neurones may produce a loss in cholecystokinin peptides that may in turn stimulate dopamine release and increase synthesis of tyrosine hydroxylase messenger RNA. Thus, CCK-en-

hanced production of tyrosine hydroxylase mRNA could compensate for a reduction in tyrosine hydroxylase messenger RNA induced by serotonin denervation in substantia nigra.

The consequences of a reduction of CCK mRNA in substantia nigra is unknown. CCK regulates the activity and actions of midbrain dopamine neurones in SNc in a complex and poorly understood manner [5]. CCK increases the firing rate of dopamine-containing neurones in the SNc and decreases their sensitivity to dopamine and dopamine agonists [41]. In the striatum, the major termination region of dopamine neurones in SNc, most studies indicate that CCK or its agonist, ceruletide, decrease the turnover of dopamine and reduce the number of dopamine receptors [5,9,21]. Since CCK may inhibit the release and actions of dopamine, a loss of CCK may lead to a potentiation of the effects of dopamine in the striatum.

The functional significance of CCK dopamine interactions has yet to be elucidated, although there has been much interest in the possible role of CCK in modulating dopamine transmission and its relevance to human conditions such as psychoses, anxiety, drug addiction and movement disorders [5,12,20,43]. However, the extent of co-existence of CCK and dopamine in human substantia nigra is a matter of some controversy [28]. We do not know whether the phenomenon that we observe here may underlie some of the neurotoxic effects of MDMA or long-term psychological effects of MDMA abuse such as psychosis [23].

Acknowledgements

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