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COLCHICINE, an axonal transport blocking agent, was unilaterally injected in the medial forebrain bundle of rats. As early as 18 h after the injection a rapid decrease in TH-mRNA level was observed in the substantia nigra and the ventral tegmental area (SN/VTA) on the injected side. In contrast, TH protein levels remained stable for 48 h, and decreased later in both cells bodies and terminals (caudate/putamen). The number of TH-immunopositive cells in SN/VTA increased after colchicine equally in both sides, excluding a neurotoxic effect. These results suggest that TH gene expression is controlled by a retrogradely transported activating factor rather than by feedback inhibition by the end product, i.e. TH protein. *NeuroReport* 9: 1529–1532 © 1998 Rapid Science Ltd.

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Mesencephalic THmRNA-reduced expression by blocking axonal transport with colchicine

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Introduction

Tyrosine hydroxylase (TH) is the rate-limiting enzyme of dopamine (DA) synthesis. The regulation of TH gene expression in substantia nigra (SN) and ventral tegmental area (VTA) remains an open question. DA cell bodies, while originating from restricted and compact areas in the mesencephalon, innervate large and various structures of the forebrain. The mechanisms by which the cell bodies are informed of the need for TH protein in the terminals are still largely unknown. Intracellular messengers could be retrogradely transported but indirect descending pathways could also be involved.¹ An intracellular mode of TH gene regulation has been proposed in the nigrostriatal system, involving the retrograde transport of neurotensin.² Following chronic administration of imipramine a reduction of mRNA coding for TH protein has recently been observed in VTA DA-cells, correlated with a decrease of DA transmission in the amygdala. In the mean time, an increase of the amount of TH protein was observed in both cell bodies and terminals.³ These results suggested that the reduced DA transmission in terminals was not a consequence of the TH gene expression impairment in the cell bodies. A message could have been generated at the terminal level, reaching the cell bodies after retrograde transportation in the axons or through heterologous descending pathway. It is possible that accumulation of the TH protein, produced by the reduction of its turnover, could play this role by a feed-back inhibitory mechanism. To

test the hypothesis of a TH inhibition of TH gene expression, we attempted to increase TH protein levels in DA cell bodies by blocking axonal transport with colchicine, injected locally and unilaterally in the medial forebrain bundle (MFB). Following colchicine injection, the TH amount was evaluated at successive times in the SN/VTA regions and the caudate putamen complex (C/P). Possible alterations in TH-mRNA were also examined in SN/VTA. Finally immunopositive-TH cells were counted in SN/VTA regions and the ipsilateral/contralateral ratio was calculated.

Materials and Methods

The animals used in this study were maintained and sacrificed in accordance with the European Communities Council Directive (86/609/EEC). Male Wistar rats were anesthetized (ketamine/xylazine mixture, 15/1.5 mg/kg) and injected in the right MFB with colchicine (Serva, 20 mg/ml in 0.6 µl NaCl 9%, MFB coordinates: 5.0 mm anterior and 2.5 mm above the ear bar, lateral: 1.3, the horizontal plane passing through the interaural axis and incisor bar). Animals were sacrificed (five per group) 8 h, 12 h, 18 h, 24 h, 48 h and 96 h after surgery. The brain was rapidly removed, frozen in isopentane at -35°C for 1 min and stored at -80°C. Serial sections were cut at 20 µm for 100 µm through the SN/VTA. Two adjacent sections were directly collected on nitrocellulose filters (Millipore, Bedford, Ma; HAHY 02412, 0.45 µm pore size). TH protein and TH-mRNA levels

were measured using autoradiography on the first and the second slice respectively. For the C/P region, only one section was collected at 100 μm intervals on nitrocellulose filter for TH protein transfer and autoradiography.

Measurements of the TH protein and TH-mRNA were made using two techniques based on transfer onto nitrocellulose filters before immunautoradiography. TH protein was detected by the method originally described by Weissmann *et al.*⁴ and modified by Leviel and Faucon Biguet.⁵ TH protein level was estimated in arbitrary units, using a series of defined quantities of TH protein (medulla homogenate) adsorbed on nitrocellulose filters for standardization. TH-mRNA on nitrocellulose filters was measured by the technique described by Lavergne *et al.*³ TH-mRNA was quantified using densitometric scanning of serial dilutions of total RNA extracted from PC12 cells dotted on nitrocellulose membrane and hybridized in the same experimental conditions.

The quantitative analysis of TH protein and TH-mRNA from autoradiogram was similar. The quantities were calculated on each slice using the following formula $Q_t = \log(D_0/D) \cdot S$, where S is the surface of the section exhibiting the signal and D the mean optical density. A region of the same size but corresponding to a non-catecholaminergic structure was taken as the blank value (D_0). Results are presented as percentages of the values obtained from the side contralateral to the injection (considered as 100%). Statistical analysis was conducted by comparing values obtained from both sides and using the two-tailed Student's t -test.

On three animals in the 18 h group, three animals in the 24 h group and a control group of three non-injected animals, the cells were labelled for TH immunoreactivity (TH_{im}) in the SN/AVT region using the method described by Riche *et al.*⁶ This was done on a third section, adjacent to the two previously mentioned but collected on a glass slide. The number of TH_{im} -positive cells were counted on each side of each slice (corrected by Abercrombie's formula⁷), and the ipsilateral/contralateral ratio was calculated.

Results

The TH-mRNA level in SN/VTA was rapidly and greatly decreased after the colchicine injection. The time course of the alterations of TH-mRNA expression is presented in Fig. 1. A 25% decrease could be detected as early as 18 h after the treatment. The difference between ipsilateral and contralateral sides was maximal at 48 h (14% of controls) and then only slightly decreased at 96 h (35% of controls). In contrast, TH protein was reduced later. The

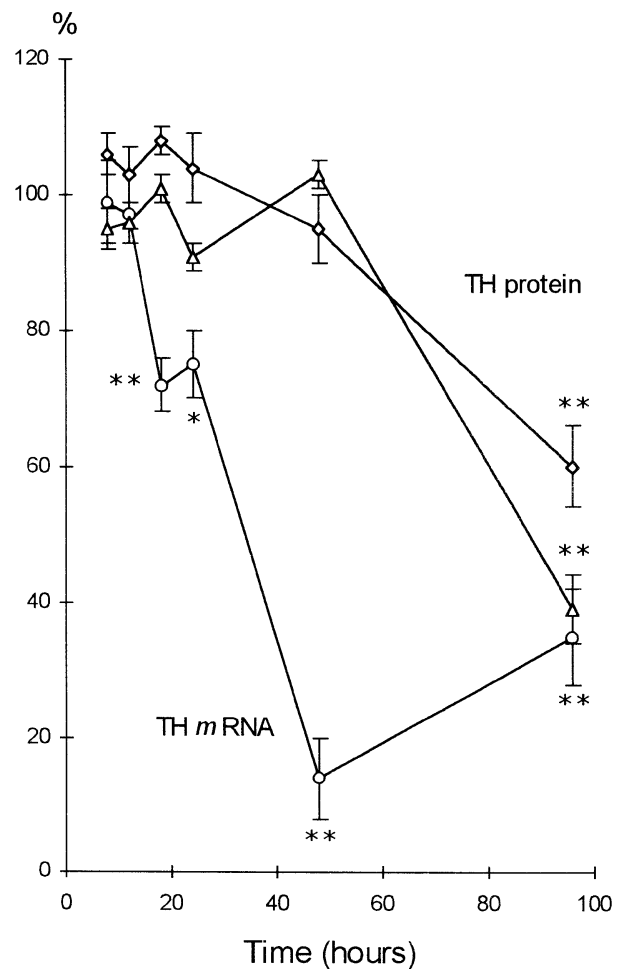


FIG. 1. Time course alterations in TH-mRNA (\circ) and TH protein (\diamond) in the SN/VTA, and TH protein levels (Δ) in C/P after colchicine injection. Each point corresponds to the sum of the amounts measured in the region of interest of all sections collected, averaged between the animals of the group and expressed as a percentage of the contralateral corresponding area (** $p < 0.01$; * $p < 0.1$, Student's t -test, $n = 5/\text{group}$).

effect was only detectable between 48 and 96 hours post-treatment. When it appeared, the profile of the decline was quite similar in both the SN/VTA and C/P regions.

After colchicine injection, no change in the morphology of cells in the SN/VTA region could be seen with light microscopy. A general increase (+39%) in TH_{im} cell number was observed at 18 h and maintained at 24 h and this effect was strictly bilateral. Indeed, the ratio between the number of cells in the both sides remained unchanged, as shown in Table 1.

Discussion

We describe in the present study the precise time course of colchicine-induced alterations in TH protein and TH-mRNA levels in the DA cells of

Table 1. Number of TH_{im} cells in SN/VTA after colchicine injection.

	No total slices	Left side	Right side	Contralateral/ipsilateral ratio
Controls	57	104.16 ± 4.03	107.11 ± 4.17	1.03 ± 0.01
18 h	62	143.32 ± 3.63	150.87 ± 3.79	1.06 ± 0.01
24 h	52	144.94 ± 3.28	147.12 ± 4.53	1.01 ± 0.02

SN/VTA. A decrease in TH-mRNA was observed as early as 18 h after colchicine injection along the axons while TH protein remained unchanged both in cell bodies and terminals. This effect concerned the ipsilateral cells only, suggesting that TH gene was down-regulated in response to blockade of axonal traffic. This also pinpoints the presence of a tonic positive control of TH gene expression in dopaminergic mesencephalic cells. Such observation does worth being further discussed but the colchicine effects should also be carefully considered with respect to the known various effects of this substance.

Colchicine exerts antimetabolic and other biological effects by the disruption of microtubules.⁸ In neurons, colchicine alters the structure of the cytoskeleton, blocking axonal transport and firing activity.⁹ This substance was described as toxic for various types of cells,¹⁰ including the granule cells of the hippocampus.^{11,12} Although the striatum is known to be poorly sensitive to colchicine neurotoxicity,¹³ neuronal destruction has been observed in this region 7 days after local injection of 2.5 µg colchicine. In our study the population of dopaminergic cells did not seem to be altered after colchicine injection. Indeed the number of TH_{im} cells remained identical in the ipsilateral and the contralateral SN/VTA. This is probably due to the fact that the substance was applied distantly from DA cell bodies and colchicine axonal transport has never been described. In addition a slight recovery in TH-mRNA level was observed 96 h after injection, suggesting that colchicine action can be reversible. A reversal of the effect of colchicine has also been described in non-granular cells 11 days after injection.¹¹

The ability of colchicine to block axonal transport in dopaminergic neurons was described as lasting for days or weeks,¹⁴ although the kinetic of association and dissociation of colchicine tubulin complex occurred earlier.¹⁵ The current data confirm the rapid effect of colchicine, since a reduction in TH-mRNA was observed as early as 18 h after toxin application.

Colchicine is well known to increase immunoreactions, and numerous neuropeptides have only been detected after local or intraventricular injections of this substance.¹⁶ This has generally been attributed to an accumulation of the antigenic molecules in the cell bodies following blockade of axonal transport. Our results are not in agreement with that view. Indeed

the number of TH_{im} cells was drastically increased (39%) but TH protein level was unmodified in both ipsilateral and contralateral SN/VTA and C/P. It is noteworthy that TH protein amount was measured by immunautoradiography after transfer onto a nitrocellulose filter and this technique has been demonstrated to produce quantitative, linear and perfectly reproducible data.³⁻⁵ In contrast, the *in situ* TH_{im} used to count the DA cells in SN/VTA cannot be considered as quantitative. Colchicine could facilitate immunoreactive *in situ* detection through a stabilization of the antigen/antibody complex but, whatever the mechanism involved, the dose required appears extremely low since the effects of an unilateral injection was observed equally in the SN/VTA of both sides. The strictly unilateral effects we observed on TH gene expression suggest that higher doses are required to disrupt efficiently the cytoskeleton and block axonal transport.

A direct and non-specific action of colchicine on TH gene expression could also be questioned. However, while alterations in the expression of various genes have been reported after colchicine treatment, the nature of these effects corresponded to increases or reductions as well.^{17,18} Thus it can be expected that the observations we report are well the consequence of axonal transport impairment and not a general non-specific effect of colchicine on the transcription. The fact that the effects observed were strictly unilateral is also in line with a specific action of colchicine in the DA neurons of one side since the contralateral SN/VTA remained unaffected. Axonal transport impairment could, however, have altered the expression of any other protein and is not specific for TH protein.

It can be concluded from present results that TH protein synthesis was reduced by colchicine injection. Indeed, the treatment did not induce TH protein accumulation in the cell bodies at the origin of the dopaminergic axons. A slight but non-significant increase could be observed, never exceeding 10% of the amount measured in the contralateral side. Thus, the absence of TH protein accumulation suggests an early reduced TH protein synthesis. This is confirmed by the simultaneous TH protein disappearance in both SN/VTA and C/P. Indeed, in C/P where the protein pool no longer contains new molecules, TH protein disappeared following the same profile as in SN/VTA, with two phases: no effect during the first 48 h and a delayed decrease thereafter. This allowed us to estimate the protein half-life as about 56 h in our experimental conditions. This could be slightly over-estimated if we consider that colchicine could have reduced DA neurons activity and, as a consequence, decreased TH protein turn-over rate.

The reduction in TH protein synthesis rate probably reflects reduced gene activity, as suggested by the reduced TH-mRNA level. Such a TH-mRNA alteration after intraventricular injection of colchicine has already been reported,¹⁹ and this also allowed us to evaluate the mRNA half-life at about 24 h in our conditions. Taken together, the present data suggest that the initial effect of the treatment affects TH-mRNA production; this is followed by TH protein disruption. It can be speculated that blocking axonal transport could have interrupted a retrograde messenger which would have normally be responsible for both gene expression and mRNA translation. A reduction in TH_{im} in SN²⁰ and impairment of TH-mRNA²¹ production was observed after DA cell axotomy. It is unlikely that TH protein itself is the messenger responsible for the gene inactivation, since no accumulation was detected in the cell bodies. The messenger is more likely to act through a tonic activating process than through feedback inhibition since its absence was responsible for the reduced gene activity observed. Some candidates could be proposed to control TH gene expression after their anterograde transport, such as neurotensin, C-Fos or trophic factors but further experiments are needed for a definitive identification of this messenger.

Conclusion

Following the blockade of axonal transport, the respective time courses in the reductions for both

TH protein and TH-mRNA levels were observed. The results suggest that both gene transcription and/or TH-mRNA translation are under the control of a sustained tonic activation originating from the terminal level and retrogradely transported. The substance mediating this activation is unlikely to be the protein itself and remains to be identified.

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