Biochemical Pharmacology, Vol. 32, No. 22, pp. 3297–3301, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

STRIATAL MET-ENKEPHALIN CONCENTRATION INCREASES FOLLOWING NIGROSTRIATAL DENERVATION

LEON J. THAL,* NANSIE S. SHARPLESS, IRA D. HIRSCHHORN, SARA G. HOROWITZ and MAYNARD H. MAKMAN

Departments of Neurology, Psychiatry, Biochemistry and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

(*Received* 10 *March* 1982; *accepted* 8 *April* 1983)

Abstract—Following specific lesion of the nigrostriatal dopaminergic pathways in rat brain, striatal met-enkephalin on the lesioned side increased to 245% of that on the non-lesioned side. This increase was evident only after a lag period of 7 days and the increase was maintained for at least 2 months after lesion. By contrast, there was no change in striatal somatostatin or vasoactive intestinal polypeptide concentration, indicating that the effect was not a generalised one. Levels of all three of these neuropeptides were unchanged in frontal cortex. These findings support the concept of a dopaminergic–enkephalinergic functional interrelationship in the striatum. In addition, the findings provide evidence that, following destruction of nigrostriatal dopaminergic neurons, not only is there a gradually developing postsynaptic dopamine receptor supersensitivity but also a compensatory alteration in the enkephalinergic system.

The enkephalins are believed to play an important role as neurotransmitters or neuromodulators in mammalian brain. This is supported by their presence and unequal distribution in brain [1–3], their release from nerve terminals following depolarization [4], their ability to interact with specific opiate receptors [5], and their role in the modification of pain perception [6]. Enkephalins are found in high concentrations in the same forebrain structures which contain high concentrations of dopamine and there is evidence suggesting a functional relationship between the enkephalinergic and dopaminergic systems [7–11]. The highest concentration of enkephalin is found in the striatum, and methionine-enkephalin is the predominant form [3, 11].

This study was designed to determine the effect of dopaminergic denervation, induced by substantia nigra lesions, on striatal met-enkephalin levels. Chronic dopaminergic receptor blockade with peripherally administered haloperidol increases striatal met-enkephalin levels [12] and increases enkephalin biosynthesis [13]. If this increase is caused by blockade of striatal dopamine receptors, then destruction of the nigrostriatal dopaminergic neurons should produce similar effects. We report here that striatal met-enkephalin level is indeed markedly increased following substantia nigra lesion. The time course of this is described. In addition, met-enkephalin level in frontal cortex and levels of somatostatin and vasoactive intestinal polypeptide (VIP) in both striatum and frontal cortex were measured in order to assess whether or not the effect of lesion was a general one or possibly selective for met-enkephalin in striatum.

* Correspondence should be sent to: Dr. Leon Thal, Department of Neurology, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461.

METHODS

Unilateral right substantial nigra lesions were produced in male Sprague–Dawley rats, 180–200 g, using standard surgical and stereotaxic techniques. 6-Hydroxydopamine hydrobromide (8 μg in 1 μl of 0.02% ascorbate) injected over 5 min. The coordinates were AP + 2.6, L − 1.1 and DV + 2.9, according to the atlas of DeGroot [14]. For animals with lesions present for a sufficient period of time to develop dopamine receptor supersensitivity (more than 2 weeks in the present study), the rate of rotation following apomorphine administration was determined by recording in an automated rotometer for 15 min to ascertain behavioral supersensitivity. Only those rats which circled contralaterally at a rate of at least 3 turns/min in response to 2.5 mg/kg (i.p.) of apomorphine were included. Animals lesioned within 2 weeks of being killed were not tested for rotation.

Rats were decapitated at various time intervals after lesioning and the striata or frontal cortex were dissected. Each tissue piece was placed immediately in 0.75 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25°) and hand homogenized. A 50-μl aliquot was quickly removed and frozen in liquid nitrogen for analysis for dopamine, 3,4-dihidroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The remainder of the homogenate was diluted to 2 ml with Tris buffer. For analysis of met-enkephalin, a 100 μl aliquot of diluted homogenate was added to 100 μl of ice-cold 0.2 M acetic acid, vortexed and centrifuged at 1100 *g*, and the clear supernatant fraction was removed. For somatostatin or VIP, 1 ml of homogenate was mixed with 1 ml of 4 M acetic acid, heated in a boiling water bath for 10 min, and then lyophilized.

Met-enkephalin was assayed in duplicate at three

different concentrations by radioimmunoassay. Rabbit antiserum to met-enkephalin and [^{125}I]met-enkephalin were purchased from the Immunonuclear Corp. The antiserum had 1.5% cross-reactivity with leu-enkephalin and negligible cross-reactivity with α-endorphin, β-endorphin, and other closely related tetrapeptides and quatrapeptides [15]. The lower limit of sensitivity was 4 pg. Standards of 0–250 pg of met-enkephalin were prepared in a bovine serum albumin phosphate buffer, pH 6.4. Sequential addition of sample or standard, rabbit antiserum to anti-met-enkephalin, and [^{125}I]met-enkephalin was followed by overnight incubation at 4°. Bound ligand was separated from free addition of an equal volume of saturated ammonium sulfate in the presence of carrier gamma-globulin followed by centrifugation. The supernatant fraction was decanted and the precipitate counted in a gamma counter.

Somatostatin was measured by a sensitive and specific radioimmunoassay previously described [16]. Samples were analyzed in triplicate at three different dilutions. Antiserum (donated by Dr. Peter Davies) was used at a final dilution of 1:48,000. [^{125}I]Somatostatin was purchased from the England Nuclear Corp.

For radioimmunoassay of VIP, the lyophilized residues were reconstituted in enough essay buffer to give approximately 0.4 mg protein/ml of caudate or 0.09 mg protein/ml for cortex, and aliquots of 0.05, 0.1 and 0.2 ml were analyzed in duplicate. The volume of each tube was adjusted to 600 μl with 0.06 M sodium phosphate buffer, pH 7.0, containing 1% bovine albumin (Sigma, RIA grade) and 1000 KIU/ml of aprotinin (Trasylol, FBA Pharmaceuticals). Rabbit antiserum to VIP (supplied by Dr. Gajanan Nilaver, Columbia University) at a final dilution of 1:200,000 was added in a volume of 100 μl, and the mixture was incubated for 24 hr at 4°. [^{125}I]VIP (prepared by a chloramine T method and purified by sequential chromatography on Sephadex G25 superfine and CM-Sephadex) was then added in a volume of 100 μl, and the mixture was incubated at 4° for 3 more days. Antibody-bound and free [^{125}I]VIP were separated with 10 mg of dextran-coated charcoal. A standard curve was generated by analyzing synthetic porcine VIP (Chemalog) over the range 0.5 to 100 fmoles. The limit of sensitivity of the assay was 1 fmole of VIP/tube. Cross-reactivity of the antiserum with other peptides was negligible [17].

Dopamine and its metabolites were measured by high performance liquid chromatography. The reserved portion (50 μl) of homogenate was mixed with 0.2 M $HClO_4$ (50 μl) and centrifuged. An aliquot (25 μl) of the clear supernatant fraction was injected directly onto the column. The chromatographic system consisted of a Biophase ODS–5 μm column and an LC-4A electrochemical detector from Bioanalytical Systems. The TL-5 glassy garbon electrode was set at a potential of +0.72 V versus Ag/AgCl reference electrode. The isocratic mobile phase (1.0 ml/min) was 0.1 M chloroacetate (pH 3.5), 1 mM EDTA, 0.2 mM sodium octyl sulfate, and 14% methanol. Quantitation was performed by determination of peak heights relative to those of external standards. Retention times were 6.2 min for dopamine, 7.6 min for DOPAC, and 17.4 min for HVA. Serotonin (13.4 min) and 5-hydroxyindoleacetic acid (12.2 min) were the only other electroactive compounds seen in the brain homogenates. norepinephrine and its metabolites eluted in the solvent front and did not interfere. Protein concentration of the homogenate was measured by the method of Lowry *et al.* [18].

RESULTS

In striata from non-lesioned control animals, met-enkephalin levels were 11.7 + 1.0 ng/mg protein (mean + S.E.M., N = 14). There was no difference between left and right striata. In rats with chronic substantia nigra lesions (killed more than 2 weeks post-lesion), the met-enkephalin concentration in the striatum on the non-lesioned side (11.0 ± 0.8 ng/mg protein) was not significantly different from that in the control animals (Table 1). However, the met-enkephalin concentration on the lesioned side (27.0 ± 2.9 ng/mg protein) had increased to 245% of that of the non-lesioned side. There were no changes in striatal somatostatin or VIP levels in the chronically lesioned animals (Table 1). Striata from animals with acute lesions (2 days post-lesion) failed to show changes in met-enkephalin, somatostatin or VIP level. In the cortex, neither chronic nor acute lesions produced an alteration in any of the three peptides studied (Table 1).

The completeness of the lesion was verified by measuring striatal dopamine, HVA and DOPAC. When compared to the non-lesioned side, the striatal dopamine concentration was reduced by 90% at 2 days following the lesion and by 98% following the chronic lesion (Table 2). There were similar large unilateral decreases in striatal HVA and DOPAC concentrations. In other studies (data not shown), dopamine was decreased by about 97% at both 4 and 7 days after lesion.

The time course for the development of increased striatal met-enkephalin levels was determined (Fig. 1). At 2 and 7 days after lesion, there was no significant change in met-enkephalin level on the lesioned side. Between days 14 and 58, there was a large increase in striatal met-enkephalin level on the lesioned side. By contrast, there was no alteration in met-enkephalin concentration on the non-lesioned side at any time point examined.

DISCUSSION

This is the first demonstration of increased striatal met-enkephalin levels following selective nigrostriatal lesion. Following hemisection at the midbrain level, others have found elevated striatal met-enkephalin levels at 30 days [19] but not at 10 days [20]. However, the non-specificity of this lesion does not allow one to distinguish which of the destroyed pathways resulted in the observed increase in met-enkephalin. By contrast, the nigrostriatal lesion appeared to be relatively selective in that there was no alteration in levels of two other neuropeptides in striatum or in the three peptides measured in frontal cortex. While the precise synaptic relationships of striatal enkephalinergic neurons are not yet known, immu-

Table 1. Neuropeptides in striatum and frontal cortex following unilateral substantia nigra lesion*

	Striatum			Frontal cortex		
	Met-enkephalin (ng/mg protein)	Somatostatin (ng/mg protein)	VIP (fmoles/mg protein)	Met-enkephalin (ng/mg protein)	Somatostatin (ng/mg protein)	VIP (fmoles/mg protein)
Acute lesion						
Control side	9.4 ± 0.8 (3)	5.4 ± 0.2 (3)	37.0 ± 3.7 (2)	0.94 ± 0.36 (3)	3.5 ± 0.3 (3)	1901 ± 333 (3)
Lesioned side	7.4 ± 1.2 (3)	7.5 ± 3.2 (3)	40.2 ± 2.6 (2)	0.64 ± 0.3 (3)	4.6 ± 0.3 (3)	2102 ± 373 (3)
Chronic lesion						
Control side	11.0 ± 0.8 (8)	8.7 ± 1.0 (3)	34.6 ± 6.6 (3)	0.97 ± 0.32 (3)	3.3 ± 0.2 (3)	1464 ± 142 (3)
Lesioned side	27.0 ± 2.9† (9)	9.4 ± 1.8 (3)	40.5 ± 3.8 (3)	1.11 ± 0.37 (3)	4.0 ± 1.1 (3)	1599 ± 377 (3)

*Animals with acute lesions were killed 2 days post-lesion while those with chronic lesions were killed more than 2 weeks after lesion. Values are means ± S.E.M. for the number of animals in parentheses.

† $P < 0.001$, when compared to the control side by Student's *t*-test.

Table 2. Dopamine and its metabolites in the striatum following unilateral substantia nigra lesion*

	Dopamine (ng/mg protein)	Homovanillic acid (ng/mg protein)	DOPAC (ng/mg protein)
Acute lesion			
Control side	77.4 ± 8.3 (6)	7.8 ± 1.3 (6)	9.5 ± 0.6 (5)
Lesioned side	7.9 ± 0.5† (6)	1.1 ± 0.3† (6)	1.9 ± 0.4† (6)
Chronic lesion			
Control side	91.1 ± 7.7 (6)	7.7 ± 1.2 (6)	11.6 ± 0.9 (6)
Lesioned side	2.2 ± 1.2† (6)	0.31 ± 0.2† (6)	0.51 ± 0.3† (6)

*Animals with acute lesions were killed 2 days post-lesion while those with chronic lesions were killed more than 2 weeks after lesion. Values are the means ± S.E.M. for the number of animals in parentheses.

† $P < 0.001$, when compared to the control side by Student's *t*-test.

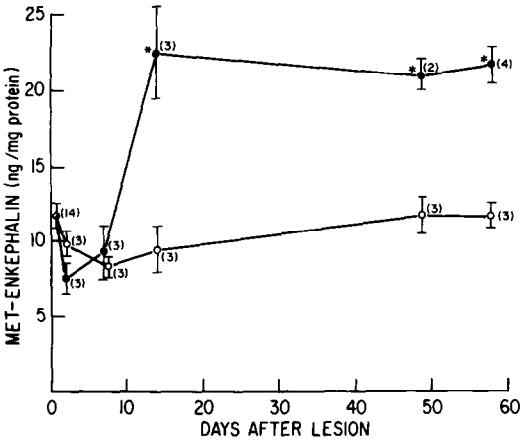

Fig. 1. Time course of striatal met-enkephalin concentration following unilateral 6-hydroxydopamine lesion of the substantia nigra. Key: (●) lesioned side, and (○) non-lesioned side. Lesion induced an overall increase in met-enkephalin levels on the lesioned side (one-way analysis of variance, $f = 1.98$, $P < 0.01$). Met-enkephalin was increased significantly on the lesioned side between 14 and 58 days post-lesion. There was no significant change in met-enkephalin concentration on the non-lesioned side. Each value is a mean ± S.E.M. for the number of animals in parentheses. An asterisk (*) signifies $P < 0.001$, when compared to day 0 non-lesioned animals.

nohistochemical studies [21, 22] and studies involving kainic acid lesion [20] suggest that striatal met-enkephalin is largely located in interneurons. Additional work has indicated that striatal met-enkephalin neurons are primarily located in caudate and putamen and project to globus pallidus [23].

The nigrostriatal lesion is similar to chronic haloperidol treatment in that haloperidol blocks dopamine receptors and thereby produces a pharmacological equivalent of dopaminergic denervation. Therefore, our results are in accord with the finding that chronic haloperidol increases striatal met-enkephalin levels [12]. The present study also provides more direct evidence for the regulation of striatal met-enkephalin neurons by nigrostriatal dopaminergic neurons.

Scopolamine administration partially blocks the haloperidol-induced increase in striatal met-enkephalin [24], suggesting cholinergic mediation of striatal met-enkephalin regulation. Hong *et al.* [24] have postulated that blockade by haloperidol of inhibitory dopamine receptors on cholinergic neurons, in turn, results in an increase in cholinergic firing, leading to stimulation of enkephalinergic neurons, increased enkephalin synthesis and release, as well as higher steady-state levels of met-enkephalin. In an analogous manner, lesion of the nigrostriatal dopamine neurons might result in increased stimulation of enkephalinergic neurons by cholinergic neurons. As alternate to the sequential influence of dopamine and acetylcholine on enkephalin level, dopamine and acetylcholine might exert separate but interrelated effects on enkephalinergic neurons. The available data are compatible with either hypothesis.

The time course for development of the increase in met-enkephalin is quite interesting. At 2 and 7 days after lesion, there was no increase in met-enkephalin levels despite over 90% depletion of dopamine. However, by 14 days after lesion, met-enkephalin levels were increased markedly. At this time, dopamine receptor number assess by antagonist [25] and agonist [26]; (Hirschhorn *et al.*, unpublished findings) radioligand binding is increased. Furthermore, in parallel studies, dopamine levels were decreased by 97% but dopamine receptor binding was not increased at 4 and 7 days after lesion (Hirschhorn *et al.*, manuscript in preparation). It is of interest that chronic treatment with haloperidol also leads only gradually to an increased level of enkephalin in the striatum [12]. Both the increase in dopamine receptor binding and the increase in enkephalin levels as reported here represent delayed or gradually developing consequences of nigrostriatal dopamine denervation. It is postulated that the increase in enkephalin immunoreactivity following denervation represents a chronic adaptation of the enkephalinergic neuron to denervation.

Acknowledgements—Supported by USPHS Grant NS09469. We thank Mariann Downing for performing the analyses of VIP and dopamine.

REFERENCES

1. J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. R. Morris, *Nature, Lond.* **258**, 577 (1975).
2. J. Hughes, H. W. Kosterlitz and T. W. Smith, *Br. J. Pharmac.* **61**, 630 (1977).
3. H. Y. Yang, J. S. Hong and E. Costa, *Neuropharmacology* **16**, 303 (1977).
4. L. L. Iversen, S. D. Iversen, F. E. Bloom, T. Vargo and R. Guillemin, *Nature, Lond.* **271**, 679 (1978).
5. J. A. H. Lord, A. A. Waterfield, J. Hughes and H. W. Kosterlitz, *Nature, Lond.* **367**, 495 (1977).
6. J. D. Belluzzi, N. Grant, V. Garsky, D. Sarantakis, C. D. Wise and L. Stein, *Nature, Lond.* **260**, 625 (1976).
7. H. Pollard, C. Llorenz-Cortes and J. C. Schwartz, *Nature, Lond.* **268**, 745 (1977).
8. G. Biggio, M. Casu, M. G. Corda, C. DiBello and G. L. Gessa, *Science* **200**, 552 (1978).
9. B. I. Diamond and R. L. Borison, *Neurology, Minneap.* **28**, 1085 (1978).
10. E. L. Gardner, R. S. Zukin and M. H. Makman, *Brain Res.* **194**, 232 (1980).
11. I. D. Hirschhorn, D. Hittner, E. L. Gardner, J. Cubells and M. H. Makman, *Brain Res.*, in press.
12. J. S. Hong, H.Y.T. Yang, W. Fratta and E. Costa, *J. Pharmac. exp. Ther.* **205**, 141 (1978).
13. J. S. Hong, H. Y. T. Yang, J. C. Gillin, A. M. DiGiulio, W. Fratta and E. Costa, *Brain Res.* **160**, 192 (1979).
14. J. D. DeGroot, *The Rat Forebrain in Stereotaxic Coordinates*. NV Noord-Hollandsche Uitgevers Maatschappy, Amsterdam (1959).
15. I. Lindberg and J. L. Dahl, *J. Neurochem.* **36**, 506 (1981).
16. P. Davies and R. Terry, *Neurobiol. Aging* **2**, 9 (1981).
17. L. E. Eiden, G. Nilaver and M. Palkovits, *Brain Res.* **231**, 472 (1982).

18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. E. Costa, A. DiGiulio, W. Fratta, J. Hong and H-Y. T. Yang, in *Catecholamines: Basic and Clinical Frontiers* (Eds. E. Usdin, I. J. Kopin and J. Barchas), p. 1020, Pergamon Press, New York (1979).
20. J. S. Hong, H. Y. T. Yang and E. Costa, *Neuropharmacology* **16**, 451 (1977).
21. T. Hokfelt, R. Edle, O. Johansson, L. Terenius and L. Stein, *Neurosci. Lett.* **5**, 25 (1977).
22. R. Simantov, M. J. Kuhar, G. R. Uhl and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **74**, 2167 (1977).
23. M. Del Fracco, G. Paxinos and A. C. Cuello, *Brain Res.* **231**, 1 (1982).
24. J. S. Hong, H. Y. T. Yang, J. C. Gillin and E. Costa, *Adv. Biochem. Psychopharmac.* **24**, 223 (1980).
25. C. Feuerstein, P. Demenge, P. Caron, G. Barrette, B. Guerin and P. Mouchet, *Brain Res.* **226**, 221 (1981).
26. I. D. Hirschhorn and M. H. Makman, *Eur. J. Pharmac.* **83**, 61 (1982).