Brain Research Bulletin, Vol. 7, pp. 683–686, 1981. Printed in the U.S.A.

Brain β-Endorphin-Like Immunoreactivity in Adult Rats Given β-Endorphin Neonatally

ROBERTA L. MOLDOW, ABBA J. KASTIN,* CHARLES S. HOLLANDER, DAVID H. COY* AND CURT A. SANDMAN†

New York University Medical Center, New York, NY 10016
**Veterans Administration Medical Center and Tulane University School of Medicine, New Orleans, LA 70146*
and
†University of California Medical Center, Irvine and Fairview Hospital, Costa Mesa, CA 92626

Received 2 October 1981

MOLDOW, R. L., A. J. KASTIN, C. S. HOLLANDER, D. H. COY AND C. A. SANDMAN. *Brain β-endorphin-like immunoreactivity in adult rats given β-endorphin neonatally.* BRAIN RES. BULL. 7(6) 683–686, 1981.—β-endorphin-like immunoreactivity was measured by radioimmunoassay in the brains of adult rats treated neonatally with β-endorphin, naloxone, or vehicle. After treatment with β-endorphin, the decreases observed in β-endorphin-like immunoreactivity in the hypothalamus, pineal, midbrain, pons-medulla, hippocampus, striatum, frontal cortex, occipital cortex, and posterior cortex were highly significant but the 23% decrease in the thalamus was not significantly different from that of control rats. Neonatal administration of naloxone only resulted in a significant decrease in β-endorphin-like immunoreactivity in the hypothalamus. In contrast, no differences were discernible in content of either β-endorphin-like immunoreactivity or ACTH-like immunoreactivity in the pituitary of rats treated with β-endorphin, naloxone, or vehicle in the neonatal period. These same rats had shown an increased threshold to painful thermal stimulation by the tail-flick test after administration of either β-endorphin or naloxone at birth. The results suggest that neonatally injected β-endorphin may alter the levels of β-endorphin-like immunoreactivity in rat brain as well as the response to pain.

β-endorphin Naloxone Neonatal Brain Behavior

NEONATAL administration of morphine attenuates morphine induced analgesia in adult animals [9, 14, 17] and early treatment with opiate antagonists potentiates the effect of morphine in adult rats [9]. Long-term effects on behavior have been reported with neonatal administration of neuropeptides such as melanocyte-stimulating hormone (MSH)[1], adrenocorticotropin (ACTH) fragments [3], thyrotropin releasing hormone (TRH) (15), and Met-enkephalin [6].

The effect of β-endorphin on the brain after peripheral administration during a critical stage in the development of opiate receptors [13] remains to be fully elucidated. Neonatal treatment with β-endorphin resulted in chronic elevation in the threshold for painful thermal stimulation in adult rats [11]. The levels of β-endorphin were, therefore, determined in the brains of these adult rats which had been treated neonatally with β-endorphin, naloxone (a specific opiate antagonist), or a control solution. Levels of β-endorphin-like immunoreactivity were decreased in the hypothalamus and extra-hypothalamic areas of the brains of rats treated from 2–7 days of age with β-endorphin.

METHOD

The response of rats used in this experiment to thermal stimuli has been previously reported [11]. From days 2–7 of age rat pups were injected subcutaneously with either β-endorphin (50 μg/rat), naloxone (100 μg/rat), or a vehicle solution. The rats were weaned on day 22 and maintained on a 12-hour light, 12-hour dark schedule. At 90 days of age, tail flick testing was begun.

Five rats were randomly picked from each group, decapitated and the brain rapidly removed. After the superficial blood vessels were carefully removed, the brains were dissected on ice by the method of Glowinski and Iverson [5] with modifications described elsewhere [4] and frozen until use. These brains had been kept at −20°C for about two years before extraction and assay. However, all three groups were treated in the same fashion.

A second study was performed with pregnant rats which were injected subcutaneously with either 100 μg/rat β-endorphin or vehicle every other day from the seventh to the twenty-first day of pregnancy. Male offspring were cross-fostered and after 60 and 180 days were tested for threshold of response to painful thermal stimuli with the tail-flick test [12]. Rats were then decapitated and the brains rapidly removed, dissected as previously described and frozen for about 1 year before extraction.

Tissue Extraction

A modification of the method of Moldow and Yalow [7,8] was used for extraction. In brief, frozen tissue was rapidly extracted at 100 mg/3 ml in cold distilled water, placed in a

boiling water bath, chilled in an ice bath, and centrifuged at 2000 G at 4°C for 30 minutes. The supernatant was removed and frozen. The tissue was re-extracted in cold 0.1 M acetic acid, placed in a boiling water bath and centrifuged at 2000 G at 4°C for 30 min. The supernatant was removed and frozen until assayed.

Radioimmunoassay of β-endorphin

Antibody production. Human β-endorphin (kindly supplied by Dr. C. H. Li) was conjugated to thyroglobulin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The mixture was dialyzed against distilled water, lyophilized, dissolved in 0.9% NaCl and Freund's complete adjuvant (Difco), emulsified, and injected intramuscularly into random-bred New Zealand white rabbits. Venous blood was obtained from the ear, and serum was stored in aliquots at −20°C.

Iodination. Human β-endorphin was iodinated by a modification of the chloramine-T method of iodinating ACTH (2). The following were added as quickly as possible: 20 μl of 0.25 M PO_4(pH 7.6); 500 μCi^{125} I (Amersham), 5 μg β-endorphin (in 5 μl 5× 10^{-3} M HCl), 10 μl chloramine-T (5.25 μg/μl) and 20 μl $Na_2S_2O_5$ (4.8 μg/μl). The labeled β-endorphin was then purified on 5 mg QUSO G-32 and eluted from the QUSO with 0.1% acetic acid in 40% acetone.

Incubation. An eight day incubating procedure at 4°C was used. Standards or aliquots of samples were diluted in assay diluent (0.1 M borate buffer, 1% bovine serum albumin (Sigma), 0.1% 2-mercaptoethanol, and 0.2% thimerosol, pH 8.4). The antiserum to β-endorphin was used at a final dilution of 1:25,000. After 24 hours incubation, ^{125} I-β-endorphin (1500 cpm/tube) was added.

Separation was achieved by the addition of 2 ml diluent and a 25 mg talc tablet per tube and centrifugation for 10 minutes at 1000 g at 4°C. The supernatant was carefully decanted and both fractions were counted in a gamma counter.

The assay is sensitive to 1 pg/tube. The antibody for β-endorphin is specific for the N-terminal of the molecule and crossreacts on a molar basis 30% with β-lipotropin, 25% with γ-endorphin, less than 1% with α-endorphin, and has no apparent crossreactivity with Met-enkephalin, Leu-enkephalin, α-MSH or ACTH. The coefficient of variation (CV) within assay was 3% and the CV between assays was 11%.

Radioimmunoassay of ACTH

Antibody production. Synthetic ACTH (1–24) was conjugated to bovine serum albumin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, dialyzed against distilled water, lyophilized, and emulsified with complete Freund's adjuvant. The emulsion was then injected intramuscularly into random-bred New Zealand white rabbits. A suitable antiserum was obtained after twice monthly injections for a period of four months.

Iodination. ACTH (1–39) was iodinated by the chloramine-T method and purified on QUSO G-32 (2).

Incubation. A seven day incubation procedure at 4°C was used. Standards of ACTH (1–39) or sample aliquots were diluted in assay diluent (0.05 M Na-K PO_4, 200 KIU/ml Trasylol, 0.4% 2-mercaptoethanol and 0.25% human serum albumin, pH 7.4). The antiserum to ACTH was used at a final dilution of 1:25,000. After 24 hr incubation, ^{125}I-ACTH (10,000 cpm/tube) was added. Separation was achieved by the addition of 2 ml assay diluent and a 25 mg talc tablet to each tube and centrifugation for 10 min at 1000 g at 4°C. The supernatant was carefully decanted and both fractions were counted in a gamma counter.

The assay is sensitive to 10 pg/tube. The antibody to ACTH crossreacts on a molar basis 0.37% with α-MSH and has no apparent crossreactivity with β-endorphin. The intraassay CV was 2% and the CV between assays was 10%.

Statistical Analyses

Comparisons of the concentrations of β-endorphin-like immunoreactivity among the various brain parts and neonatal treatment was made by two way analysis of variance followed by Duncan's New Multiple Range Test.

RESULTS

The concentration of β-endorphin-like immunoreactivity in the extra-hypothalamic areas of the adult rat brain was significantly decreased by neonatal injection of β-endorphin. As can be seen in Table 1, a significant ($p<0.01$) decrease in β-endorphin-like immunoreactivity was found in the midbrain, pons-medulla, hippocampus, striatum, frontal cortex, occipital cortex, and posterior cortex of rats treated neonatally with β-endorphin as compared to control rats or rats injected with naloxone. Although not significant, β-endorphin-like immunoreactivity in the thalamus of rats treated neonatally with β-endorphin was only 77% that of control rats. No significant difference was found between the levels of β-endorphin-like immunoreactivity in the extrahypothalamic areas of the adult brain of rats treated neonatally with naloxone or diluent.

A significant ($p<0.01$) decrease from control levels of 228.38±28.96 pg/mg β-endorphin-like immunoreactivity to 113.90±45.90 pg/mg was found in the hypothalamus of rats treated neonatally with β-endorphin. Rats treated with naloxone at birth also had a significant ($p<0.01$) decrease in β-endorphin-like immunoreactivity in the hypothalamus. β-endorphin-like immunoreactivity was non-detectable (<30 pg/pineal) in the pineals of rats treated neonatally with β-endorphin as compared to 198.0±7.2 pg/pineal of rats injected with naloxone or 202.6±11.9 pg/pineal of rats injected with vehicle.

As can be seen in Table 2, no significant difference was found in either the content of β-endorphin-like immunoreactivity or ACTH-like immunoreactivity in the pituitary of rats neonatally treated with β-endorphin, naloxone, or vehicle. ACTH levels were not measured in the brain.

In the study performed in rats treated prenatally, no significant differences were found in the response latencies of the tail flicks. The β-endorphin-like immunoreactivity in the adult brains of these rats was also not significantly different among the groups.

DISCUSSION

The results of this study indicate that neonatal peripheral injection of β-endorphin can permanently decrease the levels of β-endorphin-like immunoreactivity in the brain. The decrease in levels of β-endorphin-like immunoreactivity is parallelled by a chronic increase in the threshold for thermal stimulation measured with the tail flick test [11]. However, exposure to β-endorphin in utero resulted in no significant differences in the pain threshold. Similarly, levels of β-endorphin-like immunoreactivity were not different in the brains of these rats. Thus, it appears that only in the condition in which nociception is altered by early treatment with

TABLE 1

EFFECT OF NEONATAL TREATMENT WITH β-ENDORPHIN OR NALOXONE ON MEAN CONCENTRATION (± SEM) OF β-ENDORPHIN-LIKE IMMUNOREACTIVITY IN ADULT RAT BRAIN

Area	Neonatal Treatment Control	β-Endorphin	Naloxone
Thalamus	29.92 ± 4.45*	23.16 ± 3.31	30.56 ± 2.66
Midbrain	24.28 ± 1.62	4.68 ± 1.93†	25.82 ± 1.26
Pons-Medulla	22.65 ± 2.11	3.00 ± 0.95†	20.50 ± 1.78
Hippocampus	21.30 ± 1.39	4.88 ± 2.00†	26.60 ± 1.10
Striatum	18.56 ± 1.71	6.00 ± 1.86†	25.00 ± 3.00
Frontal Cortex	22.70 ± 1.50	8.60 ± 1.50†	24.58 ± 1.54
Occipital Cortex	22.96 ± 1.15	3.00 ± 1.06†	21.28 ± 1.02
Posterior Cortex	19.96 ± 1.49	3.30 ± 0.30†	21.88 ± 1.20

*pg/mg wet weight tissue.
†$p<0.01$ vs same part in rats receiving diluent or naloxone.

TABLE 2

EFFECT OF NEONATAL TREATMENT WITH β-ENDORPHIN OR NALOXONE ON MEAN PITUITARY CONTENT (±SEM) OF β-ENDORPHIN-LIKE IMMUNOREACTIVITY AND ACTH-LIKE IMMUNOREACTIVITY IN ADULT RATS

	Neonatal Treatment Control	β-Endorphin	Naloxone
β-endorphin-like Immunoreactivity (ng/pituitary)	1294 ± 148	1200 ± 450	1308 ± 98
ACTH-like Immunoreactivity (ng/pituitary)	473 ± 30	528 ± 51	499 ± 43

β-endorphin, changes in brain levels of β-endorphin-like immunoreactivity may also be evident.

Since we are reporting levels of β-endorphin-like immunoreactivity but not its turnover, the decrease in levels of β-endorphin-like immunoreactivity could be due to several different mechanisms including a decrease in synthesis or an increase in enzymatic degradation. It is also possible that peripheral administration of β-endorphin during a critical stage in the development of opiate receptors alters the number of opiate receptors and/or their affinity.

The distribution of β-endorphin-like immunoreactivity is remarkably consistent with the distribution of ACTH in the rat [7]. There is approximately 1000 times more β-endorphin-like immunoreactivity in the pituitary than in the hypothalamus and 10,000 more than in extrahypothalamic areas. The content of ACTH-like immunoreactivity in the pituitary gland is consistent with previous reports [7,8]. The pineal gland, an area outside the blood-brain barrier, was found to have a high content of β-endorphin-like immunoreactivity as previously reported (10). It is difficult to compare absolute levels of β-endorphin-like immunoreactivity in the brain reported here with other reports (10,16) because of differences in the crossreactivities of antibodies used and extraction techniques. In conclusion, β-endorphin-like immunoreactivity in the brains of rats treated neonatally with β-endorphin was much lower than in rats injected at the same time with diluent.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the excellent technical assistance of Mrs. M. L. Russo, Mr. J. Passarelli and Mr. M. Diaz. The assistance of Mrs. S. Dooskin and Ms. J. Price is also much appreciated.

REFERENCES

1. Beckwith, B. E., C. A. Sandman, D. Hothersall and A. J. Kastin. Influence of neonatal injections of α-MSH on learning, memory and attention in rats. *Physiol. Behav.* **18:** 63–71, 1977.
2. Berson, S. A. and R. S. Yalow. Adrenocorticotropin. In: *Methods of Investigative and Diagnostic Endocrinology*, edited by S. A. Berson and R. S. Yalow. Amsterdam: North Holland Publishing Co., 1973, pp. 371–375.
3. Champney, T. F., T. L. Sahley and C. A. Sandman. Effects of neonatal cerebral ventricular injection of ACTH 4–9 and subsequent adult injections on learning in male and female albino rats. *Pharmac. Biochem. Behav.* **5:** Suppl. 1, 3–9, 1976.

4. Christensen, C. W., C. T. Harston, A. J. Kastin, R. M. Kostrzewa and M. A. Spirtes. Investigations on α-MSH and MIF-I affects on cyclic AMP levels in rat brain. *Pharmac. Biochem. Behav.* **5:** Suppl. 1, 117–120, 1976.
5. Glowinski, J. and L. L. Iversen. Regional Studies of Catecholamines in the rat brain—I. The disposition of [^{3}H] norepinephrine, [^{3}H] dopamine and [^{3}H] DOPA in various regions of the brain. *J. Neurochem.* **13:** 665–669, 1966.
6. Kastin, A. J., R. M. Kostrzewa, A. V. Schally and D. H. Coy. Neonatal administration of Met-enkephalin facilitates maze performances of adult rats. *Pharmac. Biochem. Behav.* **13:** 883–886, 1980.
7. Moldow, R. L. and R. S. Yalow. Extrahypophyseal distribution of corticotropin as a function of brain size. *Proc. natn. Acad. Sci. U.S.A.* **75:** 994–998, 1978.
8. Moldow, R. L. and R. S. Yalow. Artifacts in the radioimmunoassay of ACTH in tissue extracts and plasma. *Hormone Metab. Res.* **12:** 103–108, 1980.
9. Paul, L., J. Diaz and B. Barley. Behavioral Effects of Chronic Narcotic Antagonist Administration to Infant Rats. *Neuropharmacology* **17:** 655–657, 1978.
10. Rossier, J., T. M. Varges, S. Minick, N. Ling, F. E. Bloom and R. Guillemin. Regional dissociation of β-endorphin and enkephalin contents in rat brain and pituitary. *Proc. natn. Acad. Sci. U.S.A.* **74:** 5162–5165, 1977.
11. Sandman, C. A., P. F. McGivern, C. Berka, J. M. Walker, D. H. Coy and A. J. Kastin. Neonatal administration of β-endorphin produces "chronic" insensitivity to thermal stimuli. *Life Sci.* **23:** 1755–1760, 1979.
12. Sandman, C. A., A. V. Schally and A. J. Kastin. Neuropeptide influences on the Central Nervous System: A psychobiological perspective. In: *Neuroendocrine Regulation and Altered Behavior*, edited by P. D. Hrdina and R. L. Singhal. London: Croom Helm, 1981, pp. 3–27.
13. Simon, E. J. and J. M. Hiller. In vitro studies on opiate receptors and their ligands. *Fedn Proc.* **37:** 141–146, 1978.
14. Sonderegger, T. and E. Zimmerman. Adult Behavior and Adrenocortical Function Following Neonatal Morphine Treatment in Rats. *Psychopharmacology* **56:** 103–109, 1978.
15. Stratton, L. O., C. A. Gibson, F. G. Kolar and A. J. Kastin. Neonatal treatment with TRH affects development, learning and emotionality in the rat. *Pharmac. Biochem. Behav.* **5:** Suppl. 1, 65–67, 1976.
16. Watson, S. J., H. Akil, C. W. Richard, III and J. D. Barchas. Evidence for two separate opiate peptide neuronal systems. *Nature* **275:** 226–228, 1978.
17. Zimmerman, E., B. Branch, A. N. Taylor, J. Young and C. N. Pang. Long lasting effects of pre-puberal administration of morphine in adult rats. In: *Narcotics and the Hypothalamus*, edited by E. Zimmerman and R. George. New York: Raven Press, 1974, pp. 183–196.