Pharmacology Biochemistry & Behavior, Vol. 10, pp. 647–650. Printed in the U.S.A.

Brain Biochemical and Behavioral Changes in 4 Weeks Old Rats After Neonatal Oxygen Deprivation

T. HEDNER, P. LUNDBORG AND J. ENGEL

Department of Pharmacology, University of Göteborg, Fack, S-400 33 Göteborg, Sweden

(Received 12 February 1979)

HEDNER, T., P. LUNDBORG AND J. ENGEL. *Brain biochemical and behavioral changes in 4 weeks old rats after neonatal oxygen deprivation.* PHARMAC. BIOCHEM. BEHAV. **10**(5) 647–650, 1979.—The acquisition of a conditioned avoidance response (CAR) and the effects on monoamine neurotransmittor synthesis was investigated in 28 days old rats after neonatal oxygen deprivation (100% N_2 for 20 min or 6% O_2–94% N_2 for 4.5 hr). The rats subjected to 6% O_2–94% N_2 for 4.5 hr at 1 day of age were markedly inferior in the CAR acquisition than the control group. The animals subjected to 20 min neonatal anoxia did not differ in avoidance responding compared to controls. Tyrosine hydroxylase and tryptophan hydroxylase activity was studied in vivo in whole brain at 28 days of age by measuring the accumulation of dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) respectively after inhibition of aromatic L-amino acid decarboxylase with NSD 1015. An inhibition of tyrosine hydroxylase and tryptophan hydroxylase activity was found after neonatal exposure to 6% O_2–94% N_2 for 4.5 hr while the rats exposed to 100% N_2 for 20 min did not differ from controls. The precursor amino acids tyrosine and tryptophan and the levels of the endogenous monoamines dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) did not differ from controls in any of the groups. It is suggested that the observed behavioral deficits after prolonged hypoxia may be due to an impaired development of central catecholamine mechanisms.

Oxygen deprivation Developing brain Conditioned avoidance response Monoamine synthesis

ONTOGENETIC studies using histochemical fluorescence techniques have shown that almost all the various groups of monoamine-containing nerve cell bodies, which are present in the adult rat, are visible at birth [19, 20, 25]. Although these monoamine neurons develop mechanisms for synthesis and storage at a very early stage during ontogeny [9, 29, 30] the brain levels of DA, NA, and 5-HT increase progressively with age and do not reach adult levels until several weeks after birth or as in the case of DA, not until adult life [9,29]. This progressive increase in the brain monoamine levels of the growing rat is in all probability a consequence of the centrifugal outgrowth of axons and terminals from the cell bodies.

Various physical and chemical agents are known to cause morphological as well as biochemical alterations in the developing brain during infancy [21,29]. Various behavioral abnormalties can similarly be induced [21,29].

Recent studies in these laboratories have demonstrated that in neonatal rats hypoxia and/or anoxia are accompanied by marked acute alterations in the synthesis of the monoamine neurotransmittors DA, NA and 5-HT [13, 14, 15, 16, 17]. In general these changes appeared to be relatively shortlasting.

It was, however, also found to be of crucial interest to investigate if also irreversible behavioral and/or biochemical brain changes could be induced by asphyxia during the neonatal period. Therefore, in the present study, infant rats have been subjected to either a short period of complete anoxia or a relatively long hypoxic period and the effects of this treatment on conditioned avoidance response and monoamine metabolism have been studied at 28 days of age.

METHOD

Animals

Pregnant Sprague-Dawley rats (Anticimex, Stockholm) were obtained and housed in the department. The animals were kept in separate cages under regulated dark-light conditions (light period 6 a.m.–6 p.m.). The time of delivery was noted within 12 hr.

At one day of postnatal age the pups were subjected to oxygen deprivation (100% N_2 for 20 min or 6% O_2–94% N_2 for 4.5 hr) in a 22-l. plastic cage. The gas mixtures were passed through the cage at a rate of about 4 l./min. Control animals were kept in a similar box exposed to room air, separated from their dams for an equivalent time. During experiments, the cages were kept on a preheated operating table (+35–36°C). Room temperature was +27°C.

Behavioral Studies

The acquisition of a conditioned avoidance response was measured by means of a two-way shuttle box. The rats were trained to avoid an electric shock in the box, with the sound of a house buzzer as warning stimulus. The shuttle box had the internal dimensions 54×22×25 cm, and was divided into two compartments by a plastic partition, with an opening 7×9 cm. The box was housed in an insulated air conditioned

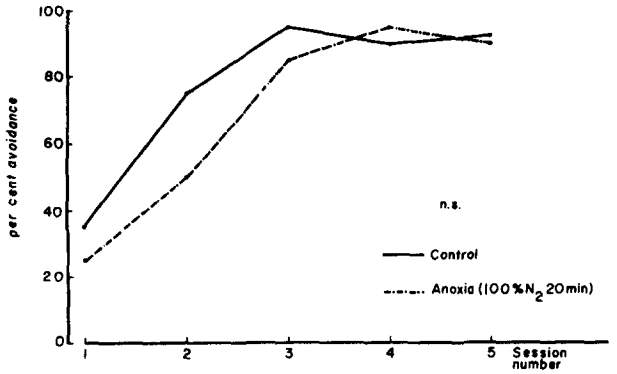

FIG. 1. Neonatal rats were exposed to 100% N_2 for 20 min at 1 day post partum. Four weeks after birth the animals were trained to perform a CAR in five consecutive daily sessions. Shown are median per cent avoidance values of 12 experimental and 9 control animals (fifth session: 3 and 2 values respectively). Statistical comparison between the two groups was made using the nonparametric multivariate analysis according to Mantel *et al.* [22].

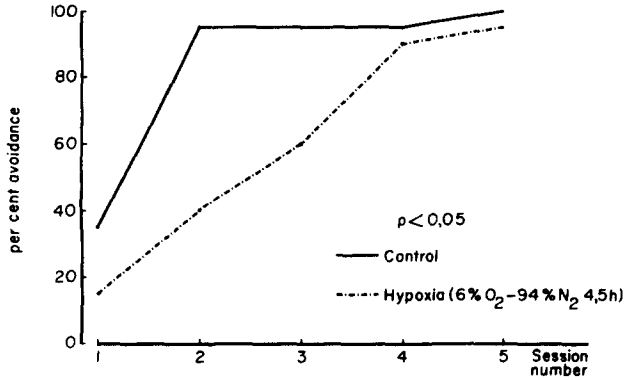

FIG. 2. Neonatal rats were exposed to 6% O_2–94% N_2 for 4.5 hr at 1 day post partum. Four weeks after birth the animals were trained to perform a CAR in five consecutive daily sessions. Shown are median per cent avoidance values of 7 experimental and 7 control animals. Statistical comparison between the two groups was made using the nonparametric multivariate analysis according to Mantel *et al.* [22].

TABLE 1

BRAIN WEIGHTS IN 28 DAYS OLD RATS AFTER NEONATAL ANOXIA OR HYPOXIA

	Control (g)	Anoxia (g)	Hypoxia (g)
Mean ± SEM	1.43 ± 0.01	1.45 ± 0.02	1.42 ± 0.01
n	(39)	(15)	(29)
		ns	ns

The rats were exposed to anoxia (100% N_2 for 20 min) or hypoxia (6% O_2–94% N_2 for 4.5 hr) at one day of postnatal age. ns=not significant in comparison with control. Statistics by *t*-test.

TABLE 2

CATECHOLAMINE AND INDOLEAMINE METABOLISM IN 28 DAYS OLD RAT BRAIN AFTER NEONATAL ANOXIA

	Control (μg/g)	Anoxia (μg/g)	*p*
Tyrosine	21.33 ± 1.08 (6)	22.57 ± 0.62 (6)	ns
DOPA	0.216 ± 0.012 (6)	0.215 ± 0.009 (6)	ns
DA	0.445 ± 0.021 (11)	0.470 ± 0.014 (9)	ns
NA	0.247 ± 0.017 (11)	0.263 ± 0.007 (9)	ns
Tryptophan	5.91 ± 0.12 (6)	6.00 ± 0.14 (5)	ns
5-HTP	0.085 ± 0.011 (6)	0.076 ± 0.003 (5)	ns
5-HT	0.166 ± 0.009 (10)	0.165 ± 0.007 (9)	ns

The rats were exposed to 100% N_2 for 20 min at 1 day of age. Animals analyzed for brain tyrosine, DOPA, tryptophan and 5-HTP were injected with NSD 1015 100 mg/kg 30 min before sacrifice. Shown are mean ± SEM. Figures within parenthesis indicate the number of experiments. Statistics by *t*-test.

enclosure, which isolated the animals from external stimuli, and the rats were observed through an one-way mirror. The temperature in the experimental chamber was +25°C.

The conditioned stimulus (CS) was the sound of the house buzzer, randomly delivered with a 0.5–2.5 min interval. The unconditioned stimulus (UCS) consisted of an intermittent shock (50 Hz, 700 V), delivered through the grid floor of either compartment over a great internal resistance (270 kOhm) in order to diminish the influence from the animals own resistance. The duration of the shock (0.5 sec) and the interval between shocks (2.0 sec) were automatically timed. The CS and the UCS were manually operated. In each trial the CS was presented for maximally 10 sec followed by the CS+UCS for a maximum of another 10 sec.

The following variables were recorded; *Conditioned avoidance response* (CAR). The rat crossed through the opening within 10 sec after the CS had been delivered. *Escape responses*. The rat crossed within 10 sec after the shock (UCS) had been delivered. *Escape failure*. The rat remained in the same compartment for the entire trial (>20 sec). *Spontaneous crosses*. A cross through the opening between trials.

Prior to the first training session, the rats were allowed to adapt to the shuttle-box for 30 min. Training sessions lasted for 20 min and consisted of 20 trials.

The littermates were given 5 daily consecutive acquisition sessions starting 28 days after birth. This has previously been shown to be the earliest age at which untreated rats can be trained to perform a CAR.

Biochemical Studies

30 min before sacrifice, all animals, in which the brain was analysed for tyrosine, DOPA, tryptophan and 5-HTP, were injected subcutaneously with NSD 1015, 100 mg/kg (3-hydroxybensylhydrazine HCl, synthetized in this laboratory by Dr. Per Lindberg) [8]. The animals analysed for brain DA, NA and 5-HT did not receive any injections.

The animals were killed by decapitation at 28 days of postnatal age. The whole brain was dissected out and immediately frozen on solid CO_2. The samples were stored in a freezer at -70°C, in no case for more than 2 months. The brain samples were homogenized in 10 ml ice cold 0.4 N perchloric acid containing 5 mg $Na_2S_2O_5$ and 20 mg EDTA. The extracts were purified on a strong cation exchange column (Dowex 50-X-4) [4] and analysed spectrofluorometrically for tyrosine, DOPA, DA, NA, tryptophan, 5-HTP and 5-HT according to previously described techniques [1, 2, 3, 5, 7, 18, 31]. The recoveries were (mean ± SEM): Tyrosine: 86 ± 3%, DOPA: 92 ± 2%, DA: 108 ± 2%, NA: 88 ± 11%, tryptophan: 82 ± 3%, 5-HTP: 73 ± 2% and 5-HT: 72 ± 1%. No corrections for recoveries have been made.

Statistics

Analysis were performed using a non-parametric multivariate analysis [22] and Student's t-test. p-values larger than 0.05 were considered not significant.

RESULTS

Brain Weights in 28 Days Old Rats

At 28 days of postnatal age, the brain weights in the groups exposed to neonatal anoxia or hypoxia were similar to the brain weights of the control group (Table 1).

Effect on Avoidance Acquisition

The rats exposed to 20 min anoxia (Fig. 1) at 1 day of postnatal age started with 22.5% CAR and reached 90.0% CAR at the fifth training session. The rats of the control group showed an initial avoidance responding of 35.0% and after the fifth session they had reached the level of 92.5% of avoidance responding. No statistical difference between the two performance curves was found.

The group of rats exposed to 4.5 hr of 6% oxygen deprivation (Fig. 2) showed an initial CAR of 15.0% compared to 35.0% CAR of the control group. At the fifth training session the groups had reached 95.0% and 100.0% of avoidance responding respectively. The acquisition curve obtained after prolonged neonatal hypoxia was significantly different ($p<0.05$) from that of the control animals.

Effect on Synthesis Rate and Brain Levels of Monoamines

Neonatal anoxia for 20 min did not effect the endogenous levels of the neurotransmittors DA, NA or 5-HT, nor their amino acid precursors and intermediate substances as measured after NSD 1015 (Table 2).

When the neonatal oxygen deprivation was prolonged (6% O_2–94% N_2 for 4.5 hr) (Table 3) the accumulation of DOPA and 5-HTP after NSD 1015 was reduced by about 20% ($p<0.005$) and 30% ($p<0.05$), respectively. No changes in the levels of tyrosine and tryptophan or DA, NA and 5-HT were observed.

DISCUSSION

In the present investigation we have found that prolonged partial diminution of neonatal oxygenation for 4.5 hr interfered with the acquisition of a conditioned avoidance response in 28 days old rats. However, the CAR peformance after a complete sudden interruption in the exchange of oxygen for 20 min during early postnatal life, was not altered compared to controls.

The activity of the initial enzymes in the catecholamine and indoleamine biosynthetic pathways, tyrosine hydroxylase and tryptophan hydroxylase respectively, are generally considered to be an appropriate index of the functional maturity of these pathways [6, 10, 11, 26]. Accordingly, during postnatal development, there is a gradual increase in tyrosine hydroxylase and tryptophan hydroxylase activity as studied in vivo by means DOPA and 5-HTP accumulation after NSD 1015 [13,14]. The NSD 1015 method seems to be an appropriate method for estimation of tyrosine and tryptophan hydroxylation respectively, as the intermediate amino acids DOPA and 5-HTP accumulate in a linear manner after inhibition of aromatic L-amino acid decarboxylase in the brain [8].

In the present study no persistent changes were noted in the endogenous levels of the amino precursors tyrosine and tryptophan or the neurotransmittors DA, NA and 5-HT either after the 20 min neonatal anoxia or the 4.5 hr prolonged hypoxia. However, after administration of NSD 1015 there was a decreased accumulation of DOPA and 5-HTP in the group of animals exposed to 4.5 hr 6% O_2–94% N_2 at 1 day of postnatal age, suggesting an impaired tyrosine hydroxylase and tryptophan hydroxylase activity respectively. No alterations in the synthetizising capacity was found in the group subjected to 20 min of neonatal anoxia.

From a pathological point of view there are two distinct forms of fetal asphyxia, the first often denoted as an acute total asphyxia, due to a complete and sudden interruption in the exchange of oxygen, while the second is manifested as a prolonged partial decrease in oxygen tension and oxygen content of fetal arterial blood. The consequences for the central nervous system from these two forms of oxygen deprivation are entirely different. A sudden total anoxia may result in a highly reproducible pattern of brain injury involving mainly structures in the brain stem [24]. This brain stem pattern of injury bears no resemblance to the pattern of injury seen typically in the human being after neonatal oxygen deprivation. The response to partial asphyxia, however, seems to be highly variable, being a function of the degree of impairment in oxygen supply to the fetus. Patterns of morphologic changes in the brain produced by partial asphyxia differ completely from those described in relation to total

TABLE 3

CATECHOLAMINE AND INDOLEAMINE METABOLISM IN 28 DAYS OLD RAT BRAIN AFTER NEONATAL HYPOXIA

	Control (μg/g)	Hypoxia (μg/g)	p
Tyrosine	17.65 ± 1.25 (10)	15.72 ± 1.26 (16)	ns
DOPA	0.191 ± 0.014 (10)	0.153 ± 0.004 (17)	<0.005
DA	0.515 ± 0.022 (11)	0.515 ± 0.018 (12)	ns
NA	0.260 ± 0.013 (11)	0.242 ± 0.019 (12)	ns
Tryptophan	5.66 ± 0.24 (10)	5.19 ± 0.15 (17)	ns
5-HTP	0.092 ± 0.012 (10)	0.066 ± 0.003 (17)	<0.05
5-HT	0.178 ± 0.014 (9)	0.192 ± 0.009 (12)	ns

The rats were exposed to 6% O_2–94% N_2 for 4.5 hr at 1 day of postnatal age. Animals analysed for brain tyrosine, DOPA, tryptophan and 5-HTP were injected with NSD 1015, 100 mg/kg, 30 min before sacrifice. Shown are mean ± SEM. Figures within parenthesis indicate the number of observations. Statistics by t-test.

asphyxia and involve hemispheral structures and paracentral regions rather than structures in the brain stem. These patterns of pathology appear to be closely similar to the pathology of perinatal brain injury as it is known to occur in the human [24].

In accordance with these morphological findings, no late effects of acute (20 min) neonatal anoxia was found in the present study. An impairment in monoamine function did however occur after prolonged hypoxia (4.5 hr) in early postnatal age. Although experiments with several species of animals have demonstrated learning deficits following hypoxia, induced either neonatally or perinatally [23, 27, 32], the possible biochemical basis of these findings remains largely unknown.

Several investigations which have been published during the last years have emphasized the importance of an undisturbed catecholamine nurotransmission for the maintenance of conditioned avoidance behavior in adult rats [12,28]. It seems therefore feasible to speculate that there is a close correlation between the persistent behavioral changes and the biochemical impairment observed in the present study in the animals exposed to a prolonged neonatal hypoxia.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council (no. 2464, 4247, 4932) "Expressens Prenatalforskningsfond", The Medical Faculty, University of Göteborg, "Svenska Sällskapet för Medicinsk Forskning", Swedish Board for Technical Development and "Stiftelsen Lars Hiertas Minne". The skillful technical assistance of Mrs Lena Nyström, Britt-Marie Otterstedt and Gabriella Salén is greatfully acknowledged. We also thank Dr. S. Ahlenius for valuable statistical advice.

REFERENCES

1. Atack, C. F. The determination of dopamine by a modification of the dihydroxyindole fluorometric assays. *Br. J. Pharmac.* **48:** 699–714, 1973.
2. Atack, C. Measurement of biogenic amines using cation exchange chromatography and fluorimetric assay. *Acta physiol. scand.* Suppl. **451:** 1–99, 1977.
3. Atack, C. and M. Lindqvist. Conjoint native and orthophtaldialdehyde-condensate assays for the fluorimetric determination of 5-hydroxyindoles in brain. *Naunyn-Schmiedeberg's Arch. exp Path. Pharmak.* **279:** 267–284, 1973.
4. Atack, C. and T. Magnusson. A procedure for the isolation of noradrenaline (together with adrenaline), dopamine, 5-hydroxytryptamine and histamine from the same tissue sample using a single column of strongly acidic cation exchange resin. *Acta pharmac. tox.* **42:** 35–57, 1978.
5. Bédard, P., Carlsson, A. and M. Lindqvist. Effect of transverse hemisection on 5-hydroxytryptamine metabolism in the rat brain. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **272:** 1–15, 1972.
6. Bennet, D. S. and N. J. Giarman. Schedule of appearance of 5-hydroxytryptamine (serotonin) and associate enzymes in the developing rat brain. *J. Neurochem.* **12:** 911–918, 1965.
7. Bertler, Å., A. Carlsson and E. Rosengren. A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* **44:** 273–292, 1958.
8. Carlsson, A., J. N. Davies, W. Kehr, M. Lindqvist and C. V. Atack. Simultaneous measurement of tyrosine and tryptophan hydroxylase activities in brain in vivo using an inhibitor of the aromatic amino acid decarboxylase. *Naunyn-Schmiedeberg's Arch. exp Path Pharmac.* **275:** 153–168, 1972.
9. Coyle, J. T. Development of the central catecholaminergic neurons of the rat. In: *Frontiers in Catecholamine Research*, edited by E. Usdin and S. Snyder. New York: Pergamon Press Inc., 1973, pp. 261–265.
10. Coyle, J. T. and J. Axelrod. Tyrosine hydroxylase in rat brain: developmental characteristics. *J. Neurochem.* **19:** 1117–1123, 1972.
11. Deguchi, T. and J. Barchas. Regional distribution and developmental change of tryptophan hydroxylase activity in rat brain. *J. Neurochem.* **19:** 927–929, 1972.
12. Engel, J. and A. Carlsson. Catecholamines and behavior. In: *Current Developments in Psychopharmacology*, **4:** edited by W. B. Essman and L. Valzelli: Spectrum Publications Inc, 1977, pp. 1–32.
13. Hedner, T., P. Lundborg and J. Engel. The effect of hypoxia on monoamine synthesis in brains of developing rats. *Biol. Neonate* **31:** 122–126, 1977.
14. Hedner, T., P. Lundborg and J. Engel. The effect of hypoxia on monoamine synthesis in brains of developing rats. II. Different length of exposure. *Biol. Neonate* **32:** 229–236, 1977.
15. Hedner, T., P. Lundborg and J. Engel. The effect of hypoxia on monoamine synthesis in brains of developing rats. III. Various O_2-levels. *Biol. Neonate* **34:** 55–60, 1978.
16. Hedner, T. and P. Lundborg. Catecholamine metabolism in neonatal rat brain during anoxia and recovery. (Accept. for publ.)
17. Hedner, T. and P. Lundborg. Serotonin metabolism in neonatal rat brain during anoxia and recovery. (Accept. for publ.)
18. Kehr, W., A. Carlsson and M. Lindqvist. A method for the determination of 3,4-dihydroxyphenylalanine (DOPA) in brain. *Naunyn-Schmiedeberg's Arch. exp Path. Pharmac.* **274:** 273–280, 1972.
19. Loizou, L. A. The development of monoamine-containing neurones in the brain of the albino rat. *J. Anat. Lond.* **104:** 588, 1969.
20. Loizou, L. A. The postnatal ontogeny of mono-amine containing neurones in the central nervous system of the albino rat. *Brain. Res.* **40:** 395–418, 1972.
21. Lundborg, P. and J. Engel. Learning deficits and selective biochemical brain changes in 4-week-old offspring of nursing rat mothers treated with neuroleptics. In: *Antipsychotic Drugs, Pharmacodynamics and Pharmacokinetics*, edited by G. Sedvall. Oxford and New York: Pergamon Press, 1975, pp. 261–269.
22. Mantel, N. and R. S. Valand. A technique of non-parametric multivariate analysis. *Biometrics* **26:** 547–558, 1970.
23. McCullough, M. L. and D. E. Blackman. The behavioral effects of prenatal hypoxia in the rat. *Devl Psychobiol.* **9:** 335–342, 1976.
24. Myers, R. E. Two patterns of perinatal brain damage and their conditions of occurrence. *Am. J. Obstet. Gynec.* **112:** 246–276, 1972.
25. Nobin, A. and A. Björklund. Histochemical demonstration of monoamine neurons/systems in the human fetal brain. In: *Frontiers in Catecholamine Research*, edited by E. Usdin and S. Snyder. New York: Pergamon Press Inc., 1973, pp. 677–681.
26. Schmidt, M. J. and E. Sanders-Buch. Tryptophan hydroxylase activity in developing rat brain. *J. Neurochem.* **18:** 2549–2551, 1971.
27. Sechzer, J. A. Memory deficit in monkeys brain damaged by asphyxia neonatorum. *Expl Neurol.* **24:** 497–507, 1969.
28. Seiden, L. S., R. M. Brown and A. J. Lewy. Brain catecholamines and behavior: Mutual interactions. In: *Chemical Modulation of Brain Function*, edited by H. C. Sabelli, New York: Raven Press, 1973, pp. 261–275.
29. Thornburg, J. E. and K. E. Moore. Pharmacologically induced modifications of behavioral and neurochemical development. In: *Perinatal Pharmacology and Therapeutics*, edited by B. L. Mirkin. New York: Academic Press, 1976, pp. 269–354.
30. Tissari, A. H. Serotoninergic mechanisms in ontogenesis. In: *Fetal Pharmacology*, edited by L. Boréus. New York: Raven Press, 1973, pp. 237–257.
31. Waalkes, T. P. and S. Udenfriend. A fluorometric method for the estimation of tyrosine in plasma and tissues. *J. Lab. clin. Med.* **50:** 733–736, 1957.
32. Windle, W. F. and M. S. Becker.Asphyxia neonatorum: An experimental study in the guinea pig. *Am. J. Obstet. Gynec.* **45:** 183–200, 1943.