Pharmacology Biochemistry and Behavior, Vol. 46, pp. 867–871, 1993
Printed in the U.S.A.

0091-3057/93 $6.00 + .00

Long-Term Effects of Postnatal Hypoxia and Flunarizine on the Dopaminergic System

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Received 15 December 1992

LUN, A., C. BERNDT, J. GROSS, H. D. FISCHER, E. BERGSTRÄSSER AND D. SCHELLER. *Long-term effects of postnatal hypoxia and flunarizine on the dopaminergic system.* PHARMACOL BIOCHEM BEHAV 46(4) 867–871, 1993. – Long-term changes of learning behavior and of the striatal dopaminergic system were observed in a rat model of early postnatal hypoxia. Striatal dopamine (DA) concentration, K^+-stimulated DA release from slices, and DA uptake into crude synaptosomal preparations (S1 fractions) were used as markers of the striatal DAergic system. Active avoidance learning was tested as behavioral criterion. Cyclodextrin and flunarizine were found to produce long-term effects on the DAergic system in control animals. While cyclodextrin normalized hypoxia-induced effects in DA release, flunarizine prevented those in DA uptake and improved avoidance learning.

Dopamine Flunarizine Hypoxia Learning behavior Long-term effect Striatum

LONG-TERM changes of conditioned avoidance learning, dopamine (DA) release from striatal slices, and low-affinity DA uptake into striatal S1 fractions were observed in a rat model of repetitive postnatal hypoxia (1,12). It is well known that hypoxia and ischemia cause alterations in the transport and the transduction systems of neurotransmitters, leading to increased intracellular Ca^{2+} concentrations that, in turn, are capable of influencing neuronal functions (5,21). This way, imbalanced Ca^{2+} homeostasis at critical developmental stages could be involved in the induction of the long-term consequences of hypoxia observed.

Flunarizine (Flu) is a calcium channel blocker and can prevent Ca^{2+}-mediated neuronal death. It antagonizes Ca^{2+} overload by a direct blockade of Ca^{2+} influx mediated by the different Ca^{2+} channel types (T, L, N) and the subsequent Na^+ influx via veratradine-sensitive Na^+ channels (18).

The aim of this study was to test the efficacy of flunarizine in a model of mild repetitive postnatal hypoxia. The K^+-stimulated DA release from striatum slices, DA uptake into striatal crude synaptosomal preparations (S1 fractions), and striatal steady-state concentrations of DA and its metabolites were used as markers of the DAergic system. The active avoidance learning was tested as a behavioral criterion. Since flunarizine is insoluble in water, dihydroxypropyl-β-cyclodextrin (Cyclo) was used as vehicle. The effects of Cyclo injection were considered additionally, because earlier studies revealed that postnatal vehicle administration itself may cause lasting neurochemical changes (1).

METHOD

Exposure to Hypoxia

Male Wistar rats (H. Meichsner, Berlin) were used. Litters for normoxic and hypoxic conditions were rearranged by randomly mixing of the offspring. Part of the rat pups plus dam were exposed to hypoxia in a low pressure chamber from the 2nd to 10th day of life for 10 h daily. The pO_2 within the chamber was 11 ± 0.3 kPa, which equals half the normal pO_2. Hypoxia was accompanied by an increased mortality rate during the first 12 days of life (hypoxia 21%, controls 10%; $p < 0.05$) and by growth retardation by 12% and 6%, respectively, at the 6th and 12th day of life ($p < 0.05$) (12). Applica-

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TABLE 1

POTASSIUM-STIMULATED (^{3}H)-DOPAMINE RELEASE FROM STRIATAL SLICES

	Normoxia	Hypoxia
Untreated	1.37 ± 0.02 [8]	1.89 ± 0.1* [8]
Cyclodextrin	1.47 ± 0.03 [8]	1.41 ± 0.02 [4]
Flu8	1.09 ± 0.06* [16]	1.00 ± 0.01* [8]

Data are given as mean values ± SEM for percent of the accumulated radiolabel. Number of observations shown in brackets.

*Difference to untreated normoxia with $p < 0.05$.

tion of flunarizine or cyclodextrin did not influence mortality rate or body growth. The rest of the litters were exposed to normoxic conditions within a similar chamber.

Drug Application

The following groups were considered both as hypoxic and normoxic animals: untreated, cyclodextrin and flunarizine administered. Flunarizine (Janssen, Lierse, Belgium) was given in dosages of 2.5 μg (Flu2) or 8 μg (Flu8) per g body weight dissolved in cyclodextrin (Janssen, Lierse, Belgium). The cyclodextrin concentration was 100 mg of Cyclo per ml acidified saline (9 g/l). Flunarizine and cyclodextrin were injected intraperitoneally (IP) in a volume of 2 μl per g body weight about 20 min before the hypoxia exposure.

DA Release

The potassium-stimulated DA release was determined at an age of 3 to 4 months as described (12). Rats were decapitated, striatum slices of 0.25 mm thickness were made using a McIlwain tissue chopper and subsequently incubated in a Krebs buffer containing (mM): NaCl (118), KCl (4.7), $MgSO_4$ (1.2), $CaCl_2$ (1.8), Na_2SO_4 (15.9), ethylenediaminetetraacetic acid (EDTA) (1.3), and glucose (5.6) at pH 7.4. After preincubation at 37°C for 5 min, the slices were loaded with [^{14}C]DA (0.03 mmol/l, specific activity 2.2 GBq/mmol, Amersham, UK) for 30 min. After washing they were transferred to a superfusion chamber and superfused with the Krebs buffer at a flow rate of 0.6 ml/min. The stimulated DA release was provoked by applying a 30-s pulse of 14 mM potassium replacing an equimolar amount of sodium chloride. A total of 10 1-min fractions was collected. The superfusion fractions and the remaining activity within the slices were counted for radioactivity. The stimulated release was expressed as the percentage of the [^{14}C]DA content present at the beginning of stimulation (19).

DA Uptake

The DA uptake was measured at the age of 3 to 4 months. Rats were decapitated and striata prepared according to Glowinski and Iversen (8). Striata were homogenized by hand in a glass Teflon homogenizer in 1 ml of 0.32 M sucrose containing (mM): Na_2HPO_4 (2), KH_2PO_4 (0.7), $MgCl_2$ (1), and Na_2EDTA (3) at pH 7.3. The homogenates were centrifuged at 1200 × g for 10 min and the supernatants (S1 fractions crude synaptosomal preparations) used for the uptake measurements. S1 fraction (15 μl) was preincubated at 37°C in 0.5 ml of incubation buffer containing (mM): NaCl (130), KCl (1.7), Na_2HPO_4 (10.4), KH_2PO_4 (1.3), $MgSO_4$ (1.3), $CaCl_2$ (1.3), Na_2EDTA (0.2), glucose (11), sucrose (17.3), ascorbic acid (1.1), and pargyline (0.13) (Sigma, St. Louis, MO) at pH 7.35. [^{3}H]DA was added at 11 different concentrations ranging from 3×10^{-8} to 10^{-4} M for 30 s. The uptake was stopped by filtration through a Sartorius nitrocellulose filter, pore size 0.45 μm. The filters with the retained crude synaptosomes were washed three times with 1 ml of incubation buffer and transferred into scintillation cocktail for counting of radioactivity. Values were corrected for blanks incubated on ice. The kinetic constants of the DA uptake (K_m for affinity and V_{max} for capacity) for the high- and low-affinity uptake sites were calculated by nonlinear regression (10).

Determination of Catecholamines

Striatal concentrations of catecholamines were determined in tissue samples from 3- to 4-month-old animals. The rats were killed by dislocation and heads were precooled in liquid nitrogen. The brains were dissected on ice and the striata homogenized by sonication in 0.5 ml of 0.2 M ice-cold perchloric acid containing 30 mg/l sodium bisulfite and 20 mg/l Na_2EDTA. The homogenates were centrifuged at 4°C for 10 min at 16,000 × g. The supernatants were stored at −20°C until the determination. Sample protein content was determined according to Lowry et al. (11).

TABLE 2

KINETIC CONSTANTS OF THE STRIATAL DA UPTAKE

	K_{m1} (μM)	V_{max1} (pmol/mg/30 s)	K_{m2} (μM)	V_{max2} (pmol/mg/30 s)
Normoxia untreated [30]	0.47 ± 0.02	114 ± 8	53 ± 2	1490 ± 39
Cyclo [7]	0.56 ± 0.32	105 ± 17	156 ± 34*	2957 ± 475*
Flu2 [7]	0.35 ± 0.30	71 ± 16	256 ± 97*	3561 ± 221*
Flu8 [11]	0.17 ± 0.13	47 ± 10	123 ± 17*	2240 ± 225*
Hypoxia untreated [18]	0.81 ± 0.03	175 ± 18	350 ± 48*	4149 ± 331*
Cyclo [8]	0.33 ± 0.08	70 ± 4	113 ± 6*	2291 ± 86*
Flu8 [7]	0.24 ± 0.28	74 ± 23	52 ± 12	1105 ± 135

Data are mean values ± SEM. Number of animals shown in brackets.

*Difference to untreated normoxia with $p < 0.05$.

TABLE 3
STRIATAL CONCENTRATIONS OF DA AND ITS METABOLITES

	DA	DOPAC	HVA	3MT
Untreated [7]	388 ± 20	30 ± 1.5	19 ± 1.5	3.3 ± 0.26
Cyclo [9]	465 ± 25*	38 ± 2.4	26 ± 1.6	3.4 ± 0.38
Flu2 [8]	416 ± 26	32 ± 3.0	22 ± 2.4	3.5 ± 0.50
Flu8 [8]	434 ± 29	33 ± 2.3	24 ± 3.0	4.2 ± 0.60
Hypoxia untreated [7]	396 ± 20	28 ± 1.2	20 ± 1.5	3.7 ± 0.38
Cyclo [7]	398 ± 16	30 ± 2.3	22 ± 1.4	2.9 ± 0.36
Flu2 [7]	428 ± 28	29 ± 2.6	21 ± 1.9	3.9 ± 0.51
Flu8 [6]	366 ± 40	26 ± 2.2	20 ± 1.4	2.2 ± 0.24

Data are mean values ± SEM in nmol/g protein. Number of animals shown in brackets.
*Difference with $p < 0.05$ from untreated normoxia (Wilcoxon test).

HPLC determination was performed using a Gilson equipment with an amperometric electrochemical detector, set to 750 mV against a Ag/AgCl reference electrode. The separation was performed at ambient temperature on a 250 × 4.6 mm Nucleosil 120 RP C18 column, particle size 5 μm, by isocratic elution. The mobile phase contained 25 mmol/l citric acid, 25 mmol/l sodium acetate, 25 mmol/l Na_2HPO_4, 0.45 mmol/l sodium salt of octanesulfonic acid (Merck, Darmstadt, Germany), 45 mg/l Na_2EDTA, and 80 g/l methanol at pH 4. The flow rate was 1.0 ml/min.

Conditioned Avoidance Learning

Training of active avoidance reaction was carried out in 2- to 3-month-old rats on 4 consecutive days (12). Fifteen stimuli were applied on each of the 4 days. After an acoustic signal (conditioned stimulus), the rats had to learn to avoid an electric shock to the feet (unconditioned stimulus) within 5 s. The intervals between the conditioned stimuli were 30 s each. The number of correct responses per stimuli at the 4th day of training was used to quantify the learning behavior (12).

TABLE 4
ACTIVE AVOIDANCE LEARNING OF ADULT RATS EXPOSED TO HYPOXIA AND TO FLUNARIZINE APPLICATION

		Correct Responses to 15 Conditioned Stimuli	
	Day	normoxia	hypoxia
Untreated	1	1.00 ± 0.21	0.14 ± 0.11
	2	6.67 ± 0.35	2.28 ± 0.22
	3	10.22 ± 0.35	4.86 ± 0.31*
	4	13.89 ± 0.22	7.86 ± 0.20*
Cyclo	1	0.38 ± 0.14	0.15 ± 0.11
	2	1.92 ± 0.18	1.61 ± 0.22
	3	6.85 ± 0.26	3.77 ± 0.27*
	4	13.85 ± 0.26	7.85 ± 0.25*
Flu2	1	0.46 ± 0.15	0.20 ± 0.12
	2	2.54 ± 0.15	1.70 ± 0.19
	3	6.23 ± 0.34	5.50 ± 0.28*
	4	11.85 ± 0.33*	8.80 ± 0.30*
Flu8	1	0.38 ± 0.15	0.15 ± 0.11
	2	2.62 ± 0.15	1.92 ± 0.22
	3	7.08 ± 0.22	6.00 ± 0.29
	4	12.46 ± 0.19*	9.38 ± 0.30*

Data are mean ± SEM of 15 animals each.
*Difference from untreated normoxic animals with $p < 0.05$.

Statistics

Mean values, standard deviations, and significance of differences between the mean values of two groups by *t*-test or Wilcoxon test were calculated. Correlation analyses were performed by the software package SPSS. The significance level was $p < 0.05$.

RESULTS

Stimulated DA Release

In accordance with previous data, postnatal hypoxia induced an increase of the K^+-stimulated DA release from striatal slices from 1.4% (normoxia) to 1.9% (hypoxia) in adult rats (Table 1). Cyclodextrin had no effect on the release in normoxic rats but it normalized the stimulated DA release of hypoxic animals. The stimulated release of flunarizine-treated animals was diminished both in normoxic and hypoxia-exposed rats.

DA Uptake

As shown in Table 2, postnatal hypoxia brought about an increase of the kinetic constants describing the low-affinity uptake component (K_{m2} and V_{max2}). Similar results were found in normoxic animals injected with cyclodextrin or flunarizine. In hypoxia-exposed rats, flunarizine but not cyclodextrin normalized the low-affinity uptake constants to the values of untreated normoxic rats.

Concentrations of DA and Its Metabolites

The results of the catecholamine measurements are summarized by Table 3. Hypoxia did not produce any long-term changes of the striatal steady-state concentrations of DA and its metabolites, dihydroxyphenylic acid (DOPAC), homovanillic acid (HVA), and 3 methoxytyramine (3-MT). Cyclo-treated normoxic rats showed elevated concentrations of DA, DOPAC, and HVA. This increase, however, seems to be incidental, because there were no changes in all the other groups tested.

Active Avoidance Learning

Results of the avoidance learning are given in Table 4. Mild chronic postnatal hypoxia impaired the learning performance from 13.9 to 7.9 correct responses to 15 conditioned stimuli. While injection of cyclodextrin did not change this pattern, flunarizine treatment was able to slightly improve the learning performance of hypoxia-exposed rats in both dosages employed, even though the level of normoxic rats was not achieved. Flunarizine application to normoxic rats decreased the conditioned avoidance reaction.

DISCUSSION

Changes of the transport and metabolism of DA were shown to be a critical factor for the genesis of behavioral and neurochemical long-term dysfunctions caused by disturbances of perinatal brain development (4,12,17,21). Repetitive alterations in neurotransmission during this critical period of brain development may modulate gene expression, synaptogenesis, and development of the dendritic tree, thus contributing to the long-term sequelae described above. Immediate-early genes, which are selectively induced by DA or DA agonists (9), may be a bridge between the short-term changes of catecholaminergic transmisson and the long-term changes, since they code for transcription factors that up- and downregulate the expression of target genes.

Those events both may result from or lead to altered neuronal Ca^{2+} transport and distribution. Therefore, it was of interest to check the efficacy of the calcium channel blocker Flu in our model of repetitive postnatal hypoxia. Flu could minimize the acute alterations in striatal catecholamine content or intracellular Ca^{2+} concentration during hypoxia (23) by interfering with transmitter release by Ca^{2+} channel blockade or specifically inhibiting DA uptake (6), thereby reducing or preventing the long-term hypoxia effects.

Our data indicate that Flu application might be of benefit in terms of some of the DA lasting effects of hypoxia. Flu normalized the hypoxia-changed pattern of the DA uptake kinetics; it improved avoidance learning of hypoxia-exposed rats and prevented the posthypoxic increase of the potassium-stimulated release although by an overshooting reduction. All this suggests that Ca^{2+} or DA transport does influence the outcome of postnatal hypoxia. The whole of the picture, however, is much more complicated because i) Flu treatement was only partiallaly effective in hypoxia-exposed animals, ii) Flu application to controls produced rather detrimental effects, and iii) vehicle injection was also shown to cause lasting changes on DA transport and metabolism (Tables 1-3), which, in the case of DA release, were even found to be preventive.

The effects of Cyclo were unexpected because a great amount of pharmacological tests did not point to any efficacy of Cyclo in adult animals (15). Based on our data, it has to be questioned that the substance is pharmacologically neutral. Cyclo has a high affinity to cholesterol and other endogenous substances, which could influence the synthetic and degradative capacity of enzymes involved in DA metabolism (20). It also seems to stimulate brain mitochondrial membrane phospholipase A_2 activity (7). During a critical developmental stage, these effects could contribute to an imprinting of neuronal functions. Yet it has to be kept in mind that any treatment of the newborn rats was connected with unspecific handling and injection stress. Lasting changes of the DA transport were seen after repetitive postnatal saline application (2). In line with this is the observation that the DA uptake kinetics of controls are very similarly affected by the application of saline, Cyclo, and Flu.

The active avoidance learning was markedly diminished by postnatal hypoxia and moderately disturbed by postnatal administration of Flu to normoxic rats. Flu application to hypoxia-exposed animals resulted in a moderate improvement of learning performance in comparison to untreated hypoxic rats. These results are similar to most others of testing preventive potencies in our model (13). Again, the application of different drugs (gangliosides, pyritinol, L-DOPA, and verapamil) during the postnatal period influenced the long-term outcome of normoxic rats in a similar manner (3,14,16,24). This supports the hypothesis of the critical developmental period during the first postnatal weeks in the rat.

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