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AGE-DEPENDENT EFFECTS OF 5,7-DIHYDROXYTRYPTAMINE ON SEROTONIN TRANSPORTER IN DIFFERENT BRAIN AREAS IN THE RAT

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Age-dependent effects of 5,7-dihydroxytryptamine on serotonin transporter in different brain areas in the rat. W. KOSTOWSKI, A. BIDZIŃSKI, P. KRZĄŚCIK, J. SZYNDLER, P. ROK, P. KOŁOMAŃSKA, A. WISŁOWSKA, M. LEHNER, A. PŁA NIK. Pol J Pharmacol, 2004, 56, 383–389.

In the present study, we investigated the [^{3}H]citalopram binding using a quantitative autoradiography following intracerebroventricular injection of 5,7-dihydroxytryptamine (5,7-DHT) in neonatal and adult male Wistar rats. One group of animals was injected with 5,7-DHT at 3 days after birth while the second group received the neurotoxin at 3 months after birth. Control group was injected with saline. Afterwards, all rats were examined at 4th months after birth to determine the serotonin (5-HT) and catecholamines concentrations using the liquid chromatography with electrochemical detection HPLC system and distribution and density of [^{3}H]citalopram binding sites in the brain using the quantitative autoradiography. A marked depletion of brain 5-HT was observed in rats lesioned either in postnatal or adult period of life. Rats lesioned in their adult period of life showed dramatic reduction of 5-HT transporter in all investigated brain areas (i.e. the frontal cortex, entorhinal cortex, hippocampus, caudate-putamen, nucleus accumbens and ventral tegmental area). On the other hand, administration of 5,7-DHT to newborn rats failed to reduce 5-HT transporter sites in the ventral tegmental area, and produced only slight or moderate reduction in the nucleus accumbens. Thus, it appears that the mesolimbic ventral tegmental area-nucleus accumbens systems are relatively more resistant to 5,7-DHT neurotoxicity in the early postnatal period.

Key words: *serotonin, serotonin transporter, neonatal 5-HT depletion, 5,7- dihydroxytryptamine*

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INTRODUCTION

Recently, the growing researchers' interest has been focused on animal models for neurodevelopmental disorders. For example, early amygdala damage has been found to affect the projections from amygdala to other brain structures in adult period of life [4]. Neonatal ventral hippocampal lesion model of behavioral disturbances in rats consistent with psychopathology of schizophrenia has been developed by Lipska and Weinberger [14]. This lesion has also been found to facilitate instrumental learning and responses for drug rewards [8]. Thus, neonatal brain insults may be interesting models for studying the etiology of psychiatric disorders. However, relatively few studies investigated the effects of early postnatal lesions on neurochemical organization of the brain in the adult period of life.

It is known that the response to injury of the developing nervous system and the mature nervous system is different. Generally, the developing brain is more vulnerable than the adult brain to various damaging treatments. On the other hand, there is more sparing or even better recovery when damaging treatment is applied to immature brain than in adult brain [11, 18]. Thus, it has been postulated that the developing brain possesses a greater potential for regeneration. Considering this discrepancy, of particular interest are studies on developmental plasticity of central serotonergic (5-HT) neurons after systemic 5,7-dihydroxytryptamine (5,7-DHT, the 5-HT neurotoxin) treatment of neonatal rats, demonstrating that so-called "prunning effect" is a general response mechanism for developing neurons [11, 16]. From these studies, it can be concluded that neurotoxin treatment produces marked denervation of the distantly situated 5-HT nerve terminal projections (i.e. the cerebral cortex and the spinal cord) whereas the cell body-near regions are more or less intact or even show an increased number of 5-HT terminals [11]. Thus, it has been postulated that the developing 5-HT neurons seem to be programmed to produce a certain quantity of nerve terminal arborizations, which they try to conserve after neurotoxin-induced injury [10].

In the present study, we examined the density of 5-HT transporter (5-HTT) by means of assessing [^{3}H]citalopram binding sites using a quantitative autoradiography following intracerebroventricular (*icv*) injection of 5,7-DHT in neonatal and adult rats. We now report that certain brain areas such as the nucleus accumbens and ventral tegmental area of newborn rats are actually less sensitive to the effect of neurotoxin than those of adult animals.

MATERIALS and METHODS

Chemicals

5,7-Dihydroxytryptamine creatinine sulfate was purchased from RBI (Natick, MA, USA) and desipramine hydrochloride (DMI) was purchased from SIGMA/RBI (St. Louis, MO, USA).

Animals

The study was performed on Wistar rats obtained from a licensed breeder (HZL, Warszawa, Poland). Animals were kept in a temperature-controlled room (20–22°C) under a standardized 12 : 12 h light-dark schedule (light on at 07:00) and 60% relative humidity, with access *ad libitum* to the granulated food and tap water.

Timed pregnant rats were singly housed in plastic cages containing wood chip bedding material. The age of newborn rats was determined by every day checking, each litter contained 8–10 pups. At 3rd day after birth (the day of birth being postnatal day 0) the sex of pups was determined and males were submitted to further experimentation as described below.

Three separate groups of animals underwent the following treatments:

1. First group: rats treated neonatally with neurotoxin. At 3 days of age rats were injected *ip* with DMI (20 mg/kg) in the volume of 1 ml/kg, followed 1 h later with *icv* injection of 5,7-DHT (70 μg per rat, dissolved in 0.1% saline solution of ascorbic acid), bilaterally in the volume of 5 μl. Then, at 10 weeks of age they received *ip* injection of saline (1 ml/kg) followed 1 h later with *icv* injection of 0.1% ascorbic acid solution in saline.

2. Second group: rats treated with neurotoxin in the adult period of life. At 3 days of age, rats were injected *ip* with saline (1 ml/kg) and 1 h later received *icv* 0.1% ascorbic acid solution in saline, and at 10 weeks of age were treated with DMI (20 mgkg *ip*) followed 1 h later with *icv* injection of 5,7-DHT (250 μg per rat, dissolved in 0.1% ascorbic acid solution in saline, bilaterally).

3. Third group: controls treated with solvents. At 3 days of age and 10 weeks of age rats received

icv the solvent (i.e. 0.1% ascorbic acid solution in saline). Each *icv* administration of the solvent was preceded (1 h) with *ip* injection of saline (1 ml/kg).

At 16 weeks of age, each group of rats was randomly divided into two equal parts. One subgroup was submitted to the autoradiographic study while the second subgroup was used for the neurochemical study (see below).

5,7-DHT administration

The details of the method of *icv* microinjections of neurotoxin in newborn and adult rats have been described elsewhere [5–7, 10]. 5,7-DHT has been shown to possess no absolute specificity in its action on 5-HT neurons but also to act on noradrenergic neurons [1, 2]. This action can be circumvented by pretreatment with DMI, thus, providing more selective effect on 5-HT neurons [11].

Newborn animals. At 3 days after birth, pups were injected *ip* initially with DMI (20 mg/kg) to protect noradrenergic neurons from neurotoxin action [11], followed 60 min later by a bilateral *icv* injection of 5,7-DHT dissolved in 0.1% ascorbic acid solution in saline (total dose of 70 μg per rat, the volume of injection of 2.5 μl, delivered with a flow rate of 1 μl/12 s). Neonates were individually removed from the litter, placed on a flat surface under a bright light. In this manner the transverse and sagital sinuses overlying the cranium as well as bregma and lambda, could be easily seen through the transparent dermis. Surgical light anesthesia was induced by diethyl ether. The needle having a polyethylene sleeve up to 2 mm from the tip was positioned 1.5 mm anterior to lambda and 2 mm lateral to the sagital plane. After the needle was lowered into lateral ventricle and neurotoxin or vehicle was injected, the needle was left in place for 30 s.

Adult animals. Male rats 10 weeks old (180 –200 g) were pretreated with DMI (20 mg/kg *ip*), and after 60 min they were anesthetized with ketamine and placed in a stereotaxic apparatus (Stoelting, USA). 5,7-DHT was infused through a Hamilton syringe bilaterally (5 μl per ventricle, total dose was 250 μg) at the following coordinates: A – 1.2 mm to bregma, V 3.5 mm, L 1.5 mm according to atlas of Pellegrino et al. [15]. Each infusion took place over a 1 min period. The needle was left *in situ* 30 s after the infusion was completed.

Autoradiography

A detalied description of the method for receptor autoradiography has been published elsewhere [13] and reported previously by us [9]. The animals were killed by decapitation, their brains were quickly removed, frozen in isopentane (–40°C) and stored at –70°C. The coronal 12 μm sections were cut on cryostat microtome at –20°C, thaw-mounted onto gelatinized glass slides and stored at –20°C until use (after 1 to 2 days). Twenty-seven and 28 slices from each structure were taken for examination in control and experimental groups, respectively. Frozen sections were brought to room temperature 30 min prior the assay. Slides were preincubated in 50 mM TRIS HCl buffer (pH = 7.4) for 60 min at 20°C to remove endogenous competitors. Then, they were incubated for 40 min at 20°C in the same TRIS HCl buffer supplemented with 1 nM [^{3}H]citalopram (85.0 Ci/mmol, Amersham). Non-specific binding was estimated in the presence of paroxetine (1.0 μM). The tissues were then rinsed in the cold buffer for 1 min and rapidly dipped in distilled water. The slides were dried under a cold stream of air, placed in X-ray cassettes and exposed to tritium-sensitive film ([^{3}H] Hyperfilm, Amersham) at 4°C together with standards ([^{3}H] Microscale, Amersham). After 6 weeks of exposure, the films were developed using Kodak LX-24 film developer, washed in water, and placed in Kodak fixer. The autoradiograms were analyzed with the image analysis system (Analytical Imaging Station, Imaging Research Inc., St. Catharines, Canada). Optical densities were converted into nCi/mg of tissue equivalent using the standard curve. [^{3}H]citalopram non-specific binding was negligible.

Neurochemical analysis

Brain 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) concentrations were assayed using a HPLC system with electrochemical detection. Neurochemical procedure was in accordance with our previous studies [10]. After completing the experiments, the rats were killed by decapitation and their brains were rapidly removed, dissected and immediately frozen (–80°C). Frozen tissue structures (frontal cortex, hippocampus, striatum and hypothalamus) were homogenized in 15 volumes of ice-cold 0.05 M perchloric acid with an internal standard added. Homogenates were centrifuged at 15 000 × g and filtered through 0.22 μm

membranes (Millipore, Milford MA, USA). Contents of monoamines were measuresd using a liquid chromatography with electrochemical detection HPLC system (Shimadzu, Japan) with LC-9A pump with a programmable flow rate, equipped with a 20 µl injection loop (Rheodyne, CA, USA). Separation of monoamines and their metabolites was carried out on a Nucleosil 7C-1B column (Macherey-Nagel, Germany) thermostated at 32°C in a Shimadzu CTO-6A column oven. An electrochemical detector (Shimadzu, L-ECD-6A) was set at + 0.8 V potential *vs.* calomel reference electrode. The mobile phase was citric acid (7.5 g/l), $Na_2HPO_4 \times 2H_2O$ (5 g/l), EDTA $Na_2 \times 2H_2O$ (10 mg/l), octanesulfonic acid (180 mg/l), and methanol (12.5% v/v). The flow was programmed from 1.0 to 1.2 ml/min over 18 min for each analytical run. The mobile phase was degassed with helium. Integration of the chromatograms was performed with a Shimadzu C-R4AX Chromatopac-computing integrator. Dihydroxybenzylamine (DHBA) was used as an internal standard. The concentrations of 5-HT, its major metabolite, 5-HIAA, as well as noradrenaline (NA) and dopamine (DA), were estimated in the frontal cortex, whole hippocampus and striatum (caudate + putamen).

Statistical analys

The statistical analysis was performed using Statistica® software package. An analysis of variance (ANOVA), followed by the multiple comparisons with the Newman-Keuls test was used to determine differences when comparing results of autoradiographic studies among the groups. Two-tailed Student's *t*-test was used to evaluate differences of biochemical (neurotransmitter concentrations) study.

RESULTS

Table 1 shows concentrations of 5-HT and 5-HIAA in the frontal cortex, hippocampus, and striatum in three groups of the investigated rats (i.e. neonatally lesioned, lesioned in adult period of life and controls). In the lesioned groups of animals, there was a similar and almost total reduction in 5-HT and 5-HIAA concentrations in all tested cerebral areas. The concentrations of NA and DA remained practically unchanged in both neonatally lesioned rats and rats lesioned in adulthood (DA data not shown).

Table 1. Monoamine concentrations (ng/g of tissue) in 5,7-DHT and control sham lesioned rats. Means ± SEM of 6–8 separate samples per group are presented. **p < 0.01 *vs.* controls. ND – not detectable values

Group	5-HT	5-HIAA	NA
CONTROL			
Frontal cortex	207.3 ± 27	188.9 ± 53	314.7 ± 27
Hippocampus	97.8 ± 29	173.0 ± 17	221.6 ± 55
Striatum	166.9 ± 35	379.9 ± 134	188.5 ± 79
ADULT			
Frontal cortex	6.2 ± 3**	ND	202.5 ± 126
Hippocampus	ND	ND	156.5 ± 47
Striatum	ND	ND	208.2 ± 39
NEONATAL			
Frontal cortex	ND	ND	255.8 ± 101
Hippocampus	ND	ND	196.2 ± 49
Striatum	11.7 ± 4**	38.5 ± 13**	206.4 ± 29

The effect of 5,7-DHT on 5-HTT density was age-dependent. Rats lesioned in their adult period of life showed significant reduction of [^{3}H]citalopram binding sites in all investigated brain structures. The ANOVA study showed significant effect of lesion (binding × group): in frontal cortex [$F(2,15) = 203.47$, $p < 0.01$], entorhinal cortex [$F(2,15) = 111.73$, $p < 0.01$], nucleus accumbens (NAC) [$F(2,15) = 4.33$, $p < 0.05$], ventral tegmental area (VTA) [$F(2,15) = 3.28$, $p < 0.05$], hippocampus CA1 area [$F(2,15) = 42.11$, $p < 0.01$], hippocampus CA3 area [$F(2,15) = 453.09$, $p < 0.01$], hippocampus, dentate gyrus [$F(2,15) = 33.65$, $p < 0.01$], caudate-putamen ($F(2,15) = 6.84$, $p < 0.04$] but not in the VTA [$F(2,15) = 3.28$, $p = 0.065$]. *Post hoc* Newman-Keuls test revealed significant difference in [^{3}H]citalopram binding in all investigated brain areas between rats lesioned in their adult period of life and control group ($p < 0.01$). On the other hand, no difference between group of rats neonatally lesioned and controls was found in the VTA and NAC (Fig. 1 and 2).

Surprisingly, adult rats lesioned neonatally showed practically no neurotoxin effect (i.e. 5-HTT density reduction) in the VTA (and relatively slight effect in the NAC.

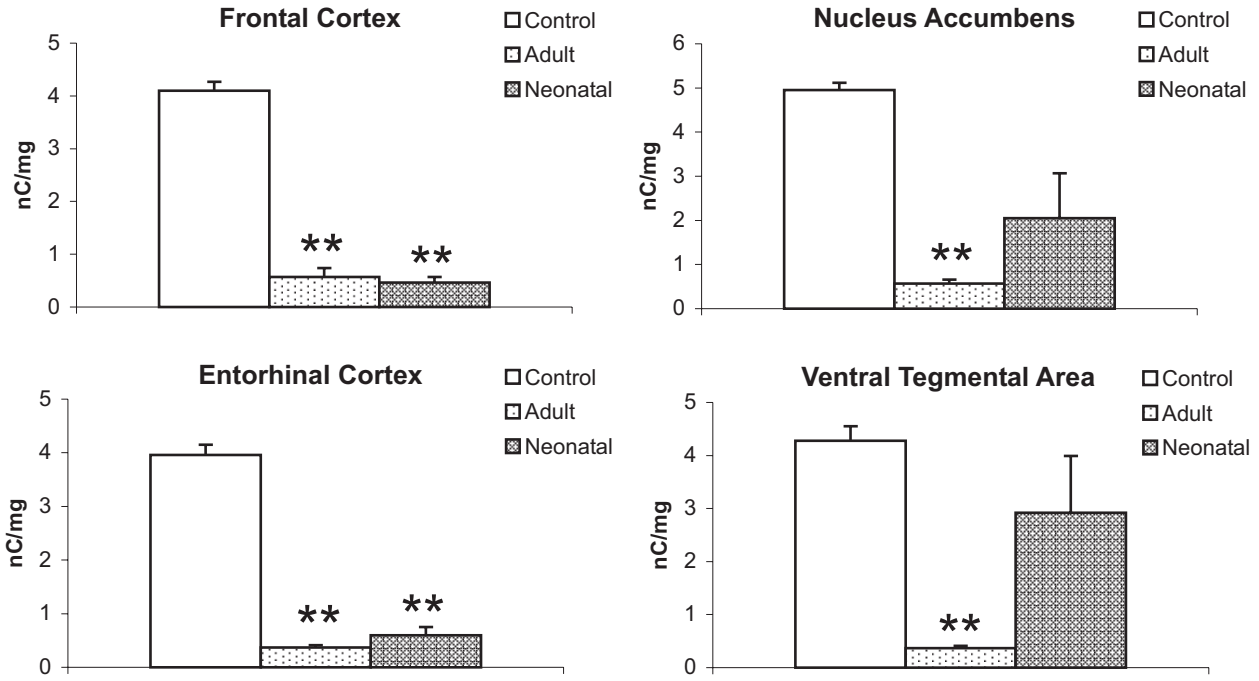

Fig. 1. Binding of [^{3}H]citalopram (nCi/mg) in different brain areas of control rats (Control), neonatally lesioned rats (Neonatal) and rats lesioned in their adult period of life (Adult). Mean values from 18 animals ± SEM. **p < 0.01, *vs.* control group (*post-hoc* Neuman-Keuls test)

DISCUSSION

The *icv* infusion of 5,7-DHT produced almost total reduction of 5-HT and 5-HIAA levels in cortical, hippocampal and striatal areas in two experimental groups of rats. Pretreatment with DMI protected catecholaminergic neurons from the toxic effect of 5,7-DHT, as evidenced by the lack of NA depletion. The present finding is in line with our previous results showing that rats treated neonatally with 5,7-DHT showed substantial and selective brain 5-HT depletion in their adult period of life [10].

The reductions in [^{3}H]citalopram binding sites most likely reflecting a decreased number of 5-HT terminals, were qualitatively similar in the frontal cortex, hippocampus (CA1, CA3, dentate gyrus), entorhinal cortex and dorsal striatum in rats treated with the neurotoxin in the early postnatal, and adult periods of life. On the other hand, 5-HT innervation of the mesolimbic structures, such as VTA and NAC, appears more resistant to 5,7-DHT injected in the early postnatal (but not adult) period of life.

Differences between 5,7-DHT effects on the VTA and NAC 5-HT innervations suggest that they might be associated with special properties of the developing 5-HT neurons that innervate these brain areas. The question, why these neurons are particularly protected during the neonatal period of life, is open to debate. Bearing in mind the study of Jonsson et al. [11] it is conceivable that relatively modest effect of 5,7-DHT in the VTA is due to conservation of axonal arborization in the area located closer to the 5-HT cell bodies (i.e. the midbrain raphe nuclei). This explanation is, however, rather unlikely or only partially correct in the case of NAC and striatal areas, which are situated more distantly to the 5-HT cell bodies. Nevertheless, the neurotoxin effects in these areas were clearly stronger than in the VTA.

Interestingly, both VTA and NAC, are the brain areas containing neurons which form the ascending dopaminergic mesolimbic system. This system plays an important role in motivational and rewarding brain processes [17, 19]. It may be well to add that interaction between 5-HT and DA during postnatal ontogeny is of importance in the functioning of dopaminergic neurotransmission in the adulthood as neonatal 5,7-DHT treatment has been reported to modify the behavioral responses induced by DA D_2 and D_1 agonists. Thus, brain D_2 receptors become sensitized after ontogenic injury to 5-HT neurons while D_1 receptors become less sensitive [6].

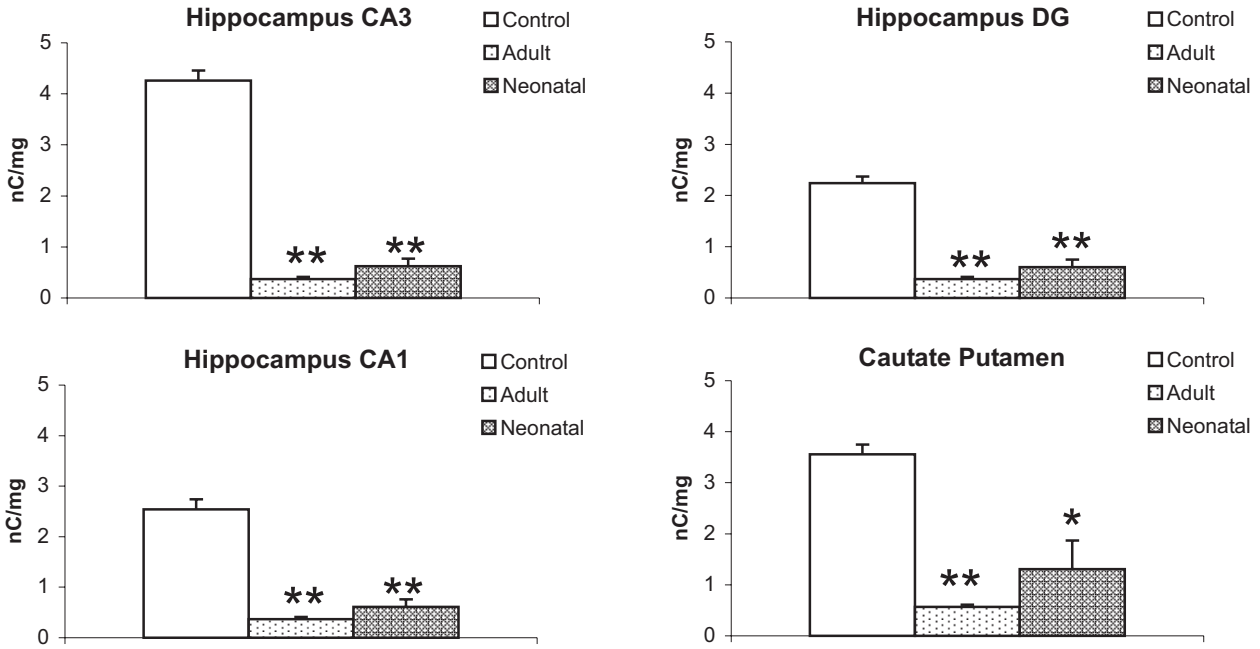

Fig. 2. Binding of [^{3}H]citalopram (nCi/mg) in different brain areas of control rats (Control), neonatally lesioned rats (Neonatal) and rats lesioned in adult period of life (Adult). Mean values from 18 animals ± SEM. Hippocampus DG – dentate gyrus of the hippocampus. *p < 0.05, **p < 0.01 *vs*. control group (*post-hoc* Neuman-Keuls test)

Previous experiments from our laboratory showed that neonatal 5,7-DHT-induced destruction of 5-HT neurons in rats produced behavioral disturbances occuring in adult period of animals' life. In particular, neonatally lesioned animals showed depressive-like behavior as assessed in the forced swim (behavioral despair) test [12], which has never been reported in rats lesioned in their adult period of life [3]. Whether or not this difference is related to the findings from the present study remains unknown.

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