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Michał Świerszcz[1], Przemysław Nowak[1], Michał Bałasz[1], Izabela Walawender[1], Jacek Kasperski[2], Dariusz Skaba[3], Elżbieta Nowak[1], Ryszard Szkilnik[1]

Effect of Neonatal Serotonin Depletion on Morphine-, Nefopam-, Indomethacin-, and Imipramine-Induced Analgesia in Tests of Thermal and Mechanical Pain in Adult Rats

Wpływ zniszczenia układu serotoninergicznego u noworodków szczurzych na percepcję bólu po podaniu morfiny, nefopamu, indometacyny lub imipraminy w testach z użyciem bodźca termicznego i mechanicznego u dorosłych zwierząt

[1] Department of Pharmacology, Medical University of Silesia, Zabrze, Poland
[2] Department of Prosthetic Dentistry, Medical University of Silesia, Zabrze, Poland
[3] Department of Conservative Dentistry and Endodontics Division of Dental Propedeutics, Medical University of Silesia, Zabrze, Poland

Abstract

Background. Previous studies have demonstrated that chemical lesion of the noradrenergic system modified the antinociceptive effects of morphine, paracetamol, and nefopam in rats. Furthermore, it has been demonstrated that the serotoninergic as well as the noradrenergic system participate in the central effect of these drugs in different ways.
Objectives. In this study the impact of serotoninergic system lesion on the antinociceptive effects of morphine, nefopam, indomethacin, and imipramine in rats was investigated.
Material and Methods. Three days after birth, control rats were pretreated with desipramine HCl (20 mg/kg *i.p.*, base) 30 min before intraventricular (*i.c.v.*) saline (0.85%)-ascorbic acid (0.1% a.a.) vehicle injection. A separate group received 5.7-dihydroxytryptamine (5,7-DHT; 70 μg in each lateral ventricle, base). When the rats attained 10 weeks of age, painful reactions were assessed by means of tail immersion and paw pressure tests. Furthermore, monoamines and their metabolite levels in some parts of the brain were determined using the HPLC/ED method.
Results. In the tail immersion test (thermal stimulus), no differences were found in the antinociceptive actions of morphine (5.0 mg/kg *s.c.*), nefopam (20 mg/kg *i.p.*), indomethacin (5 mg/kg *i.p.*), and imipramine (10 mg/kg *i.p.*) between the control and 5,7-DHT lesioned rats. In the paw pressure test (mechanical stimulus), all the examined drugs also showed a similar analgesic effect. Biochemical studies demonstrated that in the 5,7-DHT pretreated rats there was a marked decrease in serotonin (5-HT) and 5-hydroxyindoloacetic acid (5-HIAA) levels in all the examined structures (prefrontal cortex, thalamus, and spinal cord) compared with the control group ($p < 0.05$). Simultaneously, no changes in noradrenalin (NA) and dopamine (DA) concentrations were observed.
Conclusions. The analgesic activities of the morphine, nefopam, indomethacin, and imipramine are not perturbed by central serotoninergic system dysfunction (**Adv Clin Exp Med 2010, 19, 1, 33–41**).

Key words: serotoninergic system, lesion, antinociceptive drugs, rats.

Streszczenie

Wprowadzenie. Dotychczas wykazano, że przeciwbólowe działanie morfiny, paracetamolu i nefopamu zmienia się pod wpływem chemicznego uszkodzenia ośrodkowego układu noradrenergicznego u szczurów oraz że w ośrodkowych mechanizmach analgetycznego działania badanych leków uczestniczą w różnym stopniu układy serotoninergiczny i noradrenergiczny.
Cel pracy. Zbadanie wpływu chemicznej lezji ośrodkowego układu serotoninergicznego u noworodków szczurzych na przeciwbólowe działanie morfiny, nefopamu, indometacyny i imipraminy u dorosłych zwierząt.

Materiał i metody. Zwierzęta z grupy kontrolnej w 3. dniu życia otrzymały dezmetyloimipraminę (DMI; 20 mg/kg *i.p.*) i po 30 min dokomorowo (*i.c.v.*) 10 µl 0,1% roztworu kwasu askorbinowego w 0,85% chlorku sodu. Grupa z lezją układu serotoninergicznego otrzymała DMI (20 mg/kg *i.p.*) i po 30 min 5,7-dihydroksytryptaminę (DHT; 70 µg/10 µl *i.c.v.* w 0,1% roztworze kwasu askorbinowego). Gdy badane zwierzęta ukończyły 10 tygodni, wykonano testy imersji ogona oraz wycofania łapy. Dodatkowo, posługując się metodą HPLC/ED, oznaczono zawartość amin biogennych i ich metabolitów w wybranych częściach mózgu badanych zwierząt.
Wyniki. Nie stwierdzono różnicy w przeciwbólowym działaniu morfiny (5,0 mg/kg *s.c.*), nefopamu (20 mg/kg *i.p.*), indometacyny (5,0 mg/kg *i.p.*) i imipraminy (10 mg/kg *i.p.*) w teście imersji ogona (bodziec termiczny) między grupą kontrolną i 5,7-DHT. Również w teście wycofania łapy (bodziec mechaniczny) działanie przeciwbólowe stosowanych analgetyków nie różniło się między badanymi grupami zwierząt. Wykazano natomiast, że podanie 5,7-DHT w dawce 70 µg/10 µl (*i.c.v.*) u noworodków szczurzych wywołuje głęboki spadek zawartości serotoniny (5-HT) oraz jej metabolitu kwasu 5-hydroksyindolooctowego (5-HIAA) we wszystkich badanych strukturach mózgu, tj. korze przedczołowej, wzgórzu oraz rdzeniu kręgowym w porównaniu z grupą kontrolną (uzyskane różnice były statystycznie istotne; $p < 0,05$). Nie stwierdzono natomiast zmian zawartości noradrenaliny (NA), dopaminy (DA) i jej metabolitów.
Wnioski. Na podstawie przeprowadzonych badań wyciągnięto wnioski, iż zniszczenie ośrodkowego układu serotoninergicznego u szczurów nie wpływa na przeciwbólowe działanie badanych analgetyków (**Adv Clin Exp Med 2010, 19, 1, 33–41**).

Słowa kluczowe: układ serotoninergiczny, lezja, leki przeciwbólowe, szczury.

It is well established that neurons producing serotonin (5-hydroxytryptamine, 5-HT) and norepinephrin (NE) contribute to descending pain control pathways [1]. Many studies demonstrated reciprocal interactions between µ-opioid and α_2-adrenergic as well as serotoninergic 5-HT_1- and 5-HT_2-mediated mechanisms [2, 3]. The most obvious evidence of the impact of NE neurons on pain modulation includes opioid withdrawal syndrome. Abrupt cessation of opioid intake precipitates opioid withdrawal, which produces several aversive symptoms, including an abnormal increase in pain sensitivity (hyperalgesia) [4]. There is also evidence showing a correlation between the synthesis rate or release of 5-HT in the brain and the development of tolerance, also supporting a role of 5-HT in morphine tolerance [5, 6], although data in this matter are inconsistent because some reports suggest that 5-HT might not be involved in morphine tolerance [7].

As it has been demonstrated that the serotoninergic system may participate in the analgesic effects of many other compounds. The non-opiate drug nefopam acts principally by inhibiting 5-HT, NE, and dopamine (DA) uptake and its analgesic activity could also be modulated by α_1- and α_2-adrenergic receptors, the dopaminergic D_2 receptors, and the serotonergic 5-HT_{1B} and 5-HT_{2C} receptor subtypes [8]. Despite being introduced into clinical practice in 1976, the precise mechanism and biological action of this drug is still a matter of concern. Indomethacin-induced analgesia, besides its basic mechanism (inhibition of the production of prostaglandins), also depends, at least in part, on peripheral and central 5-HT modulation [9, 10]. In addition, the therapeutic effects of antidepressant drugs (e.g. imipramine) have been previously attributed to the facilitation of central monoaminergic neurotransmission [11]. Furthermore, the analgesic properties of antidepressants have been proved in clinical settings. However, their efficacy as adjuvant therapeutics varies with the the class of antidepressant employed and the type of pain condition [12]. The analgesic effects usually occur at lower doses and with an earlier onset than the pure antidepressant effects [13]. Antidepressants are thought to produce analgesia by increasing the activity of noradrenergic and serotonergic projections descending from the brain to the spinal cord to modulate the release of endogenous opioid peptides [14].

The present authors hypothesized that serotoninergic system dysfunction may result in an abnormal (reduced) antinociceptive effect evoked by analgesic drugs. Therefore, the general aim of this study was to determine whether serotoninergic system lesion may affect the antinociceptive effects of morphine, nefopam, indomethacin, and imipramine in rats.

Material and Methods

Animals and Treatment

Male Wistar rats (University Animal Department, Katowice, Poland) were housed in a temperature (22 ± 1°C) and light- (6:00 a.m. – 6:00 p.m.) controlled room and were allowed free access to food pellets and water. Their use in the present study was in accordance with the institutional guidelines that are in compliance with the principles and guidelines described in the NIH booklet Care and Use of Laboratory Animals.

Serotonergic lesion were produced by bilateral *i.c.v.* administration of 5,7-dihydroxytryptamine (5,7-DHT; 70 µg dissolved in 10 µl of 0.1% ascorbic

acid/0.9% NaCl, 5 µl per side) to 3-day-old rats. To protect the noradrenergic neurons, the rats were pretreated with 25 mg/kg desipramine HCl (*i.p.*) 30 min before 5,7-DHT injection. Control animals were also pretreated with desipramine HCl and administered with vehicle (10 µl of 0.1% ascorbic acid/0.9% NaCl, 5 µl per side) [15]. All procedures were reviewed and approved by the Local Bioethics Committee for Animal Care.

Thermal Stimulus – Tail Immersion Test

For analgesia studies with thermal stimulus, tail-flick assays were performed using the 58.5°C water tail-immersion approach. In brief, each rat was placed in a cone restrainer and the end of the tail was immersed 5 cm in a 58°C water bath. The pain threshold was measured as the time required to elicit a flick of the tail. The cutoff time was 10 s. The reaction latency (s) was used as the parameter reflecting the intensity of the pain experienced. Tail-flick results were expressed as % analgesia = = (post-drug latency – pre-drug latency) × 100/ (cutoff time – pre-drug latency) [16, 17]. The analgesic effect was measured before drug administration (after saline 1.0 ml/kg *i.p.*) and at 30, 60, 90, and 120 min after morphine (5.0 mg/kg *i.p.*), nefopam (20 mg/kg *i.p.*), indomethacin (5.0 mg/kg *i.p.*), or imipramine (10 mg/kg *i.p.*) injection.

Mechanical Stimulus – Paw Pressure Test

For the paw pressure test, performed with a paw pressure apparatus (analgesimeter, probe tip diameter: 1 mm; weight: 25 g; Ugo Basile Milan, Italy), the rats were gently wrapped in a towel. The left hind paw of the rat was placed under the weight of the apparatus and the test was started. A brisk foot withdrawal of the hind limb after constantly increasing pressure terminated the measurement and the pressure was recorded. The rats were habituated to the full procedure on two consecutive days and the experiments were conducted on the third day. The mechanical threshold was always assessed three times before drug administration to yield a mean value. A 750-g cutoff value was used to prevent tissue damage [18, 19].

The experiments were performed in a quiet room by the same investigator blinded to the treatment used. The formula used to calculate the percentage of analgesia was: % analgesia = (100 × × B)/A – 100, where A is the mean pressure (g) from three assessments before drug administration and B the pressure (g) assessed at 30, 60, 90, and 120 min after drug treatment. The analgesic effect was measured before drug administration (after saline 1.0 ml/kg i.p.) and at 30, 60, 90 and 120 min after morphine (5.0 mg/kg i.p.), nefopam (20 mg/kg i.p.), indomethacin (5.0 mg/kg i.p.), or imipramine (10 mg/kg i.p.) injection

Assay of Biogenic Amines and Their Metabolites

DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, 5-HIAA, and NE were determined by the HPLC/ED method described previously [20, 21]. Briefly, the animals were decapitated and the medial prefrontal cortex, thalamus, and spinal cord were rapidly dissected and placed on dry ice, weighed, and stored at –70°C pending assay. The samples were homogenized for 15–20 sec in ice-cold trichloroacetic acid (0.1 M) containing 0.05 mM ascorbic acid. After centrifugation (5000 × × *g*, 5 min), the supernatants were filtered through 0.2-µm cellulose membranes (Titan MSF Microspin filters; Scientific Resources Inc., Eatontown, GB) and injected onto an HPLC/ED column.

Monoamines were determined under the following conditions: a flow rate of 0.7 ml/min, temperature 22°C, oxidation potential +700 mV, 10 nA/V sensitivity, with the mobile phase composed of 75 mM NaH_2PO_4, 1.7 mM 1-octanesulphonic acid, 5 µM EDTA (Avocado, Research Chemicals Ltd), 100 µl triethylamine (Sigma), and 9.5% acetonitrile (Lab-Scan), adjusted pH 3 with phosphoric acid (Fluka). The instrumentation included a model 141 electrochemical detector with flow cell, model 302 piston pump with head 5SC, model 802 manometric module (Gilson, France), a thermostat for a STH 595 column (Dionex, Germany), Hypersil BDS C18, 10 × 4 mm, 3 µm precolumn, and a Hypersil BDS C18, 250 × 4.6 mm, 3 µm chromatographic column (ThermoQuest GB).

Chromatograms were automatically integrated by a UCI-100 universal chromatographic interface. The results of the catecholamine assay are present in ng per gram of wet tissue (ng/g).

Data Analysis

Group differences were assessed by Student's *t*-test. p values < 0.01 and < 0.05 were taken as levels of statistical significant.

Results

Tail Immersion Test

The tail immersion test demonstrated that morphine administered at a dose of 5.0 mg/kg s.c.

evoked intense and long-lasting analgesia in both tested groups (on average 75–98%). As has been noted, there were no differences in antinociceptive effect elicited by this drug between control and 5,7-DHT rats (Fig. 1a). The analgesic action of nefopam (20 mg/kg) was much less evident (compared with morphine) and the maximal effect was observed at 120 min (over 40%) (Fig. 1b). Indomethacin (5.0 mg/kg) treatment resulted in a stronger antinociceptive effect in the control than in the 5,7-DHT-lesioned rats (primarily at 30 and 60 min of testing), although the differences were not statistically significant (Fig. 1c). Also after imipramine challenge (10 mg/kg) there were no differences between the tested groups noted (Fig. 1d).

Paw Pressure Test

The paw pressure test showed that morphine (5.0 mg/kg) elicited more intense analgesia in the 5,7-DHT group than in the control rats (at 30, 60, and 90 min of the observation), although the difference were not significant (Fig. 2a). Nefopam (20 mg/kg and indomethacin (5.0 mg/kg) challenge evoked similar antinociceptive action in both tested groups (Fig. 2 b and 2c). In contrast, imipramine at a dose of 10 mg/kg did not produced analgesia in the paw pressure test (Fig. 2d).

Biogenic Amines and Their Metabolite Content

In the HPLC/ED assay, 5,7-DHT injection to neonates (3 days after birth) resulted in marked decreases in 5-HT and 5-HIAA levels in all the tested brain structures (prefrontal cortex, thalamus, and spinal cord) compared with the vehicle-treated rats ($p < 0.05$). In the prefrontal cortex, 5-HT and 5-HIAA levels dropped to 2.4% and 2.3%, in the thalamus to 15.7% and 6.3%, and in the spinal cord to 25.1% and 13.7%, respectively, of those of the control animals. NE, DA, DOPAC, and HVA were not altered by 5,7-DHT treatment in any of the tested animals (Tab. 1).

Discussion

The current study demonstrated that neonatal lesion of the central serotoninergic system in rats did not modify the antinociceptive effects of morphine, nefopam, indomethacin, and imipramine assessed by the tail-flick and paw pressure tests. As noted, the administration of 5,7-DHT neurotoxin caused a substantial reduction in the levels of 5-HT and its metabolite (5-HIAA) in the prefrontal cortex, thalamus, and spinal cord. In contrast, NE, DA, DOPAC, and HVA levels were not altered by 5,7-DHT treatment in any of the tested brain structures. Therefore this treatment, in agreement with previous studies, caused a substantial reduction in 5-HT and 5-HIAA with little or no changes in the other monoamine content [22, 23]. These findings confirm that 5,7-DHT has a demolishing effect on the central serotoniergic system in rats [24, 25]. It must be added that neurotoxins (e.g. 5,7-DHT, 6-hydroxydopamine) are chemical tools useful not only for discerning neuronal mechanisms and animal modeling of neurological disorders, but also for their use in medicine and their potential as treatments for medical disorders [26, 27].

In the past several decades, numerous studies have indicated that morphine-induced descending inhibition of nociceptive transmission depends on supraspinal 5-HT neurons [28]. However, many contradictory results concerning the role of central 5-HT neurons in morphine analgesia have also been reported. Gao et al. [29] demonstrated by means of electrophysiological studies a lack of firing responses of 5-HT neurons to morphine treatment in rats. Yoon and al. [30] also found no reciprocal interaction between 5-HT(3) receptor and opioid receptors at the spinal level. Thus, in contrast to the "textbook" explanation, these findings have strengthened the view that 5-HT neurons are neither necessary nor sufficient for the analgesic effect of opioids and are thus not an integral part of opioid-mediated pain-modulatory circuits [31]. This is in line with the present results concerning morphine-evoked analgesia in neonatally 5-HT-lesioned rats.

The present study also demonstrated that the 5,7-DHT treatment did not alter the antinociceptive effects of nefopam (20 mg/kg) and imipramine (10 mg/kg) assessed in the tail-flick and paw pressure tests. This is in contrast to expectation because it is generally accepted that the noradrenergic and serotonergic pathways are involved in both nefopam- and antidepressant-induced antinociception [32, 33]. However, Esposito et al. [34] demonstrated that pretreatment with reserpine, which depletes NE, 5-HT, and DA, significantly reduced the antinociceptive action of nefopam, pointing out that the interaction of this drug with the monoaminergic systems is important for its biological effects. At the same time they ruled out a role for 5-HT or NE because selective lesion of 5-HT (using 5,7-DHT) or NE (using DSP-4 or FLA-63) did not affect nefopam antinociception. These opposing results are difficult to reconcile, probably because of technical issues such as incomplete depletion of 5-HT or 5-HT neurons (using

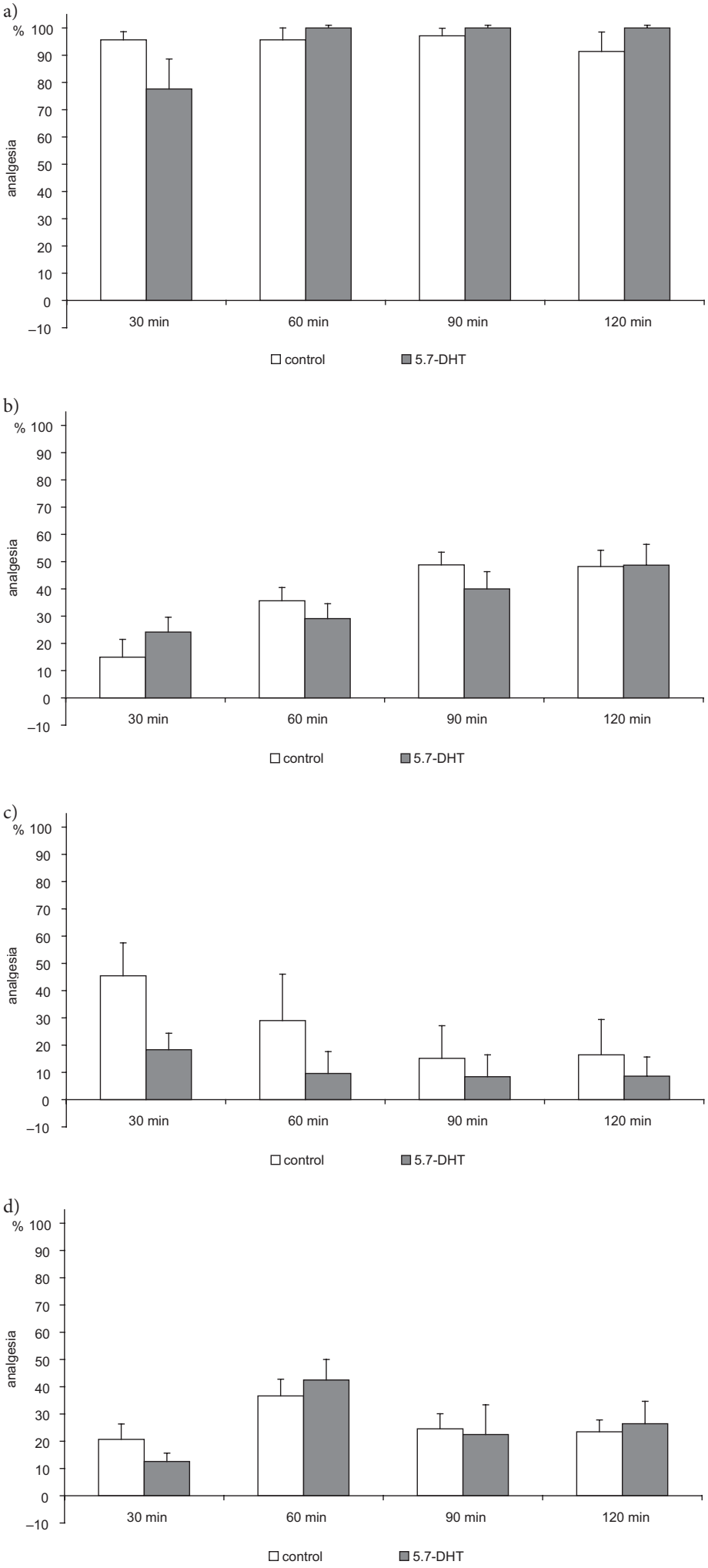

Fig. 1. Effect of 5,7-DHT treatment on analgesia assessed in the tail immersion test after morphine (5.0 mg/kg *s.c.*) (Fig. 1a), nefopam (20 mg/kg *i.p.*) (Fig. 1b), indomethacin (5.0 mg/kg *i.p.*) (Fig. 1c), and imipramine (10 mg/kg *i.p.*) (Fig. 1d) in rats (n = 10). * $p < 0.05$ control vs. 5.7-DHT

Ryc. 1. Wpływ podania 5,7-DHT na przeciwbólowe działanie morfiny (5,0 mg/kg *s.c.*) (ryc. 1a), nefopamu (20 mg/kg *i.p.*) (ryc. 1b), indometacyny (5,0 mg/kg *i.p.*) (ryc. 1c) oraz imipraminy (10 mg/kg *i.p.*) (ryc. 1d) w teście imersji ogona u szczurów (n = 10). * $p < 0,05$ grupa kontrolna *vs* 5,6-DHT

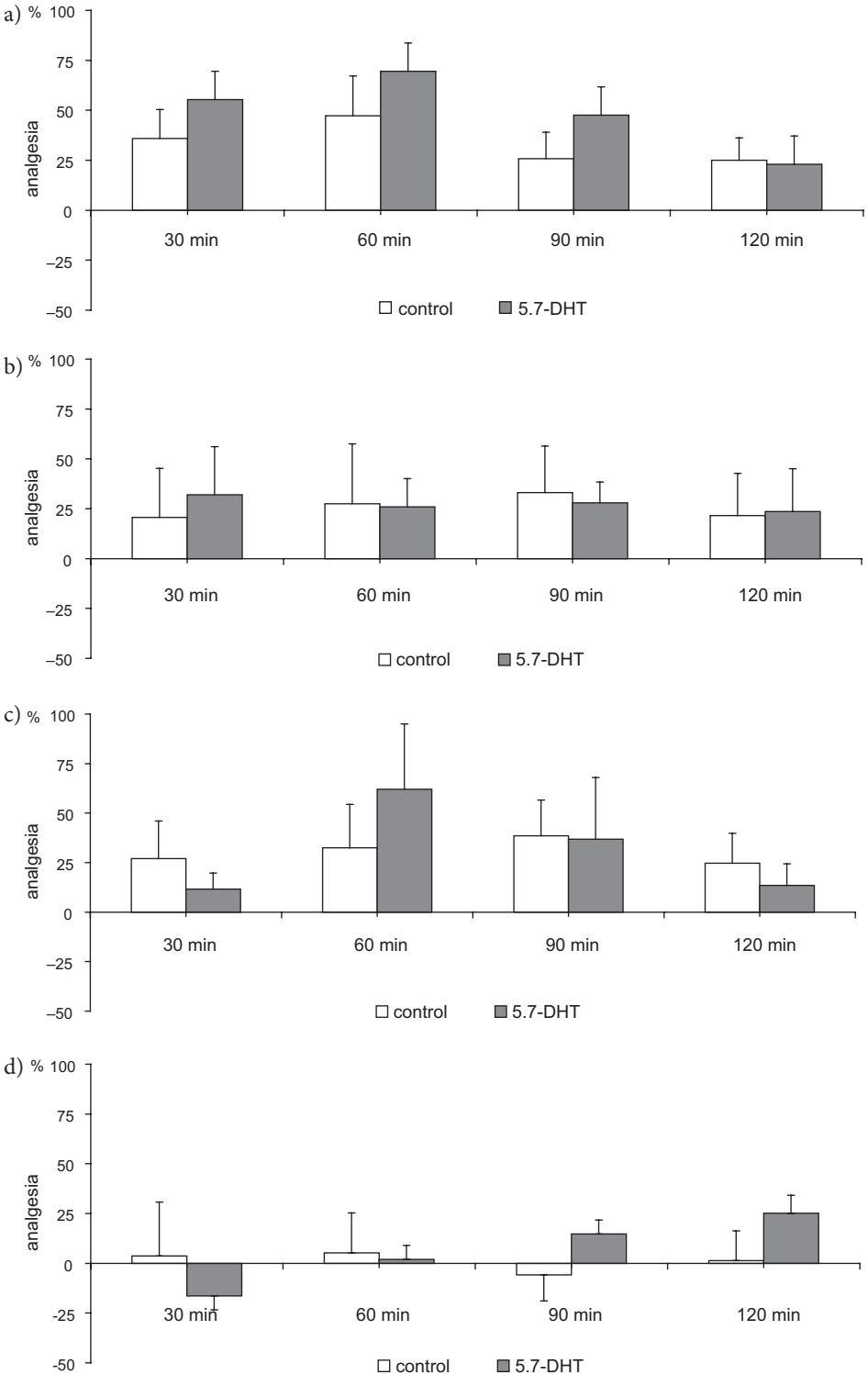

Fig. 2. Effect of 5,7-DHT treatment on analgesia assessed in the paw pressure test after morphine (5.0 mg/kg *s.c.*) (Fig. 2a), nefopam (20 mg/kg *i.p.*) (Fig. 2b), indomethacin (5.0 mg/kg *i.p.*) (Fig. 2c), and imipramine (10 mg/kg *i.p.*) (Fig. 2d) in adult rats (n = 10). Explanations as in Fig. 1

Ryc. 2. Wpływ podania 5,7-DHT na przeciwbólowe działanie morfiny (5,0 mg/kg *s.c.*) (ryc. 2a), nefopamu (20 mg/kg *i.p.*) (ryc. 2b), indometacyny (5,0 mg/kg *i.p.*) (ryc. 2c) oraz imipraminy (10 mg/kg *i.p.*) (ryc. 2d) w teście wycofania łapy u szczurów (n = 10). Objaśnienia jak w ryc. 1

5,7-DHT), nonspecific effects of drugs (reserpine), and the like. Furthermore, Sierralta et al. [35] found employing a writhing test (visceral pain examination) that pretreatment with para-chlorophenylalanine (5-HT synthesis inhibitor) significantly reduced antinociception induced by imipramine and maprotiline and did not modify the effects of zimelidine and clomipramine. On the other hand, pretreatment with alpha-methyl-tyrosine (an NE synthesis inhibitor) did not modify the antinociception induced by these drugs except maprotiline. What is interesting, concomitant pretreatment with both inhibitors significantly reduced the antinociceptive effect of all the antidepressants tested. They concluded that critical levels of both 5-HT and NA are responsible for mediating the antinociceptive effects of antidepressants.

The present authors speculated that lesion of the serotoninergic system (in the present study) was not a sufficient procedure to disclose abnor-

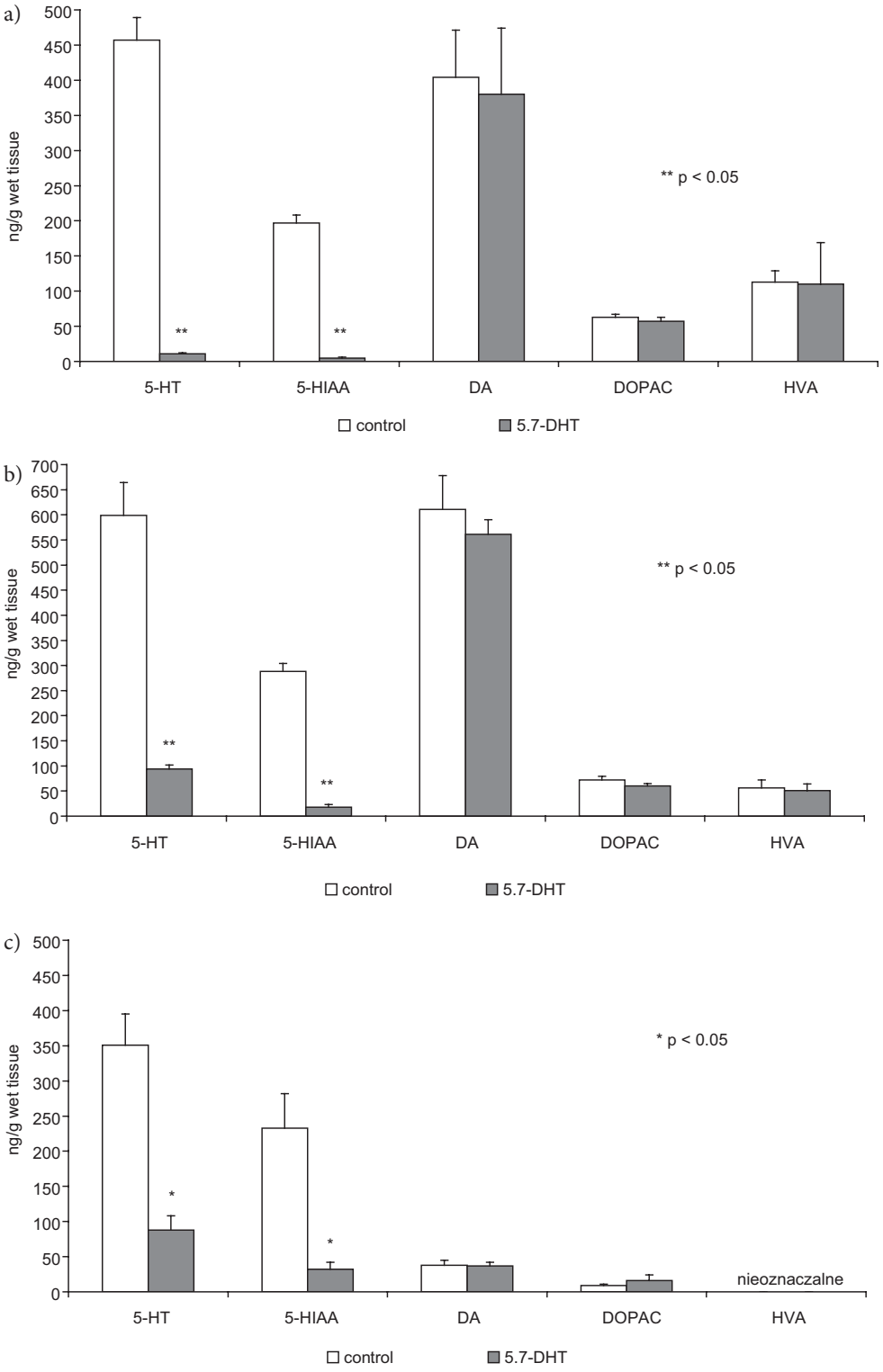

Fig. 3. Effect of 5,7-DHT treatment on biogenic amine level in the prefrontal cortex (Fig. 3a), thalamus (Fig. 3b), and spinal cord (Fig. 3c) (mean ± *SEM*, n = = 6). Explanations as in Fig. 1.
* $p < 0.05$;
** $p < 0.01$

Ryc. 3. Wpływ podania 5,7-DHT na zawartość amin biogennych w korze przedczołowej (ryc. 3a), wzgórzu (ryc. 3b) oraz rdzeniu kręgowym (ryc. 3c) (x ± SEM; n = = 6). Objaśnienia jak w ryc. 1.
* $p < 0,05$;
** $p < 0,01$

malities in pain perception after nefopam and imipramine challenge. In other words, "the signal" might be transmitted by an "intact" noradrenergic pathway. As mentioned in the introduction, indomethacin-induced analgesia, besides its basic mechanism (inhibition of production of prostaglandins), depends, at least in part, on peripheral and central 5-HT modulation [9, 10]. From own studies the authors learned that the serotoniergic system has little or no influence on indomethacin-evoked analgesia assessed by the tail flick and paw pressure tests.

In conclusion, the obtained results, although negative, indicate that 5-HT has a marginal role in the antinociception of morphine, nefopam, indomethacin, and imipramine, at least in tests employing thermal and mechanical stimuli.

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Address for correspondence:

Przemysław Nowak
Department of Pharmacology
Medical University of Silesia
H. Jordana 38
41-808 Zabrze
Poland
Tel./fax: +48 32 272 26 83
E-mail: pnowak@sum.edu.pl

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