Animal Reproduction Science 208 (2019) 106102

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Stimulatory effect of dopamine derivative, salsolinol, on pulsatile luteinizing hormone secretion in seasonally anestrous sheep: Focus on dopamine, kisspeptin and gonadotropin-releasing hormone

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ARTICLE INFO

Keywords:
Salsolinol
Luteinizing hormone
Gonadotropin-releasing hormone
Dopamine
Kisspeptin

ABSTRACT

In the present study, there was testing of the hypothesis that a centrally administered dopamine (DA) derivative, salsolinol, could affect pulsatile luteinizing hormone (LH) secretion in seasonally anestrous sheep by affecting the neuronal components of the estradiol (E2) negative feedback. In two experiments performed during early spring (increasing day length – March/April), salsolinol or Ringer-Locke solution (control) were administered into the third brain ventricle (IIIv): 1) in several injections for three consecutive days; and 2) in several hour-long infusions. In addition to determining the LH concentration (in both experiments), the abundances of gonadotropin-releasing hormone (GnRH) and kisspeptin mRNA were examined in the hypothalamus and LHβ subunit mRNA in the pituitary (Experiment 1). In Experiment 2, concentrations of DA and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in perfusates collected from the infundibular nucleus/median eminence (IN/ME) by the push-pull method. In both experiments, salsolinol increased both LH pulse frequency ($P < 0.05$) and plasma LH concentration ($P < 0.001$) compared to controls. The injected salsolinol also increased ($P < 0.05$) the abundance of GnRH mRNA in the mediobasal hypothalamus and kisspeptin mRNA in the arcuate nucleus. The two doses of infused salsolinol decreased DA to undetectable concentrations and DOPAC concentration by 60% in perfusates collected from the IN/ME. In conclusion, exogenous salsolinol functioning centrally stimulates pulsatile LH secretion in sheep during seasonal anestrus. It is suggested that salsolinol may have this effect by reducing the activity of the hypothalamic neuroendocrine dopaminergic system, which results in an increase in both kisspeptin and GnRH neurons activity.

1. Introduction

The presence of salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), a dopamine (DA) derivative, was detected in several brain regions in rats, ruminants and humans (Musshoff et al., 2000; Tóth et al., 2001; Misztal et al., 2008). Interestingly, there is production of salsolinol in the dopaminergic system in various physiological and/or pathological conditions. Results of previous studies indicate there is an association between salsolinol or its metabolites and disease progression, characterized by dysfunction of catecholaminergic neurons, such as in Parkinson's disease (Antkiewicz–Michaluk, 2002). It has also been reported that there are

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https://doi.org/10.1016/j.anireprosci.2019.06.014
Received 22 January 2019; Received in revised form 8 May 2019; Accepted 20 June 2019
Available online 21 June 2019
0378-4320/

actions of this compound in the neuropathology of chronic alcoholism, because it is a condensation product of the alcohol metabolite, acetaldehyde, and DA (Musshoff, 2002). Results of more recent studies indicate salsolinol is present in the posterior pituitary of cattle (Hashizume et al., 2008) and in the infundibular nucleus/median eminence (IN/ME) of lactating sheep (Misztal et al., 2008). There were modulatory effects of salsolinol on pituitary hormones secretion in both species (Hashizume et al., 2008; Górski et al., 2010, 2016; Hasiec et al., 2014). Knowledge about the detailed mechanism of salsolinol action in various physiological processes, however, is still limited. The structure of the salsolinol-specific receptor has not been elucidated. Results of previous studies on the modulatory effects of salsolinol in the central nervous system (CNS) indicated there were decreases in catecholamine concentrations as a consequence of treatment with this compound (Antkiewicz–Michaluk et al., 2000a; Misztal et al., 2011), which was consistent with the capacity of salsolinol to decrease tyrosine hydroxylase (TH) activity, the rate-limiting enzyme for catecholamine synthesis (Minami et al., 1992; Briggs et al., 2013).

Considering the effect of salsolinol on the dopaminergic system, the question arises whether this DA derivative can affect the gonadotropin-releasing hormone/luteinizing hormone (GnRH/LH) axis in seasonally anestrous sheep. In these animals, the seasonal lack of ovulation results from a complicated inhibitory mechanism in the CNS, in which a steroid hormone, estradiol (E2), has a primary role (Weems et al., 2015). This steroid gains the capacity to suppress the frequency of GnRH and LH pulses due to the increased responsiveness of the neural circuitry within the hypothalamus to E2 negative feedback (Karsch et al., 1980; Goodman et al., 2010). It is assumed that, in sheep, the negative feedback signal is conveyed to GnRH neurons via the afferent circuitry, because GnRH neurons lack a specific estrogen receptor (ER; Lehman et al., 1993). Results of several early studies indicated dopaminergic neurons within the retrochiasmatic area (RCh), known as the A15 group, are implicated in the seasonal regulation of GnRH secretion (Kuljis and Advis, 1989; Jansen et al., 1996; Goodman et al., 2000). It was reported that damage to A15 neurons inhibited the E2 negative feedback during anestrus in ewes (Havern et al., 1994), while direct stimulation of A15 neurons inhibited GnRH/LH pulse frequency regardless of the season (Martin and Thiery, 1987). Furthermore, E2 functioned to increase the activity of TH (Gayrard et al., 1994) and the early intermediate gene product Fos (Lehman et al., 1996) in A15 neurons during anestrus. The arcuate nucleus (ARC) kisspeptin neurons were also found to mediate signaling between DA and GnRH neurons. According to Goodman and Lehman (2012), the negative feedback action of E2 in anestrous sheep is transmitted via A15 dopaminergic neurons that suppress kisspeptin release from ARC neurons, thereby inhibiting GnRH pulse frequency.

Based on these previous findings and the described effects of salsolinol related to changes in catecholamine concentrations (Antkiewicz–Michaluk et al., 2000a; Misztal et al., 2011), the present study was conducted to test the hypothesis that centrally administered salsolinol could affect pulsatile LH secretion in seasonally anestrous sheep. In the present study, there was a focus on the hypothalamic neuroendocrine dopaminergic (NEDA) system activity, as well as *GnRH* and *kisspeptin* gene expression in the hypothalamus and *LHβ* subunit gene expression in the pituitary.

2. Materials and methods

2.1. Animal management

All animal procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals and were approved by the III Local Ethics Committee in Warsaw (Poland). Intact Polish Longwool sheep ($n = 27$), aged 2 to 3 years, maintained indoors in individual pens under natural lighting conditions (52 °N, 21 °E) were used in the experiments. Experiment 1 was performed in March and Experiment 2 in April during the natural anestrous season for this breed of sheep. While the day length increased during this time from 11 to 14 h, sheep were anovulatory without any external symptoms of estrus. The ewes were fed twice a day with a diet based on pelleted concentrate according to the recommendations of the National Research Institute of Animal Production–INRA (Strzetelski, 2009); hay and water were available *ad libitum*. Implantation of cannulas into the CNS was performed a month before the experiments.

2.2. Brain surgery

Sheep were implanted with one stainless steel guide cannula (1.2 mm o.d.) into the IIIv (Exp. 1) or two stainless steel guide cannulas: the first (1.6 mm o.d.) into the infundibular nucleus/median eminence (IN/ME), and the second (as above) into the IIIv (Exp. 2). The implantation procedure was conducted using general anesthesia (xylazine: 40 mg/kg of body weight, intravenously; xylapan and ketamine: 10–20 mg/kg of body weight, intravenously; Bioketan; Vetoquinol Biowet, Poland) through a hole drilled in the skull, in accordance with the procedure described by Traczyk and Przekop (1963). The cannulas were positioned according to the stereotaxic coordinates of the sheep hypothalamus (Welento et al., 1969), IIIv: frontal 30–31.5 mm and sagittal 0.3 to 0.5 mm (Exp. 1) and IN/ME: frontal, 30.0 mm and sagittal, 1.0 mm; IIIv: frontal 31.5 mm and sagittal, 0.3 to 0.5 mm. The guide cannulas were fixed to the skull with stainless steel screws and dental cement (Villacryl S, Zhermapol, Poland). The external opening of the canal was closed with a stainless steel cap. After the surgery, ewes were injected daily with: antibiotics for 5 days (1 g streptomycin and 1,200,000 IU benzylpenicillin; Polfa, Poland), analgesics for 4 days (metamizole sodium 50 mg/animal; Biovetalgin, Biowet Drwalew, Poland or meloxicam 1.5 mg/animal; Metacam, Boehringer Ingelheim, Germany) and with diuretics (3 ml Diurizone, Vetoquinol, France) for 3 days. The placement of the cannulae into the IIIv was confirmed by the outflow of the cerebrospinal fluid during surgery, and the placement into the IN/ME was confirmed after slaughtering with an injection of blue ink. The sheep used in the present study had correctly localized cannulas.

2.3. Drugs

Salsolinol was synthesized and kindly provided by Prof. Ferenc Fülöp from the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Hungary. The compound was dissolved in Ringer-Locke's solution (RL), aliquoted, and stored at −20 °C. A new aliquot of the drug solution was used for each injection/infusion to maintain the biological activity of the molecule during the experiment.

2.4. Experiment 1

The experiment was performed over three consecutive days: the control group ($n = 6$) received intracerebroventricular (ICV) injections of RL and the experimental group ($n = 6$) received analogous injections of salsolinol. During the first 2 days, injections were performed every 2 h, from 08:00 to 20:00, and on the third day from 08:00 to 14:00. A single dose of salsolinol was 5 μg/30 μL (daily 35 μg/animal and 20 μg/animal on the last day), and was selected based on previous studies (Górski et al., 2010). The injections were performed using a microsyringe with an approximate flow rate of 30 μL/min. In addition, on the third day, blood samples were collected every 10 min (from 10:00 to 15:00), through a catheter that was inserted into the jugular vein the day before collection. Immediately after the last blood sampling, sheep were slaughtered and the brains along with pituitaries were rapidly removed from the skull. After separation of the ME, each brain was sectioned sagittally into the cerebral hemispheres. The isolated blocks of the hypothalamus (cutting to a depth of 2 mm) were dissected into three parts: POA, anterior hypothalamus and mediobasal hypothalamus (MBH), (Ciechanowska et al., 2009) according to the stereotaxic atlas of the ovine brain (Welento et al., 1969). Landmarks included the optic chiasm, thalamus and mammillary body. Subsequently, the ARC was isolated from the basal side of the MBH by longitudinal cutting to a depth of 1 to 1.5 mm. In addition, tissue of the anterior pituitary (the ventral part of the part distalis) was collected. All tissue cuts were performed on sterile glass plates placed on ice, and the collected structures were frozen immediately in liquid nitrogen and then stored at −80 °C.

2.5. Experiment 2

The sheep were randomly divided into three groups ($n = 5$ each) and infused into the IIIv with RL (control) or with one of two doses of salsolinol. The treatment was performed in a series of five 30 min infusions, at 30 intervals, from 10:00 to 15:00. The doses of salsolinol (smaller: 5 × 1 μg/60 μL/30 min and larger: 5 × 10 μg/60 μL/30 min) were selected on the basis of a previous study (Górski et al., 2010). All infusions were performed using a BAS Bee microinjection pump (Bioanalytical Systems Inc., West Lafayette, IN, USA) and calibrated 1.0 mL gas-tight syringes. Simultaneously, perfusions of the IN/ME were conducted in each sheep, with RL by the push-pull method (Misztal et al., 2010). The tubes for perfusates contained 50 μL of 0.1 mM ascorbic acid, an antioxidant for catecholamines, and were kept in an ice bath during sampling. The flow rate was 7 μL/min and the volume of one perfusate collected during a 30 min period was about 250 μL. The total time of perfusion was 6 h including a pre–perfusion period from 09:00 to 10:00 to eliminate changes in catecholamine release caused by the insertion of the push-pull cannula and the collection period from 10:00 to 15:00. Immediately after filling, the tubes were frozen in liquid nitrogen and stored at −80 °C until assayed for catecholamines. All perfusions were performed with calibrated 1.0 mL gas-tight syringes and a CMA/100 microinjection pump (Stockholm, Sweden). Blood samples were also collected during this period, at 10 min intervals, through a catheter inserted into the jugular vein one day before the experiment. The blood volume taken each time was about 4 mL per sample (a total of about 120 mL). After centrifugation in heparinized tubes, plasma was stored at −20 °C until LH was assayed.

2.6. Catecholamine and hormone concentration analysis

The concentrations of DA and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), were analyzed in the perfusates using high-performance liquid chromatography with electrochemical detection (Tomaszewska–Zaremba et al., 2002). The limits of detection for all substances were 10 pg/100 μL.

The concentration of LH in plasma samples was assayed by the RIA double-antibody method using antiovine-LH (rabbit) and antirabbit-gammaglobulin antisera, as described in detail by Stupnicki and Madej (1976). The reference standard for LH (NIDDK-oLH-I-4, AFP-8614B) and antiovine-LH antiserum (NIDDK-anti-oLH-1, AFP-192279) were provided by Dr. A. F. Parlow (NIH, NIDDK, Torrance, CA, USA). The LH standard was iodinated using the chloramine T method and then purified on a Sephadex G-100 (Sigma) column (Stupnicki and Madej, 1976). Undiluted plasma (100 μL) was used, the range of the calibrated curve was from 0.3 to 40 ng/mL and the working dilution of antiovine LH antiserum was 1:2,000,000. The concentration of LH in plasma samples was measured using a 2470 Automatic Gamma Counter (PerkinElmer). The sensitivity of the assay was 0.06 ng/mL and the intra- and inter-assay coefficients of variation were 8.7% and 12.4% respectively.

2.7. Relative abundance of mRNA analysis

Total RNA from the hypothalamic and AP tissues was isolated using the NucleoSpin RNA II kit (MACHEREY–NAGEL GmbH and Co., Germany) according to the manufacturer's protocol. The concentration and purity of isolated RNA were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The RNA integrity was electrophoretically verified using 1.7% agarose gel stained with ethidium bromide. Complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA

Table 1
Sequences of specific primers.

Gene	Primers (5' – 3')	GenBank Acc. No.	Amplicon size
GnRH	F: GCCCTGGAGGAAAGAGAAAT R: GAGGAGAATGGGACTGGTGA	U02517	123
Kiss1	F: GGATGAACGTGCTGCTT R: CCTGTGGTTCTAGGATTCTC	GU142847	106
LHβ	F: AGATGCTCCAGGGACTGCT R: GATGCTGGTGGTGAAAGTGA	NM_001009380.1	155
PPIC	R: TGGAAAAGTCGTGCCCAAGA R: TGCTTATACCACCAGTGCCA	XM_004008676.1	158
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001190390.1	131
B2M	F: ATCCAGCGTATTCCAGAGGTC R: CTTCTCCCCGTTCTTCAGCA	NM_001009284.2	135

GnRH: gonadotropin-releasing hormone, Kiss1: kisspeptin, LHβ: luteinizing hormone–beta subunit, PPIC: peptidylprolyl isomerase C (cyclophilin C), GAPDH: glyceraldehyde-3-phosphate dehydrogenase, B2M: beta-2 microglobulin, F: forward primer, R: reverse primer. The Real-Time PCR amplification efficiencies of target and reference genes was 96–101%.

Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) according to the manufacturer's instructions. For this synthesis, 1 μg of total RNA was used in a reaction volume of 20 μL. Quantitative polymerase chain reaction (qPCR) was performed with 5 × HOT FIREPol® EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia). The PCR amplification mix contained 2 μL cDNA template, 1 μL primers (0.5 μl each, concentration of 10 pmol/ml), 3 μL buffer PCR Master Mix and 9 μL dd H2O. Reaction conditions were as follows: initial denaturation at 95 °C for 15 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 20 s (35 cycles). Specific primers for determining the expression of genes of interest (*GnRH* and *Kiss1* in the hypothalamus and *LHβ subunit* in the AP), as well as endogenous control genes (*beta-2 microglobulin* (*B2M*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *peptidylprolyl isomerase C* (*PPIC*)) were designed using Primer3 software (The Whitehead Institute, Boston, MA, USA) and are listed in Table 1. To confirm that single amplification products were amplified, the samples were stained with ethidium bromide and subjected to electrophoresis on an agarose gel before visualization under a UV light camera.

Data were analyzed with the Rotor Gene 6000 v. 1.7 software (Qiagen, Germany) using the comparative quantification option. All three endogenous control genes were assayed in each sample to compensate for cDNA concentration variation and PCR efficiency between individual tubes. The following housekeeping genes were selected as the most desirable endogenous control genes to determine the relative abundance of mRNA for the genes evaluated using the BestKeeper software (http://www.gene–quantification.de/bestkeeper.html): *PPIC* for *GnRH* and *Kiss1* in the mediobasal hypothalamus (MBH) and ARC respectively; *GAPDH* for *GnRH* and *Kiss1* in the POA and *B2M* for *LHβ* in the AP. The results are presented as relative abundance of mRNA of the target gene as compared with that for the housekeeping gene, with relative abundance for the control group set to 1.0.

2.8. Statistical analysis

Initially, all data were tested for normality by the Shapiro-Wilk normality test and then grouped into parametric and non-parametric ones. The LH and DOPAC concentrations over time were analyzed using one-way analysis of variance (ANOVA) (STATISTICA, Stat Soft, Tulsa, OK, USA) or, where appropriate (hourly estimation), by the two-way ANOVA with repeated measures, with time and treatment as factors of repeated measurements. Each analysis was followed by the use of the *post-hoc* Least Significant Difference test. Due to the small and different number of measurements in groups, the significance of differences in DA concentrations was estimated by the non-parametric Kruskal-Wallis test, followed by multiple comparisons of average ranks.

The LH pulses were identified using criteria described previously by Goodman et al. (2012): 1) the peak occurred within two samples of the previous nadir; 2) pulse amplitude exceeded assay sensitivity; and 3) the peak was two SD above the preceding and following nadirs. The analysis was performed separately for every ewe and encompassed the entire sampling period. The frequency of LH pulses was defined as the number of identified pulses per total collecting time. Pulse amplitude was defined as the difference between peak value and preceding nadir. Due to the non–parametric characteristics of the variables, the effects of treatment on both the frequency and amplitude of LH pulses were estimated using the ANOVA rank Kruskal-Wallis test. Differences in relative abundance of mRNA between experimental and control groups were determined for particular genes by non-parametric Mann-Whitney U test. Differences were considered significant at $P < 0.05$ and showing a tendency towards significance at $P < 0.10$. All data are presented as a mean ± standard error of the mean (SEM).

3. Results

3.1. Experiment 1

3.1.1. LH secretion

The mean plasma LH concentration in sheep treated ICV with salsolinol was greater than that in sheep receiving control

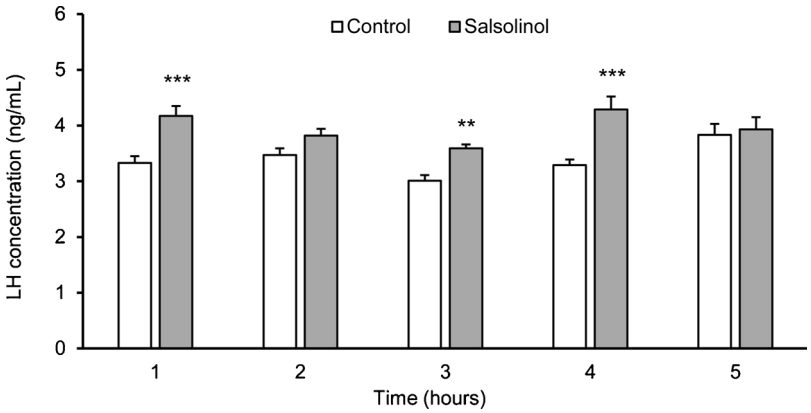

Fig. 1. Plasma LH concentrations (mean ± SEM) according to 1-h-long consecutive periods in the control ($n = 6$, white bars) compared with salsolinol-treated ($n = 6$, grey bars) ewes on the third day of the experiment; differences between groups in particular hours are indicated with $^{**}P < 0.01$ and $^{***}P < 0.001$.

injections: 3.96 ± 0.08 compared with 3.38 ± 0.06 ng/mL ($P < 0.001$), respectively. The distribution of mean LH concentrations during experimental hours indicated that marked increases in hormone secretion occurred in the 1st ($P < 0.001$), 3rd ($P < 0.01$) and 4th ($P < 0.001$) hour of the experiment (Fig. 1). Likewise, the frequency of LH pulses was greater in the salsolinol-treated group than in controls: 2.33 ± 0.21 compared with 1.50 ± 0.22 pulses/5 h ($P < 0.05$), respectively. The mean LH pulse amplitudes did not differ between the groups: 2.51 ± 0.28 compared with 2.22 ± 0.20 ng/mL (salsolinol-treated compared with control, respectively). Representative time-concentration profiles of LH in three sheep from each group are depicted in Fig. 2.

3.1.2. Relative abundance of mRNA in the hypothalamus and AP

Administration of salsolinol for 3 days into the IIIv resulted in an increase ($P < 0.05$) in the relative abundance of GnRH mRNA in the MBH, as well as kisspeptin mRNA in the ARC when compared to the tissues from ewes in which there was the control administration. In contrast, there was a tendency ($P < 0.06$) towards a decrease in the relative abundance of GnRH mRNA transcript in response to salsolinol treatment in the POA, while the relative abundance of kisspeptin transcript in this structure did not differ between the groups. There was a tendency ($P < 0.06$) for a greater relative abundance of LHβ subunit mRNA in the pituitary of salsolinol-treated sheep compared to controls. The relative abundances of mRNA transcript for GnRH in the MBH and POA, as well as for kisspeptin in the ARC and LHβ in the AP are depicted in Fig. 3.

3.2. Experiment 2

3.2.1. LH secretion

The mean plasma LH concentration in the first samples collected before the start of the infusion did not differ between the groups (data not shown). Mean plasma LH concentration and mean frequency of LH pulses increased in both groups of sheep receiving ICV infusions of salsolinol compared to values noted in the control group: 3.75 ± 0.09 and 3.78 ± 0.06 ng/mL compared with 3.01 ± 0.04 ng/mL ($P < 0.001$), and 2.80 ± 0.37 and 2.40 ± 0.40 pulses/5 h compared with 1.40 ± 0.24 pulses/5 h ($P < 0.05$), (in ewes treated with smaller and larger doses compared with controls, respectively). The distribution of mean hourly LH concentrations indicated that there were increased hormone concentration at all hours of the experiment ($P < 0.05 \downarrow P < 0.001$, Fig. 4). There were no differences in mean LH pulse amplitudes between the groups: 1.70 ± 0.21 and 1.50 ± 0.15 ng/mL compared with 1.18 ± 0.26 ng/mL (in ewes treated with the smaller and larger doses compared with controls, respectively). Representative time-concentration profiles of LH in two sheep from each group are depicted in Fig. 5.

3.2.2. DA and DOPAC concentrations

Mean perfusate DA concentrations in control sheep ranged from 72.47 ± 10.58 to 115.52 ± 22.60 pg/100 µL and did not differ statistically throughout the experiment. The DA was detected only in the 1 st perfusate in sheep infused with a smaller dose of salsolinol: 73.57 ± 13.00 pg/100 µL. The mean concentration of DA in the 1 st perfusate was 59.30 ± 8.36 pg/100 µL in sheep infused with a larger dose, and in three animals, DA was also detected in the 2nd perfusate: 37.53 ± 3.47 pg/100 µL. There were no statistical differences in mean DA concentrations in the 1 st perfusate between the groups. The concentration of DA in other perfusates was less than the detection concentration.

Mean perfusate DOPAC concentrations in the control and salsolinol-treated groups ranged as follows: from 68.30 ± 17.00 to 123.60 ± 40.00 pg/100 µL in control sheep; from 32.72 ± 4.55 to 50.30 ± 13.80 pg/100 µL in sheep infused with the smaller dose; and from 32.22 ± 5.13 to 50.12 ± 9.50 pg/100 µL in sheep infused with the larger dose. There were lesser mean DOPAC concentrations from the 2nd to the 4th hour of the salsolinol treatment period ($P < 0.05$) in comparison with controls. The distribution of mean DA and DOPAC concentrations in individual perfusates is depicted in Fig. 6.

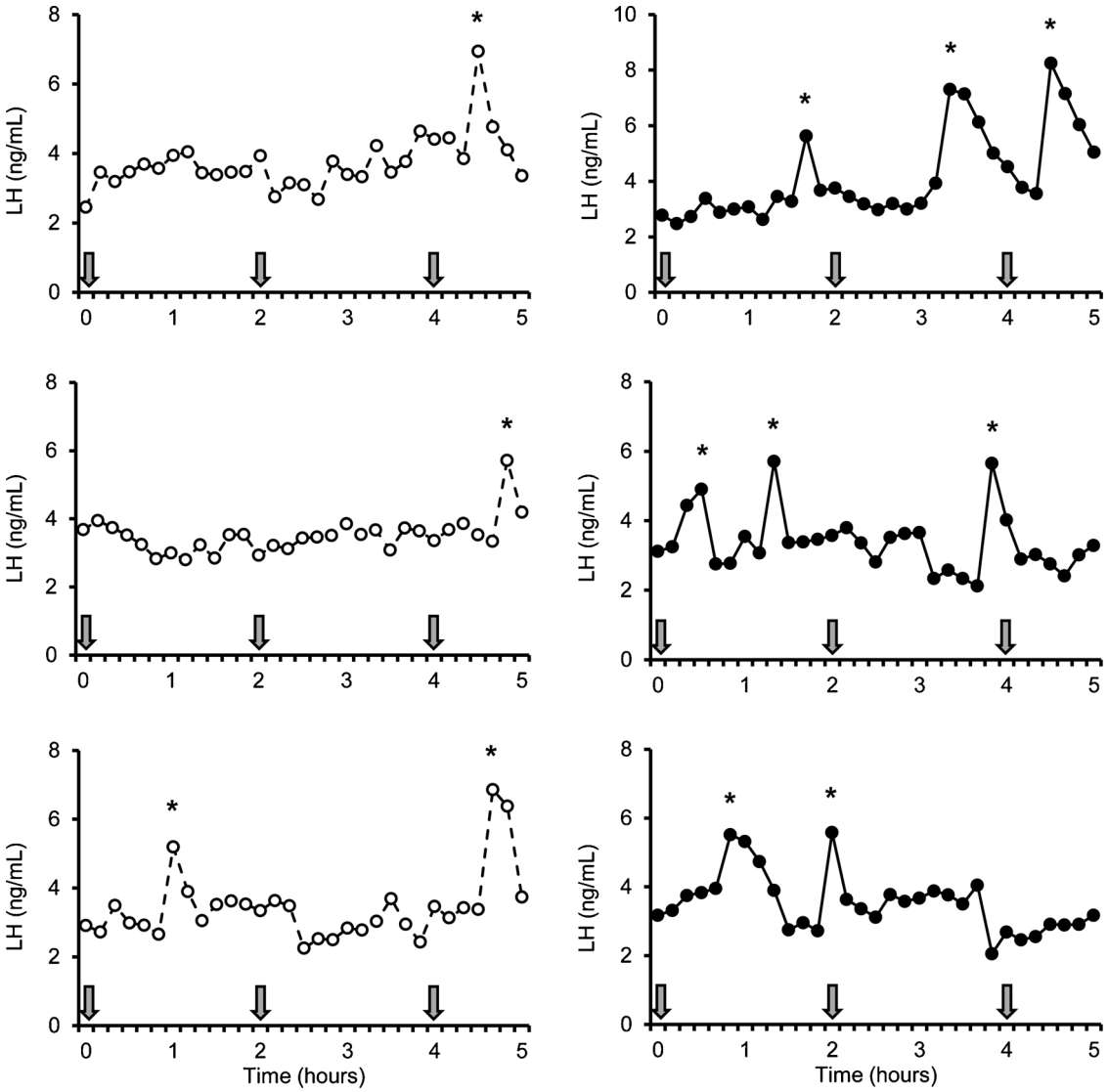

Fig. 2. Representative time-concentration profiles of LH in three sheep from each studied group following control (left panel, -○-) and salsolinol injections (right panel, -●-) during the third day of the experiment; injection times are indicated by gray arrows; LH pulses are marked with an asterisk.

4. Discussion

Pulsatile release of LH depends on the pulsatile stimulation of pituitary gonadotrophs by the hypothalamic GnRH (Clarke and Cummins, 1982). Even though there is a great dispersion of GnRH neurons in the ovine brain, the majority of GnRH cell bodies are concentrated in the discrete subsets that are located mainly in the MBH and POA (Advis et al., 1985; Caldani et al., 1988). In sheep, a population of MBH neurons might be responsible for the pulsatile secretion of GnRH, because the early intermediate gene product, Fos, was induced in these neurons by both pharmacological and physiological stimuli that also increased pulsatile LH release (Boukhliq et al., 1999). In the present study, an increase in LH pulse frequency occurred in response to the 3-day salsolinol administration, which was accompanied by an increase in the content of the GnRH transcript in the MBH, indicating there was an increased synthesis of the neuropeptide.

Both excitability and inhibition of the hypothalamic GnRH neurons are complex processes requiring numerous neurotransmitters, neuropeptides and other excitatory and inhibitory molecules (Goodman et al., 2010; Lehman et al., 2010). In addition, many of these compounds mediate the feedback effects of sex steroids in different phases of the reproductive cycle. The hypothalamic NEDA system may be important in E2 positive feedback, as well as negative feedback on GnRH neurons in sheep (Havern et al., 1994; Anderson et al., 2001). The inhibitory DA effects dominate during the anestrous season in terms of seasonal sheep breeding. Hence, a set of A15 dopaminergic neurons have a primary role, where DA functions directly on GnRH neurons through inhibitory D2 receptors at both

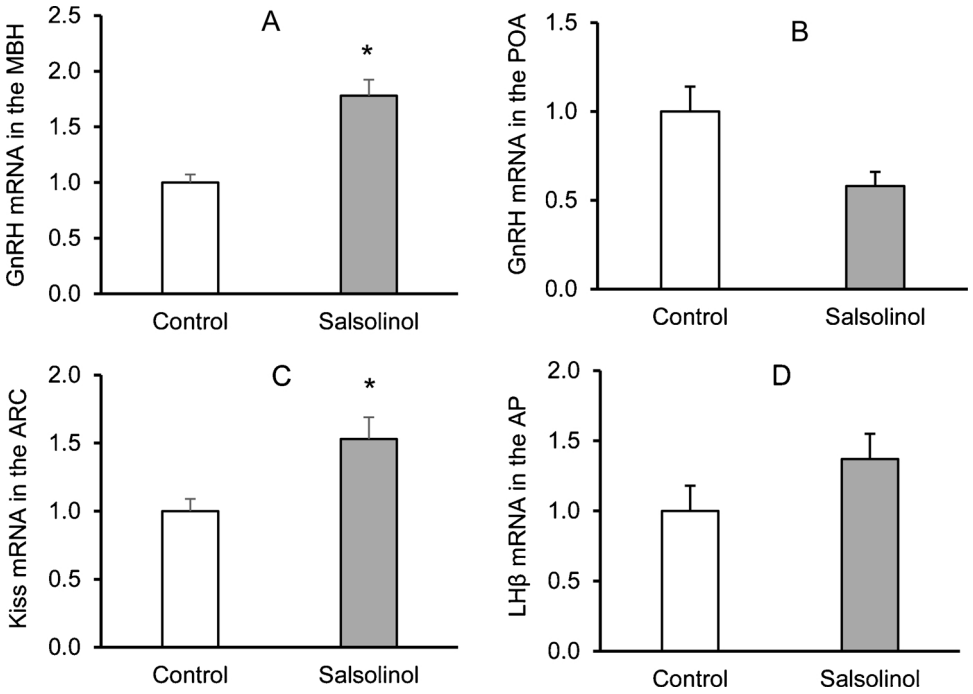

Fig. 3. Relative abundance of GnRH mRNA in the mediobasal hypothalamus (MBH, A) and preoptic area (POA, B), kisspeptin (Kiss) mRNA relative abundance in the arcuate nucleus (ARC, C) and LHβ subunit mRNA relative abundance in the anterior pituitary (AP, D) of control ($n = 6$) and salsolinol-treated ($n = 6$) sheep; data are presented as means ± SEM; differences between groups are indicated with $^{*}P < 0.05$.

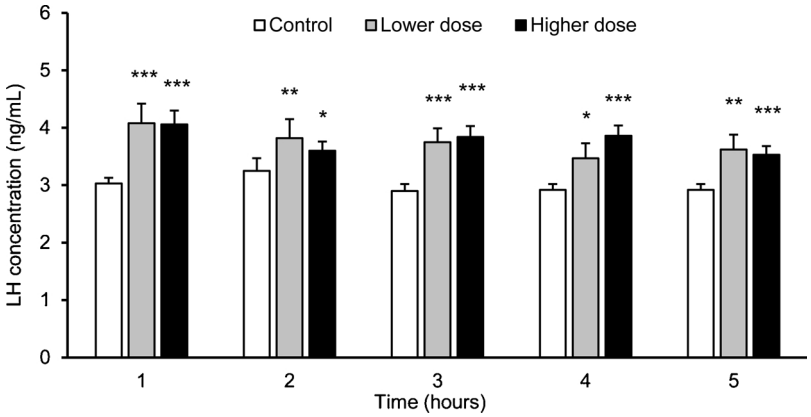

Fig. 4. Plasma LH concentrations (mean ± SEM) according to 1-h-long consecutive periods in the control ($n = 5$, white bars) compared with salsolinol-treated ewes: smaller ($n = 5$, grey bars) and larger ($n = 5$, black bars) doses; differences between groups as specific hour time points are indicated with $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

GnRH perikarya in the MBH and axons in the median eminence (Goodman et al., 2010; Goodman and Lehman, 2012). It is unlikely, however, that GnRH perikarya in the POA are directly inhibited by activation of A15 dopaminergic neurons, because the latter do not project to these areas (Goodman et al., 2010). In contrast, DA appears to be able to function in both the MBH and POA to modulate LH secretion in rodents (Rotsztejn et al., 1977; Jarjour et al., 1986). The GnRH neurons in mice contain both D1 and D2 DA receptors and, approximately half of all GnRH neurons, were inhibited by DA, with one third being tonically inhibited (Liu and Herbison, 2013). The direct action of salsolinol, as a DA derivative, on GnRH neurons in the MBH, though possible, seems rather doubtful. Salsolinol has been reported to not function through DA receptors, because it could not displace 3H-SCH23390 (D1 receptor-specific ligand) from striatal synaptosome or 3H-spiperon (D2 receptor-specific ligand) from both striatal synaptosomal (Antkiewicz–Michaluk et al., 2000a, 2000b) and pituitary homogenates (Tóth et al., 2001). Accordingly, none of the tested agonists and antagonists of different DA receptors (D1–D5) displaced 3H-salsolinol except for DA itself (Homicsko et al., 2003). Salsolinol binding, therefore, is closely related to a site where DA functions as a signaling molecule, but its properties differ from any known DA receptors. Knowledge about the structure of salsolinol-specific receptor, however, is very limited, although saturable binding sites for

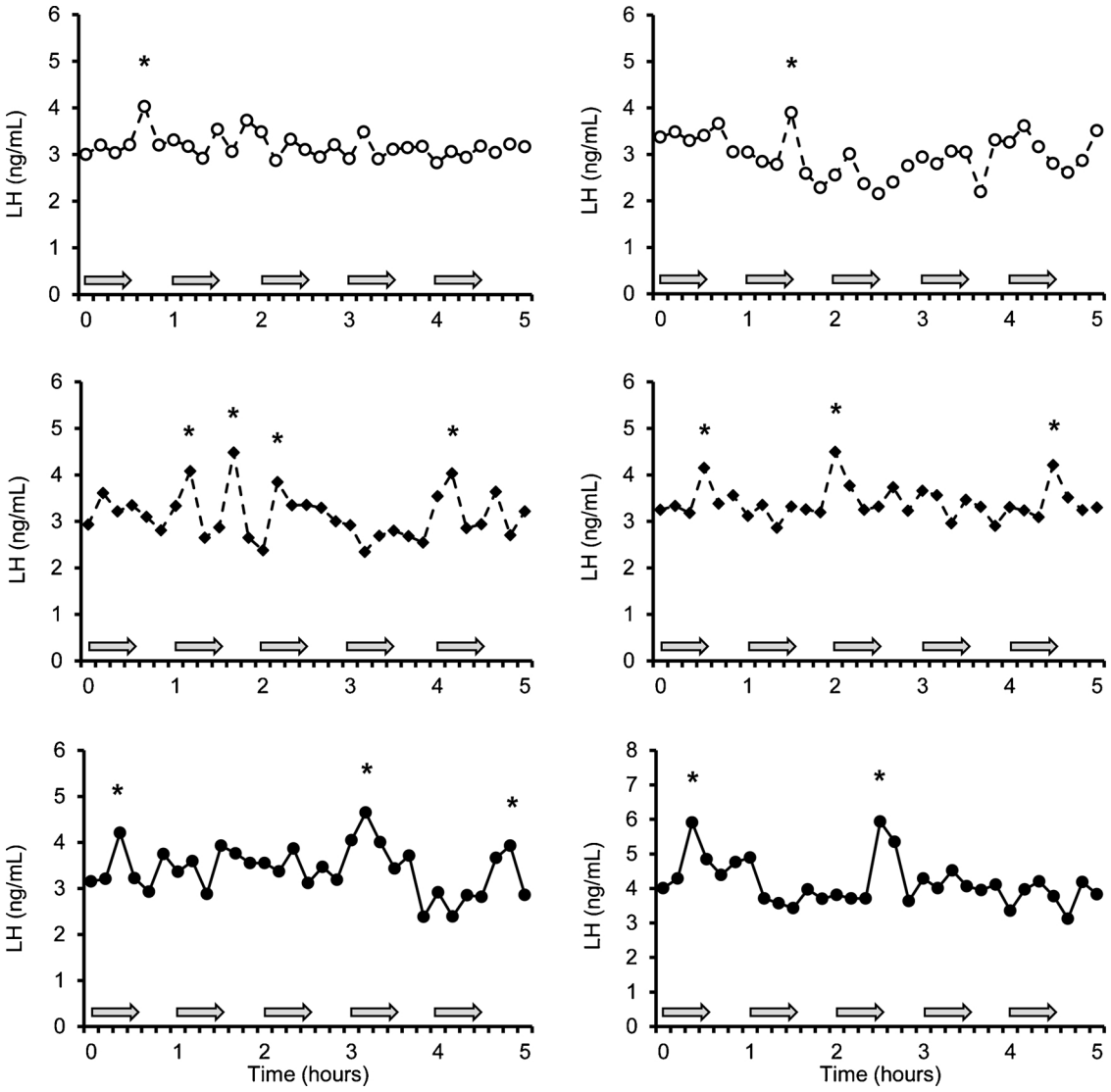

Fig. 5. Representative time-concentration profiles of LH in two sheep from each group following control (top, -○-) and salsolinol infusions: smaller (middle, -◆-) and larger (bottom, -●-) doses; the infusion periods are indicated by gray arrows; LH pulses are marked with an asterisk.

this compound have been detected in the striatum, cortex, hypothalamus, median eminence and in the neuro-intermediate lobe of the pituitary (Homicsko et al., 2003). Thus, the changes in LH secretion observed in the present study may result from the interaction of salsolinol with hypothalamic centers that control the release of GnRH, rather than from direct action on GnRH neurons.

Considering the previously described effects of salsolinol in the CNS related to changes in catecholamine concentrations (Antkiewicz–Michaluk et al., 2000a; Misztal et al., 2011), the hypothalamic NEDA system seems to be an appropriate target. Observed changes in perfusate DA and DOPAC concentrations confirm this assumption. In the present study, DA concentrations in perfusates reflect DA release into the extracellular spaces of the IN/ME. In Experiment 2, salsolinol infused into the IIIv resulted in a decrease in perfusate DA concentration, even to values that were less than the detection level. Furthermore, a decrease in perfusate DA concentration was accompanied by a decrease in DOPAC concentration, which also indicated a reduction in hypothalamic DA catabolism. In the present study, however, there was not direct identification of hypothalamic dopaminergic centers, but there are indications of a general change in NEDA activity that can occur at least in the MBH and adjacent sites. It is almost certain that the DA in perfusates collected from the IN/ME also originates from centers of regulation of prolactin secretion, i.e. dopaminergic A12 neurons, where activity is greater during the part of the year when there is lengthening photoperiod due to the increasing secretion of this hormone (Beccavin et al., 1998).

The interaction of salsolinol with the NEDA system can take place at several different anatomical locations. Results of previous studies indicate salsolinol may be neurotoxic in dopaminergic cells by inhibiting the mitochondrial enzymatic complex responsible for cellular energy supply (Storch et al., 2000). This action can induce a typical apoptotic dopaminergic cell death, which appears to

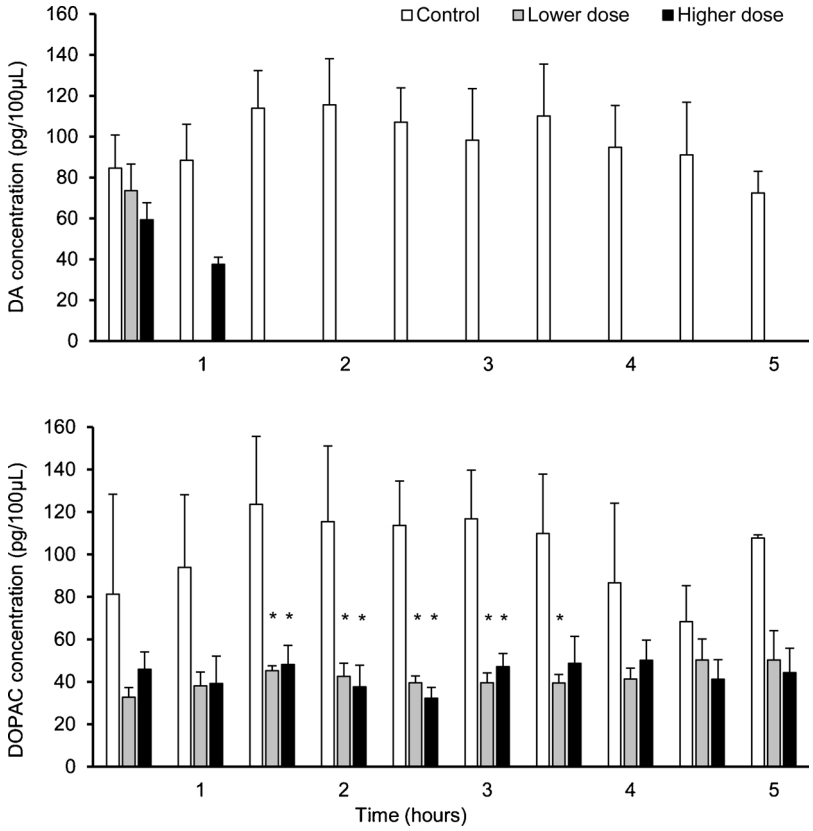

Fig. 6. DA (top) and DOPAC (bottom) concentrations (mean ± SEM) in the consecutive perfusates (every 30 min) in the control (n = 5, white bars) compared with salsolinol-treated ewes: smaller (n = 5, grey bars) and larger (n = 5, black bars) doses; differences between groups are indicated with *P < 0.05.

be mediated, at least in part, through a reactive oxygen species-activated cascade (Chun et al., 2001). Salsolinol may also regulate the neurotransmission of NEDA neurons by altered intracellular or intraterminal synthesis and/or DA distribution (Homicsko et al., 2003; Radnai et al., 2005). More recently, Briggs et al. (2013) reported that this compound inhibited the rate-limiting enzyme in DA synthesis – TH, especially its phosphorylated form – by competing with the co-factor, tetrahydrobiopterin. The potent inhibition of TH phosphorylation by salsolinol was thought to prevent the complete activation of TH and, thus, DA synthesis. The results of the present study indicate there is a marked suppression of the hypothalamic NEDA system by salsolinol, the consequence of which could be an observed increase in LH pulse frequency and plasma LH concentration in sheep in both of these experiments. The presence of DOPAC in perfusates (Experiment 2), however, although in lesser concentrations, may indicate pending DA production; hence, the LH response may be partly limited.

In addition, results of the present study are complemented by data that indicate an increase in the content of kisspeptin transcripts in the ARC. In many mammalian species, kisspeptin is one of the major stimulators of GnRH neuronal activity (Yeo, 2013). Results from electrophysiological and imaging studies indicate kisspeptin may function at the GnRH neuron cell body and dendrites, as well as GnRH neuron nerve terminals in the median eminence (Lehman et al., 2013; Yeo, 2013). In sheep, two major diencephalic sites rich in kisspeptin neurons, fibers and terminals have been identified, one located in the ARC and the other in the POA (Lehman et al., 2013). These two populations are under regulatory control by gonadal steroid hormones due to the co-localization of estrogen and progesterone receptors (Wintermantel et al., 2006; Lehman et al., 2013). An increase in relative abundance of kisspeptin mRNA in the ARC was observed in sheep before the preovulatory LH surge (Estrada et al., 2006) and after ovariectomy (Smith et al., 2007); hence, these neurons may have pivotal functions in mediating both positive and negative E2 feedback. Furthermore, inhibitory dopamine D2 receptors (D2–R) were present not only on GnRH neurons but also on approximately 80% of kisspeptin ARC neurons in anestrous sheep (Goodman et al., 2012). In this previous study, microinjections of a D2–R antagonist, sulpiride, into the ARC resulted in a dose-dependent increase in LH pulse frequency, and this effect was inhibited after ICV infusion of a kisspeptin antagonist prior to sulpiride treatment.

The stimulation of LH secretion by salsolinol, including inhibition of the hypothalamic NEDA system and increased kisspeptin synthesis in the ARC and GnRH in the MBH is consistent with the hypothesis proposed by Goodman et al. (2012) concerning the hierarchical control of GnRH/LH secretion during seasonal anestrus. It was proposed, as a result of findings in this previous study, that the negative feedback action of E2 in anestrous sheep is conveyed via A15 dopaminergic neurons that suppress kisspeptin release from ARC neurons, thereby inhibiting GnRH pulse frequency. Thus, salsolinol actions in the CNS could decrease DA release from

hypothalamic NEDA neurons, thereby inducing the synthesis of ARC kisspeptin. It should be noted that, in sheep, kisspeptin neurons in the ARC population also contain two other neuropeptides: neurokinin B and an opioid peptide, dynorphin (Goodman et al., 2007). Results of earlier studies indicate salsolinol may bind to opioid receptors, displacing other opioids (Panchenko et al., 1982; Lucchi et al., 1985). Although salsolinol in particular interacts with the μ-opioid receptor (Berríos-Cárcamo et al., 2017), it cannot be ruled out that it might disturb dynorphin (κ-opioid receptor ligand) signaling and upregulate kisspeptin and/or GnRH (Weems et al., 2018). Consideration of such a mode of salsolinol action seems more speculative at this stage. Based on the data from the two experiments in the present study, it is possible that salsolinol injected or infused into the IIIv disrupted the negative E2 signaling pathway, targeting primarily dopaminergic neurons.

In conclusion, exogenous salsolinol functioning centrally stimulates pulsatile LH secretion in sheep during seasonal anestrus. It is suggested that salsolinol exerts its effect by reducing the activity of the hypothalamic NEDA system, which results in an increase in both kisspeptin and GnRH neurons activity. Although this DA derivative is not a significant hypothalamic modulator in seasonally anestrous sheep (Misztal et al., 2008), it seems that it can be useful in studies on the negative E2 feedback.

Funding

The research was supported by the National Science Centre, Poland (grant No. 2012/05/N/NZ9/01581 – Experiment 1) and statutory subsidy provided by the Ministry of Science and Higher Education, Poland – Experiment 2.

Author contributions

TM, MH: developed the original concept and experimental design, performed the experiments, analyzed the data, edited and wrote the manuscript; DT–Z, MS, EM: performed the experiments and laboratory analyses, participated in editing and writing the manuscript. All authors approved the final version.

Data statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

The authors thank Prof. F. Fülöp (Szeged University, Hungary) for preparing and providing salsolinol, veterinary surgeons J. Rutkowski and K. Roszkowicz-Ostrowska for performing brain surgery, Dr. K. Romanowicz for help in RIA and also Mr. W. Mrozek and Mr. R. Druchniak for animal care.

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