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The influence of salsolinol on dopaminergic system activity within the mediobasal hypothalamus of anestrous sheep: A model for studies on the salsolinol–dopamine relationship

Tomasz Misztal[1,] *, Małgorzata Hasiec[1], Dorota Tomaszewska-Zaremba[2], Elżbieta Dobek[1], Ferenc Fülöp[3], and Katarzyna Romanowicz[1]

[1]Department of Endocrinology, The Kielanowski Institute of Animal Physiology and Nutrition PAS, Jablonna, Poland, *Email: t.misztal@ifzz.pan.pl; [2]Department of Neuroendocrinology, The Kielanowski Institute of Animal Physiology and Nutrition PAS, Jablonna, Poland; [3]Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Hungary

Salsolinol with its derivatives has been considered as a potential neurotoxin for the dopaminergic system in the human and rat brain. Investigating a sheep model for studies on the action of salsolinol within the central nervous system we examined whether this compound is able to affect the hypothalamic neuroendocrine dopaminergic (NEDA) system during its high seasonal activity, when sheep entered to anestrus under the long day conditions. Therefore, salsolinol was infused into the third ventricle of the brain in combination with the *in vivo* push-pull perfusion of the mediobasal hypothalamus/median eminence (MBH/ME). The effects of this drug on either perfusate noradrenaline (NA) or plasma prolactin concentration were also studied. The infusion of salsolinol resulted in rapid and permanent diminution in dopamine (DA) release into the extracellular spaces of the MBH/ME up to an undetectable level and in the 57% decrease in DA metabolite 3,4-dihydroxyphenylacetic acid concentration, compared to the control. This effect of salsolinol was accompanied by the significant enhancement of the pituitary prolactin release into circulation. The concentration of other DA metabolite, homovanillic acid, as well as NA in the MBH/ME was not affected. Thus, our results in the anestrous sheep underline the role played by salsolinol as a neuromodulator for the hypothalamic NEDA system and as a signal transmitter for the pituitary prolactin release. We suggest that the hypothalamic NEDA system of anestrous sheep during its high secretory activity may be set as a model for studies on the salsolinol-dopamine relationship.

Key words**:** anestrous sheep, dopamine, dopaminergic system, hypothalamus, prolactin release, salsolinol

INTRODUCTION

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) is an endogenous product of dopamine (DA) and acetaldehyde or pyruvate condensation (Naoi et al. 1996, 2002). This compound and its N-methylated and oxidized derivatives were at first well known for their involvement in the progression of a disease characterized by dysfunctional dopaminergic neurons (Maruyama et al. 1993, Moser et al. 1995, Antkiewicz-Michaluk 2002). N-methyl-salsolinol induced parkinsonism in rats after injection in the striatum, and the behavioral, biochemical and pathological changes were very similar to those in Parkinson's disease (Naoi et al. 1997). Antkiewicz-Michaluk and coworkers (2000) reported that chronic administration of salsolinol caused a dramatic decline in the DA level in both the striatum and substantia nigra. The more current and detailed studies showed that the racemic N-methylated derivative of salsolinol markedly accelerated DA catabolism, while the number of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra was not affected, suggesting that salsolinol may play an important role in the regulation of dopaminergic activity (Lorenc-Koci et al. 2008).

In the last decade, more attention was focused on the possible physiological role of salsolinol as a neuromodulator within the central nervous system (Vetulani

Correspondence should be addressed to T. Misztal Email: t.misztal@ifzz.pan.pl

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et al. 2003, Mravec 2006). This DA-originated compound was found to be present in the pituitary gland as well as median eminence (ME) extracts and hypothalamic perfusates of rats and ruminants (Toth et al. 2001, Hashizume et al. 2008a, Misztal et al. 2008). It was shown to be a potent stimulator of prolactin release (Toth et al. 2002, Hashizume et al. 2008b, 2010), especially during lactation (Misztal et al. 2008, 2010a, Górski et al. 2010a). Salsolinol binds specifically with the pituitary cells of lactating rats (Toth et al. 2002, Homicsko et al. 2003) and a cAMP-coupled mechanism is probably involved in the prolactin-releasing action of salsolinol at the level of lactotrophs (Randai et al. 2005).

The increased activity of the hypothalamic neuroendocrine dopaminergic (NEDA) system reflected by enhanced DA synthesis and release occurs in sheep during a period of long days (Thiery 1991), corresponding in this species to seasonal anestrus. Interestingly, the production of salsolinol, instead of DA, dominates during the early stage of lactation, which normally happen in winter and/or spring months (Misztal et al. 2008, 2010a). Our latest data (Misztal et al. 2010b) showed that the endogenous opioid peptides may be responsible for the formation and/or release of salsolinol during lactation. Contrary to that we did not find salsolinol in the perfusates collected from the MBH/ME of non-milking sheep ten weeks from the lambs weaning (May/June), when entered to seasonal anestrus (Misztal et al. 2008). Nevertheless, seasonal anestrus along with brain volume and knowledge of important neuronal sites makes from sheep an interesting model for studies of salsolinol/DA relationship. Thus, in the current study we examined whether salsolinol affects centrally the hypothalamic NEDA system, during its high seasonal activity in anestrous sheep. Therefore, salsolinol was infused into the third ventricle (IIIv) of the brain, in combination with *in vivo* push-pull perfusion of the MBH/ME (Misztal et al. 2010a,b). In addition, the effects of salsolinol on either perfusate noradrenaline (NA) or plasma prolactin concentration were studied.

METHODS

Animals and managements

All experimental procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals (1997) and approved by the Local Ethics Committee.

Ten mature Longwool sheep (3–4 years old) were used in the experiment. They were maintained indoors in individual pens under natural lighting conditions (52° N, 21° E) and fed a constant diet of commercial concentrates, with hay and water available *ad libitum*. Every sheep had implanted two stainless steel guide canullae: the first (1.6 mm o.d.) into the MBH/ME, positions: frontal, 30.0 mm and sagittal, 1.2 mm, and the second (1.2 mm o.d.) into the IIIv, positions: frontal 32.0 mm and sagittal, 0.3–0.5 mm. The implantation was performed under general anaesthesia (pentobarbital sodium 8–12 mg/kg of body mass, i.v.; Vetbutal, Biowet, Pulawy, Poland; and ketamine 6–10 mg/kg of body mass, i.v.; Bioketan, Biowet, Pulawy, Poland), through a drill hole in the skull, in accordance with the stereotaxic coordinate system for sheep hypothalamus (Welento et al. 1969) and the procedure described by Traczyk and Przekop (1963). The guide canullae were fixed to the skull with stainless steel screws and dental cement (Villacryl S, Zhermapol, Poland). The external opening to the canal was closed with a stainless steel cap. After surgery, ewes were injected daily with antibiotics (1 g streptomycin and 1 200 000 IU benzylpenicillin, Polfa, Poland) for five days and with diuretics (3 ml Diurizone, Vetoquinol, France) for three days. The placement of the canullae was confirmed after slaughtering by the infusion of a small volume of blue ink.

Experimental design

The experiment was performed during a period of long days (May), corresponding in this breed to seasonal anestrus. Salsolinol was synthesized and kindly provided by Prof. Ferenc Fülöp from the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Hungary. It was dissolved in Ringer-Locke (RL) solution, divided into portions and stored at −20°C. The sheep were randomly divided into two groups and infused into the IIIv with a RL solution (control, n=5) or with salsolinol (total 5 µg/animal, n=5). Treatment was performed in a series of five 30-min infusions, at 30-min intervals. The dose of salsolinol (5 × 1 µg/60 µl/30 min) was chosen on the base of our previous study (Gorski et al. 2010a). All infusions were done from 10:00 AM to 03:00 PM, using a BAS Bee™ microinjection pump (Bioanalytical

Systems Inc., West Lafayette, IN, USA) and calibrated 1.0-ml gas-tight syringes. A new portion of salsolinol was used each time to maintain the stability of the molecule during the experiment.

Simultaneously, in every sheep, perfusions of the MBH/ME were made with the RL by the push-pull method (Misztal et al. 2010a,b). 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 30-min period was about 250 µl. The total time of perfusion was 6 h including a pre-perfusion period from 09:00 AM to 10:00 AM to eliminate the changes in catecholamines release caused by the insertion of the push-pull cannula and the collection period from 10:00 AM to 03:00 PM. Immediately after filling, the tubes were frozen in liquid nitrogen and stored at −80°C until assayed for catecholamines. All perfusions were done 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 a day before the experiment. The blood volume taken each time was about 4 ml per sample (total about 120 ml). After centrifugation in heparinized tubes, plasma was stored at −20°C until prolactin was assayed.

Analytical techniques

The concentrations of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as NA, in the perfusates were analyzed using high-performance liquid chromatography with electrochemical detection (Tomaszewska-Zaremba et al. 2002). The limits of detection for all substances were 5 pg/50 µl.

The prolactin concentration in the plasma was assayed by the radioimmunoassay double-antibody method, using antiovine-prolactin and antirabbit-gammaglobulin antisera as described by Wolinska and coauthors (1977). The prolactin standard was synthesized and kindly provided by Prof. Kazimierz Kochman from our Institute (Kochman and Kochman 1977). The assay sensitivity for prolactin was 2 ng/ml and the intra- and interassay coefficients of variation were 9% and 12%, respectively.

Statistical analysis

The effects of the treatments on the catecholaminergic system component concentration, i.e. DA, DOPAC, HVA and NA, as well as plasma prolactin concentration were examined by one-way analysis of variance (STATISTICA, StatSoft, Inc., Tulsa, OK, USA). The Tukey *post-hoc* test was used to compare the differences between concentrations of catecholamines in the consecutive perfusates and between prolactin concentrations in the 30-min periods of the experiment, corresponding to the consecutive perfusates. All data are expressed as means ± SE.

RESULTS

Perfusate DA, DOPAC, HVA and NA concentrations

The mean concentration of DA in perfusates collected from the MBH/ME of control sheep during the experimental period was 101.32 ± 8.46 pg/50 µl. Due to high fluctuations in the release of DA in the individual animals, there were no significant differences in the mean concentrations of this compound between the consecutive perfusates (Fig. 1). In the salsolinol-infused group, DA was detectable only in the first perfusate and the concentration ranged from 48.23 to 90.91 pg/50 µl, showing a tendency to decrease. In the next perfusates, DA concentration was maintained below the limit of detection. No salsolinol concentration was detected in the perfusates collected from the control sheep.

The mean concentration of DOPAC in perfusates collected from the MBH/ME of control group was 97.42 ± 5.37 pg/50 µl and was significantly ($P<0.001$) higher than in salsolinol-treated group 41.53 ± 5.26 pg/50 µl. In the consecutive perfusates, the significant ($P<0.05$) differences in DOPAC concentrations between groups were noted during the mid-phase of the experiment (Fig. 2). In case of HVA, there were no significant differences in the mean perfusate concentration between groups, 471.84 ± 64.36 vs. 506.10 ± 58.10 pg/50 µl, for control and salsolinol-infused group, respectively. The concentrations of HVA in the consecutive perfusates did not differ significantly within and between groups (Fig. 3).

Mean concentrations of NA in perfusates collected from the MBH/ME were similar in the control and

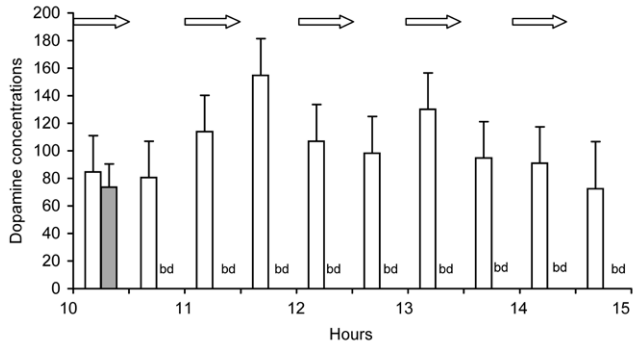

Fig. 1. Mean dopamine concentrations (pg/50 µl) in consecutive perfusates (30-min) collected from the mediobasal hypothalamus/median eminence of anestrous sheep during control (white bars) and salsolinol (grey bars) infusions. The series of infusions are indicated by white arrows. bd., below detection

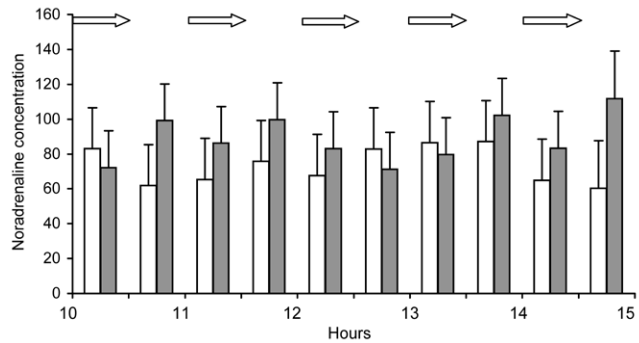

Fig. 4. Mean noradrenaline concentrations (pg/50 µl) in consecutive perfusates (30-min) collected from the mediobasal hypothalamus/median eminence of anestrous sheep during control (white bars) and salsolinol (grey bars) infusions. The series of infusions are indicated by white arrows.

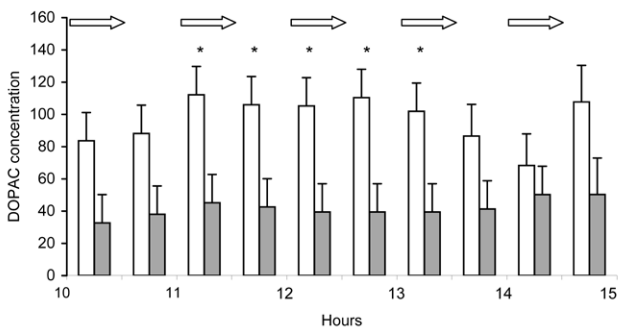

Fig. 2. Mean 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations (pg/50 µl) in consecutive perfusates (30-min) collected from the mediobasal hypothalamus/median eminence of anestrous sheep during control (white bars) and salsolinol (grey bars) infusions. The series of infusions are indicated by white arrows. * $P<0.05$.

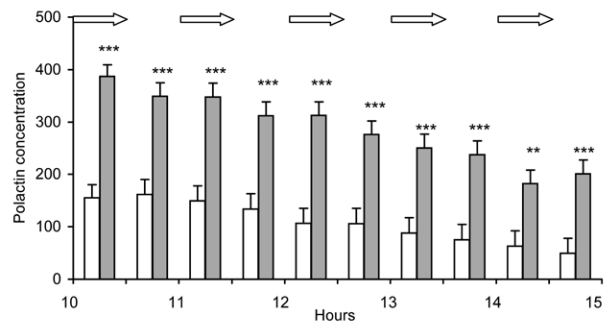

Fig. 5. Mean plasma prolactin concentrations (ng/ml) in anestrous sheep during the consecutive 30-min periods in time of control (white bars) and salsolinol (grey bars) infusions. The series of infusions are indicated by white arrows. ** $P<0.01$; *** $P<0.001$.

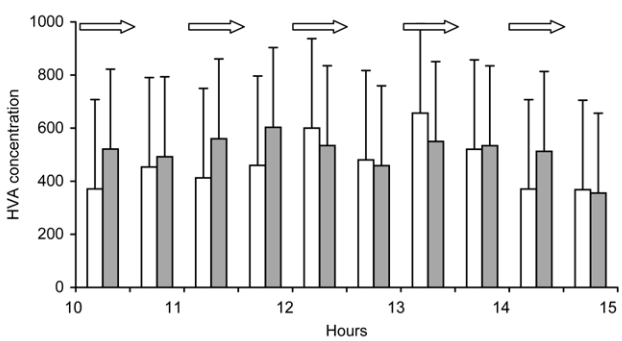

Fig. 3. Mean homovanillic acid (HVA) concentrations (pg/50 µl) in consecutive perfusates (30-min) collected from the mediobasal hypothalamus/median eminence of anestrous sheep during control (white bars) and salsolinol (grey bars) infusions. The series of infusions are indicated by white arrows.

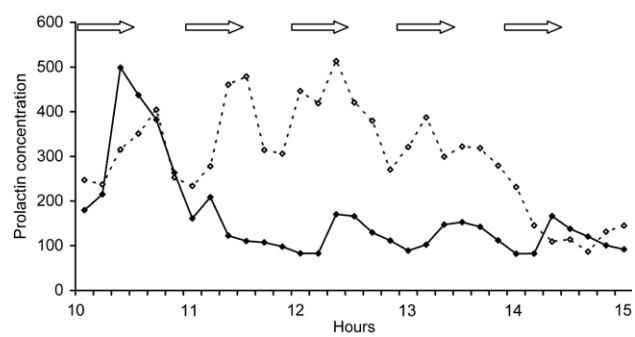

Fig. 6. Different patterns of prolactin response to salsolinol infusions in two anestrous sheep. The series of infusions are indicated by white arrows.

salsolinol-infused groups: 73.87 ± 7.02 and 87.92 ± 6.33 pg/50 µl, respectively. Although visually an increase in NA level was observed following salsolinol treatment, there were no statistical differences in NA concentrations in the consecutive perfusates within and between groups (Fig. 4).

Plasma prolactin concentration

Mean plasma prolactin concentration in the control group was 110.28 ± 9.64 ng/ml and in the salsolinol-infused group it was significantly higher, 288.84 ± 8.62 ng/ml ($P<0.001$).

The distribution of mean plasma prolactin concentrations in 30-min periods within the control group showed that prolactin level significantly ($P<0.05$ to $P<0.01$) increased during the initial phase but decreased gradually following the duration of the experiment. In sheep infused with salsolinol, prolactin concentrations in all 30-min periods were significantly higher ($P<0.01$ to $P<0.001$) than those in the respective periods in controls, showing also a gradual but significant decrease in the final phase of the experiment (Fig. 5). Different patterns of prolactin response to salsolinol are shown on Figure 6.

DISCUSSION

The results of the current study demonstrated that salsolinol effectively suppressed the DA release into the extracellular spaces of the MBH/ME in sheep during a period of high secretory activity of the hypothalamic NEDA system. This effect of salsolinol was accompanied by the enhancement of the pituitary prolactin release into the circulation. The release of NA into the extracellular spaces of the MBH/ME was not affected in this *in vivo* model of the experiment.

The perfusate DA as well as plasma prolactin concentrations, measured in the current study, were similar to these reported earlier in non-lactating anestrous sheep maintained under the long day conditions (Misztal et al. 1997). The fluctuation in the DA release, noted in individual animals, does not allow any significant differences between the concentrations in consecutive perfusates, but based on DOPAC levels it seems that higher dopaminergic activity occurred around mid-day. Numerous studies on salsolinol action within the central nervous system (CNS) demonstrated primarily its effect on the dopaminergic system in connection with suspected involvement of this compound in the etiology of Parkinson's disease. Salsolinol and especially its N-methylated and oxidized derivatives were shown to decrease DA and DA metabolite concentrations (Zhu et al. 2008) as well as to inhibit tyrosine hydroxylase activity – the rate limiting enzyme in DA synthesis (Patsenka and Antkiewicz-Michaluk 2004) in the rat brain striatum. Salsolinol was also shown to increase the production of reactive oxygen species and reduce intracellular ATP levels in dopaminergic SH-SY5Y cells (Wanpen et al. 2004). Finally, N-methyl-salsolinol induced apoptosis by the activation of the apoptotic cascade initiated in mitochondria, leading to the depletion of DA neurons (Naoi et al. 2002). This wide spectrum of salsolinergic activity with reference to the dopaminergic system may set this compound and its derivatives as the regulators of neurotransmission and/or neurotoxins. As to our results, salsolinol effectively diminished DA release within the MBH/ME, followed by the reduction of DA metabolism to DOPAC. It is noteworthy that a decline in DA in the extracellular spaces of the MBH/ME was very fast and in the term of 30-minutes the DA concentration was already below a detectable level, while DOPAC concentration remained low up to the end of the experiment. Due to the high individual variability in perfusate HVA concentrations, there were no statistical differences between the groups. A visual decrease in HVA concentrations in response to salsolinol was observed only in two animals. Thus, HVA seems to be a weak marker of salsolinol's effect on DA metabolism in the sheep during the long-day period.

The *in vitro* studies by Szekacs and others (2007a, b) showed that salsolinol altered neither the basal nor the stimulation-induced dopamine release from individual ME obtained from lactating rats. No relevant changes were found also in NA release following salsolinol treatment in the ME, or in the neurointermediate lobe of the pituitary gland (Szekacs et al. 2007a,b). It seems noteworthy however that the synthesis and release of DA within the hypothalamus/ME is strongly reduced during lactation in both rats and sheep (Anderson et al. 2006, Misztal et al. 2008, 2010b). In case of our acyclic and non-lactating animals, only the NA release within the MBH/ME was not affected significantly by salsolinol. On the other hand, in peripheral organs, as in the salivary gland, atrium, spleen and pancreas, of rats treated with salsolinol, there was a dose related decrease in DA concentration, while NA

concentration also did not change leading to an increase in NA/DA ratio (Szekacs et al. 2007a,b).

The other important observation of this study confirming a proper action of salsolinol within the CNS is that infused compound stimulated the release of the pituitary prolactin to the circulation. Interestingly, although a response of prolactin differed individually, it was almost immediate all the time to each series of salsolinol infusion. Thus, the observed increases in prolactin release could result not only from the suppressed activity of the dopaminergic system, but also from a direct stimulatory influence of salsolinol on the pituitary lactotrophs. Homicsko and coauthors (2003) demonstrated that salsolinol binding sites are located in the anterior pituitary (AP) and a stimulatory effect of salsolinol on prolactin was also demonstrated in the *in vitro* study on the rat and bovine AP lactotrophs, proving the existence of salsolinol receptors in the pituitary cells (Toth et al. 2001, Hashizume et al. 2008a). Supporting this thesis, Randai and coworkers (2005) showed that the prolactin-releasing action of salsolinol is mediated probably by a cAMP-coupled mechanism at the level of lactotrophs. It is noteworthy that for the drug infusion in our study, the tip of the infusing cannula was placed behind the ME, allowing passage of the infused compounds through the cerebrospinal fluid of the third ventricle, as well as close to the blood vessels of the hypothalamo-pituitary system (Skipor and Thiery 2008). In turn, the long lasting decrease in plasma prolactin concentration throughout the experiment in both control and salsolinol-treated groups may reflect a gradual habituation of the animals to the experimental conditions, as prolactin is very susceptible to experimental stress, and also a depletion of the AP cells from the hormone.

The presence of salsolinol in both the hypothalamus and pituitary gland as well as its stimulatory effect on prolactin release were demonstrated in the last decade either in rodents or ruminants, females or males and in young or adult animals (Toth et al. 2001, 2002, Hashizume et al. 2008a, 2010, Misztal et al. 2008, 2010a, Gorski et al. 2010a). The physiological significance of this compound in the different physiological conditions is not precisely ascertained. According to Bodnar and coauthors (2004), salsolinol seems to play a pivotal role in the mechanism of suckling-induced prolactin release. Indeed, we have previously shown high fluctuations in salsolinol concentration at the MBH/ME level before and during suckling stimulus in sheep, which were closely related to the changes in the plasma concentration of prolactin (Misztal et al. 2008). Moreover, the suppression of prolactin surge stimulated by suckling or salsolinol treatment is possible by the use of a structural analogue of salsolinol, 1-methyl-3,4-dihydroisoqinoline, known from the antagonizing effects (Bodnar et al. 2004, Misztal et al. 2010a). Contrary to that, no salsolinol concentration was measured in the samples collected in the current study from the MBH/ME of control anestrous animals. The lack of salsolinol was reported also previously in non-milking sheep ten weeks after weaning lambs, when entered to seasonal anestrus (Misztal et al. 2008). It suggests that the synthesis and/or release of salsolinol in the sheep hypothalamus occurs specifically, at least during the period of lactation (Misztal et al. 2008, 2010a,b), to stimulate the secretion of lactogenic hormones, prolactin and growth hormone (Gorski et al. 2010a,b). Nevertheless, the data cited above and these obtained in the current study underline the role for salsolinol as a neuromodulator within the CNS and as a signal transmitter for the pituitary prolactin release.

CONCLUSION

Salsolinol effectively suppresses an activity of the hypothalamic neuroendocrine dopaminergic system in sheep during seasonal anestrus what may predispose this model for studies on the salsolinol/dopamine relationship.

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