Access of dopamine to the median eminence and brain throughout local vascular pathways in sheep

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SUMMARY

In female sheep, estradiol-dependent dopaminergic inhibition exerted by the A15 nucleus during long days (LD) results in a blockade of reproductive activity. This effect could involve the GnRH cell bodies or their terminals in the median eminence (ME). However, a vast majority of terminals of the A15 nucleus are located in neurohypophysis and only a few in the ME. Previously we demonstrated that tritiated dopamine (DA) was transferred from the venous blood of the cavernous sinus to the arterial blood supplying the brain. In the present paper, we tested the hypothesis that the transferred dopamine could reach further the brain and ME. Using isolated sheep heads harvested on short days vs. long days, we examined radioactivity in brain tissues following infusion of tritiated dopamine into the cavernous sinus. The experiment was performed in ovariectomized ewes treated with estradiol (E₂) or vehicle. The mean level of radioactivity in brain was affected by season (p<0.001) and E₂.
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(p<0.05) and was the highest during LD in E₂-treated animals. In the next experiment on isolated sheep head we measured dopamine and its metabolites levels in blood, brain and pituitary after infusion of non-radiolabelled dopamine. We observed an increase (p<0.01) in dopamine concentration in arterial blood but not in the brain. The pituitary was the only structure examined in which a tendency (p=0.06) towards increased dopamine concentration following dopamine infusion was observed. Thus, even if part of DA released from terminals within the posterior and intermediate lobes of the pituitary reaches the vessels of the ME through local vascular pathways, it is unlikely that it could affect the LHRH terminals located in ME. In addition, our results suggest that brain capillaries in the isolated head are able to maintain a functional blood brain barrier. Reproductive Biology 2004 (1): 91-106.

**Key words:** counter-current transfer, dopamine, season, estradiol, sheep, brain

**INTRODUCTION**

Decreased LH release during long days (LD) results from a diminished frequency of the GnRH pulsatile stimulation in the sheep. This is due to an increase in hypothalamic sensitivity to negative feedback [9, 15] exerted by estradiol (E₂). Lesions [12, 24] and microdialysis studies [7] have established a role for the A15 dopaminergic nucleus in mediating the inhibition of the GnRH neurons by E₂ during anestrus. The site of action of the A15 nucleus on GnRH activity remains to be established. Firstly, an axosomatic inhibition from A15 could take place on the GnRH pericarions [14, 23]. Additionally, the inhibition may be of the presynaptic type, occurring in the terminals of neurons in the median eminence through the D2 receptor [1, 5]. This presynaptic inhibition seems less likely since a vast majority of terminals of the A15 nucleus have been observed in the neurohypophysis and only a few have been found in the median eminence [8]. Consequently, the inhibition of GnRH terminals in the median eminence could depend upon the few terminals of A15 in this site [8, 14], or could be mediated indirectly via an unidentified intermediate. Finally, an additional, humoral link between the terminals of A15 nucleus in the neurohypophysis and the median eminence is possible.

In a previous experiment using the isolated sheep head perfused with autologous blood, we demonstrated that tritiated dopamine (³H-DA) infused into the cavernous sinus is transferred in a retrograde fashion to the arterial blood [20]. Thus, dopa-
mine released from terminals within the posterior and intermediate lobes of the pituitary could reach the median eminence (ME) and brain through local vascular pathways. Therefore, the aim of this study was to examine the degree of dopamine access to the ME and brain through local vascular pathways during anestrus (high A15 nucleus activity) and the breeding season (low A15 nucleus activity) in both estradiol treated and untreated ewes.

**MATERIALS AND METHODS**

This study was conducted in accordance with protocol No8/2000/N approved by local Ethics Committee for Animal Experiments.

**Surgical protocol**

Adult, crossbred ewes were ovariectomized under xylazine anaesthesia (0.025 ml kg$^{-1}$, i.m.; Rometar, Spofa, Praha, Czech Republic) in mid-anoestrous (exp. 1 and 2) or in early breeding season (exp. 1) four-five weeks before the experiment. In both seasonal groups, half of the animals received a subcutaneous $E_2$ implant ($E_2^+$) while the remaining animals received an empty implant ($E_2^-$). The $E_2$ implants, inserted during ovariectomy, were made from silastic tubing [13] and maintained the plasma $E_2$ concentrations of 5-8 pg ml$^{-1}$ [3].

**Experimental protocol**

All experiments were performed on the perfused head isolated from animals after sacrifice [19]. The isolated head was connected to the blood flow system by right common carotid artery. Blood samples for DA identification were collected from the catheter inserted into the left carotid artery. Immediately after the experiment (the end of rinsing) the brain was removed from the skull. Using a modified stereotaxic atlas from the sheep brain [22], tissue samples were dissected as follows: the stalk median eminence containing the proximal part of the pituitary stalk and pars tuberalis in sheep (SME), the optic chiasma (OCH), the preoptic area (POA), the caudate nucleus (CN), the anterior hypothalamus (AH), the mediobasal hypothalamus (MBH), the posterior hypothalamus (PH), the cortex (Cx) and the pituitary (Pit).
Experiment 1. Effect of season and estradiol on DA access to the ME and brain through local vascular pathways

Half of the ewes were used during the seasonal anoestrus on LD and the other half during the short days (SD) of the breeding season. There were four experimental groups of E\textsubscript{2} or placebo-treated animals receiving \textsuperscript{3}H-DA infusion into the cavernous sinus: LD-E\textsubscript{2}+ (n=5), LD-E\textsubscript{2}- (n=6), SD-E\textsubscript{2}+ (n=7) and SD-E\textsubscript{2}- (n=7).

\textsuperscript{3}H-DA (7.35 x 10\textsuperscript{7}dpm; [2,4,6 -\textsuperscript{3}H] dopamine, 60 Ci mmol\textsuperscript{-1}) purchased from Nycomed Amersham, Bucks, UK, was dissolved in 10 ml of multielectrolytic liquid (Solfin, Polfa, Kutno, Poland), and 5 ml of the solution was infused for 5 min (1 ml min\textsuperscript{-1}) into each angularis oculi vein. Approximately 90 ng of the \textsuperscript{3}H-DA was infused for 5 min into both angularis oculi veins. After the \textsuperscript{3}H-DA infusion, the same volume of Solfin was infused for 5 min to rinse cathethers and vessels. Brains were removed from the skull and dissected.

Apart from the 25 experimental animals, corresponding control tissues (control samples) were collected from three additional untreated ewes and processed in a similar way to the experimental samples. All tissue samples were weighed and then solubilized overnight in 0.5 ml of Tissue Solubilizer (Soluene-350, Packard Instruments Company, USA) at 50\degree C for background estimation. The tissues samples were decolorized using 0.3 – 0.5 ml of 30\% H\textsubscript{2}O\textsubscript{2} which was added dropwise with swirling and followed by 170 µl of acetic acid. The liquid scintillation cocktail (10 ml, Insta Gel Plus, Packard) was added to the vials and radioactivity was counted (LS 5000 TD, Beckman Instruments, USA). Each sample was counted for 10 min using a program with automatic quench compensation. Mean values (dpm) of the control samples (background) were subtracted from values estimated for the corresponding experimental samples and then radioactivity was expressed per 100 mg of tissue.

Experiment 2. Identification of DA and/or its metabolites in blood and tissues

The low amount of \textsuperscript{3}H-DA (90 ng) used in exp. 1 did not allow for DA and dopamine metabolite identification in tissues. Therefore, an additional experiment was performed. Studies were performed during LD since pituitary and brain tissues accumulated the highest level of radioactivity using sheep heads harvested during
long days. Solfin or dopamine in Solfin was infused into the cavernous sinus. We had four experimental groups of E₂-treated or E₂-untreated animals receiving DA or Solfin (S) infusion: DA-E₂⁺ (n=7), S-E₂⁺ (n=6), DA-E₂⁻ (n=7) and S-E₂⁻ (n=7).

Non-radiolabelled DA for infusion was prepared ex tempore from 1 ml of stock solution (1mg ml⁻¹ of 0.1 N HCl) and 9 ml of Solfin. Five ml of the solution was infused for 5 min (1ml min⁻¹) into each angularis oculi vein. This dose of DA (1 mg) was 10⁴ fold higher than that used in the experiment with radiolabelled DA but has been demonstrated to have no effect on the cerebral blood flow in sheep when given intravenously [16]. In the control group, Solfin was infused instead of DA.

After the DA infusion, the same volume of Solfin was infused for 5 min for rinsing DA from cathethers and vessels. The duration of infusion in exp. 2 was selected on the basis of the maximum radioactivity observed in the blood in our previous study [20]. Two minutes prior to as well as 1, 3 and 5 min after the beginning of DA or Solfin infusion, blood samples from the carotid rete (within 1 min) were collected in tubes containing 100 µl of 1N HCl to prevent oxidation and kept on ice. Plasma samples were stored at −80°C until assayed by high-performance liquid chromatography (HPLC). Brain tissue samples (2-3 mm³) were placed in two ml plastic tubes (4°C) containing 150 µl of an aqueous solution of 0.4% perchloric acid, 0.1% EDTA, 0.1% sodium metabisulfite and 0.01% ethanol, ex tempore prepared to prevent oxidation of amines and to precipitate proteins. The tissues were stored in this solution at −80°C until assayed by HPLC.

Catecholamines determination in blood plasma

Catecholamines were extracted from plasma on alumina. A 250 µl of 0.25 % sodium bisulfite, 250 µl of 2M TRIS (solution A, pH 8.8) and 6 ng of dihydroxybenzylamine (DHBA in 0.01N HCl) as an internal standard were added to 2 ml Eppendorf tubes before the addition of 1 ml of thawed plasma and 20 mg of activated alumina. The samples were then shaken (rotative agitation) at 4°C for 15 min. After decantation, alumina was washed three times with 1 ml of solution C (1 ml of EDTA 10 %, 0.75 ml of solution A, H₂O ad 100 ml). Each time, samples were shaken for 2 min.

Catecholamines were eluted from alumina with 100 µl of acidic solution (25 µl of EDTA 10%, 130 µl of acetic acid, H₂O ad 10 ml), shaken (rotative
agitation) at 4°C for 15 min and then centrifuged at 11,000 g. Supernatant was stored at –80°C until assayed by HPLC. Amines were assayed by HPLC equipped with an electrochemical detector (Hewlett-Packard HP 1049A). An amount of 45 µl supernatant was injected into a C18 reverse-phase column (3 µm, Hypersil BDS).

The mobile phase consisted of 0.05 M citrate–sodium phosphate buffer (pH 3.5) containing 0.165 g octane sulphonate, 0.05 g EDTA in 1 litre. It contained 1.5% methanol (Merck) and 1.0 % acetonitrile (Merck). The flow rate was 1 ml/min. This mobile phase was degassed, filtered through a 0.22 µm filter, and the pH value was adjusted to 3.5 with orthophosphoric acid or NaOH.

Separated catecholamines were detected with a glassy carbon electrode at oxidizing potential of 0.65 V vs Ag/AgCl reference electrode. Catecholamine standards prepared in 0.25 M perchloric acid were processed in parallel with plasma samples for quantitative comparison. The chemicals used for standards: dopamine (DA), 3-4 dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and dihydroxybenzylamine (DHBA) were purchased from Sigma.

**Determination of catecholamines in tissues**

Thawed tissues were sonicated (three sequences of 10 s, with repeated cooling on ice between the sequences). The tubes were centrifuged (11,000 g, 4°C, 45 min) and supernatant was collected and stored at –80°C until assayed by HPLC. The pellets were used to determine the protein content [2]. An amount of 10 µl supernatant was injected into a C18 reverse-phase column (3 µm spheres, spherisorb ODS2, 2.1 *150 mm-Waters).

The HPLC mobile phase consisted of 7 g sodium acetate, 100 mg EDTA, 200 mg sodium octane sulfonate in 1 litre of distilled water containing 5% methanol, and the flow rate was 0.2 ml min⁻¹. This mobile phase was degassed, filtered through a 0.22 µm filter, and the pH value was adjusted to 4.95 with orthophosphoric acid. The HPLC was coupled to an electrochemical detector (Decade ANTEC) equipped to a glassy carbon electrode, at a potential of 0.70 V vs Ag/AgCl reference electrode. The chemical used for standards (NA, AD, DA, DOPAC, HVA and 5-hydroxyindolacetic acid, 5HIAAA) were purchased from Sigma.
In Experiment 1, the effects of the season and estradiol treatment on the $^3$H-DA transfer (exp. 1) were analyzed by ANOVA either in all collected brain structures or in each structure individually. The blood transfer of DA (exp. 2) was analyzed by ANOVA followed by Bonferroni’s test. The effect of DA infusion on catecholamine concentrations in brain structures, SME and pituitary (exp. 2) was performed by ANOVA.

**RESULTS**

**Experiment 1**

The levels of radioactivity measured in the brain structures, SME and pituitary gland (Pit) are presented in Figure 1. Estradiol treatment ($p<0.05$) and long days ($p<0.001$) significantly increased (estradiol x season, $p<0.05$) the radioactivity
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Table 1. Radioactivity recovered (mean±SEM) in brain, SME and pituitary after \textsuperscript{3}H-DA infusion into the cavernous sinus during the long (LD) and short days (SD) in estradiol treated (E2+) and untreated (E2-) ewes.

<table>
<thead>
<tr>
<th></th>
<th>LD-E2+</th>
<th>LD-E2-</th>
<th>SD-E2+</th>
<th>SD-E2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (%)</td>
<td>0.59±0.187</td>
<td>0.29±0.152</td>
<td>0.27±0.09</td>
<td>0.15±0.06</td>
</tr>
<tr>
<td>SME (%)</td>
<td>0.0042±0.029</td>
<td>0.0007±0.006</td>
<td>0.0005±0.001</td>
<td>0.0010±0.005</td>
</tr>
<tr>
<td>Pituitary (%)</td>
<td>0.070±0.19</td>
<td>0.040±0.07</td>
<td>0.033±0.058</td>
<td>0.064±0.27</td>
</tr>
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Data are presented as a percentage of total (7.35 x 10\textsuperscript{7} dpm) dose infused.

in the brain structures considered together (without SME and Pit). The mean (±SEM) radioactivity in all brain structures (without SME and Pit) decreased as follows: LD-E\textsubscript{2}+ (429 ± 59 dpm 100mg\textsuperscript{-1} of tissues) > LD-E\textsubscript{2}- (212 ± 54) > SD-E\textsubscript{2}+ (201 ± 32) > SD-E\textsubscript{2}- (128 ± 23). Estradiol treatment significantly increased (p<0.05) the radioactivity in the CN. Long days increased (p<0.05) the radioactivity in MBH and Cx. In all tissues except POA and CN, the highest level of radioactivity was observed in LD-E\textsubscript{2}+ ewes. Table 1 present the recovery of radioactivity infused into the cavernous sinus calculated for the whole brain, SME and the pituitary.

Experiment 2

\textit{DA identification in the blood.} Following the infusion of DA into the cavernous sinus, its concentration in arterial blood collected from the common carotid artery increased significantly from the mean (± SEM) level of 25.3 ± 8.0 pg ml\textsuperscript{-1} (time 0) to the level of 120.5 ± 44.5 pg ml\textsuperscript{-1} in the 5 min of the experiment (p<0.01). In the control group (Solfin infusion) DA concentration in arterial blood taken from carotid artery ranged from 1.9 ± 1.9 pg ml\textsuperscript{-1} to 18.0 ± 13.2 pg ml\textsuperscript{-1} (fig. 2).

\textit{Catecholamines and their metabolites in brain structures,} SME and pituitary. The pattern of DA and its metabolites distribution in brain differed from structure to structure, while individual variations among groups in particular structure were rather low (figs. 3 and 4). Higher (p<0.01) concentration of 5-HIAA (metabolite of serotonin) was demonstrated in posterior hypothalamus (PH; fig. 3) than in preoptic area (POA) and anterior hypothalamus (AH). Dopamine concentrations
in Cx, CN and SME were high and ranged from 2 to 22 pg µg⁻¹. No differences were found among groups (fig. 4).

All other structures showed low concentration of DA (figs. 3 and 4). The effect of DA infusion tended to be significant only in the pituitary (ANOVA, p = 0.06). In the POA, DOPAC was detectable in three out of seven animals in DA-E₂⁺-group and in three out of seven in DA-E₂⁻-group. In contrast, this DA metabolite was not found in groups receiving Solfin.

DISCUSSION

Infusion of ^3^H-DA into the cavernous sinus significantly increased the radioactivity in all brain tissues, SME and pituitary in comparison with control samples. This demonstrated that ^3^H-DA reached the examined structures throughout local vascular pathways and was accumulated in tissues. However, the low amount of
Fig. 3. Concentration (mean±SEM) of dopamine (DA), dopamine metabolites (DOPAC, HVA) and serotonin metabolite (5-HIAA) in three diencephalic structures: preoptic area (POA), anterior hypothalamus (AH) and posterior hypothalamus (PH). Tissues were collected after the end of dopamine (DA) or Solfin (S) infusion followed by 5 min rinsing with Solfin. Note the differences in concentrations of monoamines/metabolites between structures and the absence of differences among the treatment groups. Long days (LD), estradiol treated (E2+) ewes, untreated (E2-) ewes; dopamine (DA), 3-4 dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), hydroxyindolacetic acid (5-HIAA).
Fig. 4. Concentration of dopamine (mean±SEM) within dopamine-rich structures: cortex (Cx), caudate nucleus (CN) and stalk median eminence (SME) as well as within three structures with lower dopamine concentration: ophtalmic chiasma (OCH), mediobasal hypothalamus (MBH) and pituitary (Pit). Note the effect of exogenous dopamine infusion on DA concentration in the pituitary.

$^{3}$H-DA (90 ng) used in this experiment did not allow us to determine the exact chemical identity of the labelled molecule uptaken. Therefore, an additional experiment (exp. 2) was performed. Much higher dose (1 mg) of non-radiolabelled DA was infused and concentrations of DA and its metabolites in the brain, SME and pituitary were measured using HPLC.

Following infusion of DA into the cavernous sinus, we observed differences in the concentration of DA and its metabolites among anatomical brain structures. This was in agreement with data obtained by other authors [22, 27]. In addition, within the same brain structures we noticed only limited variations among groups.
No effect of infusion of exogenous DA into the cavernous sinus on concentration of DA and its metabolites was found in examined tissues. This suggests that DA and its metabolites measured in this study were brain-derived.

The pituitary was the only structure in which a tendency towards an increased concentration of DA in the ewes receiving DA (fig. 4) was observed. Being outside of the brain, the pituitary is not protected by the blood-brain barrier and therefore, DA may pass unaltered from the bloodstream to this gland. Low level of endogenous DA found in the pituitary might have resulted from difficulties in proper sectioning of the posterior and intermediate lobes of the pituitary (localisation of A15 terminals). Surprisingly, we did not observed any increase of DA concentration in SME following DA infusion in both E2-treated and untreated animals (fig. 4). It is possible that amount of DA transferred (tab. 1) was negligible when compared to the endogenous catecholamine concentration. Thus, even if part of DA released from terminals within the posterior and intermediate lobes of the pituitary reaches the vasculature of the pituitary or ME, it is unlikely that it could affect the LHRH terminals located in ME. Therefore, our data do not support the possibility that DA derived from the posterior pituitary contributes to the inhibitory tone exerted on LHRH terminals.

The results of the second experiment indicate that endothelial cells from the brain capillaries are functional and able to maintain the blood-brain barrier in the isolated head of sheep. Among other functions, capillaries from the brain prevent the entry of DA due to the activity of monoamine oxidase-B. Monoamine oxidase-B catalyses the production of DOPAC from DA, but in our experiment we did not observe increased concentration of DOPAC within the brain structures of animals receiving DA infusion. The persistance of the blood-brain barrier function after perfusion lasting 20 minutes in our model seems to be rather surprising. However, the use of ex vivo choroid plexus from the same species is a reliable model for the study of blood-CSF permeability [17, 26]. It appears that our isolated sheep head model may also be applied to examine penetration of molecules into the brain.

Significant increase in DA concentration in arterial blood during DA infusion into the cavernous sinus clearly indicated that DA was transferred from the venous to arterial blood in intact form. This is in agreement with previous studies performed on female pigs [10, 11]. When Solfin was infused instead of DA, we observed gradual decrease in DA concentration in arterial blood that reflects the lack of
DA de novo production by the brain. Previously we demonstrated that transfer of DA from venous to arterial blood is affected by estradiol and season [20]. In the current study we did not observe any differences in DA transfer in $E_2$-treated and untreated animals. This may result from the differences in sensitivity of methods used in both experiments: $^3$H-DA vs DA infusion.

Mechanism of $E_2$ action and way by which the season affects the DA transfer remain to be identified. In rats, it has been postulated that $E_2$ and melatonin may play a role in cerebral vascular tone and, therefore, in the regulation of blood flow to the brain [18]. In rat arteries forming the Circle of Willis, estrogen replacement decreases the number of melatonin binding sites compared to those in ovariectomized rats [18]. Melatonin was shown to affect vascular reactivity of the vessels involved in thermoregulation in rats [4, 28]. Vessel response was dependent on the phase of the estrous cycle and, therefore, on circulating levels of $E_2$ [6]. Large blood vessels such as arteries or veins show presence of melatonin binding sites [4]. Melatonin receptors or binding sites have not yet been found in brain capillaries. In contrast, melatonin binding sites are currently observed in choroid plexus, the other component of the brain barriers from mammals [21, 29, 30] that may provide an anatomical support for direct action of the pineal hormone.

The results of the first experiment showed that a mean level of radioactivity in the brain changed in relation to season and $E_2$ treatment. The following trend in radioactivity was observed: LD-$E_2^+$ > LD-$E_2^-$ > SD-$E_2^+$ > SD-$E_2^-$. Differences in the radioactivity level in examined structures, especially within hypothalamus follow the dopaminergic tone of the MBH and SME in ewes. In sheep, DA content in SME is high during long days [22, 27] when $E_2$ stimulates the endogenous synthesis of DA in the A15 nucleus. In our previous paper [20], we demonstrated that transfer of $^3$H-DA from the venous to arterial blood was lowest during LD in $E_2$-treated ewes. In contrast, the opposite was observed in the examined brain structures. In the model of the isolated head, the amount of radioactivity in the blood collected from the left carotid artery reflects the efficiency of the counter-current transfer minus the tissue uptake. Consequently, the lowest radioactivity in the blood collected from $E_2$-treated ewes during LD may reflect the high uptake of DA. Even though the exact chemical identity of the radiolabelled compound measured in the brain in the first experiment remains unknown, obtained results allow us to suggest that season and hormonal status may modulate penetration
of molecules from the peripheral blood plasma to the brain. This agrees with the recent in vivo findings demonstrating that the penetration of progesterone within the brain of female sheep changes with photoperiod [25].

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