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Original article

Thyroid hormones in the cerebrospinal fluid of the third ventricle of adult female sheep during different periods of reproductive activity

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Abstract

Thyroid hormones (THs) are obligatory for transition from breeding season to anestrus in sheep. In this process, THs act during a very limited time of the year and primarily within the brain. In ewes chronically equipped for sampling cerebrospinal fluid (CSF) from the third ventricle, we have characterized the concentrations of total and free thyroxine (T4), triiodothyronine (T3), and total reverse T3 (rT3) in the CSF during breeding season, anestrus and during a critical period required for transition to anestrus (December-March). The total T4, T3, rT3 and free T3 average concentrations (± SEM) in CSF were 1.5 ± 0.07 ng/ml, 14.5 ± 1.2 pg/ml, 43 ± 7.4 pg/ml, and 0.6 ± 0.05 pg/ml, respectively, and all were significantly lower ($p < 0.001$) than in blood plasma except free T4 (12.6 ± 1.1 pg/ml), which was similar to that in plasma. There was a seasonal trend ($p < 0.05$) in the concentration of total T3 (highest in December) and free T4 (highest in November) in the CSF that does not follow that in blood plasma. During the period of transition to anestrus the CSF total T3/TT4 molar ratio and free T3/ T4 ratio were significantly lower ($p < 0.05$ and $p < 0.01$, respectively) than in blood plasma, while the total rT3/T4 ratio was significantly higher ($p < 0.01$) at the end of this period (March). Additionally, the CSF total rT3 concentrations were also significantly correlated with the CSF total T4 levels ($r = 0.57$; $p < 0.05$). In conclusion, the CSF in sheep may serve as a considerable source of thyroid hormones for neuroendocrine events. The lack of significant changes in THs concentrations in the CSF during the period of transition to anestrus indicate that neither seasonal changes of THs circulating in the blood plasma nor THs circulating in the CSF actively drive the transition to anestrus.

Key words: ewe, cerebrospinal fluid, thyroxine, triiodothyronine, reverse triiodothyronine

Introduction

Thyroid hormones (THs) are crucial for the proper function of the central nervous system in vertebrates; they are involved in brain development (Porterfield and Hendrich 1993), plasticity (Lehman et al. 1997), and the endogenous seasonal rhythms of neuroendocrine reproductive activity in several photoperiodic species such as birds, deer, hamsters, and sheep (Nicholls et al. 1988, Shi and Barrell 1992,

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Karsch et al. 1995, Yasuo et al. 2006). In sheep, THs are obligatory for at least one aspect of this endogenous rhythm: neuroendocrine changes that lead to the transition from breeding season to anestrus (Moenter et al. 1991, Webster et al. 1991, Dahl et al. 1994). In this process, THs act during a very limited time of the year (Thrun et al. 1997) and primarily within the brain (Viguie et al. 1999).

The majority of THs are released from the thyroid gland in the form of thyroxine, a prohormone, whereas biologically active triiodothyronine (T3) is mainly generated in extrathyroidal tissues by enzymatic deiodination of T4. The activating enzyme, deiodinase type II (DIO2), is located in the brain and converts T4 to T3, while the inactivating enzyme, deiodinase type III (DIO3), also located in the brain, converts T4 and T3 to biologically inactive reverse T3 (rT3) and diiodothyronine (T2), respectively (Kohrle 1999). In seasonal breeders, expression of the *DIO2* and *DIO3* genes in the hypothalamus is highly localized to the ependymal layer of the third ventricle, in the specialized glial cells known as tanycytes (Yasuo et al. 2006, Barrett et al. 2007, Hanon et al. 2008). This location suggests that the cerebrospinal fluid (CSF) may be a source of T4 that could be transduced to the hypothalamus and/or pituitary gland through T3 released from the tanycyte processes into specific hypothalamic nuclei and/or the pituitary portal plexus. To act at a cellular level in the brain, THs must cross the blood-brain barrier and the blood-cerebrospinal fluid barrier (BCSF-B). A recent study in rabbits demonstrated that the distribution of T4 from the CSF into the brain may be carrier-mediated and dependent on transthyretin (TTR) which is unidirectionally secreted by the choroid plexus into the CSF (Schreiber et al. 1990, Southwell et al. 1993, Kassem et al. 2006). The study found that TTR enhances T4 uptake into the ependymal region of the ventricles, which suggests that even distribution of T4 within this tissue is not only dependent on the free fraction of T4 in the CSF, but also on the T4 bound to TTR. The involvement of DIO2 and DIO3 from tanycytes in regulation of seasonality of reproduction is now well established (Barrett et al. 2007, Watanabe et al. 2007), but studies have not been performed that focus on THs at the level of the CSF, especially during the period of transition to anestrus. Just recently we demonstrated that local changes in T3 production in the hypothalamus are reflected in the CSF but not in circulating blood T3 concentrations (Skipor et al. 2010). Therefore the aim of the study was 1) to evaluate the impact of season on the concentrations of total and free T4 and T3, as well as the total concentration of rT3 in the CSF of the third ventricle and compare these with blood plasma and 2) to examine whether any characteristic changes occurred in the CSF TH levels during a critical period of temporary responsiveness of the neuroendocrine system to TH action, which is required for seasonal changes in reproductive activity in ewes.

Materials and Methods

Animals and ventricular surgery

The experiments were performed on adult ewes (3-4 years old, 50-60 kg body weight, n = 10) of the seasonal Polish Lowland breed. Animals were maintained indoors in pens under natural lighting conditions and fed a constant diet of hay, straw, and commercial concentrates, with water and mineral licks available *ad libitum*. During the experiment, ewes were kept in comfortable cages where they could lie down and have access to hay. To prevent the stress of social isolation, ewes had visual contact with other sheep. All animal procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals (1997) and approved by the Local Ethics Committee. Under general anesthesia (pentobarbital sodium 8-12 mg/kg body mass, i.v.; Vetbutal, Biowet, Puławy, Poland; and ketamine 6-10 mg/kg body mass, i.v.; Bioketan, Biowet, Puławy, Poland) ewes were implanted with a stainless steel guide cannula (1.2 mm o.d., 1.0 mm i.d.) in the third ventricle of the brain, one month before the experiments, as described previously (Skipor et al. 2010). Correct placement of the guide cannula was confirmed by outflow of a small amount of CSF during the surgery. 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.

CSF and jugular blood collection

To collect a sample of CSF, the stainless steel catheter (1.0 mm o.d., 0.8 mm i.d.) was carefully introduced into the guide cannula. After the outflow of CSF was achieved, the catheter was connected to a special cannula-Eppendorf tube system joined to the PHD 2000 infuse/withdraw pump (Hugo Sachs Elektronik Harvard Apparatus, Germany). The total time of CSF collection was four hours, and the outflow rate was 20 µl/min. Collection tubes were kept in an ice bath during sampling and immediately after filling. Sample tubes were stored at -80°C until their contents were assayed for thyroid hormones.

Ovarian cyclicity was detected using measurements of progesterone in the jugular blood taken once a week from November to June (Exp. 1) or November to March (Exp. 2). For thyroid hormone measurements, blood samples were collected at the beginning

and end of CSF collection. After centrifugation in heparinized tubes, plasma was stored at -20°C until it was assayed.

Exp 1: Concentrations of THs in the CSF of the third ventricle and effects of the season

To evaluate the concentrations of total and free T4 and T3, as well as the total concentration of rT3, in the CSF of the third ventricle and to measure the effects of the season on the level of TH in the CSF, five ewes were prepared for CSF collection as described above. In every ewe, four CSF collections from the third ventricle were performed, at a one-month interval during periods of decreasing (November and December 2006) and increasing (April and May 2007) day length, corresponding to the breeding and anestrous periods, respectively.

Exp 2: Thyroid hormones in the CSF during a period of transition from breeding season to anestrus

To characterize the level of thyroid hormones in the CSF during the time of transition from the breeding season to anestrus, another group of five ewes were subjected to the series of CSF collection at one-month intervals from December 2007 to March 2008.

Analytical techniques

The progesterone concentration was assayed by a direct RIA method used routinely in our laboratory, with a sensitivity of 6.2 pg/sample. Total T4 (TT4) and T3 (TT3) in blood were assayed in 20-µl and 50-µl single plasma aliquots, respectively, using the RIA-gnost® TT4 and TT3 kits (CIS bio International, GIF-SUR-YVETTE, CEDEX, France) validated for use in sheep (Skipor et al. 2010). The same kits were used for detection of TT4 and TT3 in CSF. Because the TT4 and TT3 concentrations in individual CSF samples were close to or below the limit of detection of the assays CSF samples were lyophilized before assay and then TT4 and TT3 were measured in 0.5 ml and 1 ml of concentrated sample, respectively. The concentrations of TT4 and TT3 in the CSF samples were extrapolated from a standard curve prepared using barbitone buffer. The detection limits in blood plasma were determined to be 5.35 ng/ml and 0.1 ng/ml for TT4 and TT3, respectively. In the CSF, the detection limit was 1.8 pg/ml for TT4 and 50 pg/ml for TT3. Free fractions of T4 (fT4) and T3 (fT3) were measured in 100-µl and 50-µl single aliquots of blood plasma and in 130-µl and 100-µl single aliquots of CSF using the RIA-gnost® fT4 and fT3 kits (CIS bio International). Measurements in CSF samples were corrected by subtracting the amount of fT4 or fT3 measured in blank CSF samples (free of thyroid hormones), which were included in each assay. The detection limit of the assay was 0.9 pg/ml for fT4 and 0.2 pg/ml for fT3. Reverse T3 (rT3) was measured in 100-µl single aliquots of blood plasma using the RIA rT3 kit (BIOCODE-HYCEL, Belgium). For rT3 measurements in the CSF, one 250-µl aliquot of lyophilized CSF was reconstituted with 100 µl of distilled water, and 100 µl of the concentrated sample (concentration factor 2.5) was used. The minimum detection level of the rT3 kit was 25 pg/ml. Samples of CSF were analyzed in batches to avoid inter-assay variation. The intra-assay variations were 4.6%, 4.2%, 2.2%, 2.0% and 1.5% for TT4, fT4, TT3, fT3 and rT3, respectively. The inter-assay variations for plasma samples were 5.8%, 12.8%, 2.1% and 2.9% for TT4, fT4, TT3 and fT3, respectively.

Data analysis and statistics

The data are expressed as the mean ± SEM for all ewes in each collection period. One-way ANOVA and repeated measure ANOVA, both followed by a *post-hoc* Tukey's test (Graph Pad Prism, San Diego, USA), were used to determine the differences in the TH concentrations between collection periods (months) in Exp. 1 and Exp. 2, respectively. The relationship between variables was analyzed using a Pearson's correlation coefficient. Statistical significance was assumed at $p < 0.05$. Molecular ratios were calculated after conversion of individual data according to the following factors: T4, pg/ml = pmol/liter = 1.283; T3 and rT3, pg/ml = pmol/liter = 1.536. A two-way ANOVA followed by a *post-hoc* Bonferroni test (Graph Pad Prism, San Diego, USA) was used to determine the effect of time and type of body fluid (blood or CSF) collection. The reproductive activity of the ewes was defined according to O'Callaghan et al. (1992).

Results

In experiment 1, all sheep sampled in November and the beginning of December were cycling. Seasonal suppression of the estrous cycle occurred in late December for three ewes and in January/February of the next year for the remaining two ewes. Afterwards, the plasma progesterone level in all animals remained below 0.5 ng/ml, with the exception of episodic elevations up to 0.7 ng/ml, noted in two ewes (data not presented). The TT4, TT3 and rT3 average concen-

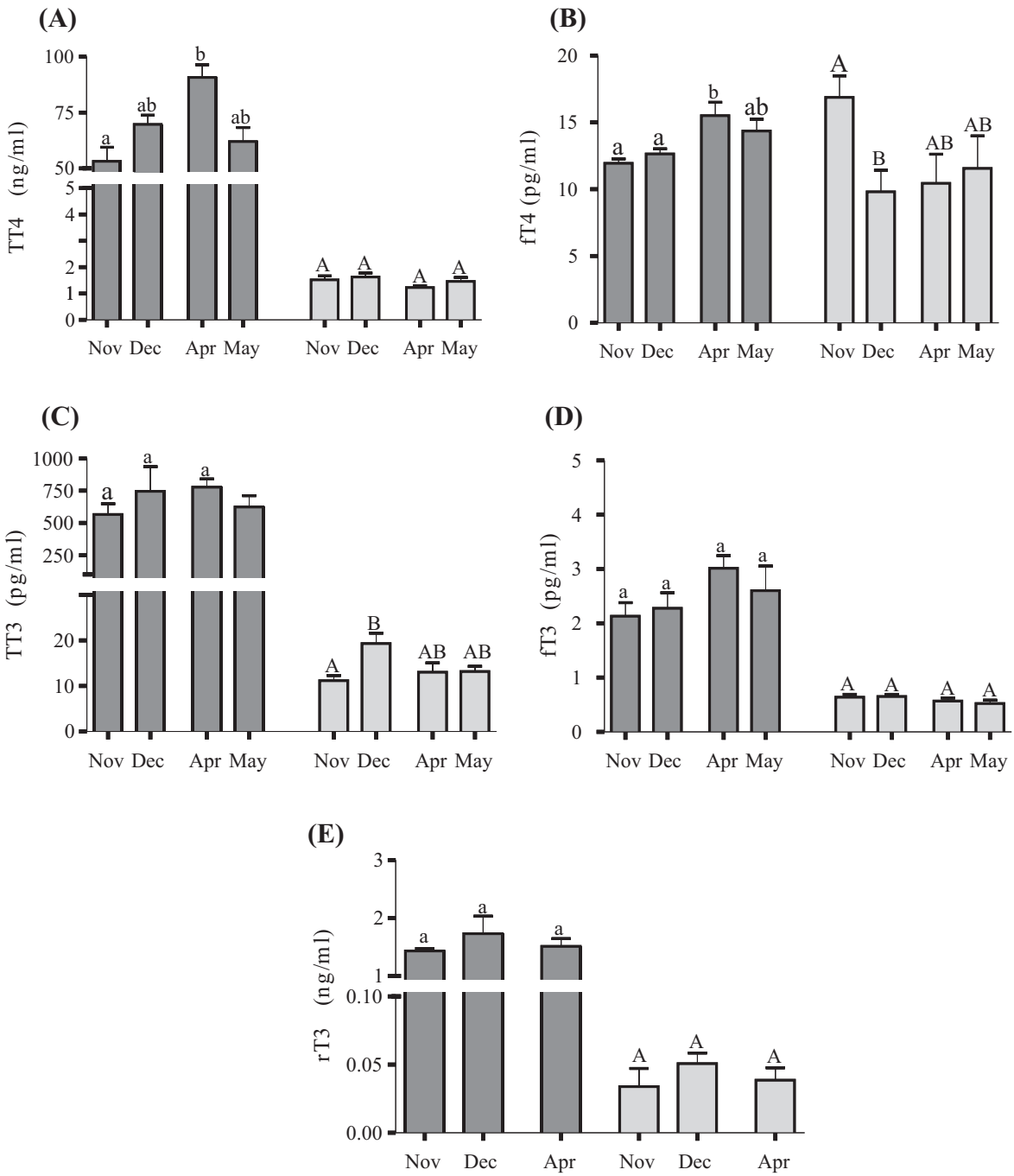

Fig. 1. Mean (±SEM) concentrations of thyroid hormones in the blood plasma (dark gray bars) and CSF (light gray bars) of sheep during the reproductive season (Nov and Dec) and seasonal anestrus (Apr and May). A – total thyroxine (TT4), B – free thyroxine (fT4), C – total triiodothyronine (TT3), D – free triiodothyronine (fT3) and E- total reverse triiodothyronine (rT3). Due to the lower volume of CSF collected in May rT3 was not measured in plasma and CSF samples collected in May. Different lowercase letters and capital letters indicate significant differences ($p < 0.05$) for mean thyroid hormone concentrations in blood plasma and CSF, respectively.

trations in CSF were 1.5 ± 0.07 ng/ml, 14.5 ± 1.2 pg/ml and 43 ± 7.4 pg/ml, respectively; this was 44-, 46- and 38-fold less than the concentrations present in blood plasma (66.7 ± 8 ng/ml, 670 ± 14 pg/ml, 1.6 ± 0.17 ng/ml, respectively). In contrast, the level of free T4 (12.6 ± 1.1 pg/ml) in CSF was similar to that in plasma (13.2 ± 0.4 pg/ml), while the concentration of free T3 in CSF (0.6 ± 0.05 pg/ml) was four-fold lower than the plasma concentration (2.5 ± 0.2 pg/ml). There was 0.02% and 0.94% of free T4 in blood plasma and CSF, and 0.37% and 4.37% of free T3 in blood and CSF, respectively. The seasonal patterns of TT4, fT4,

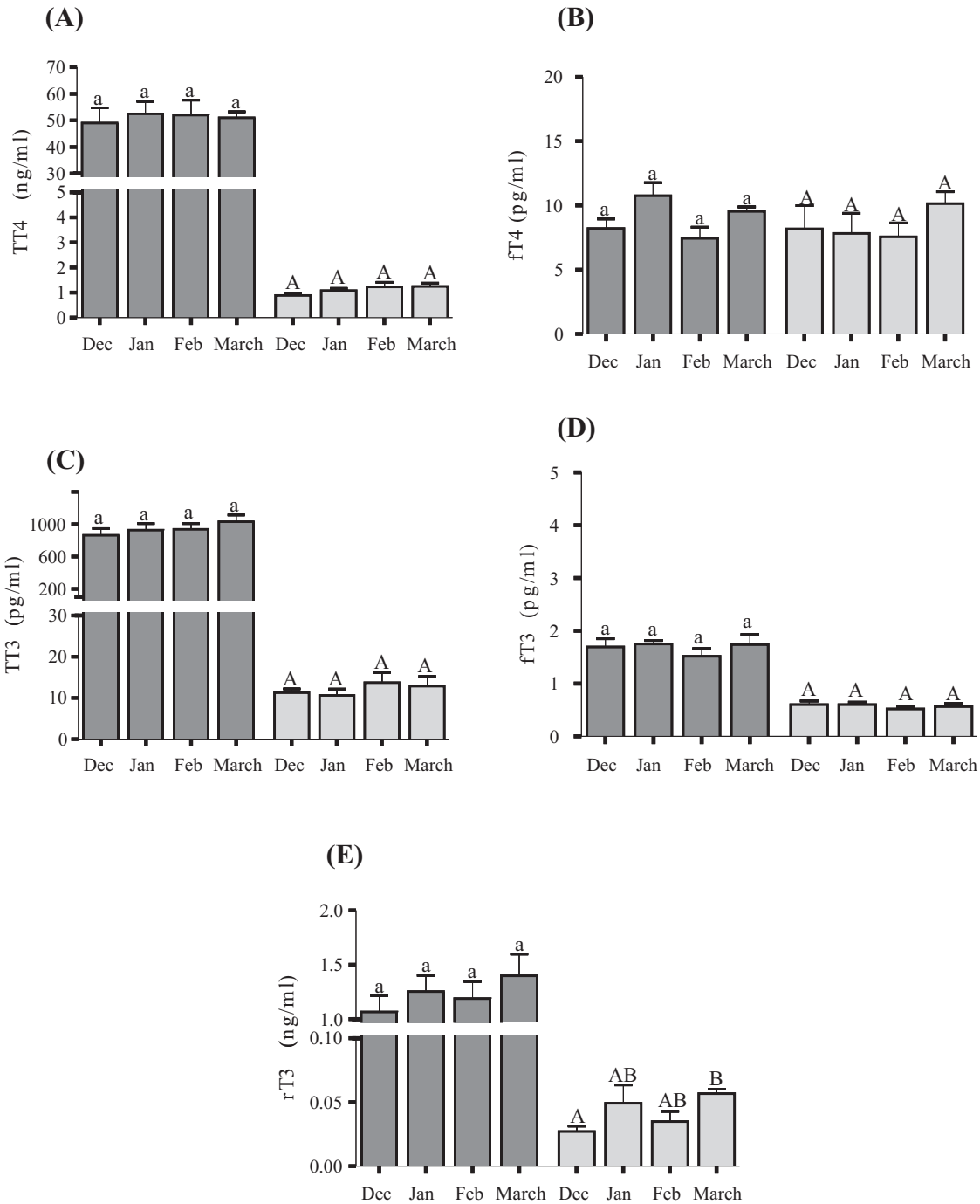

Fig. 2. Mean (±SEM) concentration of thyroid hormones in the blood plasma (dark gray bars) and CSF (light gray bars) in ewes during a transition from breeding season to anestrus (Dec to Mar). A – total thyroxine (TT4), C – total triiodothyronine (TT3), B – free thyroxine (fT4), D – free triiodothyronine (fT3), E – total reverse triiodothyronine (rT3). Different lowercase letters and capital letters indicate significant differences ($p<0.05$) for mean thyroid hormone concentrations in blood plasma and CSF, respectively.

TT3, fT3 and rT3 concentrations in blood plasma and CSF of the sampled ewes are illustrated in Fig. 1. The mean level of circulating TT4, fT4, TT3, fT3 reached the maximum value in early spring (by April). Furthermore, total and free T4 levels in plasma were significantly affected depending on the season ($p < 0.05$).

In CSF, the seasonal pattern of thyroid hormone concentrations differed from that in plasma. In December, the TT3 level in CSF was significantly ($p < 0.05$) higher compared to November but similar to that measured in April and May (Fig. 1C). Additionally, it was accompanied by a significantly

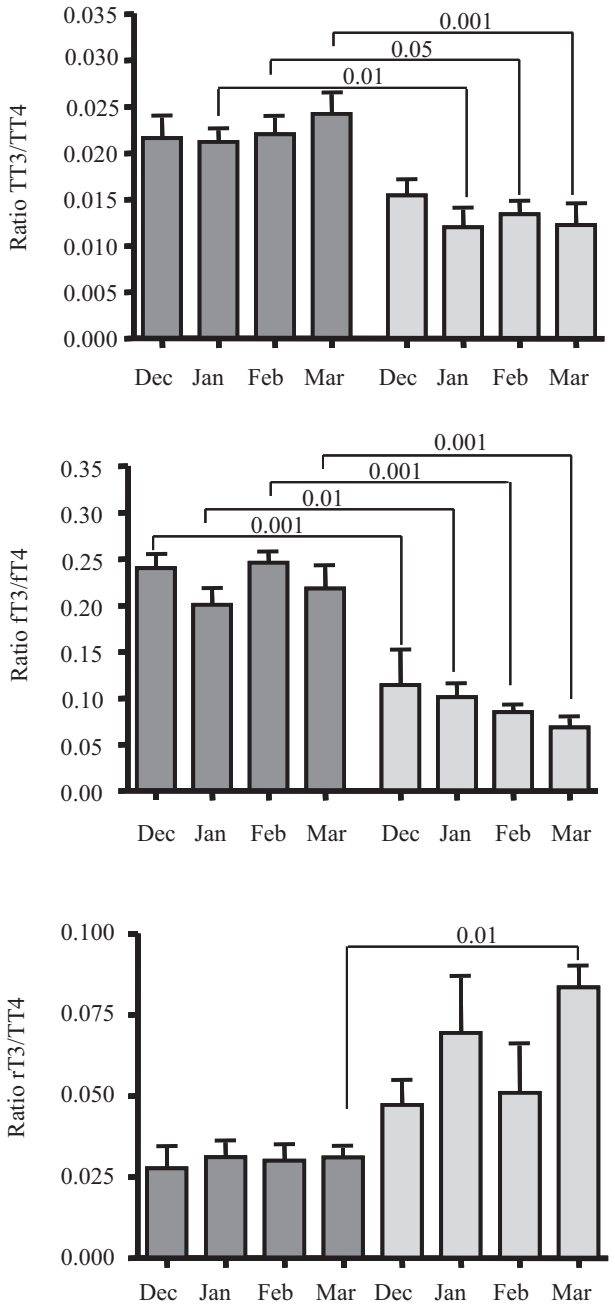

Fig. 3. Mean (±SEM) molecular ratios of TT3/TT4 (upper), fT3/fT4 (middle) and rT3/TT4 (bottom) in blood plasma (dark gray bars) and CSF (light gray bars) in ewes during a transition from breeding season to anestrus (Dec to Mar). TT4 – total thyroxine, TT3 – total triiodothyronine, fT4 – free thyroxine, fT3 – free triiodothyronine and rT3 – total reverse triiodothyronine.

($p < 0.05$) higher level of free T4 in November than December (Fig. 1B). There were no seasonal differences in the CSF concentrations of TT4, fT3 and rT3.

In experiment 2, all sheep sampled in early December were cycling. Seasonal suppression of the estrous cycle occurred in one ewe in late December, two in February, and two in March (data not presented). Afterwards, the plasma progesterone level in all animals remained below 0.5 ng/ml. Overall means of TT4, TT3, fT3, (1.1 ± 0.08 ng/ml, 12.1 ± 0.7 pg/ml, 0.6 ± 0.02 pg/ml) and rT3 (42.0 ± 6.7 pg/ml) concentrations in he CSF (Fig. 2) were significantly ($p < 0.001$) lower than those in plasma (51.1 ± 0.77 ng/ml, 0.9 ± 0.03 ng/ml, 1.7 ± 0.05 pg/ml, and 1.2 ± 0.07 ng/ml, respectively). In contrast, the mean fT4 levels in the CSF (8.43 ± 0.58 pg/ml) were similar to those measured in the blood (9.0 ± 0.73 pg/ml). In the CSF, the free fraction of T4 constituted 0.76% of TT4, while the free fraction of T3 was significantly higher and constituted 4.78% of TT3. The percentages of free T4 and T3 in blood plasma were calculated at 0.02% and 0.18%, respectively. The patterns of TT4, fT4, TT3, fT3 and rT3 concentrations in the CSF and blood plasma of sampled ewes are illustrated in Fig. 2. In March, the rT3 level in CSF was significantly ($p < 0.05$) higher compared to December but similar to that measured in January and February. Aditionally, total rT3 concentrations in the CSF were significantly correlated with TT4 ($r = 0.57$; $p < 0.05$). There were significant ($p < 0.001$) differences between plasma and CSF molar ratios of TT3/TT4, fT3/fT4 and rT3/TT4. Molar ratios for TT3/TT4 (except December) and fT3/fT4 in the CSF were significantly lower than in the plasma, while the rT3/TT4 molar ratio was higher ($p < 0.01$) compared with that evaluated in the plasma, but only in March (Fig. 3).

Discussion

This is the first comprehensive study reporting the concentrations of total and free fractions of T4 and T3, as well as total rT3 in the CSF of the third ventricle in ewes. More importantly, it is the first to characterize the seasonal changes in the concentrations of these hormones and to correlate the CSF levels with those in the plasma. In our study, the levels of THs measured in the CSF rank in the same order as the levels found in human CSF (Thompson et al. 1982, Sampaolo et al. 2005). Furthermore, despite CSF collection sites differing from those used in previous studies, CSF TT4 concentrations in our study were similar to those measured in sheep CSF (1.9 ± 0.5 ng/ml) collected from the cisterna magna (Viguie et al. 1999).

Compared with the total fraction of thyroid hormones found in our study, concentrations of free T4 in the CSF were similar to those measured in the plasma, while free T3 concentrations in the CSF were 4-fold lower than in the plasma. In general, there is no agreement concerning the concentrations of total and free T4 in the CSF (Hagen and Solberg 1974, Thompson et al. 1982, Kirkegaard and Faber 1991). In these previous studies, free T4 in the CSF was reported to be present at 1- to 5-fold higher concentrations than

in the serum. Calculating the percentage of free T3 and T4 in both compartments, we found that the percentage of free THs in blood plasma was lower than in the CSF. This may be explained by differences between the levels of blood and CSF proteins that can bind THs. There is no thyroxine-binding globulin gene expression in the choroid plexus in sheep (Tsykin and Schreiber 1993); therefore, the main binding proteins in the CSF are TTR and albumins, but they exist in lower concentrations compared to plasma (Chopra et al. 1978). Interestingly, we found that the free fraction of T3 in the CSF was about 4.5-6.2 times higher than free T4 and was similar to that found in human CSF (Thompson et al. 1982). This may be explained by a higher affinity of TTR for T4 than for T3. Indeed, mammalian TTR has a 4-fold higher affinity for T4 than T3 in sheep and an 8-fold higher affinity in rats (Chang et al. 1999). Binding of T4 to TTR in the CSF prevents its removal from the CSF, as TTR significantly inhibited radiolabeled T4 efflux across the choroid plexus from the CSF to the blood side (Chen et al. 2006). Moreover, the availability of TTR in the CSF is also correlated with an increased uptake of T4 into the brain that is based on receptor-mediated endocytosis. Indeed, ependymal cells are able to endocytose TTR bound to T4 from the CSF, and the TTR receptor has been found in ependymoma cells (Kuchler-Bopp et al. 1998). It has been postulated that the ependyma may act as a reservoir for T4 in the brain that are important for conditions of increased hormone demand by various regions of the brain (Kassem et al. 2006). It is important to note that the levels of THs in the CSF are not correlated with their plasma concentration, which may protect against altered brain tissue availability of THs.

The results of the present study showed a significant elevation of TT3 concentrations in the CSF and a decrease of fT4 levels in December, which may be linked with local changes of T4 conversion to T3. However, recently published data demonstrated that in Soay sheep, *DIO2* is induced by long day exposure (Hanon et al. 2008). On the other hand, if an increase of TT3 in the CSF reflects a local synthesis, one may expect parallel elevation of fT3. We did not notice any significant increase in CSF fT3 levels or in the CSF fT3/fT4 molar ratio. In seasonal sheep, the responsiveness of the neuroendocrine system, which regulates reproductive activity to thyroid hormones is strongly limited to the particular time of the year. The minimal effective duration of exposure to THs required for the transition to anestrus was estimated to be 60-90 days (Thrun et al. 1997). In thyroidectomized ewes, replacement of T4 beginning in late December was found to be the only time of year that THs were effective in seasonal changes of reproductive activity (Thrun et al. 1997). In our study we began the CSF collection in December and finished in March, when all ewes entered anestrus according to the decrease in progesterone levels. Interestingly, within this period we did not observe any significant fluctuations in TH concentrations in the CSF, but we found a significant decrease of TT3/TT4 and an increase of the rT3/ TT4 molar ratio compared with those in the blood plasma. Moreover, the correlation between rT3 and TT4 in the CSF, found in our studies, indicates that the amount of rT3 in the CSF is linked to T4 metabolism in the brain. This indicates that in ewes some local changes in the conversion of T4 to T3 or to rT3 may occur in a time-dependent fashion. It has been demonstrated that rT3 itself is a very effective competitive inhibitor of DIO2-mediated deiodination of T4 to T3 and has also been reported to be a physiological regulator of DIO2 activity (Kohrle 1999, Yasuo et al. 2005). Studies of Japanese quail have demonstrated that reciprocal expression of *DIO2* and *DIO3* genes in the mediobasal hypothalamus is critical for photoperiodically-induced gonadal growth (Yasuo et al. 2005). In the LD-breeding Siberian hamster, expression of *DIO3* in the ependymal layer lining the third ventricle is affected by photoperiod; it is present during SD and absent during LD exposure (Barrett et al. 2007). However, further studies are necessary to clarify whether changes in *DIO2* and *DIO3* expression or activity in the tanycytes provide a mechanism that accounts for temporal responsiveness of the neuroendocrine system to thyroid hormones in ewes.

The location of tanycytes at the blood-CSF interface, their endocytotic potential, and their high expression of *DIO2* and *DIO3* transcript raise the possibility that tanycytes extract T4 from the bloodstream of portal capillaries or capillaries in the arcuate nucleus through the end processes terminating on these vessels and from the CSF via apical specializations after T4 has traversed the choroid plexus. The products of tanycyte metabolism, which include the conversion of T4 to T3 and rT3, may be released back into the CSF, bloodstream, median eminence, and/or the adjacent arcuate nucleus, thereby providing a source of T3 for the CNS. T3 released into the CSF could diffuse into the parenchyma of the brain by volume transmission (Agnati et al. 1995). This has been postulated to be particularly important for providing T3 to hypophysiotrophic thyrotropin releasing hormone (TRH) neurons in the paraventricular nucleus (PVN), which do not posses DIO2 activity (Lechan and Fekete 2007). Also ependymocytes that express α and β isoforms of nuclear receptors to T3 (Graff et al. 1993) may utilize T3 present in high amounts as a free hormone in the CSF.

In summary, we present the first comprehensive study reporting the concentrations of total and free fractions of T4 and T3, as well as the total concentration of rT3 in the CSF of the third ventricle of ewes. The results demonstrate that, in ewes, seasonal

changes of TH concentrations in the CSF do not follow that observed in the blood plasma. The lack of significant changes in TH concentrations in the CSF during the period of transition to anestrus indicate that neither seasonal changes of THs circulating in the blood plasma (Dahl et al. 1994) nor THs circulating in the CSF actively drive the transition to anestrus. The extent to which changes in TT3/TT4 and rT3/TT4 molar ratios in CSF during this period are connected with temporal changes in thyroxine converting enzymes remains to be determined. In sheep, the CSF of the third ventricle may serve as a considerable source of thyroid hormones for the neuroendocrine events, thereby highlighting a possible role for the CSF as an active medium for neuroendocrine regulation (Skipor and Thiery 2008).

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