

Differential expression and seasonal modulation of VGF peptides in sheep pituitary

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Abstract

The inducible gene *vgf* and its peptide products are relevant to the neuroendocrine regulation of homeostasis and reproduction in rodents. We show here that in the anterior pituitary of female sheep the somatotrope, gonadotrope, and lactotrope/thyrotrope cell populations each expressed *vgf* mRNA, but displayed a distinct profile of VGF immunoreactive peptides. ProVGF C-terminus and VGF₄₄₃₋₅₈₈ immunoreactivities were found in lactotropes and thyrotropes, often in a subcellular location restricted to the Golgi area and suggestive of rapid peptide (or proVGF) release upon biosynthesis, while high molecular weight bands consistent with proVGF were shown in pituitary extracts. Distinct seasonal changes were revealed, proVGF C-terminus immunoreactive cells being largely identified as lactotropes during the summer ($83.7 \pm 2.1\%$ (mean \pm S.E.M.) versus $27.0 \pm 1.9\%$ during the winter), as opposed to thyrotropes during the winter

($73.0 \pm 1.9\%$ versus $16.3 \pm 2.1\%$ during the summer). Conversely, antisera to peptides adjacent to the VGF₅₅₃₋₅₅₅ 'Arg-Pro-Arg' cleavage site, and to the N-terminus of the proVGF-derived peptide V, selectively labeled gonadotropes, indicating processing to small peptides not retaining the proVGF C-terminus in such cells. Finally, a peptide related to the VGF₄₋₂₄₀ region was immunostained in somatotropes, shown in a Western blot as a band of relative molecular mass of approximately 16 000. In conclusion, a complex, endocrine cell-type-specific processing of proVGF was revealed. Further to the known inducibility of *vgf* mRNA upon a range of stimuli, discreet, selective modulations of VGF-peptide profile/s are suggested, possibly involved in specific neuro/endocrine or modulatory mechanisms.

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Introduction

The *vgf* gene (non-acronymic denomination) was discovered in PC12 rat pheochromocytoma cells because of its specific, 'delayed early' response to nerve growth factor (NGF) occurring at a later stage compared with immediate-early genes (Levi *et al.* 1985, Salton *et al.* 2000). *In vivo*, brain derived neurotrophic factor (BDNF) was also shown to modulate *vgf* gene expression (Salton *et al.* 2000, Alder *et al.* 2003). The relevant gene product is the proVGF polypeptide, or VGF protein, composed of 617 or 615 amino acids (in rat/mouse and in man respectively, with over 85% identity; Canu *et al.* 1997, Salton *et al.* 2000) and migrating as an approximately 90 000 relative molecular mass band in Western blots. Ten stretches of paired basic amino acid residues are conserved across the rat, mouse and human coding sequences (Salton *et al.* 2000) and represent potential cleavage sites.

Processing of proVGF to intermediate and small molecular weight products has been shown *in vivo* (Possenti *et al.* 1989, Trani *et al.* 1995, 2002), and an endogenous peptide corresponding to the C-terminal 30 amino acid segment of human proVGF has been identified from bovine posterior pituitary (peptide V; Liu *et al.* 1994). Peptides derived from the proVGF C-terminal region were shown to be preferentially released in response to secretory stimuli from PC12 cells (Possenti *et al.* 1989), cerebellar granule cells (Trani *et al.* 1995), and insulinoma INS-1 cells (Possenti *et al.* 1999). In addition, processing at the VGF₅₅₃₋₅₅₅ 'Arg-Pro-Arg' cleavage site has been shown, the resulting peptides displaying a TLQPP (Thr-Leu-Gln-Pro-Pro) sequence at their N-terminus (Trani *et al.* 2002).

In animal tissues, VGF immunoreactivity was found to be restricted to central and peripheral neurons (Van den Pol *et al.* 1989, 1994, Ferri *et al.* 1992), as well as endocrine cells of pituitary, adrenal medulla, gut and pancreas

(Ferri *et al.* 1992, 1995, Possenti *et al.* 1999). Distinct *vgf* gene induction was demonstrated in several models, such as in the mouse hypothalamic arcuate nucleus in response to fasting (Hahm *et al.* 2002), and in the suprachiasmatic nucleus in connection with light stimulation (Wisor & Takahashi 1997). In the female rat anterior pituitary, VGF immunoreactivity was apparently restricted to gonadotropes and lactotropes, with striking VGF peptide/s degranulation at estrus in association with an increase in *vgf* mRNA (Ferri *et al.* 1995). Deletion of the mouse VGF gene (knock-out) resulted in thin, small, hypermetabolic, hyperactive and relatively infertile animals (Hahm *et al.* 1999). VGF-deficient mice showed derangement of the hypothalamic outflow pathways that regulate peripheral metabolic tissues and energy homeostasis (Hahm *et al.* 2002), while the relevance of pituitary changes in connection with the observed reproductive alterations remains to be clarified (Hahm *et al.* 1999). Most recently, C-terminal proVGF-derived peptides were shown to induce penile erection upon injection into the rat paraventricular nucleus (Succu *et al.* 2004). On the whole, a potential role has been proposed for proVGF-derived peptide/s as modulators in neuroendocrine secretory mechanisms, and/or as released neuro/endocrine messengers themselves (Salton *et al.* 2000).

In view of the striking modulation of *vgf* mRNA and VGF peptides in rat pituitary over the estrous cycle (see above; Ferri *et al.* 1995), we decided to investigate the sheep pituitary in connection with the seasonal pituitary changes occurring in such seasonal breeding species. Since localization and modulation studies have so far been largely limited to peptides containing the C-terminal portion of proVGF, we developed and applied antisera to a range of further peptide stretches, mainly in connection with proVGF processing sites.

Materials and Methods

Animals and tissue samples

Samples were taken shortly *post-mortem* from female sheep of the Sardinian breed (aged 5–7 years, all groups) slaughtered for meat at a local abattoir during January–February ($n=7$), June–July ($n=10$), and October ($n=5$). Hypophyses were divided into antero-posterior, vertical slices (2–3 mm thick), immersion fixed in paraformaldehyde (40 g/l in 0.1 mol/l PO_4 buffer, pH 7.2, 0–4 °C for 3 h), then washed in phosphate buffered saline (PBS: 10 mol/l PO_4 , pH 7.2–7.4, 150 mmol/l NaCl) containing sucrose (200 mmol/l) and NaN_3 (3 mmol/l). Slices were oriented in aluminum foil moulds (one each, sectioned surface down) in embedding medium (PBS containing 65 g/l polyvinyl alcohol 56–98, 10 g/l Tween–20, 40 g/l polyethylene glycol 400, 15 mmol/l NaN_3 ; Cocco *et al.* 2003) and snap-frozen in melting freon, cooled with liquid nitrogen. Cryosections (at a 5–6 μm setting) were obtained

using a Microm HM-560 cold-blade cryomicrotome (Microm, Walldorf, Germany), and were collected on slides coated with either poly-L-lysine (relative molecular mass >500 000, 1 g/l, in distilled water; Sigma, Milan, Italy) for immunocytochemistry, or 3-aminopropyltriethoxysilane (90 mmol/l, in acetone; Sigma) for *in situ* hybridization, air dried (1–8 h), wrapped in aluminum foil and stored in the vapor phase of a liquid nitrogen tank until used.

For Western blot, one of the above pituitary slices (roughly 25–30% of the gland) was taken from each of 6 sheep (January–February, $n=3$; July, $n=3$) and chopped with a scalpel. Tissue was dropped into a pre-heated tube containing distilled water (approximately 10 ml/g tissue), kept in a vigorously boiling water bath for 15 min, and stored frozen. Upon addition of phenylmethylsulfonyl fluoride (5 mmol/l final concentration; Sigma), extracts were homogenized and briefly centrifuged (3000 g). Supernatants were heated in a boiling water bath (15 min), spun (12 000 g, 15 min), decanted and stored at –20 °C overnight upon addition of 6 volumes of an ethanol/methanol/acetone mixture (2/1/1 proportions, v/v). After centrifugation (12 000 g, 30 min), pellets were dissolved using 4 mol/l urea in 35 mmol/l sodium dodecyl sulfate (SDS). Total proteins were determined using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA).

Antisera

VGF antisera were raised against (i) the rat and human proVGF C-terminus, (ii) two internally cleaved VGF peptides, sequenced from bovine and rat tissues respectively (Liu *et al.* 1994, Trani *et al.* 2002) and (iii) three VGF-beta-galactosidase fusion proteins encompassing longer domains within the rat proVGF. The topography of such antigens within the proVGF precursor is shown in Fig. 1, while all antisera and antibodies used are listed in Table 1.

In the C-terminal region of proVGF, a single amino acid substitution is found at position –3 (from C-terminus; Fig. 1), with an arginine (Arg₆₁₃) in man, as opposed to a histidine (His₆₁₅) in rat and mouse (Salton *et al.* 2000). The relevant rat and human nonapeptides were synthesized (Affiniti-Biomol, Exeter, Devon, UK) and conjugated to bovine thyroglobulin via an additional N-terminal D-tyrosine for immunizations. The human VGF_{586–595} sequence corresponds to the N-terminal part of bovine peptide V (Liu *et al.* 1994), while the rat VGF_{556–565} peptide corresponds to the N-terminal portion of VGF fragment/s found in rat brain (Trani *et al.* 2002). For both such peptides, at least the N-terminal five amino acids are identical in the corresponding regions of human and rat proVGF. The relevant peptides were synthesized (Affiniti-Biomol), and conjugated to keyhole limpet hemocyanin (KLH) via an additional C-terminal cysteine, to expose their N-terminal region during immunizations. A fusion protein composed of a small, 21-amino acid

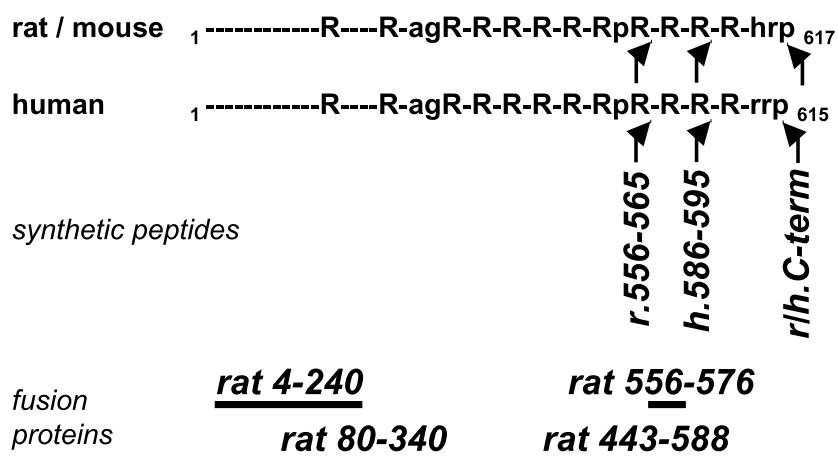


Figure 1 VGF peptides and fusion proteins used to raise antisera. Putative and confirmed processing sites within rat/mouse and human proVGF are indicated as R (2 or more Arg/Lys basic residues), RpR (rat Arg₅₅₃-Pro₅₅₄-Arg₅₅₅ processing site; Trani *et al.* 2002), or agR (putative processing-amidation site: rat Ala₃₀₉-Gly₃₁₀-Arg₃₁₁-Arg₃₁₂); the last 3 C-terminal amino acids are indicated in single-letter notation. Synthetic peptide antigens were conjugated (i) via N-terminal D-tyrosine to yield exposed C-termini, or (ii) via C-terminal cysteine to expose their N-termini during immunization (arrowheads are bent according to expected antiserum reactivity). r.556-565, rat VGF₅₅₆₋₅₆₅; h.586-595, human VGF₅₈₆₋₅₉₅; r/h.C-term, rat and human proVGF C-terminus.

VGF peptide (rat VGF₅₅₆₋₅₇₆, also named TLQP-21), plus glutathione S-transferase (GST, from *Schistosoma japonicum* species), was obtained using the pGEX-4T-3 plasmid vector (Amersham Pharmacia, Milan, Italy) and used for guinea-pig immunizations. The VGF-beta-galactosidase fusion protein antisera (against rat VGF₄₋₂₄₀,

rat VGF₈₀₋₃₄₀ and rat VGF₄₄₃₋₅₈₈) have been described previously in detail (Possenti *et al.* 1989, Ferri *et al.* 1992).

The hormone antisera and antibodies used (Table 1) showed negligible (<0.5%) cross-reactivity with other pituitary hormones or subunits in RIA, and resulted in consistent tissue labeling when compared in double/triple

Table 1 Antisera and antibodies used in the study

	Antigen	Species	Reference
VGF peptides			
Rat VGF ₆₀₉₋₆₁₇ *	<i>b. thyrogl</i> -dY-IEHVLLHRP	Rabbit	Ferri <i>et al.</i> 1995
Human VGF ₆₀₇₋₆₁₅ *	<i>b. thyrogl</i> -dY-IEHVLLRRP	Rabbit	This paper
Human VGF ₅₈₆₋₅₉₅	AQEEAEAEER-C-KLH	Guinea pig	This paper
Rat VGF ₅₅₆₋₅₆₅	TLQPPASSRR-C-KLH	Guinea pig	This paper
Rat VGF ₅₅₆₋₅₇₆	GST-rat VGF ₅₅₆₋₅₇₆	Guinea pig	This paper
Rat VGF ₄₋₂₄₀	<i>beta-galact</i> -rat VGF ₄₋₂₄₀	Rabbit	Possenti <i>et al.</i> 1989
Rat VGF ₈₀₋₃₄₀ **	<i>beta-galact</i> -rat VGF ₈₀₋₃₄₀	Rabbit	Ferri <i>et al.</i> 1992
Rat VGF ₄₄₃₋₅₈₈	<i>beta-galact</i> -rat VGF ₄₄₃₋₅₈₈	Rabbit	Ferri <i>et al.</i> 1992
Hormones			
ACTH	Human ACTH	Rabbit	NIDDK
ACTH	Human ACTH	Mouse	Berger <i>et al.</i> 1988
FSH	Human beta FSH	Mouse	Berger <i>et al.</i> 1988, 1990
FSH	Human beta FSH	Rabbit	NIDDK
GH	Human GH	Rabbit	NIDDK
GH	Rat GH	Monkey	NIDDK
LH	Human beta LH	Mouse	Berger <i>et al.</i> 1988, 1990
Prolactin	Bovine PRL	Mouse	Berger <i>et al.</i> 1988
TSH	Human TSH (whole molecule)	Guinea pig	NIDDK

b. thyrogl, bovine thyroglobulin; *beta-galact*, beta-galactosidase; *KLH*, keyhole limpet haemocyanin; *GST*, glutathione-S-transferase; amino acids in single-letter notation, dY= α -tyrosine; ACTH, adrenocorticotropin; *rat/human proVGF C-terminus; **mainly used for Western blot.

immunofluorescence, with no undue co-localization within the different pituitary cell types. Regarding gonadotropes, double labeling with luteinizing hormone (LH) and follicle-stimulating hormone (FSH) antisera/antibodies resulted in 65–70% double-labeled cells, fewer cells being only LH (about 20–25%) or only FSH immunoreactive (10–12%). Since the thyrotropin (TSH) antiserum was raised against whole TSH, it could contain alpha subunit antibodies, thus potentially also labeling gonadotropes. Such antiserum was revealed with the AMCA fluorochrome staining (blue, see below) and compared with a mixture of anti-beta-LH and anti-beta-FSH mouse antibodies (both revealed in green). Any pituitary cell labeled by the anti-LH/FSH mixture (green) was identified as a gonadotrope (whether or not it was also labeled with AMCA, i.e. blue), while blue-only cells were assumed to be thyrotropes.

Immunohistochemistry and labeled cell quantitation

Sections were soaked in Triton X-100 (0.1% in H₂O for 1 h; Merck, Milan, Italy), incubated overnight with anti-VGF antiserum/a (at room temperature), then with cyanine 3-18 (Cy3)-conjugated anti-guinea pig or anti-rabbit antibody, as relevant (1 h). All VGF antisera effectively immunostained the endocrine cells in sheep pituitary, except for the VGF_{80–340} antiserum, which resulted in weak labeling and was thus mainly used in Western blot. Antisera raised against comparatively short peptide stretches (rat VGF_{556–565} and human VGF_{586–595}, as well as the fusion protein encompassing rat VGF_{556–576}) appeared to react preferentially with the relevant, cleaved peptides (see below). In a few additional experiments we used far higher than usual concentrations of such antisera (up to tenfold) in order to test their reactivity with the proVGF precursor.

Endocrine cell populations were characterized on the basis of their hormonal content/s by double and triple immunofluorescence, including one or two anti-pituitary hormone antisera/antibodies in the primary incubation step. Secondary antibodies used (from donkey; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were conjugated with one of the following: (i) cyanine 3-18 (Cy3, emitting in yellow-red), (ii) 7-amino-4-methylcoumarin-3-acetic acid (AMCA, blue), (iii) either cyanine 2-18 (Cy2, green) or biotin, followed by Alexa 488-labeled avidin (green; Molecular Probes, Leiden, The Netherlands). All antisera and antibodies were diluted in PBS containing normal donkey and sheep sera (3% each), while conjugated avidin was dissolved in PBS containing 10 g/l bovine serum albumin. Absorption controls were carried out using the relevant peptides and fusion proteins (up to 50 nmol/ml), the homologous peptide resulting in virtual prevention of immunostaining, while unrelated peptides and carrier protein had no significant effect. Routine controls included substitution of each primary

antiserum, in turn, with non-immune serum or PBS. Preparations were observed using an Olympus BX60 fluorescence microscope equipped with a PM30 photographic system (Olympus, Milan, Italy) and a Fuji FinePix S2 PRO digital camera (Fujifilm, Milan, Italy).

As described below (see Results section), three patterns of immunolocalization were shown with the antisera to (i) C-terminus and VGF_{443–588}, (ii) VGF_{556–565}, VGF_{556–576} and VGF_{586–595}, and (iii) VGF_{4–240}. Hence, the C-terminus, VGF_{556–565}, and VGF_{4–240} antisera were used for quantitation, which was carried out on preparations double/triple immunostained for VGF and the relevant pituitary hormone/s. Pilot experiments were subjectively assessed by two independent observers in a blind fashion, and revealed distinct differences between summer and winter specimens with the C-terminus and VGF_{443–588} antisera, but not with the others. Quantitation was then carried out by a single observer, with labeled cells showing a visible (unlabeled) nuclear profile being counted on 3 non-adjacent sections each from either 13 sheep pituitaries for the C-terminus antiserum (summer and winter, $n=5$ each; autumn, $n=3$), or 4 sheep pituitaries for the VGF_{4–240} and VGF_{556–565} antisera (summer and winter, $n=2$ each). Data were expressed as means \pm S.E.M. throughout. Statistical analysis was carried out by one-way ANOVA, followed by post-hoc multiple comparison tests (StatistiXL software, www.statistXL.com).

In situ hybridization

An approximately 600 bp fragment of rat VGF cDNA (BamHI-XhoI 235–854 bp from the ATG of the coding sequence) was cloned in both orientations, downstream of the T7 polymerase promoter. Labeled antisense and sense probes were obtained by *in vitro* transcription of the linearized plasmids with T7 polymerase, using Megascript labeling kits (Ambion, Austin, TX, USA) and digoxigenin-11-UTP (Roche, Monza, Italy). The hybridization buffer contained 50% deionized formamide, 1% blocking solution (Roche), 5 mmol/l EDTA, 5 \times SSC, 0.1% Chaps (Sigma), 0.1 mg/ml heparin (Sigma), 1 mg/ml yeast total RNA, and 10 mg/ml denatured sheared herring sperm DNA (reagents from either Roche or Sigma, as relevant; see Moorman *et al.* 2001).

Sections were treated with Triton X-100 (0.1% in PBS), then digested with proteinase K (1 μ g/ml, in PBS containing 1 mmol/l EDTA, for 20 min; ICN, Milan, Italy) followed by treatment with glycine (25 mmol/l, in PBS for 5 min) and post-fixation with paraformaldehyde (4% in PBS for 5 min). After a pre-incubation step with hybridization buffer (at 70 °C for 1 h), preparations were hybridized overnight with the relevant probe dissolved in hybridization buffer (at 70 °C). After RNase digestion (100 μ g/ml; from ICN) and stringency washes (at 70 °C; 2 \times SSC, 2 \times 10 min; 1 \times SSC, 0.5 \times SSC, 0.25 \times SSC, 10 min each; 1 \times SSC=150 mmol/l NaCl, 15 mmol/l

sodium citrate, pH 7.2), the bound probe was revealed using alkaline phosphatase-labeled anti-digoxigenin Fab fragment antibodies (Roche) diluted in AP1 buffer (1 mol/l Tris-HCl, pH 7.6, containing 5 mol/l NaCl, 0.5 mol/l MgCl₂ and 30 g/l bovine serum albumin). Overnight development was carried out using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium kit, with inclusion of levamisole, according to the kit's instructions (Vector, Burlingame, CA, USA). Controls included incubation with sense probe, probe omission, as well as pre-digestion of sections with RNase (100 µg/ml, 37 °C for 1 h). Preparations were observed as described for immunohistochemistry.

Western blot

Extracted proteins (approximately 20 µg) were loaded onto gels, and NuPAGE 10% bis/tris gels were run and blotted with XCell SureLock, according to the procedure's protocol (NuPAGE System, Invitrogen, San Giuliano Milanese, Italy). The relevant peptides and fusion proteins were effectively used for preabsorption controls with each of the VGF antisera (up to about 50 nmol/ml). In view of the limited retention of low molecular weight peptides in Western blot, pilot runs were carried out loading rat VGF₅₅₆₋₅₇₆ (21 amino acid, or TLQP-21), or synthetic rat VGF₅₈₆₋₆₁₇ (30 amino acid rat sequence, corresponding to bovine peptide V; Affiniti-Biomol). As expected, such small peptides were scarcely retained and resulted in no detectable signal with the relevant antisera. On this basis, and in view of the immunocytochemical findings, use of the VGF₅₅₆₋₅₆₅, VGF₅₅₆₋₅₇₆ and VGF₅₈₆₋₅₉₅ antisera in Western blot was not pursued further.

Results

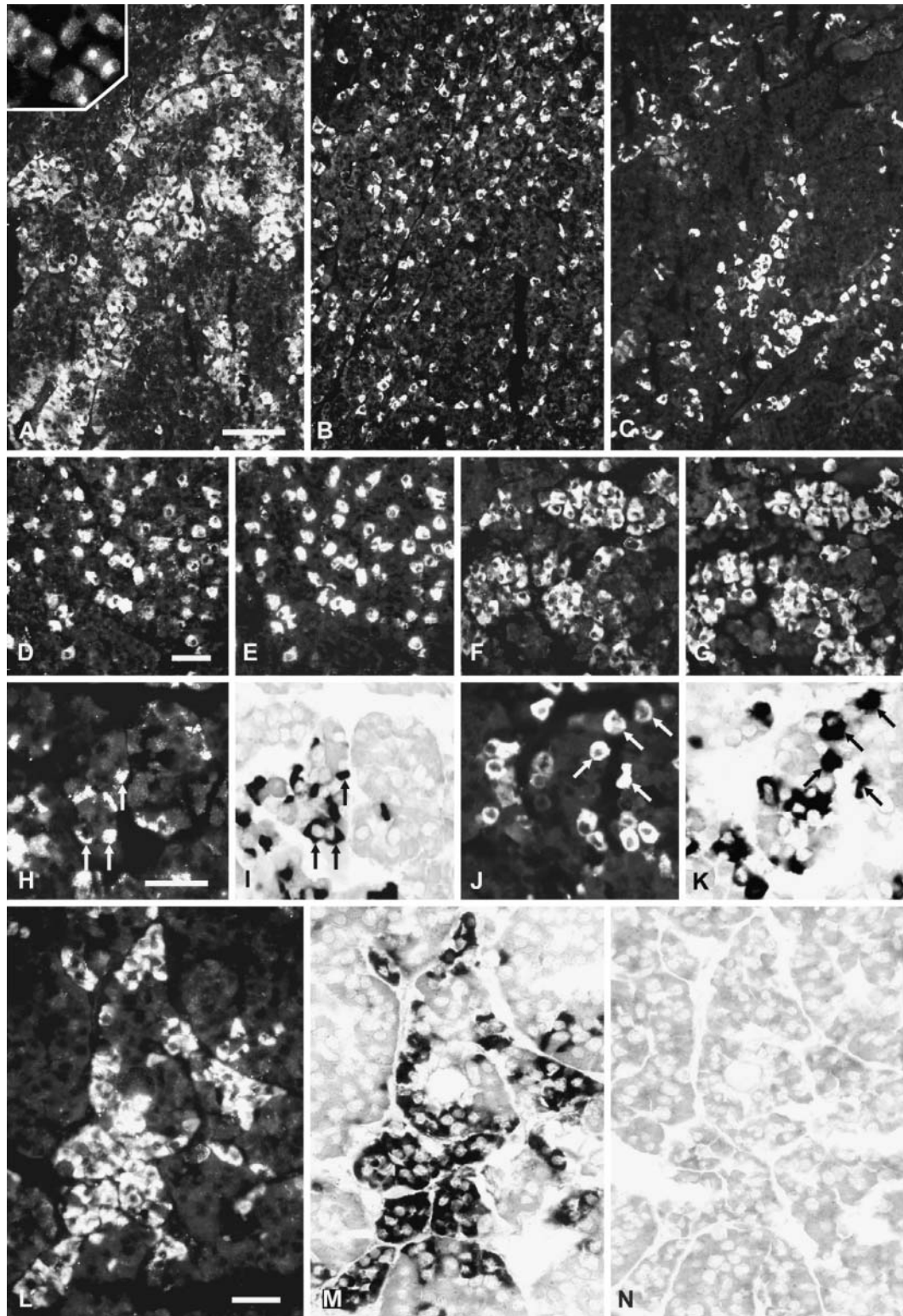
Using the various VGF antisera, endocrine cells were labeled in varied numbers and intensities in female sheep anterior pituitary. The rat C-terminus (rat VGF₆₀₉₋₆₁₇) antiserum resulted in sparse labeling, while the one raised against the corresponding human sequence (human VGF₆₀₇₋₆₁₅) showed reproducible, bright immunostaining and was henceforth used. When the distribution of VGF immunoreactivity was studied systematically, three main differential patterns became apparent (Fig. 2A-C). The human proVGF C-terminus (VGF₆₀₇₋₆₁₅, Fig. 2A) and VGF₄₄₃₋₅₈₈ antisera mainly decorated strands of pituitary endocrine cells, often distinctly granular in appearance. At close inspection, many such cells showed a sharply localized area of intracellular labeling, close to the cell nucleus and identified as the *bona fide* Golgi area (Fig. 2A, insert). Conversely, antisera raised against sequences adjacent to the VGF₅₅₃₋₅₅₅ 'Arg-Pro-Arg' internal cleavage site (rat VGF₅₅₆₋₅₆₅, Fig. 2B, and rat VGF₅₅₆₋₅₇₆ antisera), and to

the N-terminus of peptide V (anti-human VGF₅₈₆₋₅₉₅) revealed numerous, singly distributed endocrine cells of medium size and with moderate to bright intensity. Finally, immunoreactivity for the VGF₄₋₂₄₀ fusion protein was largely found in scattered groups and clusters of cells, of fairly consistent labeling intensity (Fig. 2C).

In double and triple immunolabeling experiments, the above patterns were linked to specific endocrine cell populations. Labeling with the proVGF C-terminus (VGF₆₀₇₋₆₁₅) and VGF₄₄₃₋₅₈₈ antisera was co-localized with prolactin and/or TSH (Fig. 3A-H), but not with the other hormones tested. When samples taken at different times over the year were compared, proVGF C-terminus immunoreactive cells were largely identified as lactotropes during the summer, with the addition of a fraction of thyrotropes, while an almost reverse pattern was found in winter (Fig. 4, upper panel). Most of such changes could be accounted for by an increase in VGF peptide-expressing lactotropes during summer and autumn (40–50%, as opposed to about 15% during winter; Fig. 4, lower panel), while the proportion of proVGF C-terminus immunoreactive thyrotropes showed a less evident increase from summer and autumn to winter (Fig. 4, lower panel). Conversely, cleavage-site-related peptides (VGF₅₅₆₋₅₆₅, VGF₅₈₆₋₅₉₅ and VGF₅₅₆₋₅₇₆ antisera) were detected in most gonadotropes, co-localized with LH (Fig. 2D,E, Fig. 4, lower panel) and/or FSH, but not in other pituitary endocrine cell types. Finally, VGF₄₋₂₄₀ peptide immunoreactivity was revealed in the vast majority of somatotropes (Fig. 2F,G, Fig. 4, lower panel), and in a small fraction of corticotropes (3–5%; not shown), but did not colocalize with LH/FSH, prolactin or TSH. No distinct seasonal changes were revealed for VGF₅₅₆₋₅₆₅, VGF₅₅₆₋₅₇₆, VGF₅₈₆₋₅₉₅, or VGF₄₋₂₄₀ immunoreactivity.

As mentioned, absorption controls confirmed specificity of the immunostaining obtained with the various VGF antisera. Antisera generated against peptides adjacent to proVGF cleavage sites (rat VGF₅₅₆₋₅₆₅, rat VGF₅₅₆₋₅₇₆ and human VGF₅₈₆₋₅₉₅) were tested further. When these were used under standard conditions, i.e. at dilutions which reliably provided bright labeling and high signal-to-noise ratio, they all resulted in immunolabeling completely separate from that obtained with the other VGF antisera. Conversely, when the same antisera were applied at 5–10 times higher concentrations, co-labeling with the proVGF C-terminus antiserum was shown in many endocrine cells, suggestive of a low-key, concentration-dependent reactivity with the proVGF precursor (not shown).

In *in situ* hybridization experiments, *vgf* mRNA was revealed in a large number of pituitary endocrine cells. When studied in parallel with the relevant VGF peptides by immunostaining of adjacent sections, *vgf* mRNA was consistently revealed in most growth hormone (GH) cells (Fig. 2L,M). Labeling intensity for the same mRNA varied in VGF peptide immunoreactive gonadotropes,



lactotropes and thyrotropes (Fig. 2H–K), each showing intensely labeled, faint and hardly labeled cells within the same pituitary, in virtually all animals. When possible seasonal changes were addressed, the large number of diverse *vfg* mRNA-containing cells and their varied labeling extent made it hardly possible to reveal clear cut differences. RNase and sense probe preparations were devoid of localized signal (Fig. 2N).

In Western blot, the proVGF C-terminus (VGF_{607–615}) and VGF_{443–588} antisera mostly showed large forms of approximately 90 000 relative molecular mass (Fig. 3I, lanes 1–4), in agreement with the expected migration of the human proVGF precursor. Only minor amounts of additional, low relative molecular mass bands down to approximately 20 000 were revealed with the VGF_{443–588} antiserum (Fig. 3I, lanes 1,2). A single, approximately 16 000 relative molecular mass band was revealed using the VGF_{4–240}, or VGF_{80–340} antisera (Fig. 3I, lanes 5–8), with minor reactivity in the high molecular mass range corresponding to proVGF.

Discussion

On the basis of the present study, *vfg* mRNA and its derived peptide/s are well represented in sheep pituitary. In lactotropes and thyrotropes, proVGF precursor appeared to be the major intracellular product, as shown by the results obtained with proVGF C-terminus and VGF_{443–588} antibodies in both Western blot and immunohistochemistry. Striking seasonal changes were revealed, the vast majority of proVGF C-terminus (and VGF_{443–588}) immunoreactive cells being identified as lactotropes during the summer, and as thyrotropes during the winter. Conversely, processing to smaller peptides appeared to occur in gonadotropes, as shown by labeling for peptides cleaved at the VGF_{553–555} 'Arg-Pro-Arg' site, as well as for the N-terminus of peptide V, while proVGF C-terminus antibodies were negative. A novel VGF peptide was shown in somatotropes, of approximately 16 000 relative molecular mass in Western blots. In view of its reactivity with both VGF_{4–240} and VGF_{80–340} antisera, such a peptide band was probably related to the corresponding VGF_{80–240} overlap region. No apparent seasonal changes were revealed for VGF peptides contained in either

gonadotropes, or somatotropes. Regarding corticotropes, a minor fraction (3–5%) was labeled by VGF_{4–240} antibodies, while all other VGF antisera used were negative.

Thus, selective, differential VGF peptide profiles were displayed by the major hypophyseal endocrine cell types, with the virtual exception of corticotropes. The latter 'negative' finding, however, ought to be interpreted with caution, since several regions of proVGF have been little studied. In addition, a few further VGF-derived peptides have been identified, but would not be recognized by the antisera we used. These include proVGF N-terminus and VGF_{378–397}-related peptides identified from the human cerebrospinal fluid (Stark *et al.* 2001, Carrette *et al.* 2003). Furthermore, the sheep *vfg* gene has not yet been sequenced, hence its coding region might differ from the highly conserved sequence found in rat, mouse and human genes (Salton *et al.* 2000). At the proVGF C-terminus, a single amino acid substitution is shown at position –3, an arginine (Arg₅₁₃) being found in human proVGF as opposed to a histidine (His₅₁₅) in rat/mouse (Salton *et al.* 2000). The far better reactivity that we found with human VGF_{607–615} antibodies, as opposed to those raised against the corresponding rat VGF_{609–617} peptide, suggests that the sheep VGF precursor may be closely related to human proVGF in such a region. The latter observation is in keeping with the reported sequence of peptide V, which was isolated from the bovine pituitary and proved identical to the C-terminal 30 amino acid domain of human proVGF (Liu *et al.* 1994). In several rat neuroendocrine tissues, both VGF_{4–240} and VGF_{80–340} antisera revealed bands of relative molecular mass of approximately 90 000 in Western blots, corresponding to proVGF (Posenti *et al.* 1989, Trani *et al.* 1995). As shown for the human gene (Salton *et al.* 2000), amino acid substitutions might be present in the comparatively N-terminal portions of sheep proVGF compared with the rat sequence, and could explain the low reactivity of the above antisera with sheep proVGF.

Proteolytic precursor processing in neuroendocrine cells largely occurs in secretory granules, via endoproteases that act upon recognition of specific stretches of basic amino acid residues and their surrounding sequence/s. The best characterized prohormone processing enzymes acting on proVGF are PC1/3 and PC2 (Trani *et al.* 2002). These are expressed in endocrine and neuroendocrine cells, being

Figure 2 VGF immunohistochemistry and *in situ* hybridization. (A–C) The distinct differential localization shown using different VGF antisera. (A) The proVGF C-terminus antiserum (VGF_{607–615}) revealed multiple strands of pituitary endocrine cells, often with a localized area of labeling identified as the *bona fide* Golgi region (insert). (B) Peptides adjacent to VGF cleavage sites (here VGF_{556–565}) were brightly immunostained in single, scattered cells. (C) The VGF_{4–240} antiserum mainly decorated groups and clusters of endocrine cells. Consecutive sections, immunofluorescence with Cy3-labeling; scale bar=100 µm, except for insert where the same bar =30 µm. (D–G) Cleaved VGF peptide immunoreactivity was selectively localized in gonadotropes (D, VGF_{586–595} antiserum, versus E, LH), while the VGF_{4–240} antiserum (F) largely labeled somatotropes (G, GH). Double immunofluorescence; scale bar=30 µm. (H–N) *vfg* gene expression versus VGF peptides. *vfg* mRNA labeling intensity varied in VGF-immunoreactive lactotropes and thyrotropes (H, VGF_{607–615} antiserum versus I, *vfg* mRNA), or gonadotropes (J, VGF_{556–565}, versus K, *vfg* mRNA), while it was fairly consistent in somatotropes (L, VGF_{4–240}, versus M, *vfg* mRNA). N, sense-probe control. Adjacent serial sections, indirect immunofluorescence and *in situ* hybridization; scale bars=20 µm.

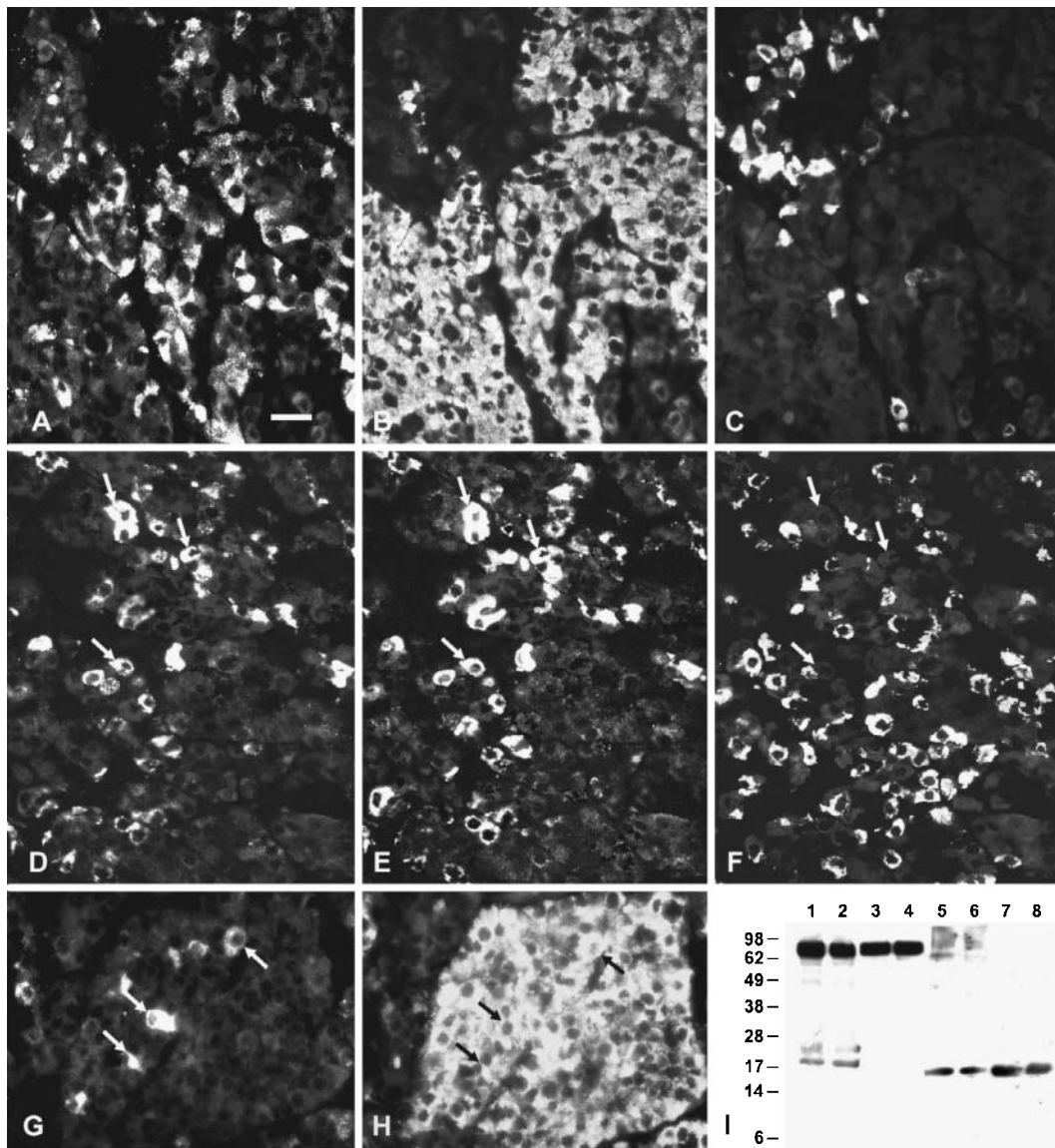


Figure 3 Seasonal changes in VGF localization and Western blots. (A-C) During the summer, proVGF C-terminus immunoreactive cells were mostly identified as lactotropes (A, VGF₆₀₇₋₆₁₅, versus B, prolactin), while only a few were thyrotropes (A, VGF₆₀₇₋₆₁₅, versus C, TSH). (D-H) During the winter, the same VGF peptide/s were largely found in thyrotropes (D, VGF₆₀₇₋₆₁₅, versus E, TSH; F, LH, for comparison), with the addition of a small number of lactotropes (G, VGF₆₀₇₋₆₁₅, versus H, prolactin). Triple/double immunofluorescence; scale bar=30 μ m. (I) In Western blot, VGF₄₄₃₋₅₈₈ and C-terminus antisera (lanes 1, 2 and 3, 4 respectively) showed high molecular mass bands, compatible with the proVGF precursor, only minor amounts of low molecular mass forms were revealed by the former antiserum. VGF₄₋₂₄₀ and VGF₈₀₋₃₄₀ antisera (lanes 5, 6, and 7, 8 respectively) revealed an approximately 16 000 relative molecular mass band, while scarcely reacting with proVGF. Extracts from two sheep pituitaries, taken during the summer (lanes 1, 3, 5, 7), and winter (lanes 2, 4, 6, 8) are shown.

especially abundant within the pituitary in corticotropes, gonadotropes and thyrotropes (Takumi *et al.* 1998). PC2 and PC1 convertases were localized to different secretory granules in rat gonadotropes (Uehara *et al.* 2001). Our findings suggest that sheep gonadotropes may be similarly provided with the latter convertases. In the rat, the

secretory granule protein chromogranin A and its derived beta-granin and WE-14 peptide fluctuated in FSH- and LH-containing gonadotropes over the estrous cycle (McVicar *et al.* 2003), in keeping with the similar modulation we observed for C-terminus VGF immunoreactivity in the same species (Ferri *et al.* 1995). In addition

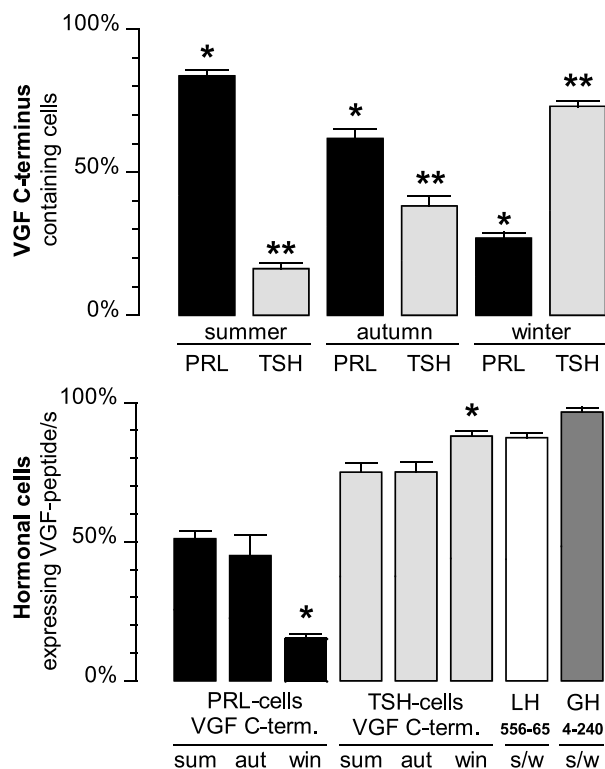


Figure 4 Quantitation of VGF immunoreactive cells. (Upper panel) Cells labeled by the proVGF C-terminus antiserum were mostly identified as lactotropes during the summer, as opposed to thyrotropes during the winter (PRL, lactotropes; TSH, thyrotropes; * or **, $P < 0.001$, summer versus autumn/winter, and autumn versus winter). (Lower panel) Expression of immunoreactive VGF peptides in the main pituitary endocrine cells types (PRL, lactotropes; TSH, thyrotropes; LH, gonadotropes; GH, somatotropes; VGF C-term., 556–65, 4–240: VGF_{607–615}, VGF_{556–565} and VGF_{4–240} immunoreactive peptides respectively; sum, summer; aut, autumn; win, winter; s/w, average summer and winter; * $P < 0.05$, winter versus summer/autumn).

to pituitary gonadotropes, proVGF C-terminus antisera effectively revealed VGF cleaved peptides in various other rat tissues (Trani *et al.* 1995), and permitted the determination of proVGF processing by PC1/3 and PC2 convertases (Trani *et al.* 2002). In addition, processing was demonstrated at the Arg₅₅₃-Pro₅₅₄-Arg₅₅₅ site (Trani *et al.* 2002), resulting in the production of peptide/s displaying a TLQPP sequence at their N-terminus. Our findings with TLQPP-related antisera (VGF_{556–565} and VGF_{556–576}), as well as with the antiserum to the N-terminus of bovine peptide V (VGF_{586–595}; Liu *et al.* 1994) suggest that the relevant peptides are produced in sheep gonadotropes. Pending their precise identification, such TLQPP-containing and peptide V-related peptides appear not to extend all the way to the proVGF C-terminus, although they could not be studied further by Western blot due to their comparatively small size.

The seasonal changes revealed for proVGF-related peptide/s across sheep lactotropes and thyrotropes may be related to the known seasonal modulation of prolactin, as well as of TSH and thyroid hormone secretion. In sheep, lactotrope cells are increasingly stimulated from winter to summer, serum prolactin being higher during long days (from spring to summer) and lower during short days (from autumn to winter; Pelletier 1973, Ravault 1976). In the same species, the pituitary pars tuberalis is probably involved in stimulating prolactin secretion (Graham *et al.* 2002), while prolactin may be involved in seasonal inhibition of gonadotropin release via a paracrine intra-pituitary mechanism (Tortonese *et al.* 1998), probably also involving dopamine (Gregory *et al.* 2004). Conversely, thyrotropes can be stimulated in response to reduced ambient luminosity, in apparent connection with a higher need for heat production via increased thyroid hormone release during the winter months (Hassi *et al.* 2001). Thyroid hormones show seasonal modulation in sheep, and are necessary during an interval late in the breeding season to promote seasonal reproductive suppression (Thrun *et al.* 1997). In the present study, we used samples from slaughterhouse sheep of the Sardinian breed, which were of undetermined reproductive state – they probably included cyclic as well as lactating animals, as seasonally appropriate. In sheep of the Sardinian breed, ovarian activity increases in connection with decreasing day length, with the highest sexual activity in adult animals occurring during November and the lowest in April (Manunta & Casu 1968). Recent parturition, or the presence of males also affect cyclical activity (Cappai *et al.* 1984). Seasonal modulation of prolactin secretion has been confirmed in the Sardinian breed of sheep (Carcangiu *et al.* 1995), while TSH has been little studied.

Further to seasonal modulation, restriction of proVGF C-terminus (and VGF_{443–588}) immunostaining to the Golgi area in many lactotropes may suggest stimulated biosynthesis and release of proVGF, and/or of other, as yet unknown, VGF peptide/s. A similar picture was shown in rat pituitary in the morning following the estrous peak secretion of LH, both in gonadotropes degranulated of their previous VGF content, as well as in lactotropes, in parallel with raised pituitary *vgf* mRNA levels (Ferri *et al.* 1995). It is conceivable that proVGF and/or certain of its product/s are involved in secretory mechanisms operating under conditions of enhanced stimulation, in which circumstances they undergo almost complete release from their cells of expression. Further to a possible involvement in hormonal release mechanisms, VGF peptides themselves could act as endocrine messengers, proVGF possibly representing a multifunctional precursor (Salton *et al.* 2000). Furthermore, paracrine roles ought to be considered for VGF peptides, as appears to be the case for certain pro-opiomelanocortin-derived and several other peptides (Denef & van Bael 1998).

In the rat, VGF is also well represented in several hypothalamic nuclei, including the suprachiasmatic, supraoptic, paraventricular and arcuate nuclei (Van den Pol *et al.* 1989, 1994). In the hamster suprachiasmatic nucleus, *vfg* mRNA is induced by light in the caudal portion, with delayed kinetics, while showing circadian rhythmicity in the rostral portion (Wisor & Takahashi 1997). While little is known of comparable VGF responses in farm animals, including sheep, the latter finding may be relevant to seasonal modulatory and reproductive mechanisms. A specific investigation of the sheep's pituitary pars tuberalis may also be of interest, in view of its involvement in seasonal rhythmicity (Lincoln *et al.* 2003). As to the regulation of VGF peptide/s expression, the *vfg* gene was originally recognised due to its responsiveness to NGF in PC12 cells (Levi *et al.* 1985), hence it is relevant to note that both NGF and its receptors are found not only in many areas of the nervous system, but also in the pituitary (Patterson & Childs 1994). In different tissues and species, however, *vfg* and its products may also respond to BDNF (Alder *et al.* 2003) and to a range of other factors (Salton *et al.* 2000).

In conclusion, proVGF appears to be widely expressed, but differentially processed, in most pituitary cell types. *In vivo* and *in vitro* release studies, as well as precise identification of VGF peptide products, will enable their testing for endocrine and/or local, paracrine regulatory roles. In parallel, it will be relevant to address the regulation of VGF peptide expression and processing in pituitary endocrine cells, primarily by hypothalamic releasing/inhibiting hormones, as well as by several neurotrophins and related factors.

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