0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(3):1415–1418

Presence of Functional Oxytocin Receptors in Cultured Human Myoblasts

CHRISTOPHE BRETON, CHRISTINE HAENGGELI, CLAUDE BARBERIS, FREDDY HEITZ, CHARLES R. BADER, LAURENT BERNHEIM AND ELIANE TRIBOLLET

Département de Biologie Animale, Université de Lilles I, 59650 Villeneuve d'Ascq Cedex, France (CB), Département de Physiologie, Centre Médical Universitaire, 1 rue Michel Servet, 1211 Geneva 4, Switzerland (CH, FH, LB, ET), INSERM, U.469, CCIPE, 141 rue de la Cardonille, 34094 Montpellier Cedex 5, France (CB), Division de Recherche Clinique Neuro-Musculaire, Département des Neurosciences Cliniques et Dermatologie, Hôpital Cantonal Universitaire, 24 rue Micheli-du-Crest, 1211 Geneva 14, Switzerland (CRB)

ABSTRACT

In the present report, we provide for the first time evidence that functional oxytocin receptors (OTRs) are present in human myoblasts obtained from clonal cultures of postnatal satellite cells. First, binding studies performed with a non selective vasopressin (AVP) and oxytocin (OT) radioligand indicated the presence of a single class of binding sites. Second, OTR mRNA was detected by RT-PCR analysis whereas transcripts for AVP V_{1a}, V_{1b} or V_2 receptors ($V_{1a}R$, $V_{1b}R$ and V_2R respectively) were not detected. Third, the presence of functionnal OTRs was evidenced by showing that agonist substances having a high affinity for the human OTR, namely OT, AVP and [Thr_4,Gly_7]OT, increased the rate of myoblasts fusion and myotubes formation in the cultures, whereas F180, a $V_{1a}R$ selective agonist, and dDAVP, a V_2R agonist had no significant effect on the fusion process. In addition, we show by RT-PCR and immunocytochemistry that the OT gene is expressed in cultured myoblasts. Taken together, our data suggest that OT may act as a paracrine/autocrine agent that stimulates the fusion of human myoblasts *in vitro*. *In vivo*, OT may be involved in the differenciation of human skeletal muscle during postnatal growth, and possibly its regeneration following injury.

Growth-promoting effects of neurohypophyseal hormones have been reported in a variety of peripheral cell types (1). Vasopressin (AVP) was the first to be described as an *in vivo* and *in vitro* mitogenic factor, presumably acting via the AVP V_{1a} receptor ($V_{1a}R$). Over the past years, an increasing number of studies have also demonstrated a growth-regulating effect of OT mediated by the OT receptor (OTR) in different cell types, among which myoepithelial cells in the mammary gland (2), uterine smooth muscle cells (3), vascular endothelial cells (4, 5), osteoblasts (6), trophoblast and choriocarcinoma cell lines (7). Concerning skeletal muscle, AVP was shown to increase the differentiation and the rate of fusion of rat L6 myoblasts and primary mouse satellite cells whereas it did not affect their proliferation (8). Pharmacological experiments suggested the presence of V_{1a}Rs in rat L6 and mouse myoblasts (8, 9). In addition, AVP-like immunoreactivity was evidenced in the embryonic human muscle (10). In the present report, we describe results obtained with clonal cultures of human myoblasts, derived from isolated postnatal satellite cells. We show that OTR is the unique receptor subtype expressed in this preparation, that its activation by agonist binding stimulates the rate of myoblasts fusion, and that the OT gene is also expressed by cultured myoblasts.

Materials and Methods

Preparation of clonal cultures. Satellite cells were isolated from samples of human skeletal muscles as described previously (11). Single satellite cells were grown in a proliferating medium consisting of F10 nutrient medium supplemented with 15% FCS, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, 10 ng/ml epidermal growth factor, 0.39 mg/ml dexamethasone, 0.18 mg/ml insulin and 0.1 μg/ml gentamicin. When myoblasts reached confluency, fusion could be induced by substituting the proliferation medium with a low nutrient medium consisting of DMEM supplemented with 0.5 mg/ml BSA, 10 ng/ml epidermal growth factor, 10 μg/ml insulin and 1 μg/ml gentamicin. In these conditions, approximately 70% of the myoblasts will eventually fuse to form multinucleated myoblasts, irrespective of the time spent in differentiation medium.

Binding assays. Dose-dependent binding assays were performed on living cultures with the linear antagonist HO-Phenylacetyl^1-D-Tyr(Me)^2-Phe^2-Gly^4-Asn^5-Arg^6-Pro^7-Arg^8-NH_2 (HO-LVA, table 1) radioiodinated at position 1 to a specific activity of 2000 Ci/mmol as previously described (15). Preliminary experiments conducted on myoblasts at different stages of differentiation showed no major differences in the amount of ^{125}I-HO-LVA binding. Consequently, all experiments were conducted in 35 mm culture dishes on confluent myoblasts non-exposed to differentiation medium. Cultures were first rinsed (5 min and 10 min) with 170 mM Tris-HCl buffer (pH 7.4) supplemented with 1mg/ml BSA, then incubated for 1 h at room temperature with a medium (170 mM Tris-HCl (pH 7.4), 1 mg/ml BSA, 0.25 mg/ml bacitracin, 1 mM $MgCl_2$, 1 M tyrosine, 1 M phenylalanine) containing ^{125}I-HO-LVA at concentrations ranging from 0.5 pM to 1 nM. Non-specific binding was determined in the presence of 1 μM non radioactive OT or AVP. Cells were rinsed by two 5-min washes in ice-cold incubation medium and solubilized with 800 μl/dish of 0.1 N NaOH. Radioactivity in the extracts was determined by scintillation counting. Three separate experiments were conducted on myoblasts from 3 different clones. Results were analyzed with a non-linear model fitting program (Ligand, Biosoft, Cambridge, Cambs, UK).

Corresponding author: Dr. Eliane Tribollet
E-mail: eliane.tribollet@medecine.unige.ch
Received 10/24/01. Accepted 11/30/01.

Table 1. Affinities (Ki in nM) of ligands used in this study for human OT and AVP receptors.

	OTR	$V_{1a}R$	$V_{1b}R$	V_2R
OT	0.79[a]	64[b]	1782[b]	167[b]
AVP	1.65[a]	1.7[b]	1.1[b]	1.1[b]
[Thr^{4}Gly^7]OT	13	305	>·10000	>10000
F-180	800[c]	5.8[c]	3800[c]	>10000[c]
dDAVP	89[b]	21[b]	22[b]	2.7[b]
HO-LVA	0.4[b]	0.5[b]	2.2[b]	428[b]

Values were obtained using CHO cells transfected with the 4 receptor subtypes. They are taken from refs 12 (a), 13 (b) and 14 (c), and from C. Barberis (unpublished data).

RT-PCR analysis. Total mRNA was extracted from myoblasts at confluency, non-exposed to differentiation medium, by using the Trizol reagent. RNA from Chinese hamster ovary (CHO) cells expressing the human OT, V_{1a}, V_{1b} and V_2 receptors from transfected plasmid, and RNA from human uterus tissue (kindly provided by HH Zingg, Montreal) served as control. cDNA was synthetized as previously described (15). Primers used had the following sequences:

OTR forward, 5'-GGGCGCGTGGCCCTGGCGCGTGTCA-3'
OTR reverse, 5'-CGTGGATGGCTGGGAGCAGCTCCTCTG-3'
$V_{1a}R$ forward, 5'-GTCGCGCCAGAGCAAGGGTGCAGAGC-3'
$V_{1a}R$ reverse, 5'-GAA GATTTAGGCGAGTCCTTCCACAT-3'
V_2R forward, 5'-CCACCATGCTCATGGCGTCCACCACT-3'
V_2R reverse, 5'-GAAGCGGTCGGTGGC CTTCCAGGC CA-3'
$V_{1b}R$ forward, 5'-CGGGTCAGCAGCATCAACACCATCTCA-3'
$V_{1b}R$ reverse, 5'-GGGCCGCGGTAACAG GTG GCTGTTGA-3'
OT forward, 5'-CCGGACCTCGACGTGCGCAAGTG-3'
OT reverse, 5'-GGCAGGTAGTTCTCC TCCTGCAGCG-3'.

The predicted size of the amplification products of OTR, $V_{1a}R$, V_2R and $V_{1b}R$ primers pairs were 408, 468, 319, and 254 bp, respectively, covering amino acids 252-388 of OTR, 252-407 of $V_{1a}R$, 1-105 of V_2R and 264-359 of $V_{1b}R$ (17). For OT peptide amplification, the primer pair was designed to amplify a 146 bp cDNA fragment corresponding to residues 34-82 (17).

Effect of OT, AVP and related compounds on myoblasts fusion. Confluent myoblasts received differentiation medium supplemented or not with OT, AVP, and related agonist or antagonist compounds at concentrations ranging between 10-200 nM. Preliminary experiments showed that none of these compounds influenced the final steady-state fusion. Therefore, the degree of fusion was evaluated during the fusion process, 20-48 hours following transfer of the cells in the differentiation medium. To this end, cultures were fixed 5 min in 100% ethanol, stained with Haematoxylin for 10 min and the fusion index was calculated by dividing the number of nuclei located within myotubes by the total number of nuclei (18). At least 2 dishes per condition were quantified. For each dish, nuclei were counted in 10 randomly chosen microscopic fields at magnification 240X (one field contains between 50 and 150 nuclei). Cells were considered to be fused only if cytoplasmic continuity was evident. Altogether, 16 experiments were performed and 12 clones examined. The affinities for the human OTR and AVPRs of OT, AVP and of the other substances used are described in Table 1. Results are expressed as means ± S.E.M. Student's t test was used to compare data obtained in control and treated cultures. Differences between values were considered to be significant when $P < 0.01$ level.

Immunocytochemistry. Confluent myoblasts non-exposed to differentiation medium were fixed 1h in 4% paraformaldehyde dissolved in 0.1M phosphate buffer (PBS; pH 7.4), washed 20 min in PBS, and incubated overnight at 4°C with a rabbit polyclonal antibody recognizing both OT- and AVP-associated neurophysins (AB948, Chemicon), diluted 1/500 in PBS containing 0.3% Triton X-100 and 0.1 M L-Lysine. Following 2 washings of 10 min in PBS, cells were further incubated for 2h at room temperature with a Cy3-conjugated donkey anti-rabbit antibody (n° 711-155-252, Jackson) diluted at 1/200. Three different clones were tested.

Chemicals. F-10, DMEM, gentamicin and Trizol were purchase from Gibco BRL (Gaithersburg, MD); FCS from Ready System (Zurzach, Switzerland) or Inotech (Dottikon, Switzerland); BSA, dexamethasone, fetuin and insulin from Sigma; Epidermal growth factor from Inotech; OT, AVP, [Thr^4,Gly^7]OT and dDAVP from Bachem (Budendorf, Switzerland). HO-LVA was a gift from Dr. M. Manning (Toledo, OH, USA) and F-180 was provided to us by Ferring Research (San Diego, CA, USA).

Results

Saturation studies performed with ^{125}I-HO-LVA demonstrated the presence of high affinity binding sites in human myoblasts (Fig. 1). Experiments were performed with 3 different clones, yielding a mean dissociation constant (K_d) of 319 ± 80 pM and a mean maximal binding capacity (B_{max}) of 55 ± 19 fmoles/dish. The Hill coefficient was 0.99 to 1.00 which is consistent with the presence of a single class of binding sites. Each dish contained 695000 ± 25050 myoblasts (n = 6) at the time of the experiment. From cell counts and B_{max} values, it can be estimated that each myoblast cell contained 95280 ± 2648 ^{125}I-HO-LVA binding sites, which is in the same range than the number of OT binding sites estimated *per* myoepithelial cell in the lactating rat mammary gland with ^{3}H-OT (19). Since in the human, ^{125}I-HO-LVA has a similar high affinity for the OTR and $V_{1a}R$ (Table 1), binding sites detected may correspond to either one of these receptors.

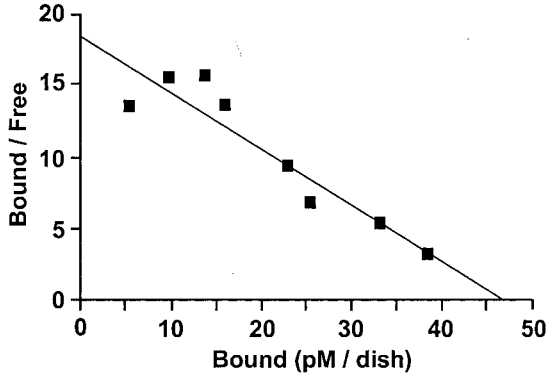

Fig. 1. Scatchard analysis of ^{125}I-HO-LVA binding in human myoblasts. Values are from one experiment. Specific binding was measured for ^{125}I-HO-LVA concentrations ranging from 25 to 500 pM. Each value has been calculated by measuring total binding in 3 culture dishes and non specific binding in one culture dish. The correlation coefficient of linear regression was 0.99, K_d was 266 pM and B_{max} was 46.7 pmoles/dish.

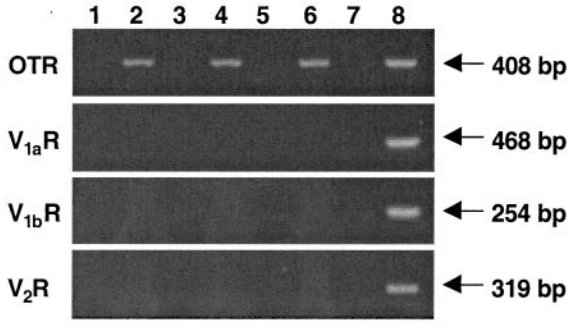

Fig. 2. RT-PCR analysis for OTR and AVPRs mRNA expression. RT-PCR was performed using either no RNA (negative controls, lanes 1, 3, 5 and 7), 1 ng of total RNA from CHO cells expressing the 4 subtypes of human receptors (positive control, lane 8), or 3 μg of RNA extracted from myoblasts from three different clonal cultures (lanes 2, 4 and 6). The sizes of the amplified products are indicated in number of base pairs on the right.

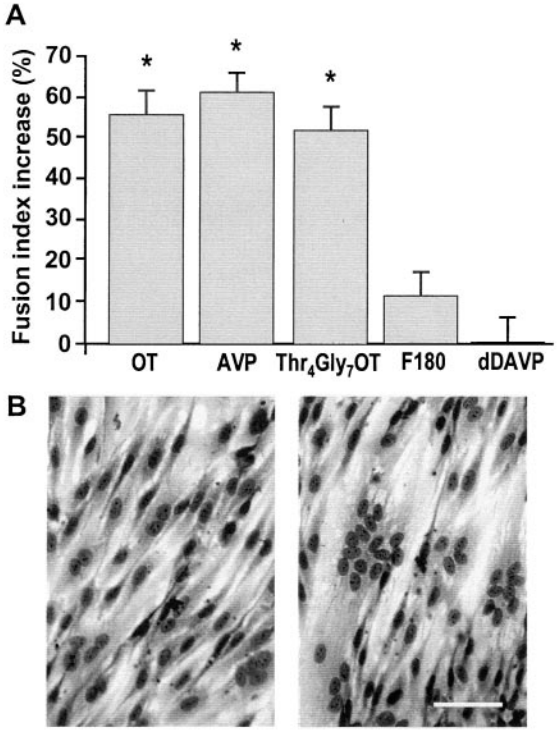

Fig. 3. Effect of OT, AVP and related compounds on fusion. A, fusion increase induced by OT, AVP and related compounds. Data were obtained from 3 different clones, 22-32 hours after transfer of confluent myoblasts to differentiation medium. Error bars represent fluctuations between the microscopic fields. B, microphotographs of Haematoxyline-stained cultures after 22 h in differentiation medium in the control condition (left picture) or in the presence of OT at 100 nM (right picture). $*P < 0.001$ with respect to controls. Scale bar = 50 μM.

We used RT-PCR analysis to determine which receptor genes were expressed in cultured human myoblasts. As a control, PCR amplifications were performed from RNA of transfected CHO cells expressing the four structurally related types of human receptors. Results are summarized in Fig. 2. The expression of OTR mRNA was demonstrated in myoblasts of 3 different clones. In contrast, V_{1a}, V_{1b} and V_2 receptor mRNAs were not detectable. Subcloning and sequencing of the PCR products confirmed that the cDNA sequences obtained from myoblasts and transfected CHO cells were identical and corresponded to sequences of the human OTR.

To determine whether receptors detected were functionnal, and to further assess that they were OTRs, we treated myoblasts during the fusion process with OT, AVP and other agonists compounds having an enhanced selectivity for either the OTR or one of the 3 subtypes of AVPRs. Results obtained with the compounds at 100 nM are summarized in Fig. 3. Both OT and AVP increased the rate of fusion by more than 50%. The selective OTR agonist [Thr^4,Gly^7]OT had a similar ability to speed up the rate of fusion. In contrast, the V_{1a} selective agonist F180 and the V_2 agonist dDAVP had no significant effect. The non selective antagonist HO-LVA prevented the fusion-promoting effect of AVP whereas it had no effect when applied alone (data not shown). Neither OT nor AVP had any effect on myoblasts proliferation (data not shown).

In order to assess whether the peptide OT could be produced by cultured human myoblasts, we used RT-PCR analysis and immunocytochemistry. RT-PCR analysis yielded amplification products of the same size in the uterus (Fig 4A, lane 2) and in myoblasts (Fig. 4A, lane 4). Subcloning and sequencing of these products confirmed that they corresponded to the human OT peptide transcript. Myoblasts were found strongly immunoreactive with an antibody which recognize both OT- and AVP- associated neurophysins (Fig. 4B).

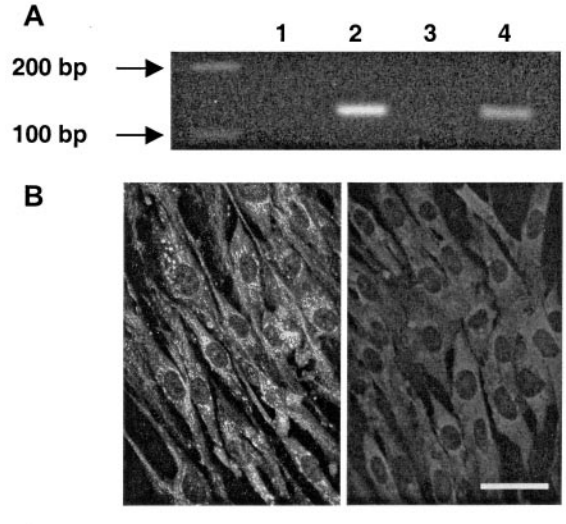

Fig. 4. Analysis of OT gene expression. A, RT-PCR analysis. Amplification was performed using either no RNA (negative controls, lanes 1 and 3), 3μg of total human uterus RNA (positive control, lane 2) or 3μg of total RNA from myoblasts (lane 5). B, immunocytochemistry. Myoblasts were strongly immunoreactive for neurophysin (left photograph). No signal was present when myoblasts were incubated only with the Cy3-coupled second antibody (right picture). Scale bar = 25 μM.

Discussion

The present findings are the first demonstration that cultured human myoblasts express functional OTRs. Furthermore, we also detected OT peptide transcripts as well as neurophysin immunoreactivity in these cells, which suggest the presence of an intrinsic OT/OTR system. OT appears, therefore, as a putative paracrine/autocrine agent that might regulate the differentiation of human skeletal muscle cells. To confirm this hypothesis, future studies should aim at demonstrating that OT is released by the cultured human myoblasts and is present in the culture medium.

Our results are in good agreement with those of Nervi et al. (9) showing that both AVP and OT stimulate the differentiation and fusion of rodent myoblasts. However, these authors suggested that the effect of neurohypophyseal peptides was mediated by a V_1R subtype, whereas we provide both pharmacological and molecular evidence that OTR is the unique receptor subtype expressed in human myoblasts. This apparent discrepancy may possibly be explained by the different origin of myoblasts used, rodent *versus* human. In line with this hypothesis, species-related differences have been reported regarding the type of ionic channels involved in the fusion process of human (20) or rodent myogenic cells (21,22).

OTR-dependent myoblast fusion may be associated with modulation of the expression of some cytoskeletal components, which could be involved. Given that OTRs are functionally coupled to phospholipase C, they may influence human myoblasts differentiation by modulating the intracellular Ca^{2+} concentration, which plays a crucial role in the process of myoblast fusion (20).

Aknowledgments

The authors are most grateful to Mrs A. Marguerat, Mrs M. Berti and Mr P. Brawand for their excellent technical assistance, to Dr M. Manning for the gift of HO-LVA and to Dr P. Rivière (Ferring Research, San Diego, CA, USA) for providing us with F180. This work was supported by the Swiss National Science Foundation (grant 31-55332.98 to E.T.and grant 31-065409.01 to L.B.), the Institut de la Santé et de la Recherche Médicale and the Fondation Suisse pour la Recherche sur les Maladies Musculaires.

References

1. **Carter DA, Choi KF, Murphy D** 1993 Neurohypophysial peptides as regulators of growth and development. J Mol Neurosc 4:11-19

2. **Sapino A, Macri L, Tonda L, Bussolati G** 1993 Oxytocin enhances myoepithelial cell differentiation and proliferation in the mouse mammary gland. Endocrinology 133:838-842

3. **Tahara A, Tsukad J, Tomur Y, Wada K, Kusayama T, Ishii N, Yatsu T, Uchida W, Tanaka A** 2000 Pharmacological characterization of the oxytocin receptor in human uterine smooth muscle cells. Br J Pharmacol 129:131-139

4. **Thibonnier M, Conarty DM, Preston JA, Plesnicher CL, Dweik RA, Erzurum SC** 1999 Human vascular endothelial cells express oxytocin receptors. Endocrinology 140:1301-1309

5. **Jankowski M, Wang D, Hajjar F, Mukaddam-Daher S, McCann SM, Gutkowska J** 2000 Oxytocin and its receptors are synthetized in the rat vasculature. Proc Natl Acad Sci USA 97:6207-6211

6. **Copland JA, Ives KL, Simmons DJ, Soloff MS** 1999 Functional oxytocin receptors discovered in human osteoblasts. Endocrinology 140:4371-4374

7. **Cassoni P, Sapino A, Munaron L, Deaglio S, Chini B, Graziani A, Ahmed A, Bussoloti G** 2001 Activation of functional oxytocin receptors stimulates cell proliferation in human trophoblast and choriocarcinoma cell lines. Endocrinology 142:1130-1136

8. **Nervi C, Benedetti L, Minasi A, Molinaro M, Adamo S** 1995 Arginine-vasopressin induces differentiation of skeletal myogenic cells and up-regulation of myogenin and Myf-5. Cell Growth Differ 6:81-89

9. **Wakelam MJO, Patterson S, Hanley MR** 1987 L6 skeletal muscle cells have functional V1-vasopressin receptors coupled to stimulated inositol phospholipid metabolism. FEBS Lett,210:181-184

10. **Smith A, Stephen RI, Arkley MM, Mcintosh N** 1992 Immunoreactive arginine vasopressin in human fetal and neonatal skeletal muscle. Early Human Dev 28:215-222

11. **Baroffio A, Aubry J-P, Kaelin A, Krause RM, Hamann M, Bader CR** 1993 Purification of human muscle satellite cells by flow cytometry. Muscle Nerve 16:498-505

12. **Chini B, Mouillac B, Balestre MN, Trumpp-Kallmeyer S, Hoflack, Hibert M, Andriolo M, Pupier S, Jard S, Barberis C** 1996 Two aromatic residues regulate the response of the human oxytocin receptor to the partial agonist arginine vasopressin. FEBS Lett 397:201-206

13. **Thibonnier M, Berti-Mattera LN, Dulin N, Conarty DM, Mattera R** 1998 Signal transduction pathways of the human V1-vascular, V2-renal, V3-pituitary vasopressin and oxytocin receptors. In: Urban IJA, Burbach JPH, DeWied D (eds) Progress in Brain Research. Elsevier Science B.V.; vol 119:147-161

14. **Andres M, Trueba M, Guillon G** 2001 Pharmacological characterization of F-180: a selective human V_{1a} vasopressin receptor agonist of high affinity. British J Pharmacol (in press)

15. **Barberis C, Balestre MN, Jard S, Tribollet E, Arsenijevic Y, Dreifuss JJ, Bankowski K, Manning M, Chan WY, Schlosser SS, Holsboer F, Elands J** 1995 Characterization of a novel linear radioiodinated vasopressin antagonist: an exellent radioligand for vasopressin V_{1a} receptors. Neuroendocrinology 62:135-146

16. **Breton C, Pechoux D, Morel G, Zingg HH** 1995 Oxytocin receptor mRNA : Characterization, regulation and cellular localization in rat pituitary gland. Endocrinology 136:2928-2936

17. **Gimpl G and Fahrenholz F** 2001 The oxytocin receptor system: structure, function, and regulation. Physiol Reviews 81:629-683

18. **Fisher-Lougheed J, Liu JH, Espinos E, Mordasini D, Bader CR, Belin D, Bernheim L** 2001 Human myoblast fusion requires expression of fonctional inward rectifier Kir2.1 channels. J Cell Biol 153:677-686

19. **Schroeder BT, Chakraborty J, Soloff MS** 1977 Binding of [^{3}H]oxytocin to cells isolated from the mammary gland of the lactating rat. J Cell Biol 74:428-440

20. **Bijlenga P, Liu JH, Espinos E, Haenggeli CA, Fisher-Lougheed J, Bader CR, Bernheim L** 2000 T-alpha 1H Ca^{2+} channels are involved in Ca^{2+} signaling during terminal differentiation (fusion) of human myoblasts. Proc Natl Acad Sci USA 97:7627-7632

21. **Constantin B, Cognard C, Raymond G** 1996 Myoblast fusion requires cytosolic calcium elevation but not activation of voltage-dependent calcium channels. Cell Calcium 19:365-374

22. **Seigneurin-Venin S, Parrish E, Marty I, Rieger F, Romey G, Villaz M, Garcia L** 1996 Involvment of the dihydropyridine receptor and internal Ca^{2+} stores in myoblast fusion. Experimental Cell Research 223: 301-307