

Summary of the Ph.D. thesis

**Different roles of α_2 -AR subtypes in the uterine function
in vitro in the rat**

Adrienn Gál

Department of Pharmacodynamics and Biopharmacy
Faculty of Pharmacy, University of Szeged

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Ph.D program: Pharmacology, Biopharmacy and Clinical Therapy

Program leader: George Falkay Ph.D., D.Sc.

Department: Department of Pharmacodynamics and Biopharmacy

Supervisor: Róbert Gáspár Ph.D. .

Exam board

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Introduction

Tocolysis is one of the greatest challenges in obstetrical practice. In spite of tremendous efforts, the statistics indicate that preterm birth is the main cause of neonatal morbidity and mortality. While babies born near term may have few or none of problems, babies born before week 32 may have a number of complications. In some cases, these complications may be fairly mild, while in other cases, they are severe and may lead to long-term medical problems or even death.

It has been established that the adrenergic system plays an important role in the regulation of myometrial contractility during pregnancy. Currently, β_2 -adrenergic-receptor (β_2 -AR) agonists are still one of the most frequently used tocolytics. In rat, besides the β_2 receptors, the α_1 -ARs have been found to have a great impact on myometrial contractility. However, The role of α_2 -ARs is not fully clear from the aspect of myometrial contractility and cervical resistance.

The α_2 -ARs are coupled to the pertussis toxin PTX sensitive G_i proteins whose activation leads to the inhibition of the adenylate cyclase enzyme, the reduction of the intracellular cAMP concentration, the inhibition of voltage-gated Ca^{2+} channels, the activation of inwardly rectifying K^+ channels and finally, the physiological answer. Three α_2 -AR subtypes (α_{2A} -AR, α_{2B} -AR and α_{2C} -AR) have been identified in the human myometrium, while only α_{2A} -AR and α_{2B} -AR were found in the rat uterus. To date there has been no investigation of the roles of the different α_2 -AR subtypes in the control of uterine function. The existence of α_2 -AR subtype-selective compounds offers a good possibility for the determination of the functions of the given subtypes.

Aims

1. Identification of the myometrial and the cervical α_2 -AR subtype mRNA and protein expressions by using RT-PCR and Western-blot techniques, respectively.
2. By isolated organ methods, the investigation of the role of the α_2 -AR subtypes in the noradrenaline (NA) evoked myometrial contractions (day 22 of pregnancy) and the cervical resistance (gestation days 18, 20, 21 and 22) by subtype-specific antagonists *in vitro*.

3. The measurement of the cAMP release after receptor stimulations by NA in the presence of the subtype-specific α_2 -AR antagonists.
4. Investigation of the effect of the drugs on overstimulated myometrial samples by using induced labour model.

Methods

RT-PCR studies

The tissues were rapidly removed, frozen in liquid nitrogen and then stored at -70 °C until total RNA extraction. Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform. After precipitation with isopropanol, the RNA was washed 3 times with ice-cold 75% ethanol and then dried. The PCR was carried out with 5 μ l cDNA, 25 μ l ReadyMix REDTaq PCR reaction mix, 2 μ l 50 pM sense and antisense primers of the α_2 -adrenoceptor subtypes and 16 μ l DNase- and RNase-free distilled water. The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel.

Western blotting studies

20 μ g of protein per well was subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels in Series Standard Dual Cooled Units. Proteins were transferred from gels to nitrocellulose membranes, using a semidry blotting technique. The membranes were blocked overnight at 4 °C with 5% non-fat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% Tween. After washing, the blots were incubated for 1 h at room temperature on a shaker, with α_{2A} -, α_{2B} -, and α_{2C} -adrenoceptor and β -actin polyclonal in the blocking buffer. Immunoreactive bands were visualized with the WesternBreeze Chromogenic Western blot immune detection kit and quantified. Protein bands were identified via the MagicMark Western Protein Standard.

Measurement of myometrial contractility

Myometrial tissue samples were removed from non-pregnant, 22-day-pregnant and labour-induced 20-day-pregnant rats. The tissues were prepared and incubated according to earlier studies. Contractions were elicited with NA (10^{-8} – 3×10^{-5} M) and cumulative concentration-response curves were constructed in the presence of propranolol (10^{-5} M) and doxazosin (10^{-7} M) in order to avoid β - and α_1 -adrenergic actions. Concentration - response curves were fitted and areas under curves (AUCs) were evaluated and analysed with the Prism 4.0 computer program.

Induction of premature labour in pregnant rat

In the experimental premature labour procedure, the 19-day-pregnant rats were treated with s.c. antiprogesterone (mifepristone) at 3 mg/animal at 9.00 a.m. At 4.00 p.m., prostaglandin E_2 (0.5 mg/animal) was administered intravaginally. Our preliminary results had revealed that, after this treatment, the pregnant animals delivered between 9.00 and 10.00 a.m. on day 20 of pregnancy. Accordingly, the animals were sacrificed and uterine tissues were removed at 9.00 a.m., ensuring that the pregnant myometrium was very close to, but not after delivery.

Measurement of cervical resistance

Cervical tissues were removed from late-pregnant (gestational day 18, 20, 21 or 22) rats. After incubation, the cervical resistance was investigated by gradual increase of the tension in the tissues. The cervixes were stretched in incremental steps (between 1 and 12 g) and allowed to relax for 5 min. In the evaluation of the cervical resistance, the initial tension of the cervix was plotted versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. A steeper slope reflected higher resistance.

Before the stretching the samples were incubated with 10^{-6} M of the subtype-selective α_2 -AR antagonist (BRL 44408 for α_{2A} , ARC 239 for α_{2B} and spiroxatrine for α_{2C}) in the presence of propranolol (10^{-5} M) and doxazosine (10^{-6} M) to avoid the activation of β - and α_1 -ARs. Control values were registered in the presence of or without NA.

When the effect of the G_i -protein inhibitor pertussis toxin (PTX) was investigated on 18- and 20-day-pregnant cervixes, the samples were pretreated with PTX (400 ng/ml) for 2 hours before stretching.

cAMP studies

IBMX (10^{-3} M), doxazosin, propranolol and the investigated subtype-selective α_2 -AR antagonists were incubated with the tissues for 20 min, and NA was then added for 10 min to stimulate cAMP generation. At the end of the NA incubation period, forskolin (10^{-5} M) was added for another 10 min. When the effect of the G_i -protein inhibitor PTX on the cAMP accumulation was investigated, the samples were pretreated with PTX (400 ng/ml) for 2 h before the IBMX incubation

The tissue samples were next ground under liquid nitrogen weighed, homogenized in ice-cold 5% trichloroacetic acid and centrifuged at 600 g for 10 min. The supernatant was extracted with water-saturated diethyl ether. After drying, the extracts were stored at -70 °C. cAMP accumulation was measured with a cAMP Enzyme Immunoassay Kit.

Results

RT-PCR studies

RT-PCR studies revealed the mRNAs of all three α_2 -AR subtypes in both the non-pregnant and the 22-day-pregnant rat myometrium. In the pregnant myometrium a strong α_{2B} -AR mRNA predominance was found.

In the cervix, all the three α_2 -AR subtype mRNAs are expressed each day in the cervical samples; however, differences were detected between the levels of expression on the different days. The mRNA expressions of each subtype were found to be elevated on days 20 and 21. On day 22, however, the α_{2A} - and α_{2B} -AR mRNA levels remained unchanged, while the expression of α_{2C} -AR mRNA was lower

Western blotting studies

The Western blotting analysis gave a result for the non-pregnant myometrium similar to that from the RT-PCR studies. In the pregnant myometrium, the predominant subtype protein was the α_{2B} -AR, while the optical density of the α_{2A} -AR protein was significantly lower than that of the α_{2C} -AR.

In case of the cervical samples the analysis revealed the presence of the proteins of all three α_2 -AR subtypes on each investigated day. The protein expressions of the α_2 -AR subtypes were significantly increased on day 21, whereas decreases were observed on day 22

Isolated organ studies

Effects of α_2 -AR subtype antagonists on the myometrial contractions

NA in the concentration range 10^{-8} - $10^{-4.5}$ M did not exert a contractile effect on the non-pregnant uterine rings. In the 22-day-pregnant myometrium, NA concentration-dependently increased the contractions, and these were increased by the α_{2A} -AR antagonist BRL 44408 and the α_{2C} -AR antagonist spiroxatrine. The $\alpha_{2B/C}$ -AR antagonist ARC 239 significantly decreased the maximum effect of NA. (Fig.1.)

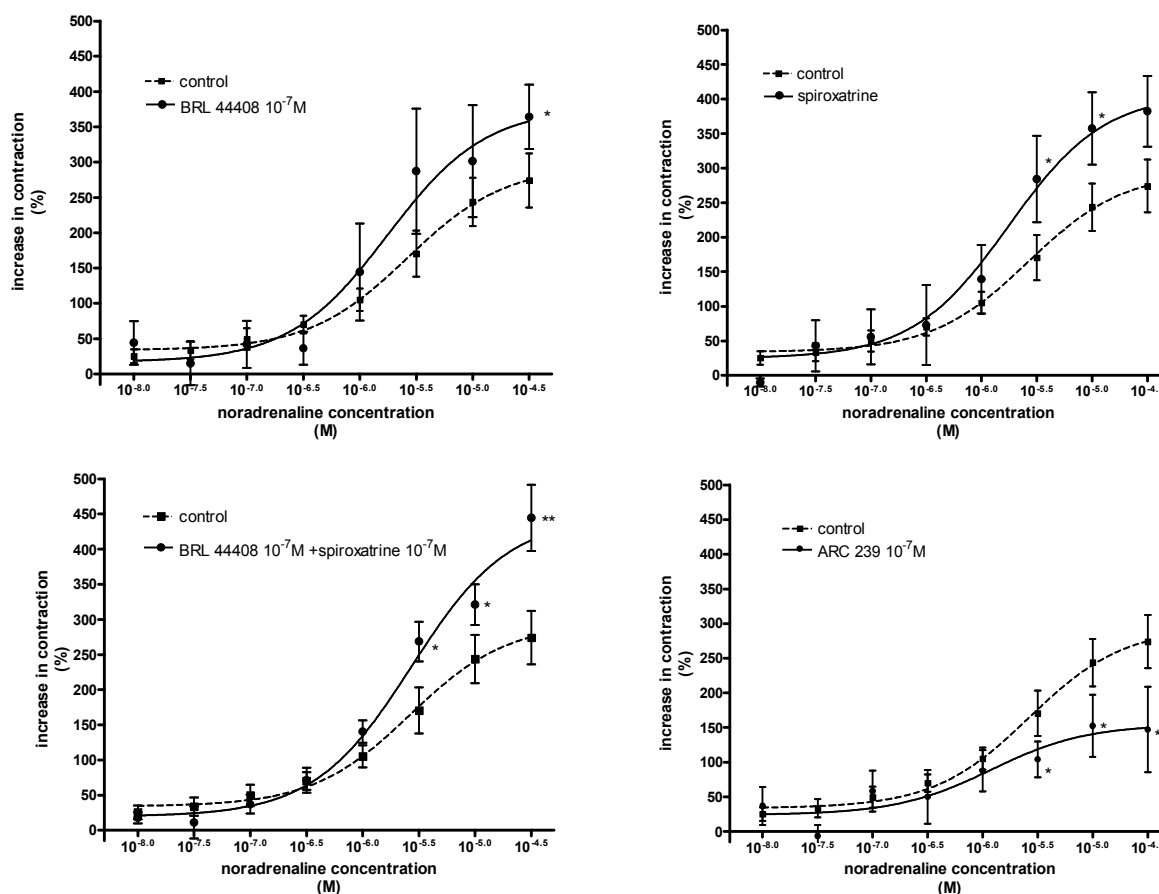


Figure 1. Effects of the subtype-selective α_2 -AR antagonists on the NA-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath (n = 8). *: p<0.05; **: p<0.01.

Isolated organ studies with myometrium from hormonally-induced preterm birth

NA enhanced the contractions of labour-induced uterine rings, although its effect was less than that in the 22-day-pregnant animals. ARC 239 blocked the NA-evoked contractions

Effects of α_2 -AR subtype antagonists on the cervical resistance

We found that on day 18, NA enhanced the cervical resistance, both alone and also in the presence of ARC 239, while BRL 44408, spiroxatrine and their combination blocked the effect of NA. The opposite effect was found on day 20. On day 21, the resistance-increasing action of NA was maintained only in the presence of spiroxatrine. NA, however, did not alter the cervical resistance in the presence of or without the antagonists on day 22. (Fig.2)

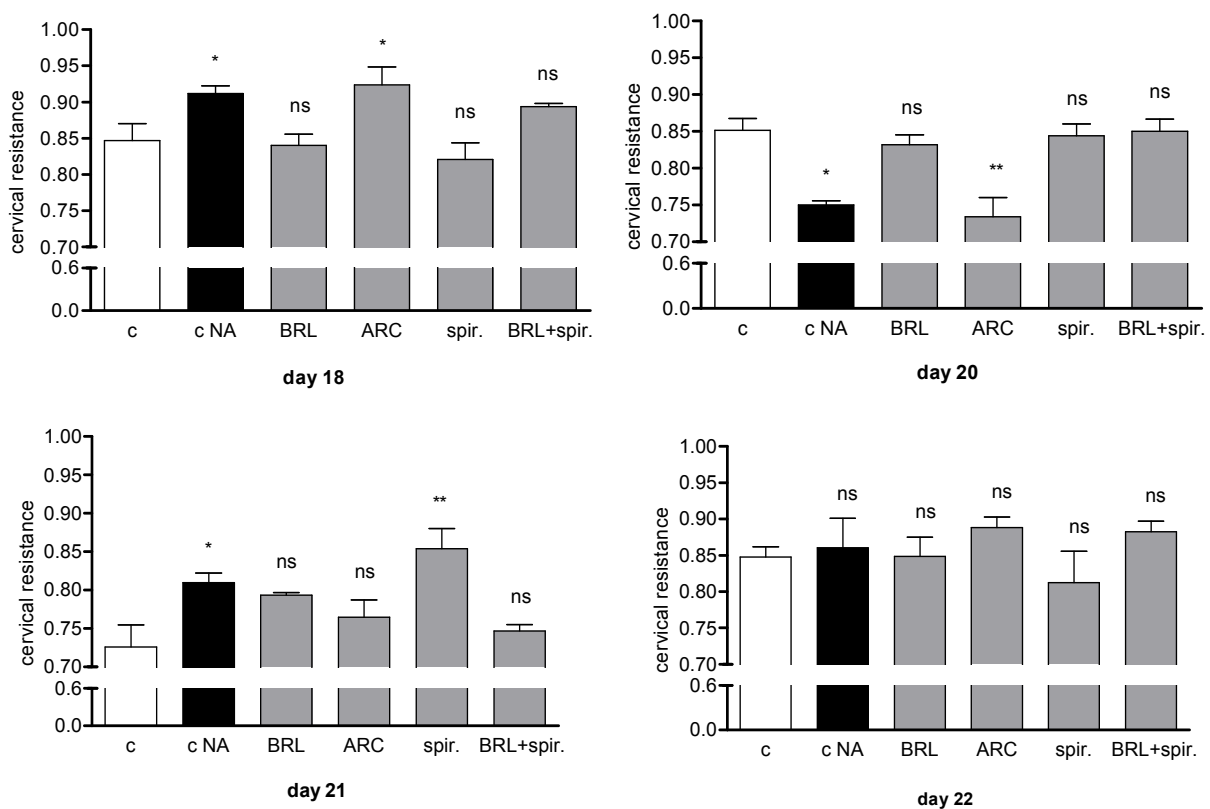


Figure 2. Effects of α_2 -AR subtype antagonists on the resistance of 18-, 20-, 21- and 22-day-pregnant rat cervixes in vitro (n=8). *: p<0.05; **: p<0.01.

PTX significantly decreased the cervical tone as compared with the control level without PTX on days 18 and 21. PTX also decreased the resistance-increasing effect of NA, both alone and in the presence of ARC 239, and spiroxatrine, respectively. On day 20, PTX did not alter the basal cervical resistance or the effects of NA and NA+ARC 239 on the cervical tone

cAMP studies

Effects of subtype-selective α_2 -AR antagonists on myometrial cAMP level

In the pregnant myometrium, only the ARC 239 was able to increase the cAMP level produced by NA, while BRL 44408, spiroxatrine and the combination BRL 44408 + spiroxatrine caused significant decreases in the amount of myometrial cAMP. (Fig.3.)

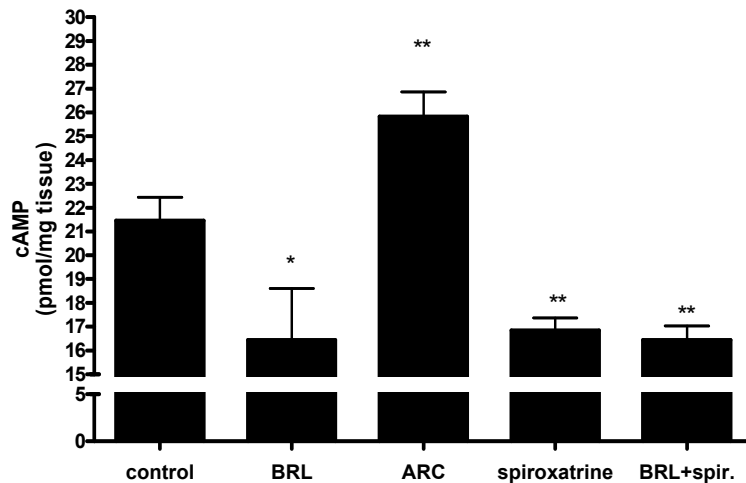


Figure 3. Effects of the subtype-selective α_2 -AR antagonists on the myometrial cAMP level (pmol/mg tissue \pm S.E.M.) stimulated by NA in the presence of isobutylmethylxanthine and forskolin (control) in the 22-day-pregnant rat (n = 6). *: p<0.05; **: p<0.01.

Effects of subtype-selective α_2 -AR antagonists on cervical cAMP level

NA treatment significantly decreased the intracellular cAMP production on day 18, and the same effect was found in the presence of ARC 239. An elevated cAMP accumulation was seen on day 20 both by NA alone and in the presence of ARC 239. On day 21, NA decreased the level of intracellular cAMP, and an analogous effect was displayed in the presence of spiroxatrine. Nevertheless, the antagonists did not influence the amount of cAMP in the presence of NA on day 22.

The investigations carried out in the presence of PTX were in complete accordance with the results in the isolated organ studies.

Discussion

The exact mechanism(s) of the initiation of labour are still poorly understood. So far numerous extensive experiments have been carried out to investigate the roles of the α_1 - and β_2 -ARs in the control of the uterine function; however, no study has been performed related to the effect of the α_2 -AR subtypes in the labour. The main focus of our study was to clarify the role of these subtypes in the control of myometrial contractions and cervical resistance in the late pregnant rat, *in vitro*.

We proved the presence of all three α_2 -AR subtypes in both the myometrium and the cervix by RT-PCR and Western blotting techniques. The functional consequences of the stimulation of the different α_2 -AR subtypes were detected in parallel with the generation of intracellular cAMP, while the changes in the tissue cAMP level are crucial in the control of the smooth muscle contraction and relaxation. In the presence of the antagonists, we found that the changes were in complete accordance with the alterations in the intracellular cAMP levels.

Myometrium. In the presence of BRL 44408 the contractions were increased (and the cAMP level was decreased), which suggests that in the late-pregnant myometrium α_{2A} -ARs mediate relaxation. We proved the same in the presence of the α_{2C} -AR blocker spiroxatrine, consequently the α_{2A} - and the α_{2C} -ARs are responsible for relaxation. In the presence of ARC 239, only the α_{2A} -ARs remained free; the stimulation of this subtype decreased the effect of NA, which results support our earlier assumption, that the α_{2A} -ARs are responsible for relaxation. In order to clarify the role of the α_{2B} -ARs, we used the combination of BRL 4408 + spiroxatrine, which blocked the α_{2A} - and α_{2C} -ARs; the stimulation of the free α_{2B} -ARs also increased the uterine contractions. The uterus-contracting effect of NA can be explained as a resultant effect mediated by the AR subtypes.

	Contractile response		
	α_{2A}	α_{2B}	α_{2C}
day 22	↓	↑	↓

The induced labour model was designed to investigate the effects of ARC 239 (which had a most effective relaxing effect) on overstimulated uterine tissue. Its effect on the NA-evoked contractions was also convincing; it can be promising in the therapy of premature labour.

Cervix. On day 18, in the presence of ARC 239 only the α_{2A} -ARs remained free; the

stimulation of this subtype by NA increased the resistance with a reduction in the cervical cAMP level, while stimulation of the same resulted in the opposite effect on day 20.

The α_2 -ARs are classically described as coupling to the PTX-sensitive G_i -protein, but under certain circumstances α_2 -ARs can also couple to G_s -proteins, leading to activation of adenylyl cyclase. Based on our experiences, we suggest that the α_{2A} -ARs (and possibly α_{2C} -ARs) can couple to both G_i - and G_s -proteins in the cervix too. In order to clarify this assumption, we repeated the investigations in the presence of PTX. On day 18, in the presence of PTX, the cervical resistance was decreased. The effects of NA alone (non-selective α_2 -AR stimulation) and in the presence of the $\alpha_{2B/C}$ -AR blocker ARC 239 (selective α_{2A} -AR stimulation) were eliminated by PTX. This indicates the predominance of G_i -coupling of α_{2A} -ARs (and possibly α_{2C} -ARs) on this day. On day 20, the PTX pre-treatment did not alter the basic cervical tone, the cAMP level or the effects of NA alone and in the presence of ARC 239. These results suggest that on this day there is a G_s -protein predominance in the coupling of α_{2A} - (and α_{2C} -) ARs. On day 21, we detected a resistance-increasing effect of NA. This effect could be maintained in the presence of spiroxatrine. This means that the effect of NA is mediated through the α_{2A} - and α_{2B} -ARs. After PTX treatment, the effects were disappeared. Accordingly, on day 21, the agonist action may be mediated predominantly by coupling to G_i -proteins. On 22 day of gestation, however, NA had no effect on the cervical resistance in the presence of the antagonists. We have a good reason to suppose that the α_2 -ARs are uncoupled from the G-proteins on this day.

	α_{2A}		α_{2B}		α_{2C}	
	G protein coupling	resistance	G protein coupling	resistance	G protein coupling	resistance
day 18	G_i	↑	-	-	G_i	↑
day 20	G_s	↓	-	-	G_s	↓
day 21	G_i	↑	G_i	↑	-	-
day 22	-	-	-	-	-	-

In the light of our experiences it seems very probably that the blockade of the α_2 -AR subtypes will not be sufficient to stop the whole preterm labour process, but their combination with more potent inhibitor drugs may have clinical benefits.

Annex

Publications related to the Ph.D. thesis

1. Róbert Gáspár, **Adrienn Gál**, Márta Gálik, Eszter Ducza, Renáta Minorics, Zoltán Kolarovszki-Sipiczki, Anna Klukovits and George Falkay: Different roles of α_2 -AR subtypes in non-pregnant and late-pregnant uterine contractility *in vitro* in the rat. *Neurochemistry International* 2007; 51(5). 311-318. **IF.: 3,228**
2. **Adrienn Gál**, Eszter Ducza, Renáta Minorics, Anna Klukovits, Márta Gálik, George Falkay and Róbert Gáspár: The roles of α_2 -AR subtypes in the control of cervical resistance in the late-pregnant rat. *European Journal of Pharmacology* 2009; 615(1-3). 193-200. **IF.: 2,787**
3. **Gál Adrienn**, Kolarovszki-Sipiczki Zoltán, Gálik Márta, Ducza Eszter, Minorics Renáta, Klukovits Anna, Falkay György, Gáspár Róbert: Az ARC 239 hatása a miometrium és a cervix működésére patkányban, *in vitro*. *Acta Pharmaceutica Hungarica* 2009; 79(2). 75-80.

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2. **A Gál**, R. Gáspár, M. Gálik, E. Ducza, R. Minorics, Z. Kolarovszki-Sipiczki, A. Klukovits and G. Falkay: Different roles of alpha2-adrenergic receptor subtypes in the pregnant uterine contractility *in vitro* in the rat. The First Conference of PhD Students in Medicine and Pharmacy, July 9-11, 2008, Targu Mures, Romania
3. Gáspár R., Gálik M., **Gál A.**, Ducza E., Minorics R., Kolarovszki-Sipiczki Z., Falkay G.: Different roles of alpha2-adrenergic receptor subtypes in the pregnant uterine contractility in the rat. 15th World Congress of Pharmacology, July 2-7, 2006, Beijing, China

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5. Gáspár R., Kolarovszki-Sipiczki Z., **Gál A.**, Minorics R., Gálik M., Ducza E., Falkay G.: Alpha2-AR subtypes as new targets for tocolytic therapy. 1st International Symposium in Hot topics & Controversies in Perinatal Medicine, June 16-19, 2005, Rhodes Island, Greece

Other publications

6. Renáta Minorics, Róbert Gáspár, **Adrienn Gál**, Anna Klukovits and George Falkay: Progesterone decreases the relaxing effect of the β_3 -adrenergic receptor agonist BRL 37344 in the pregnant rat myometrium. *Reproduction* 2009; 138(2):383-390. **IF.: 3,073**
7. Maria N. Modica, Giuseppe Romeo, Loredana Salerno, Valeria Pittalà, Maria A. Siracusa, Ilario Mereghetti, Alfredo Cagnotto, Tiziana Mennini, Róbert Gáspár, **Adrienn Gál**, George Falkay, Márta Palkó, Gábor Maksay, Ferenc Fülöp: Synthesis and receptor binding of new thieno[2,3-d]-pyrimidines as selective ligands of 5-HT(3) receptors. *Archiv der Pharmazie* 2008; 341: 333-343. **IF.: 1,429**

Other abstracts

8. R. Gáspár, I. Zupkó, **A. Gál**, G. Spiegl, G. Csík and G. Falkay: Effects of streptozotocin-induced diabetes on the uterine denervation and contractility in pregnant rats. The 5th International Symposium on Diabetes and Pregnancy (DIP 2009), March 26-28, 2009, Sorrento, Italy
9. R. Minorics, R. Gáspár, **A. Gál** and G. Falkay: Can the β_3 -adrenergic receptor be a novel target in the tocolytic therapy? 2nd Meeting of the Diczfalusy Foundation: Prevention in Women's Health, September 30 - October 1, 2008, Szeged, Hungary
10. Minorics R, Gáspár R, **Gál A**, Klukovits A, Falkay G.: Progesterone decreases the relaxing effect of BRL37344, a β_3 -adrenergic receptor agonist in the pregnant rat myometrium. Prevention in Women's Health 2nd Meeting of the Egon & Ann Diczfalusy Foundation 2008, September 30 - October 1, Szeged, Hungary.
11. R. Minorics, R. Gáspár, **A. Gál** and G. Falkay: Can the β_3 -adrenergic receptor be a novel target in the tocolytic therapy? EACPT 2007, August 28 – September 2, Amsterdam, The Netherlands
12. **Gál Adrienn**, Kolarovszki-Sipiczki Zoltán, Gálik Márta, Ducza Eszter, Minorics Renáta, Klukovits Anna, Falkay György, Gáspár Róbert: Az ARC 239 hatása a