

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

**Investigation of pregnancy induced adrenergic denervation in  
the rat**

**PhD Thesis**

**Dénes Csonka**

**Supervisor:**

**Prof. George Falkay, PhD, DSc**

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## **Introduction**

Pregnancy is one of the most mysterious phenomenon of our life. There are many unresolved questions covering all stages of pregnancy. Plenty of biochemical, physiological and pharmacological methods are used to answer the open questions of physiological as well as pathological conditions affecting pregnancy e.g. preterm birth, hypertensive disorders in pregnancy and gestational diabetes.

During pregnancy, dramatic changes take place in all physiological functions both in mammals and humans. One of these changes is the remodeling of uterine innervation, which is a well described but not completely understood, therefore intensively investigated phenomenon.

Throughout pregnancy both the vasculature and the smooth muscle of uterus is denervating, and it is slowly reinnervating after delivery. Changes in the innervation during pregnancy are paralleled by a dramatic reduction in noradrenaline levels in the uterus, as well as in the activity of the noradrenaline synthesizing enzymes, tyrosine hydroxylase and dopa-decarboxylase. Reductions of noradrenaline levels were shown to result from degeneration of sympathetic terminal branches and not solely from a dilution of intact nerves in the enlarged uterus.

As adrenergic system - besides prostaglandins and oxytocin - has a key function in regulating the contractility of pregnant uterus, it is extensively investigated. We chose to examine adrenergic system since it is considered the most important in the regulation of uterine contractility and this is the system which is targeted routinely during tocolysis.

After investigating and exploring functional changes in the adrenergic innervation of both uterus and cervix during pregnancy, we decided to investigate how could denervation be influenced from a pharmacological point of view and if our technique is suitable for this or not.

Since hypertension disorders are one of the most important complications of pregnancy, affecting approximately 10% of all pregnancies and contributing greatly to maternal and perinatal morbidity and mortality throughout the world, and  $\alpha$ -methyldopa is one of the most frequently used antihypertensive agent in

pregnancy, we have decided to investigate its effect on the pregnancy-induced adrenergic denervation of rat uterus.

### Aims

- Our aim was to set up a novel experimental approach which is suitable for investigating this process from a purely functional view, not only during pregnancy but in the postpartum period which is less frequently examined.
- We hypothesized that the loss of function of adrenergic nerves may be detected earlier than the structural changes by immunohistochemical methods.
- It was also our aim to determine the recovery of function after delivery and to compare these findings to immunohistochemical results.
- We planned to investigate how  $\alpha$ -methyl dopa acts on adrenergic neurons and what are the postsynaptic consequences of this effect.
- Furthermore the fact that the information on the myometrial action of  $\alpha$ -methyl dopa is very limited, supported our aim to investigate its effects on myometrial noradrenaline release and contractility.

### Materials and Methods

- *Experimental animals*

Sprague–Dawley rats (200–250 g for females) were mated in a special cage in the early morning. Rats were killed by cervical dislocation. Non-pregnant rats used in the experiments were all in the estrus state of their cycle. In the  $\alpha$ -methyl dopa treatment experiments the animals in the active group were treated with daily 200 mg/kg  $\alpha$ -methyl dopa intraperitoneally for 7 days and the experiment was performed on the 7<sup>th</sup> day. Control animals were treated with physiological saline.

- *Release of [<sup>3</sup>H]noradrenaline*

Samples of uterine and cervical tissue (20–30 mg) were dissected; the samples from the implantation and inter-implantation sites were processed separately. Wet weights of the samples were measured, they were minced and incubated with  $10^{-7}$  M [<sup>3</sup>H]noradrenaline at 37°C for 60 min. The samples were then washed three times with de Jongh buffer, the pieces were superfused continuously for 60 min with de Jongh buffer containing pargyline, desipramine and deoxycorticosterone (each 10  $\mu$ M). During the experiments carried out with lidocain, the concentration of lidocain in the buffer was 50  $\mu$ M. The solution was maintained at 37°C and equilibrated throughout the experiment with O<sub>2</sub> containing 5% (v/v) CO<sub>2</sub>. After a 60-min wash-out period, a total of 22 3-min fractions were collected. At the end of the experiment, the tissue samples were solubilized in 1ml Solvable for 3 h at 60°C. The [<sup>3</sup>H] content in each 3-min fraction and tissue solution was determined with a liquid scintillation spectrometer. Electrical field stimulation (EFS) consisting of squarewave pulses was applied to the tissues, using a programmable stimulator. EFS was applied twice after the wash-out period, during fractions 5 and 15. Each period of stimulation consisted of 360 pulses with parameters which are suitable for selective neural stimulation. The [<sup>3</sup>H] noradrenaline contents in the fractions were expressed as fractional release. Peak releases were calculated by subtraction of the radioactivity of the fourth and fourteenth fractions from that of the fifth and fifteenth fractions, respectively. The tissue activity (expressed in dpm/mg tissue) was used to describe the uptake capacity of the sample for [<sup>3</sup>H]noradrenaline.

- *Isolated organ studies*

Uterine rings were taken from the uterine horns of pregnant or non-pregnant, treated or non-treated rats. Two muscle rings were sliced from both horns of the uterus and mounted vertically in a tissue bath containing 10 ml de Jongh buffer. Tissue samples were equilibrated for 90 minutes. The areas under the curves were analyzed for a 5-min period after each administration of the tested substances.

- *Determination of contractility changes*

Cumulative dose-response curves were constructed for noradrenaline in the concentration range  $1 \times 10^{-10}$  –  $1 \times 10^{-5}$  M. The chamber contained propranolol

( $10^{-6}$  M) to block the relaxation component mediated by  $\beta$ -adrenergic receptors. After equilibration, noradrenaline was added to the chamber cumulatively, in a total of 11 different concentrations and the contractility answers were recorded for 5 minutes after each administration. At the end of the experiment, KCl (70 mM) was added to the chamber and the evoked contractions were considered as maximal tone and recorded also for 5 min. The contractions induced by noradrenaline were expressed as a percentage of the KCl evoked contractions.

To characterize the effects of  $\alpha$ -methyldopa on the  $\beta$ -adrenergic receptor-mediated myometrial relaxation, a similar experimental design was used, but the chamber did not contain propranolol. The terbutaline concentration range was  $10^{-8}$  –  $10^{-5}$  M (altogether 7 concentrations). KCl (50 mM) was added to the chamber before the start of the experiment in order to elicit an initial tension of the uterine rings which was regarded as 100% of the motor activity.

#### *Radioligand-binding studies*

- *Membrane preparation*

Uteri were homogenized in 6–10 volumes of 10 mM Tris-HCl buffer. The homogenate was centrifuged at  $20,000\times g$  for 10 min and the pellet was recentrifuged. The supernatants were collected and centrifuged at  $50,000\times g$  for 60 min and the pellet was used for saturation experiments. The protein concentration of the membrane fraction was measured by the method of Bradford with bovine serum albumin as standard.

- *Saturation binding experiments*

It was performed by incubating the cell membrane fraction with a range of concentrations of [ $^3$ H]RX 821002 (0.2–8.0 nM,  $\alpha_2$ -adrenoceptors) and [ $^3$ H]ICI 118,551 (0.5–10 nM,  $\beta_2$ -adrenoceptors) at 25 °C for 45 min. At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration. The bound radioactivity was determined in a scintillation cocktail in a liquid scintillation counter. The nonspecific binding was measured with 10  $\mu$ M unlabeled yohimbine and alprenolol. The specific binding was calculated as the difference between the total and the nonspecific binding, and was plotted as a function of the free radioligand concentration.

## **Results**

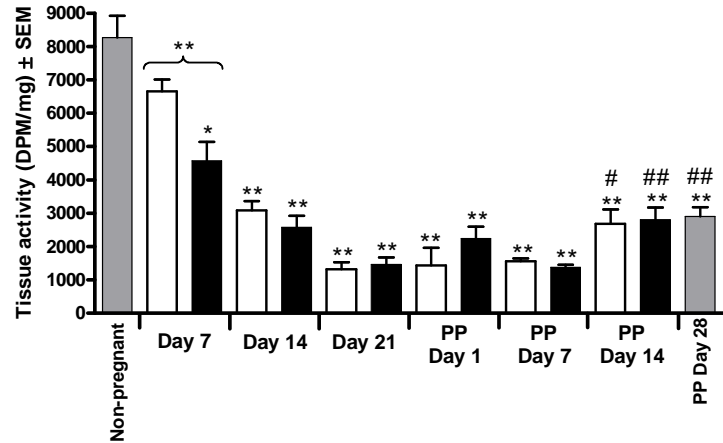
- *Results of sodium channel blockade on EFS-induced noradrenaline release*

In order to confirm that the EFS-induced noradrenaline release is sensitive to sodium channel blockade, 50  $\mu$ M lidocaine was added to the buffer used for perfusion. Lidocaine completely abolished the electrically evoked noradrenaline release which supports our hypothesis that electric field stimulation with the current parameters evokes noradrenalin release selectively from neuronal elements.

- *Results of tissue activity determination*

We used tissue activity to describe the [ $^3$ H]noradrenaline uptake capacity of the sample. It was found that tissue activity was highest in the non-pregnant state both in myometrial and cervical tissue. In early pregnancy, on day 7, transmitter uptake was significantly lower for implantation sites than for the inter-implantational part of myometrium (Figure 1). The difference in denervation of the implantational and inter-implantational sites diminished by mid pregnancy (Day 14), no site-specific difference could be detected in later stages of pregnancy and in postpartum period either. This decline in labeled neurotransmitter uptake reached its minimum at the end of pregnancy. As far as the postpartum period was concerned, tissue activity of the uterus at both the implantation and inter-implantation sites remained lower than before pregnancy throughout the 28 days of the investigation, however a slow but detectable reinnervation was found in the uterus. In contrast with the period of pregnancy, no site-dependent difference was observed in the myometrium.

In non-pregnant state, uterus and cervix has similar tissue activity, but during pregnancy the decrease of tissue activity in cervix is less pronounced than in uterus. However, cervical tissue showed a similar trend, although decrease in uptake capacity was not significantly lower on Day 7 comparing to non-pregnant state, but by Day 14 this decrease resulted in a significantly lower tissue activity. Activities of the postpartum cervical samples up to day 14 were not significantly higher than the day 21 value.



**Figure 1.** [<sup>3</sup>H]Noradrenaline-uptake capacity of myometrium during gestation and the postpartum (PP) period. \* and \*\* denote  $P < 0.05$  and  $P < 0.01$  as compared with the non-pregnant value, respectively; # and ## denote  $P < 0.05$  and  $P < 0.01$  as compared with the day-21 values, respectively. Black bars, implantation sites; white columns, inter-implantation sites.

- *Results of stimulated [<sup>3</sup>H]noradrenaline release*

EFS evoked a substantial [<sup>3</sup>H]noradrenaline release in both the uterus (Figure 2) and the cervix excised from non-pregnant rats. We have applied two stimulations in order to obtain information on the release capacity of the examined tissues. The second stimulus evoked a smaller neurotransmitter release comparing to the first peak. In myometrial tissue, a gradual decrease was detected in the peak evoked by EFS during the gestation, and a gradual, but not so extensive increase could be measured during the first 28 days of the postpartum period. A substantial and significant difference was found between the implantation and inter-implantation sites of the uterus in the early pregnant state, indicating that the loss of the adrenergic nerve function starts in the implantation area.

Concerning the cervical samples, a gradual tendency of the EFS-evoked [<sup>3</sup>H]noradrenaline release to decrease was observed during pregnancy, but these changes were not significant statistically. The EFS-evoked release of [<sup>3</sup>H]noradrenaline from the cervical samples was significantly suppressed in the

early postpartum period, and approximated the non-pregnant level 14 days after delivery.

*Results of experiments concerning the myometrial effect of  $\alpha$ -methyl dopa*

- *Tissue radioactivity determination*

Treatment with  $\alpha$ -methyl dopa decreased the amount of labeled noradrenaline in both non-pregnant and early pregnant animals. Although the difference between the tissue radioactivities from animals in term were also relevant but statistically not significant compared to the non-treated tissues.

- *Results of stimulated [<sup>3</sup>H]noradrenaline release of  $\alpha$ -methyl dopa*

Electric field stimulation (EFS) evoked a substantial [<sup>3</sup>H]noradrenaline release in the uterus isolated from non-pregnant rats (Figure 3),  $\alpha$ -methyl dopa-treatment almost completely abolished this [<sup>3</sup>H]noradrenaline release. On day 7 of pregnancy the [<sup>3</sup>H]noradrenaline release was decreased at both implantation and inter-implantation sites due to the pregnancy-induced adrenergic denervation.  $\alpha$ -Methyl dopa treatment further decreased this EFS-evoked [<sup>3</sup>H]noradrenaline release at both sites. At the end of pregnancy (day 21) the amount of [<sup>3</sup>H]noradrenaline released from the  $\alpha$ -methyl dopa-treated animals was not changed as compared with the control level.

- *Results of isolated organ experiments*

Noradrenaline did not have any effect on the non-pregnant uterus and the  $\alpha$ -methyl dopa-treated non-pregnant uterus exhibited a higher spontaneous activity without being more sensitive to  $\alpha$ -adrenergic stimulation (Figure 4). On day 7 of pregnancy, noradrenaline evoked weak dose-dependent contractions in the control group, but more profound contractions in the uteri of  $\alpha$ -methyl dopa-treated rats (Figure 4B). At term pregnancy (day 21), noradrenaline increased the contractions in a dose-dependent manner. There was no difference in the noradrenaline-evoked contractions between the control and the  $\alpha$ -methyl dopa-treated group on day 21.

In the next set of experiments, we examined the myometrial relaxing effect of terbutaline, we found that terbutaline inhibited the KCl elicited contractions in a dose-dependent manner (Figure 5).

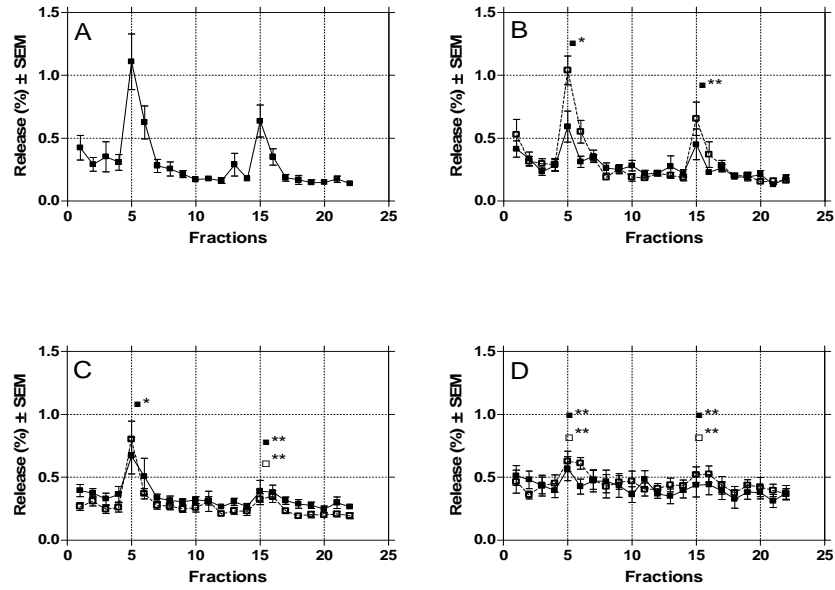


Figure 2. EFS-evoked fractional [<sup>3</sup>H]noradrenaline release from myometrial samples at oestrus (A), and on days 7 (B), 14 (C) and 21 (D) of pregnancy. \* and \*\* denote P < 0.05 and P < 0.01 as compared with the non-pregnant value, respectively. During gestation, ■ and □ indicate release from the implantation and inter-implantation sites, respectively

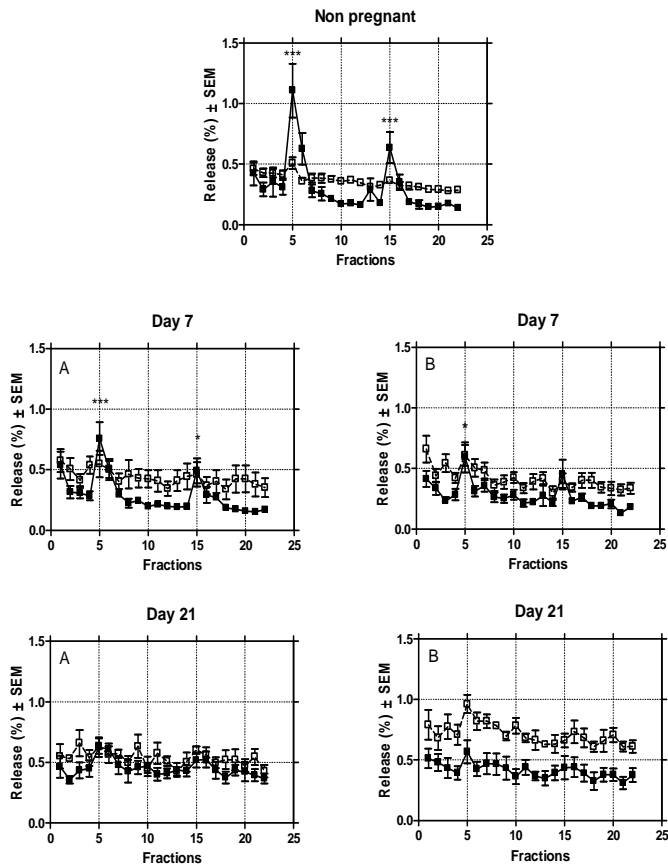


Figure 3. The effects of  $\alpha$ -methyl dopa treatment on the EFS-evoked fractional [ $^3\text{H}$ ]noradrenaline release from myometrial samples at estrus (upper panel), on day 7 (middle panels) A – at inter-implantational sites B – implantational sites and on day 21 (lower panels) A – inter-implantational sites B - at implantational sites. \* and \*\*\* denote  $P < 0.05$  and  $P < 0.001$  as compared with the nontreated value, respectively. ■ indicates control values, and □ indicates  $\alpha$ -methyl dopa-treated values.

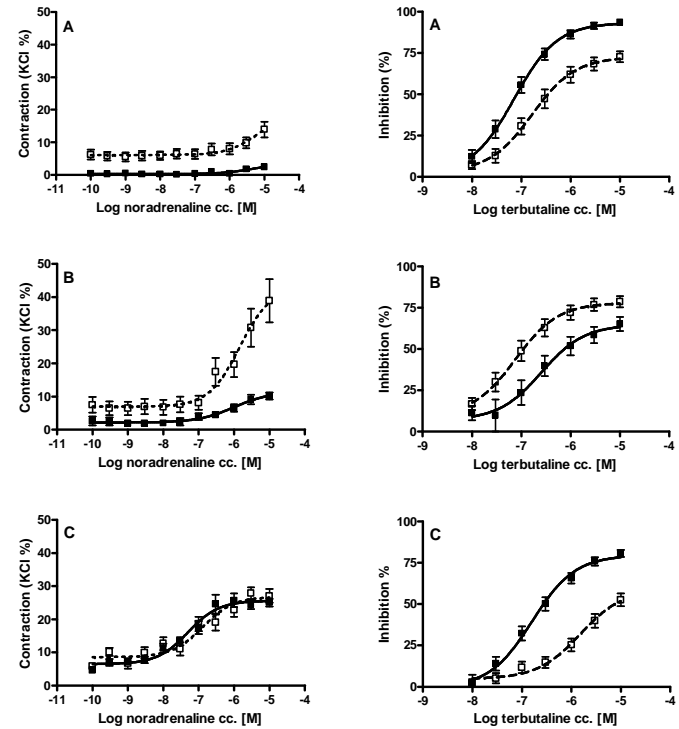


Figure 4. The effects of  $\alpha$ -methyl dopa treatment on the myometrial contractility evoked by noradrenaline at estrus (A), and on day 7 (B) and on day 21 (C). ■ indicates control values, and □ indicates  $\alpha$ -methyl dopa-treated values

Figure 5. The effects of  $\alpha$ -methyl dopa treatment on the relaxation effect of terbutaline at estrus (A), and on day 7 (B) and on day 21 (C). ■ indicates control values, and □ indicates  $\alpha$ -methyl dopa-treated values.

$\alpha$ -Methyl dopa treatment shifted the dose-response curve of terbutaline slightly to the right and decreased its maximal effect significantly in the non-pregnant animals. On day 7 of pregnancy,  $\alpha$ -methyl dopa treatment resulted in a higher maximal effect. At term,  $\alpha$ -methyl dopa treatment significantly and substantially decreased the relaxant effect of terbutaline, as evidenced by a decreased maximal effect and a higher  $\text{EC}_{50}$  value.

- *Results of radioligand-binding experiments*

In an effort to find explanation to our results received in isolated organ experiments we decided to measure the density and affinity of  $\alpha_2$  and  $\beta_2$  adrenergic receptors using radioligand binding technique. As for the  $\alpha_2$ -adrenergic receptors, both  $B_{max}$  and  $K_d$  were higher on day 7 of pregnancy than in the non-pregnant state, but both parameters had returned to the non-pregnant value at term in pregnancy. The only treatment-dependent significant change was a decrease in  $K_d$  measured on day 7 of pregnancy. No gestation-dependent change in the density of  $\beta$ -adrenergic receptors was detected, while  $K_d$  was increased by the end of pregnancy, and there was a treatment-dependent significant increase on Day 21.

### **Discussion**

Degeneration of the adrenergic nerves in the uterus during pregnancy is a well-described phenomenon in several species like guinea-pig, rat and human. It is not clear at present how this denervational phenomenon is evoked, and whether it is believed to be restricted only to the uterus but all of the relevant data are coming from histochemical data, however this was never investigated earlier from a functional point of view. We examined the decrease in the function of adrenergic neurons, and our functional approach is concluded to have one major advantage. We could detect a significant decrease in the function of the myometrial adrenergic nerves as early as day 7 of pregnancy. At the end of the first third of pregnancy, there were significant differences in noradrenaline release and uptake between the implantation and inter-implantation sites. This suggests that there are also foetoplacental factors responsible for the pregnancy-induced adrenergic denervation. This functional approach also has a disadvantage: we were not able to differentiate between muscle tissue and vessels.

Our results clearly reveal a substantial deterioration of adrenergic functions in the cervix, disclosed by the transmitter uptake capacity, but not by the EFS-evoked release. It could be suggested, therefore, that this capacity is a more sensitive feature of the adrenergic nerve function than the transmitter release.

Early reinnervation is detected immunohistochemically 48 h after delivery. However, our results indicate that the functions of the noradrenergic nerves in the myometrium and cervix have not recovered completely by postpartum weeks 4 and 2, respectively. Our results contribute to an understanding of the phenomenon, as the functional deterioration has been shown to start earlier than the structural denervation, and the restoration requires a longer period.

$\alpha$ -Methyldopa, a commonly used agent in the treatment of hypertension during pregnancy, is considered one of the safest drugs because of the huge amount of experience relating to its clinical use. Despite the large body of clinical experience, and its long use as an antihypertensive, its mechanism of action has been reconsidered several times since the recognition of its antihypertensive effect. According to the most widely accepted concept,  $\alpha$ -methyldopa exerts its antihypertensive action through the stimulation of  $\alpha_2$ -adrenergic receptors within the central nervous system, causing a decrease in the efferent sympathetic tone. In spite of the several decades of obstetrical use of  $\alpha$ -methyldopa, the available information on a direct uterotropic effect is very limited. Our transmitter liberation results revealed that  $\alpha$ -methyldopa all but abolished the electrically evoked noradrenaline peak in the estrus and in the early pregnant state. In the present experiments, treatment with  $\alpha$ -methyldopa was associated with a more marked inhibition of liberation at the inter-implantational sites, while in the case of implantational sites only the first stimulation resulted in a significantly smaller amount of liberated noradrenaline.

However, the release is similarly abolished from both  $\alpha$ -methyldopa-treated sites on the 7<sup>th</sup> day of pregnancy, indicating that the difference in the significance of the release pattern is caused by the more marked physiological denervation at the implantational sites. Hence, it could be speculated that  $\alpha$ -methyldopa treatment can “speed up” the physiological degeneration of the adrenergic fibers of the pregnant uterus. The results of the tissue radioactivity also favor this concept. By the time of late pregnancy this denervation has progressed, leading to the ineffectiveness of electrical stimulation, and thus the  $\alpha$ -methyldopa treatment cannot inhibit the release. The mechanism of the transmitter release-inhibiting effect of  $\alpha$ -methyldopa is an unanswered question, but it is in good agreement

with the finding that the  $\alpha_2$ -agonist dexmedetomidin, infused locally, decreased the liberation of noradrenaline in the nucleus accumbens of the mouse. It was proved that this effect is mediated through the  $\alpha_{2A}$  type of adrenergic receptors.

The contractions mediated by  $\alpha$ -adrenergic receptors were not affected by  $\alpha$ -methyldopa in non-pregnant and late-pregnant rats, but were significantly increased in early pregnancy. Treatment resulted in the lower efficacy and potency of terbutaline in non-pregnant and late-pregnant animals, while no change was found in the early-pregnant rats. Combining these findings, we concluded the same change for all 3 stages of gestation: the overall effects of sympathomimetics were shifted toward an increased contractility either by a decreased  $\beta$ -receptor-mediated relaxation or by a potentiated  $\alpha$ -receptor-mediated contraction. The reasons for these gestational age and treatment-dependent changes in the effects of the adrenergic agonists are not understood. Independently of the underlying mechanism,  $\alpha$ -methyldopa-induced alterations in the pharmacological effects of sympathomimetics can be of therapeutic importance, presuming that a similar final effect can develop in the human body. As many over-the-counter preparations contain  $\alpha$ -receptor agonists (e.g. nasal decongestants), their usage can seriously increase the uterine contractility when the concomitant pregnancy-induced hypertension is treated with  $\alpha$ -methyldopa. On the other hand, the decreased relaxant effect of terbutaline indicates that  $\alpha$ -methyldopa can make  $\beta$ -mimetic-based tocolytic therapy less effective, thereby increasing the danger of premature delivery.

As there are no direct data that exclude modulation of the effects of adrenergic agonists similar to our finding, sympathomimetics should be used with great care in pregnant women treated with  $\alpha$ -methyldopa.

Our results contribute to an understanding of the pregnancy induced denervation, and the outcome of our experiments leads us to conclude that superfusion can be utilized as a model system for investigation of the effects of pharmacological manipulation and pathological states (for example, pregnancy-induced hypertension or gestational diabetes) on the denervation procedure.

## List of scientific publications related to the thesis

I. Zupkó I, Csonka D, Falkay G:

A rat model for functional characterization of pregnancy-induced denervation and postpartum reinnervation in the myometrium and cervix: a superfusion study. *Reproduction*. 2005 Nov;130(5):743-9.

II. Csonka D, Zupkó I, Minorics R, Márki A, Csík G, Falkay G:

The effects of alpha-methyldopa on myometrial noradrenaline release and myometrial contractility in rat. *Acta Obstet Gynecol Scand*. 2007;86(8):986-94.

III. Csonka D, Kormányos Z, Csík G, Zupkó I, Falkay G:

Szuperfúziós technika alkalmazása a myometriális adrenerg transzmisszió vizsgálatára  
*Magy Nőorv L* 71: 121-6 (2008)



Lectures and poster presentations:

- Csonka D**, Zupkó I, Falkay G: A terhesség által kiváltott adrenerg denerváció funkcionális vizsgálata patkány uteruson. Ph.D. Tudományos Napok 2004, Budapest, 2004a. IV. 8-9.
- Csonka D**, Zupkó I, Falkay G: Adrenerg denerváció vizsgálata szuperfúziós technikával terhes patkány uteruson és cervixen. A Magyar Tudomány Ünnepe Szegeden, Szeged, 2004b. XI. 2-19.
- Csonka D**, Zupkó I, Csík G, Falkay G: Az  $\alpha$ -metildopa myometriális simaizomzatra kifejtett hatásának funkcionális vizsgálata patkányon. Ph.D. Tudományos Napok 2005, Budapest, 2005a. IV. 14-15.
- Csonka D**, Zupkó I, Csík G, Falkay G: The effect of alpha-methyl-dopa on the myometrium in rat. 1<sup>st</sup> International Symposium in Hot Topics & Controversies in Perinatal Medicine, Rhodes Island, Greece, 2005b. VI. 16-19.
- Csonka D**, Kormányos Z, Zupkó I, Falkay G: A hormonális státusz hatása a noradrenalin felszabadulásra humán myometriumban. Congressus Pharmaceuticus Hungaricus XIII., Budapest, 2006a. V. 25-27.
- Csonka D**, Kormányos Z, Zupkó I, Falkay G: Hormone replacement therapy decreases noradrenaline release in human myometrium. 15th World Congress of Pharmacology, Beijing, China, 2006b. VII. 2-7. [*Acta Pharmacol Sin* 27 Suppl 1: P420063 (2006)]
- Csonka D**, Márki Á, Csík G, Zupkó I, Falkay G: Functional investigation of myometrial adrenergic transmission in pregnant rats: Superfusion studies. International Workshop - Adrenergic Transmission: from Benchside to Bedside, Visegrád, 2006c. X. 6-7.
- Csonka D**, Zupkó I, Márki Á, Falkay G: Az  $\alpha$ -metildopa myometriális simaizomzatra kifejtett hatásának funkcionális vizsgálata patkányon. Ph.D. Tudományos Nap, Szeged, 2006d. V. 3.
- Zupkó I, **Csonka D**, Falkay G: Functional investigation of pregnancy-induced adrenergic denervation of the uterus in the rat. 4th Congress of the Federation of the European Pharmacological Societies, Porto, Portugal, 2004a. VII. 14-17. [*Fund Clin Pharmacol* 18 Suppl 1: 39 (2004)]
- Zupkó I, **Csonka D**, Falkay G: Superfusional investigation of adrenergic innervation of the uterus during pregnancy and postpartum period in the rat. 10th Symposium of the Austrian Pharmacological Society, Vienna, Austria, 2004b. IX. 23-16. [*Pharmacology* 72: 146 (2004)]
- Zupkó I, **Csonka D**, Csík G, Falkay G: The effect of  $\alpha$ -methyl-dopa treatment on the myometrial noradrenaline release and contractility of the rat. A Magyar Experimentális Farmakológia Tavaszi Szimpóziuma, Budapest, 2005. VI. 6-7.