

# Developmental Changes of Some G-protein Coupled Receptors Affected by c-fos Knock-out

**Beneš J.<sup>1</sup>, Nováková M.<sup>1</sup>, Kvetňanský R.<sup>2</sup>, Mysliveček J.<sup>1</sup>**

<sup>1</sup>Institute of Physiology of the First Faculty of Medicine, Charles University in Prague, Czech Republic;

<sup>2</sup>Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia

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**Mailing Address:** Jaromír Mysliveček, MD., PhD., Institute of Physiology of the First Faculty of Medicine, Charles University, Albertov 5, 128 00 Prague, Czech Republic, Phone: +420 224 968 485, Fax: +420 224 918 816, e-mail: jmys@lf1.cuni.cz

**Abstract:** The role of c-fos gene disruption on binding characteristics of selected G protein-coupled receptors has been investigated. The following receptors were studied: muscarinic receptors (MR),  $\alpha_1$ -adrenoceptors (AAR),  $\beta$ -adrenoceptors (BAR),  $D_1$ -like dopamine receptors ( $D_1R$ ),  $D_2$ -like dopamine receptors ( $D_2R$ ). We have tested the hypothesis that c-fos gene disruption would not influence the receptor density in central nervous system (CNS: brain cortex and cerebellum) and in the periphery (lung, heart). We found that both in the periphery and in the CNS there were important changes in receptor density. Both MR and BAR were increased in the lung and heart. The effects of c-fos gene disruption in CNS were more selective. In general, the receptors that activate Gq-phospholipase C-proteinkinase C pathway (AAR, MR) were affected, while the others (that activate/or inhibit adenylyl cyclase: BAR,  $D_1R$ ,  $D_2R$ ) were not. These results show that disruption of c-fos gene could significantly change the expression of G protein-coupled receptors. Moreover, these changes could be comprehended as one of the adaptive mechanisms that help the organism to cope with c-fos gene disruption.

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## Introduction

In the last decade, the progress in the gene disruption technology allowed to study the effects of single gene knockout on different molecules involved in the signalization cascade activated via G-protein coupled receptors (GPCR), i.e. muscarinic receptors, adrenoceptors, dopamine receptors. Targeting these signalization cascades, many knockout mice have been developed, i.e. all ( $M_1$ – $M_5$ ) muscarinic receptor knockout mice [1], and also acetylcholinesterase (AcChE) knockout mice [2]. Recently, we have shown [3] that (AcChE  $-/-$ ) mice reveal not only changes in the number of muscarinic receptors in the heart, lung, cortex and cerebellum, but also of adrenoceptors and  $D_2$ -like dopamin receptors. Similarly, CRH KO (corticotropin releasing hormone knockout) mice had changed basal levels of muscarinic receptor,  $\alpha$ -adrenoceptor and  $\beta$ -adrenoceptors in the lung [4]. Moreover, the receptor behavior under stress differed from that of wild type counterparts.

Now we wondered to know if the disruption of c-fos gene could affect the properties of muscarinic receptors,  $\alpha_1$ -adrenoceptors,  $\beta$ -adrenoceptors,  $D_1$ -like dopamine receptors and  $D_2$ -like dopamine receptors in the lung, heart, cerebral cortex and cerebellum of mice.

c-Fos is a molecule that can be viewed from more angles. First, it is a third messenger activating the target genes [5]. Second, it can be comprehended as an inducible transcription factor [6]. Third, it is a product of an immediate early gene [7]. Fourth, it is a ubiquitous molecule that can give us the picture of cell activation [8]. It is important to note that all these aspects are not clearly bordered. In respect to G-protein coupled receptors, c-Fos is activated by multiple pathways. I.e. it can be activated both by proteinkinase C activation (that is caused by

diacylglycerol increase from enhanced phospholipase C activity) and by  $\text{Ca}^{2+}$  increase (that is caused by inositoltrisphosphate increase from enhanced phospholipase C activity) [9]. The expression of c-Fos can be enhanced by muscarinic receptors [10],  $\alpha$ -adrenoceptors,  $\beta$ -adrenoceptors [11],  $\text{D}_1$ -like dopamine receptors,  $\text{D}_2$ -like dopamine receptors and many others [12].

On the other hand, our knowledge about the reverse pathways (i.e. about the effects of c-Fos protein on the receptor – G-protein coupled receptors, respectively) is limited. Therefore, we have employed the mice with targeted disruption of c-fos gene [13] in order to investigate the effects of c-Fos protein on some target (or late onset) genes (i.e. on G-protein coupled receptors expression).

The receptors of our interest were of multiple activated pathways, and of different effects on the target tissue. Muscarinic receptors (i.e. those mainly expressed in the central nervous system – odd numbered receptors) and  $\alpha_1$ -adrenergic receptors activate phospholipase C via Gq protein and then protein kinase C that can affect the c-Fos protein expression in the cell.  $\text{D}_1$ -like dopamine receptors and  $\beta$ -adrenoceptors activate adenylyl cyclase via Gs protein. On the other hand,  $\text{D}_2$ -like dopamine receptors inhibit adenylyl cyclase via Gi protein.

Therefore, with respect to limits of investigated set of receptors, we could deduce the role of c-Fos in receptor pathways activation. In general, we targeted our attention to two types of tissue: to central nervous system, where the role of muscarinic, dopaminergic and adrenergic receptors is much more independent with respect to the final effect in the target structure, and on peripheral tissue, where the role of receptors is more mutually interconnected. Therefore the effects in periphery can be explained by regulation of receptors between each other (i.e. by heterologous regulation) while in the central nervous system the c-fos gene disruption could be explained by target effects of c-fos on the receptors.

## **Material and methods**

### *Animals*

Male control (WT, +/+) and c-fos knock-out (KO, -/-) mice (20–25 g) were used. Knock-out line was originally obtained from Institute for Molecular Pathology, University of Vienna, Austria. Mice were sacrificed by decapitation and exsanguinations and lung, heart ventricles, cerebral cortex and cerebellum were dissected, flash frozen in liquid nitrogen and stored at  $-70\text{ }^\circ\text{C}$  for the further analysis.

### *Binding experiments*

Binding experiments were performed similarly as described previously [14]. Briefly, in preliminary experiments, the receptors were bound with increasing concentrations of radioligand in order to ascertain:

- a) the saturating concentration of radioligand, and
- b) the receptor affinity to radioligand, expressed as dissociation constant ( $K_D$ ).

The radioligands used were:  $^3\text{H}$ -prazosin (specific for  $\alpha_1$ -adrenoceptors),  $^3\text{H}$ -CGP12177 (specific for  $\beta$ -adrenoceptors),  $^3\text{H}$ -QNB (specific for muscarinic receptors),  $^3\text{H}$ -SCH 23990 (specific for  $D_1$ -like dopamine receptors), and  $^3\text{H}$ -spiperon (specific for  $D_2$ -like dopamine receptors).

Then, simplified saturation binding experiments with one saturating concentration of radioligand were used in order to determine the receptor density ( $B_{\max}$ ). Following formula was used:  $B_{\max} = B \times ([L] + K_D) / [L]$  (1), where  $B$  = bound of radioligand [fmol/mg of protein],  $L$  = radioligand concentration [fmol/l], and  $K_D$  =  $K_D$  [fmol/l] of the radioligand. Homogenates were incubated in duplicates in Tris-EDTA buffer (Tris-HCl 50 mmol/l, EDTA 2 mmol/l, pH adjusted to 7.4) with following single fully saturating concentration of the radioligand: 2000 pmol/l of  $^3\text{H}$ -prazosine for  $\alpha_1$ -adrenoceptors, 2000 pmol/l of  $^3\text{H}$ -CGP 12177 for  $\beta$ -adrenergic receptors, 1500 pmol/l  $^3\text{H}$ -QNB for muscarinic receptors, 4000 pmol/l of  $^3\text{H}$ -SCH23390 for  $D_1$ -like dopamine receptors and 4000 pmol/l of  $^3\text{H}$ -spiperon for  $D_2$ -like dopamine receptors.

#### Material

$^3\text{H}$ -prazosine [7-metoxo-] (3.21 TBq/mmol),  $-(\text{-})4\text{-}(3\text{-tert-butylamino-2-hydroxypropoxy})\text{-}[5,7\text{H}]\text{benzimidazol-2-one}$  ( $^3\text{H}$ -CGP 12177, 1.22 TBq/mmol),  $^3\text{H}$ -Quinuclidinyl benzilate, L-[benzilic-4,4'- $^3\text{H}$ ]- ( $^3\text{H}$ -QNB, 1.35 TBq/mmol),  $^3\text{H}$ -SCH23390, [N-methyl- $^3\text{H}$ ] (3.15 TBq/mmol),  $^3\text{H}$ -spiperon (8-[4-(p-fluorophenyl)-4-oxo[2,3(n)-butyl]-1-phenyl 1,3,8-triazospiro[4,5]decan-4-one [benzene ring- $^3\text{H}$ ] (0.56TBq/mmol) were purchased from Perkin-Elmer, Boston, MA, USA.

#### Data treatment

Radioligand binding data have been evaluated using GraphPad software. Statistical significance of differences between means was evaluated with Student t-test.

#### Results

In the peripheral tissue, there was substantial increase in both muscarinic receptors and  $\beta$ -adrenoceptors. In detail, lung muscarinic receptors (that are of  $M_2$  and  $M_3$  subtype, see Figure 1) were increased to 305% of values ascertained in wild type animals, while  $\beta$ -adrenoceptors were increased to 259%. In the heart (Figure 2), there muscarinic receptors (almost all muscarinic receptors belong to  $M_2$  subtype) in c-fos knock-outs rose to 172% and  $\beta$ -adrenoceptors rose to 155%.

In the cerebral cortex (Figure 3), there was increase in number of  $\alpha_1$ -adrenoceptor and muscarinic receptor binding sites (to 184%, and 234%, respectively). Other receptors, i.e.  $\beta$ -adrenoceptors,  $D_1$ -like dopamine receptors

and D<sub>2</sub>-like dopamine receptors did not change in comparison to wild type animals, although there was noticeable increase in D<sub>2</sub>-like dopamine receptors.

In cerebellum (Figure 4), as in the structure with important connections to motion regulation and equilibrium maintenance, we have been able to find only the changes in  $\alpha_1$ -adrenoceptor number. No other receptor of our interest was affected.

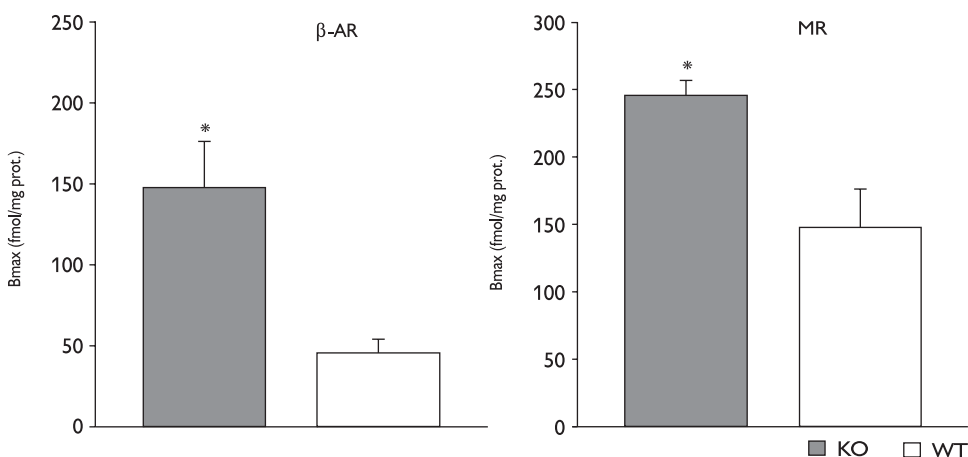


Figure 1 – Changes in  $\beta$ -adrenoceptors ( $\beta$ -AR) and muscarinic receptors (MR) in lung of mice with *c-fos* gene disruption. Ordinate: receptor densities (*B*<sub>max</sub>, fmol/mg of protein). KO knock-out mice, WT wild type mice. \*  $p < 0.05$

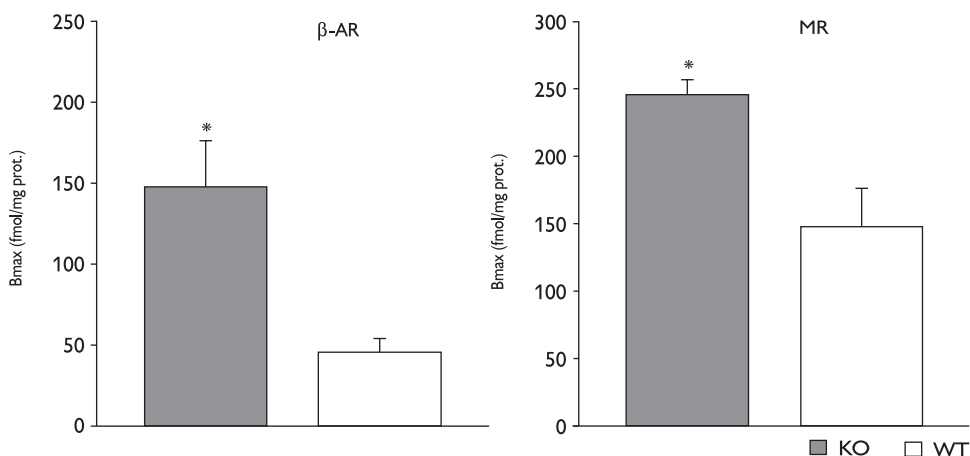


Figure 2 – Changes in  $\beta$ -adrenoceptors ( $\beta$ -AR) and muscarinic receptors (MR) in heart of mice with *c-fos* gene disruption. Ordinate: receptor densities (*B*<sub>max</sub>, fmol/mg of protein). KO knock-out mice, WT wild type mice. \*  $p < 0.05$

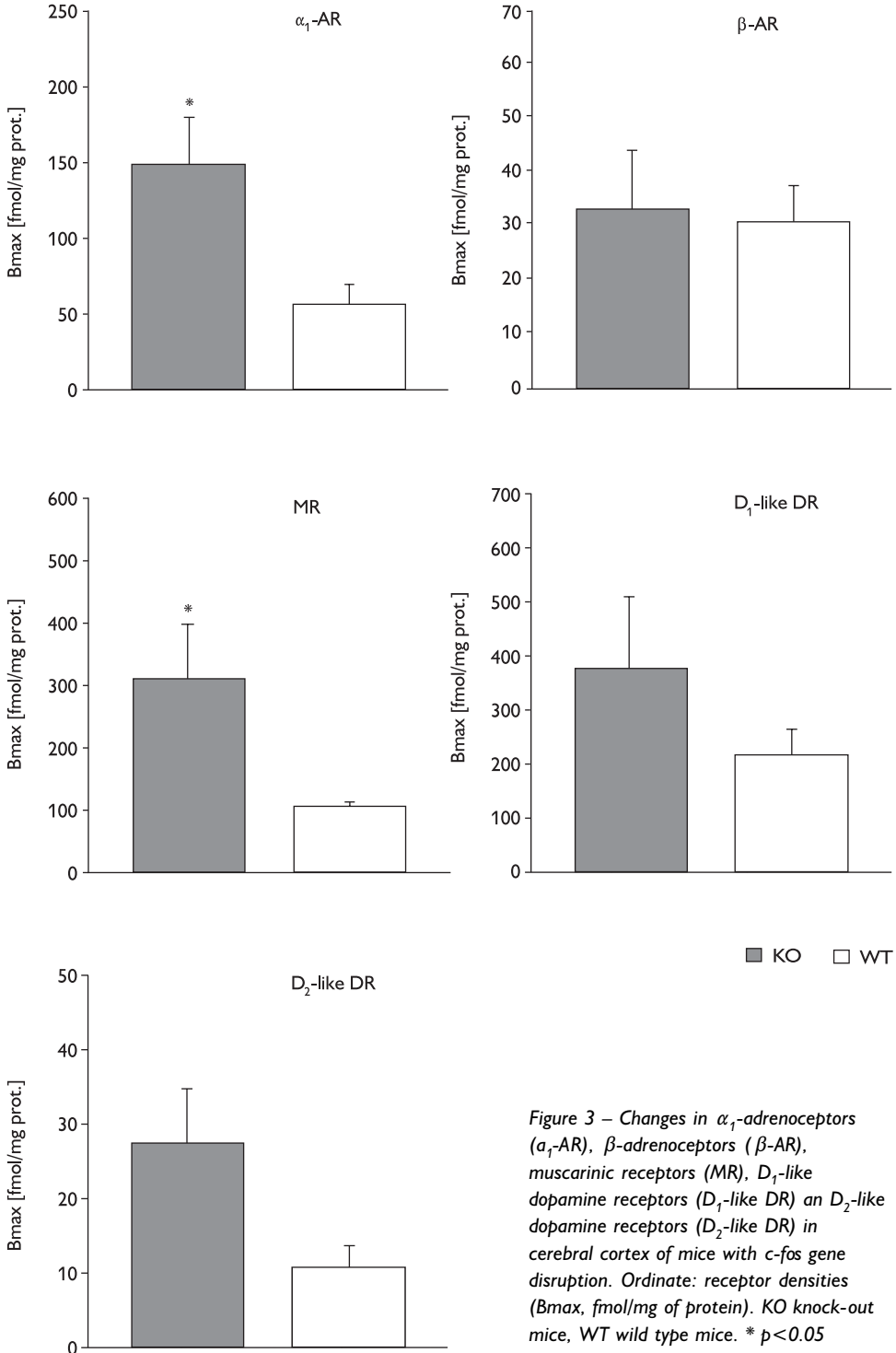


Figure 3 – Changes in  $\alpha_1$ -adrenoceptors ( $\alpha_1$ -AR),  $\beta$ -adrenoceptors ( $\beta$ -AR), muscarinic receptors (MR), D<sub>1</sub>-like dopamine receptors (D<sub>1</sub>-like DR) and D<sub>2</sub>-like dopamine receptors (D<sub>2</sub>-like DR) in cerebral cortex of mice with c-fos gene disruption. Ordinate: receptor densities (B<sub>max</sub>, fmol/mg of protein). KO knock-out mice, WT wild type mice. \* p < 0.05

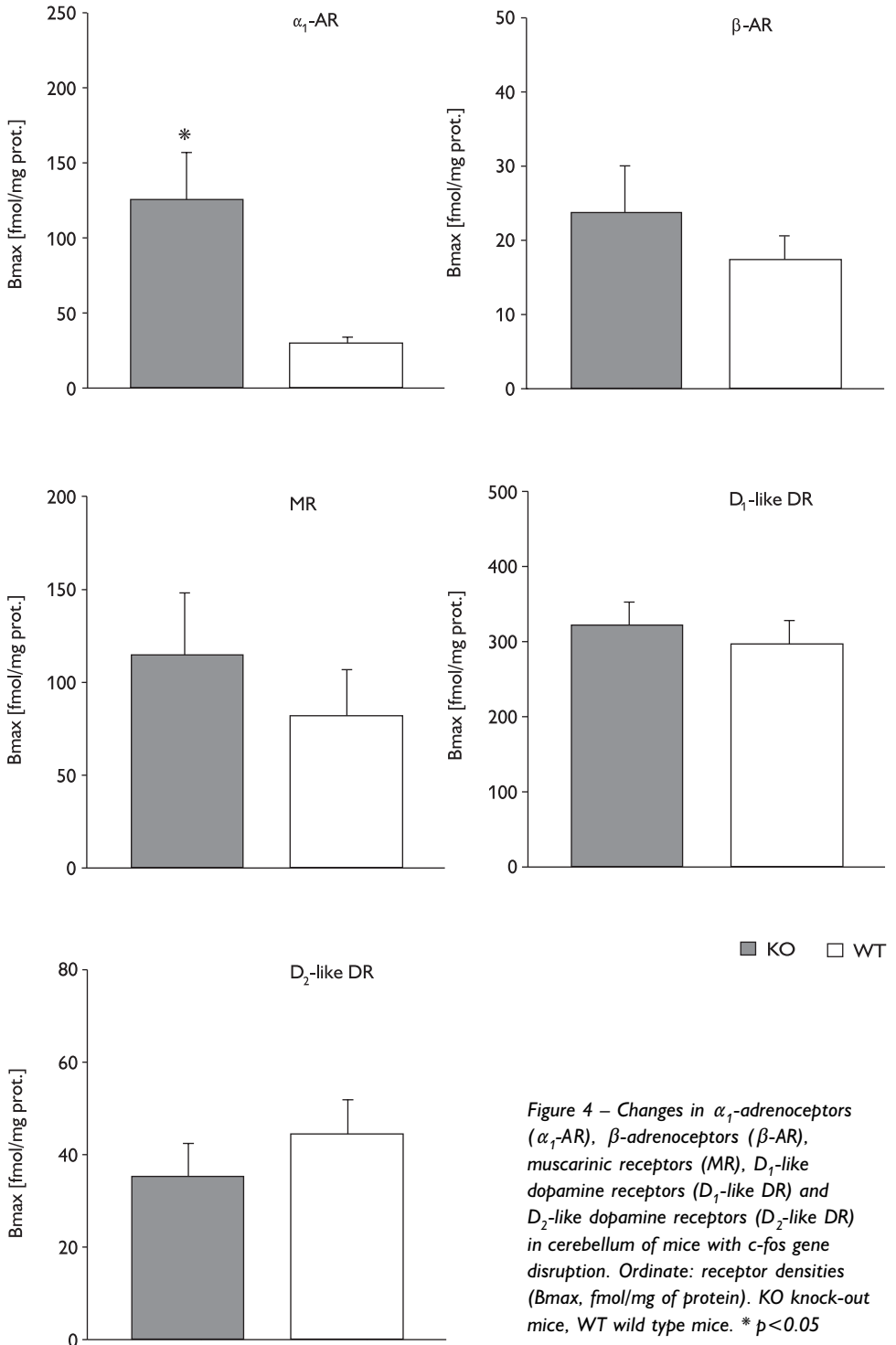


Figure 4 – Changes in  $\alpha_1$ -adrenoceptors ( $\alpha_1$ -AR),  $\beta$ -adrenoceptors ( $\beta$ -AR), muscarinic receptors (MR), D<sub>1</sub>-like dopamine receptors (D<sub>1</sub>-like DR) and D<sub>2</sub>-like dopamine receptors (D<sub>2</sub>-like DR) in cerebellum of mice with *c-fos* gene disruption. Ordinate: receptor densities (B<sub>max</sub>, fmol/mg of protein). KO knock-out mice, WT wild type mice. \* *p* < 0.05

## Discussion

Our results show that disruption of c-fos gene can dramatically change the number of binding sites (the functional receptor expression, in fact) both in the peripheral tissue and also in the central nervous system. These findings are new and, to our knowledge, have not yet been published.

Data about changed number of binding sites in the peripheral tissue are difficult to explain. One could hypothesize about what role c-Fos plays in the receptor expression in cells affected by autonomous nervous system. Moreover, c-fos plays an important role in the development of cardiac hypertrophy [15]. Therefore it is possible to speculate about its role of increased receptor number due to disrupted c-fos gene expression.

The changes of  $\beta$ -adrenoceptors in the peripheral tissue can be explained by two ways: first, it could be due to cross-regulation between  $\alpha$ -adrenoceptor and  $\beta$ -adrenoceptors or second, it could be caused by the changes in sympathetic/parasympathetic centres in the central nervous system that affect the release of sympathetic/parasympathetic transmitters.

The data about the cerebral cortex are much more interesting. These results imply the role of c-fos in regulation of receptors activating phospholipase C and consequently protein kinase C. This pathway can be comprehended as the major process in c-fos activation. Therefore it can be very important that these receptors are affected by c-fos gene disruption but the others (activating non-phospholipase C signalling pathways) do not.

Another explanation of these changes is the possibility of changed expression (function) of another transcription factor (c-Jun) that is in close connection to c-Fos function.

The next possibility is the role of c-fos in the regulation of genes affecting the acetylcholine synthesis (cholin acetyl transferase, ChAT) and availability (the expression of vesicular acetylcholine transporter, VAChT) in the neuronal cleft [16]. Then the changes of other receptors are explainable by heterologous regulation of these receptors, the event of so called “fine tuning” of receptor mediated signal.

On the other hand, in cerebellum we have only able to found the changes in  $\alpha_1$ -adrenoceptor number. This can be caused by different reasons: by the complexity of cerebellar connections to other structures in the central nervous system or by the fact that the localization of the receptors is different (in comparison to the cerebral cortex). For example, the receptors could be localized presynaptically/postsynaptically (cerebral cortex/cerebellum) and it can play different role in determination of their density in the adulthood. On the other hand, the changes of muscarinic receptors in the cortex that are mainly of  $M_1$  subtype are not surprising (as this subtype stimulate Gq-phospholipase C-protein kinase pathway). The muscarinic receptors in the cerebellum belong mainly to  $M_2$  subtype what could be reason for their unchanged status in c-fos KO



animals. Unfortunately, the identification of muscarinic receptor subtypes is very complicated. There is no subtype specific antagonist for any of five muscarinic subtypes (except of MT7 toxin for M<sub>1</sub> muscarinic receptors). Therefore, further study is needed to determine these subtypes.

In summary, the possibility to work with animals lacking c-fos allowed us to determine the possible targets of this gene/protein. It can be muscarinic receptors and  $\beta$ -adrenoceptors in the peripheral tissue. Surprisingly,  $\beta$ -adrenoceptors were not affected by this gene disruption in the cerebral cortex. Similarly, the other G protein-coupled receptors that activate adenylyl cyclase (D<sub>1</sub>-like dopamine receptors) were not affected too. In that context, it may be also important that only receptors activating phospholipase C/protein kinase C pathway were affected by c-fos gene disruption. On the other hand, much more data are needed for precise interpretation of our findings. Despite that, the fact that there can be connections between c-Fos and G-protein-coupled receptors is new and can be of great importance in the signalization regulation through these receptors.

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