Amino Acids (2000) 19: 23–31

Amino
Acids


# Margatoxin and iberiotoxin, two selective potassium channel inhibitors, induce c-fos like protein and mRNA in rat organotypic dorsal striatal slices

**A. Saria**[1], **H. S. Fischer**[1], **C. Humpel**[1], **A. Pfattner**[1], **D. S. Schatz**[1], and **R. Schuligoi**[2]

[1] Division of Neurochemistry, Department of Psychiatry, University Hospital Innsbruck, Austria
[2] Department of Experimental and Clinical Pharmacology, University of Graz, Austria

Accepted September 20, 1999

**Summary.** The isolated single organotypic slice model allows to investigate the effects of drugs and toxins on the expression of transcription factors in the striatum without dopaminergic and glutamatergic interactions. In this study the effects of margatoxin and iberiotoxin on the expression of c-fos mRNA by in situ hybridization as well as on c-fos like protein by immunohistochemistry in isolated dorsal striatum after 10 days in culture were investigated. C-fos mRNA dose-dependently increased 30 min after incubation with margatoxin and iberiotoxin. Expression of c-fos like protein was transiently detected 3 h afterwards. This effect is independent from extrinsic neuronal circuitry as dopamine neurons were found to be absent in the cultured slices. It is concluded that inhibition of voltage-gated as well as calcium-activated (Slo) potassium channels leads to activation of gene transcription in striatal neurons which may trigger long-term changes in transmitter plasticity.

**Keywords:** Amino acids – Scorpion toxins – Striatum – Gene expression

## Introduction

The functional role of high-conductance calcium-activated potassium channels (or Slo channels) in the mammalian brain is not well understood. Their putative co-localization with voltage-gated calcium channels suggests that they function as feedback regulators of intracellular calcium concentration and thus allows Slo-channels to regulate the amount of neurotransmitter released from presynaptic nerve terminals by modulating the duration of the presynaptic action potential (Robitaille and Charlton, 1992; Robitaille et al., 1993). Iberiotoxin (IbTX), a selective inhibitor of Slo channels has been shown to increase acetylcholine release at the

neuromuscular junction (Vatanpour and Harvey, 1995), but not in striatal slices in vitro (Fischer and Saria, 1999). However, significant amounts of Slo-channel protein and mRNA are found in basal ganglia (Knaus et al., 1996). The scorpion toxin margatoxin (MgTX) shares 41% sequence homology with IbTX and is a potent inhibitor of voltage-gated potassium channels of the subtype Kv1.2 and Kv1.3 (Garcia-Calvo et al., 1993; Knaus et al., 1995). MgTX was recently found to increase the release of dopamine (Saria et al., 1998) and acetylcholine (Fischer and Saria, 1999) in rat striatal slices. Immediate early genes (IEG) act as transcription factors, which are considered to be important initial factors for plastic and potential cytoskeletal changes in response to neurotransmitters, pharmaceuticals, toxins or drugs of abuse. Because c-fos is transiently induced by extracellular stimulation in nervous cells and tissue, c-fos induction may in general be a molecular marker for neuronal activity (Morgan and Curran, 1989; Zhang, 1992). Its induction has been used as a cellular marker to map neurons and circuits activated by various drugs. It has been shown, for example, that amphetamine and cocaine cause rapid induction of c-fos in the striatum in different patterns in the striosome-matrix compartments, and that both drugs are sensitive to dopamine receptor blockade (Graybiel et al., 1990; Moratalla et al., 1993; Young et al., 1991). Dragunow et al. (1991) have reported that methylenedioxy metamphetamine (ecstasy) also induces c-fos in the caudate-putamen, nucleus accumbens and olfactory tubercle in rats in vivo which was reversed by the non-competitive NMDA antagonist MK-801.

The aim of this study was to investigate the induction of c-fos like protein and c-fos mRNA in 10 day old isolated dorsal striatal slices after exposure to scorpion toxins selective for voltage-gated and calcium-activated potassium channels. We have recently shown that this model represents an isolated system without afferent dopaminergic innervation (Schatz et al., 1999). The effects of scorpion toxins on c-fos expression have therefore been studied in absence of dopaminergic and glutamatergic interactions from cortical and mesencephalic inputs.

## Material and methods

### *Organotypic cultures of striatal slices*

Organotypic cultures were established as described elsewhere (Hutter et al., 1996; Stoppini et al., 1991). The dorsal caudate-putamen of postnatal day 10 (P10) rats was dissected under aseptic conditions, 400-$\mu$m thick slices were cut with a tissue chopper (McIllwain), and the slices placed on a Millicell-CM 0.4$\mu$m culture plate (Millipore, 5–6 slices per membrane). Slices were cultured in Petri dishes at 37°C and 5% $CO_2$ with 1 ml/Petri dish of the following culture medium: 50% minimal essential medium/Hepes (Gibco), 25% heat inactivated horse serum (Gibco), 25% Hank's solution (Gibco), 2 mM $NaHCO_3$ (Merck), 6.5 mg/ml glucose (Merck), 2 mM glutamine (Merck), pH 7.2. After 14 days in culture slices were incubated with vehicle (control) or drugs. At this time point it has been found (Schatz et al., 1999) that there is no dopamine present in the slices. The medium was changed three times a week.

## *In situ hybridization*

In situ hybridization was performed as described recently (Sirinathsinghji and Dunnett, 1994). Striatal slices were carefully removed from the membrane applied onto slides (PobeOn™ slides, Fisher Biotech, USA) and frozen on $CO_2$-cooled glass plates. Slices were stored at −20°C until use, thawed to room temperature, air dried, fixed for 5 min in 4% phosphate-buffered paraformaldehyde, rinsed in phosphate-buffered saline for 2 min, dehydrated in a series of alcohol and stored in 95% ethanol at 4°C until analysis. For in situ hybridization procedure the sections were removed from the alcohols, air dried at room temperature and then incubated overnight at 42°C in a humidified chamber with the radiolabelled probe in hybridization buffer (Sirinathsinghji and Dunnett, 1994). The c-fos oligodeoxyribonucleotide probe (48 bases long, obtained from British Biotechnology, Oxon, U. K.) was labeled at the 3′ end with [$^{35}$S]deoxyadenosine-5′-($\alpha$-thio)triphosphate (1,300 Ci/mmol; New England Nuclear, Vienna, Austria) and terminal deoxynucleotidyl transferase enzyme (Boehringer Mannheim, Vienna, Austria). After incubation at 37°C for 15 min, the labelled probe was purified by a spin column procedure using Sephadex G-50, its specific activity being 1.0–1.4 $\times$ $10^9$ c.p.m./$\mu$g. After application of 100 $\mu$l of hybridization buffer with the labelled probe ($3 \times 10^3$ c.p.m./$\mu$l) to each slide, the sections were covered with parafilm and incubated overnight at 42°C in a humidified chamber to enable the hybridization process to take place. The sections were then washed for 1 h with standard saline citrate at 55°C, dehydrated through a series of alcohols and dipped in Ilford K5 autoradiographic emulsion diluted 1:1 with water. After four to five weeks of exposure, the autoradiograms were developed and the sections counterstained with Methylene Blue to permit identification of cell nuclei. The specificity of the probe was proven by the absence of any hybridization signal when an excess of cold c-fos oligodeoxyribonucleotide was used in the hybridization process. The autoradiograms were examined by use of a light microscope (Axiophot, Carl Zeiss, Oberkochen, Germany) and a computerized image analysis system (MCID M4, Imaging Research, St. Catharines, Ontario, Canada).

## *c-fos like immunohistochemistry*

Immunohistochemistry (IHC) using the avidin-biotin technique was performed as described previously (Humpel et al., 1996; Zhang et al., 1992). Slices were fixed for 3 h at 4°C with a fixative consisting of 2% paraformaldehyde (w/v) and 15% of saturated picric acid (v/v) in 0.1 M PBS (Zamboni and DeMartino, 1967), incubated overnight in 20% sucrose/phosphate-buffered saline and carefully removed from the membrane. All immunohistochemical detections were performed on whole mount slices using the free floating technique. The slices were washed three times in 0.05 M tris-buffered saline (TBS) for 5 min at room temperature and incubated with either tyrosine hydroxylase (TH) specific antiserum or the c-fos specific antiserum (rabbit polyclonal IgG, Santa Cruz Biotechnology) at room temperature overnight. The antiserum was diluted (1:1,000) in 0.05 M TBS containing 0.1% Triton X-100. Slices were washed three times in TBS again and incubated with secondary anti-rabbit biotinylated antibody (1:100, Vectastain) for 1 h at room temperature. After washing, slices were incubated with Vectastain reagent (AB-complex) for 1 h, washed again and the signal detected using 3,3′-diaminobenzidine (0.5 mg/ml) as a substrate. Slices were mounted on gelatine-coated glass slides, air dried and mounted with Entellan. To control the specificity of the immunostaining, free floating striatal sections were incubated with c-fos antibody pre-adsorbed with the synthetic peptide (c-fos peptide-2, residues 4–17, human, Oncogene Science, INC, Cambridge). The preadsorption was carried out with the diluted peptide (500 $\mu$g/ml, 1:500) at 4°C overnight. Some sections were incubated without the primary antiserum to test non-specific staining due to the secondary antibody.

### *Evaluation of sections and statistics*

Immunolabelled sections were analyzed by means of a computer-assisted image analysis system (Image pro Plus Software, connected to an Olympus BX60 microscope via a Sony video camera using a 10× magnification). Representative sections of about 1 mm$^2$ were quantified by counting stained cell nuclei and by measuring different intensities on a scale from 1–4. The hybridization signal in the organotypic cultures was quantified by counting 20 representative neurons per slice and averaging their proportional area covered by grains. Multistatistical analysis was carried out by multifactorial ANOVA, followed by one way ANOVA with a subsequent Scheffe post hoc test to compare control animals with the respective treated animals.

## Results

Tyrosine hydroxylase immunostaining (TH-LI) decreased gradually in striatal slices with time in culture. Figure 1 illustrates this decrease at different antiserum concentrations. After 10 days in culture, margatoxin as well as iberiotoxin increased c-fos mRNA in a concentration-dependent manner in striatal slices, as shown by in-situ hybridization (Fig. 2). The maximum response was reached at 3 nM of either toxin (data not shown). The highest signal was observed 30 minutes after starting the incubation, followed by a rapid decrease within few hours (Fig. 3). The induction of c-fos mRNA was accompanied by an increase in the number of cells staining for c-fos protein, as determined with immunohistochemistry (Fig. 4). The increase of c-fos protein was somewhat delayed compared with c-fos mRNA, reaching a maximum at 3 hrs incubation time (Fig. 5). Thereafter, the immunostaining

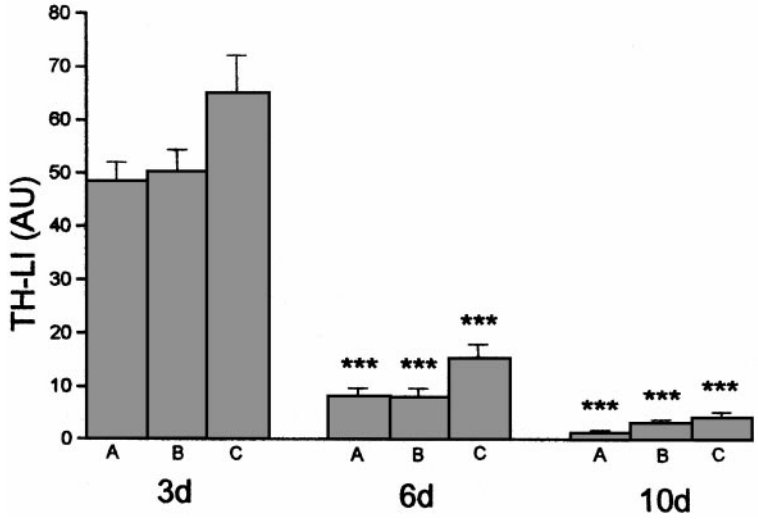


**Fig. 1.** Tyrosine hydroxylase immunostaining (arbitrary units) in organotypic striatal slices after 3, 6, and 10 days in culture. *A*, *B* and *C* represent different dilutions of the antiserum (1:1,000, 1:1,000 with post-fixation and 1:4,000, respectively). *** significantly different ($P < 0.001$) from 3d values

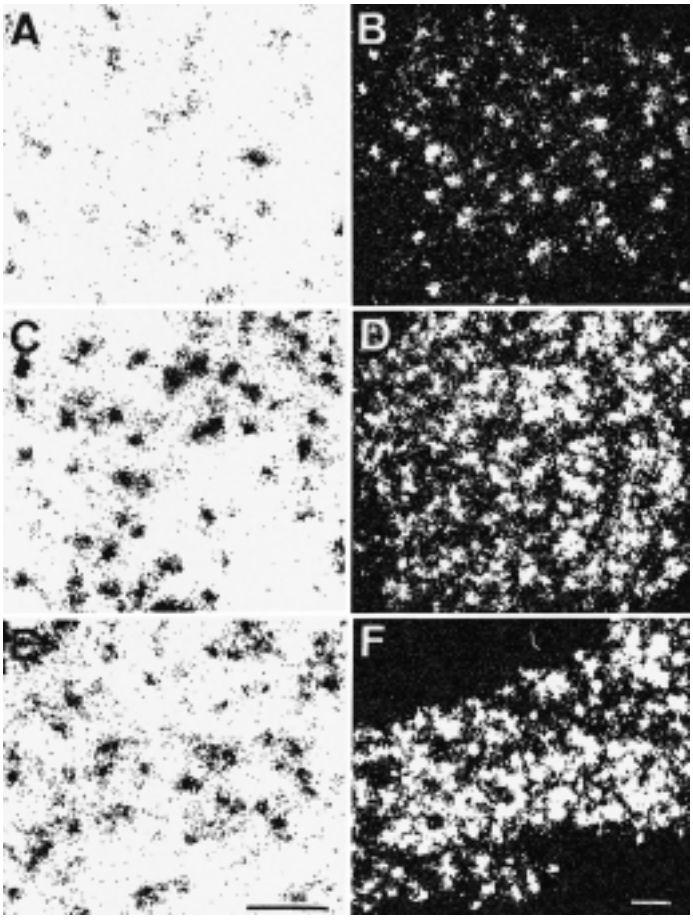


**Fig. 2.** Detection of c-fos mRNA by in situ hybridization in 10 day old dorsal striatal slices. Bright field micrographs show the typical mRNA positive black silver grains over methylene blue counterstained cells (**A**,**C**,**E**). Dark field photomicrographs show white grains (**B**,**D**,**F**). Incubation with (**A**,**B**) saline or (**C**,**D**) 30 min after stimulation with margatoxin or (**E**,**F**) iberiotoxin (10 nM each). Bar indicates 11 $\mu$m

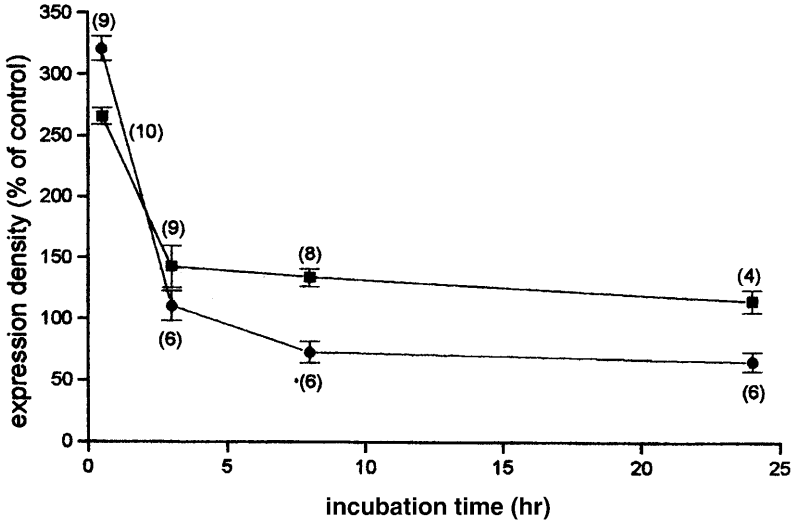


**Fig. 3.** Time course of the expression density of c-fos mRNA in 10 day old dorsal striatal slices after incubation with 10 nM margatoxin (circles) or 10 nM iberiotoxin (squares). Means ± s.e.m., n in parentheses

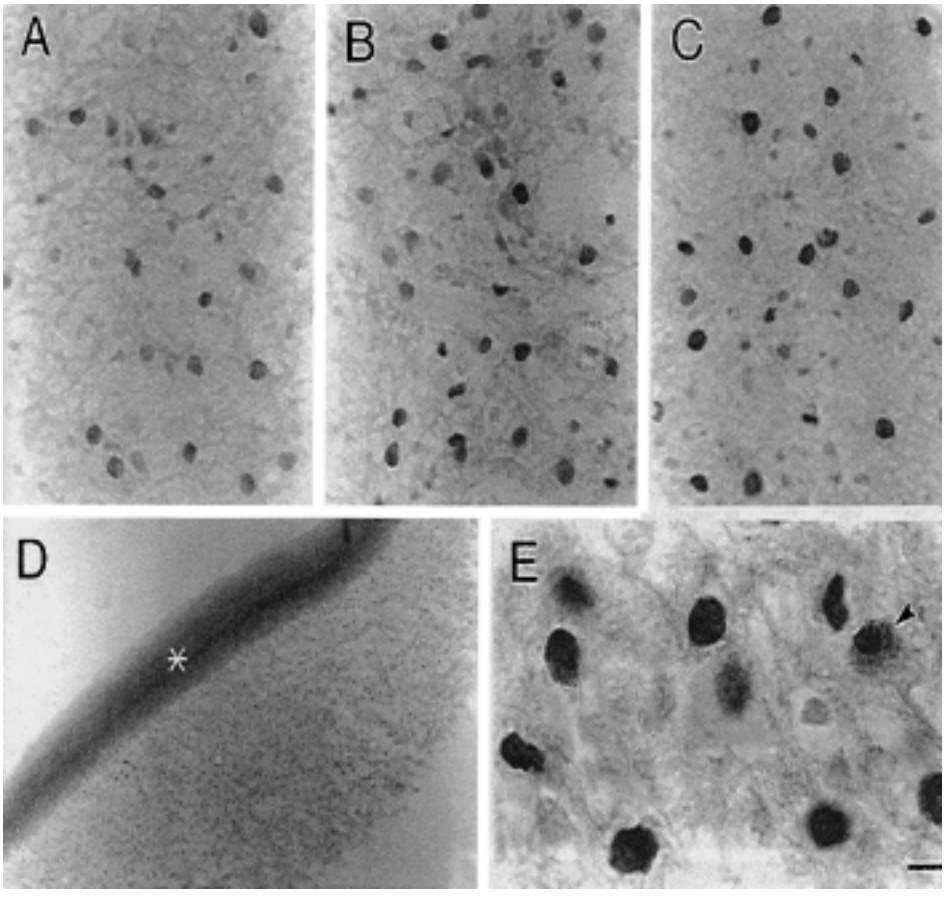


**Fig. 4.** C-fos like immunoreactivity in 10 day old dorsal striatal slices. **A** Slices were incubated for 3 h with vehicle, or 30 nM margatoxin (**B**) or with 30 nM iberiotoxin (**C**). **D** Lower magnification of **C**, asterisk indicates millicell membrane. **E** Higher magnification of **C**, bar indicates 6.6 $\mu$m, arrowhead indicates cell with predominant staining of the nucleus

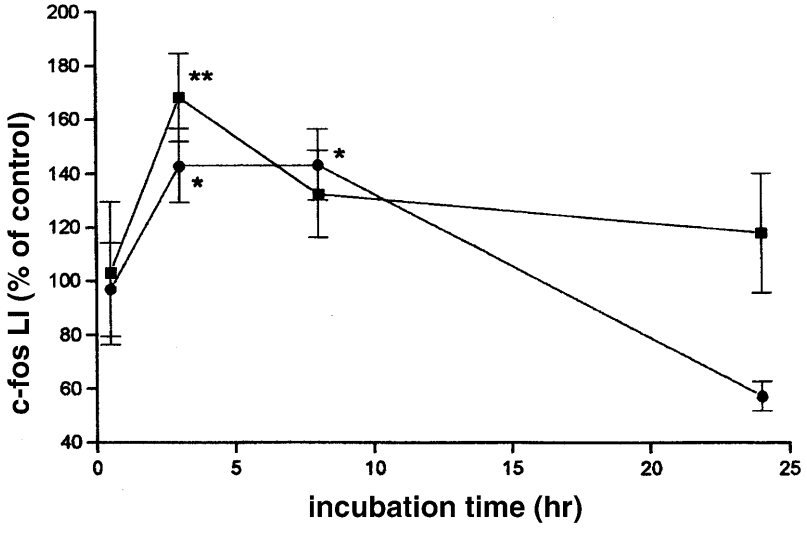


**Fig. 5.** Time course of c-fos immunoreactivity after incubation with 30 nM margatoxin (circles) or 30 nM iberiotoxin (squares). Means ± s.e.m., n in parentheses. * $p < 0.05$, ** $p < 0.01$ vs. control (saline)

declined to levels not significantly different from the control levels within 24 hrs (Fig. 5).

## Discussion

The organotypic slice model used in this study has thoroughly been characterized and was proven to be an excellent model system for studying isolated brain regions in vitro. It has recently been described as a powerful model (Humpel et al., 1996; Stoppini et al., 1991) and is suitable to study the expression of transcription factors (Liu et al., 1995). Therefore, this model offers the chance to investigate neuronal activity in the isolated rat striatum after exposure to drugs without mesostriatal and corticostriatal circuitry, thereby distinguishing direct striatal from indirect effects.

It could be shown that in 6–14 day old isolated striatal slices the tyrosine-hydroxylase immunoreactivity was significantly reduced (this paper and Humpel et al., 1996) and that dopamine levels were not detectable any more after 7 days or longer (Schatz et al., 1999). As the remaining tyrosine-hydroxylase staining cannot be interpreted quantitatively, and is no proof of active tyrosine hydroxylase, it may represent small fragments of inactive enzyme, since dopamine fell below the detection limit after 7 days in culture (Schatz et al., 1999). Since due to the high sensitivity of our assay, this represents more than 99% depletion of dopamine, the lack of measurable dopamine indicates the completion of the degeneration of dopaminergic axons and terminals. After this time point (10 days) we investigated the effect of margatoxin and iberiotoxin using this isolated slice model. It could be shown that both toxins induce expression of c-fos mRNA and a transient increase of c-fos like protein in isolated striatal slices. Our data indicate that this c-fos expression appears independently from the dopaminergic input of the nigrostriatal system and independently from the glutamatergic input of the corticostriatal system. Thus both toxins may directly induce c-fos expression in the dorsal striatum without the extrinsic neuronal circuitry and the neurotransmitters dopamine or glutamate.

The type of striatal cells expressing c-fos after margatoxin or iberiotoxin stimulation cannot be identified by the present data. Recently it was reported, that cocaine induced c-fos in striatal substance P (SP)-containing neurons (Kosofsky et al., 1995). Furthermore, we showed that about one third of all SP neurons, which mainly are GABAergic nigrostriatal projections, exhibited c-fos immunoreactive nuclei after stimulation with ecstasy (Schatz et al., in preparation). This suggests, that c-fos expression is induced in neuronal cells and that SP neurons are one of the cell subtypes within the striatum, that may also be involved in c-fos expression after margatoxin and iberiotoxin. Interestingly, a high density of Slo-channels was found in the substantia nigra, especially in the terminal region of striato-nigral GABA neurons containing substance P (Knaus et al., 1996). However, other types of neurons are likely to be involved and c-fos expression in glial cells cannot be excluded.

Margatoxin has been shown to stimulate dopamine and acetylcholine release in the rat striatum (Saria et al., 1998; Fischer and Saria, 1999), whereas iberiotoxin did not increase the release of neurotransmitters. The exact mechanism of c-fos induction is unclear, but our data indicate that the margatoxin-induced c-fos expression may be related to inhibition of voltage-gated potassium channels which leads to prolongation of the action potential and, subsequently, increased transmitter release. Since the isolated single slice model lacks dopamine (Humpel et al., 1996), the margatoxin-induced c-fos expression is not due to dopamine release and our findings point to a different mechanism. If MgTX-induced c-fos induction is a result of interference with Kv1.2 or Kv1.3 potassium channels, this means that the channels must be active under spontaneous depolarization conditions in organotypic striatal slices in culture. The mechanism of iberiotoxin-induced c-fos expression remains unclear at present. However, our data show for the first time that Slo-channels are active in striatal slices in culture, although they may not be involved directly in transmitter release. The induction of c-fos may therefore be an indirect indicator of another function of Slo channels. In this context it is interesting to note that iberiotoxin was shown to inhibit metabotropic glutamate receptor agonist-induced hyperpolarization and corresponding outward currents in the basolateral amygdala in rats (Holmes et al., 1996). Such type of mechanisms could be involved in changes of the intracellular signal transduction cascades and subsequent expression of c-fos.

In conclusion, our results show, that inhibition of voltage-gated as well as of calcium activated potassium channels increase c-fos in the striatum. The presence of afferent inputs such as dopamine or glutamate does not seem to be necessary for the induction of c-fos. Our findings point to a direct influence of margatoxin- and iberiotoxin-sensitive potassium channels on gene transcription in the striatum, which may underlie long-term neuronal plasticity and behavioral changes.

## Acknowledgements

This study was supported by the Austrian Science Foundation (SFB 00206). We thank Iris Berger and Astrid Saria for excellent technical assistance.

## References

Fischer HS, Saria A (1999) Voltage-gated, margatoxin-sensitive potassium channels, but not calcium-gated, iberiotoxin-sensitive potassium channels modulate acetylcholine release in rat striatal slices. Neurosci Lett 263: 1–3

Graybiel AM, Moratalla R, Robertson HA (1990) Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum. Proc Natl Acad Sci USA 87: 6912–6916

Holmes KH, Keele NB, Arvanov VL, Shinnick-Gallagher P (1996) Metabotropic glutamate receptor agonist-induced hyperpolarization in rat basolateral amygdala neurons: receptor characterization and ion channels. J Neurophysiol 765: 3059–3069

Hutter P, Johansson M, Saria A, Humpel C (1996) Acute and chronic noradrenergic regulation of neurotrophin messenger RNA expression in rat hippocampus: evidence from lesions and organotypic cultures. Neurosci 70: 15–29

Knaus HG, Schwarzer C, Koch ROA, Eberhart A, Kaczorowski GJ, Glossmann H, Wunder F, Pongs O, Garcia ML, Sperk G (1996) Distribution of high-conductance Ca2+-activated K+- channels in rat brain: targeting to axons and nerve terminals. J Neurosci 16: 955–963

Kosofsky BE, Genova LM, Hyman SE (1995) Substance P phenotype defines specificity of c-fos induction by cocaine in developing rat striatum. J Comp Neurol 351: 41–50

Moratalla R, Vickers EA, Robertson HA, Cochran BH (1993) Coordinate expression of c-fos and junB is induced in the rat striatum by cocaine. J Neurosci 13: 423–433

Morgan JI, Curran R (1989) Stimulus-transcription coupling in neurons: role of cellular immediate early genes. TINS 12: 459–462

Saria A, Seidl CV, Fischer HS, Koch ROA, Telser S, Wanner SG, Humpel C, Garcia ML, Knaus HG (1998) Margatoxin increases dopamine release in rat striatum via voltage-gated K+ channels. Eur J Pharmacol 343: 193–200

Schatz DS, Kaufmann WA, Saria A, Humpel C (1999) Dopamine neurons in a simple GDNF-treated meso-striatal organotypic co-culture model. Exp Brain Res (in press)

Sirinathsinghji DJS, Dunnett SB (1994) Imaging gene expression in neural grafts. In: Molecular imaging in neuroscience: a practical approach. Oxford University Press, Oxford, pp 43–73

Stoppini L, Buchs PA, Muller D (1991) A simple methode for organotypic cultures of nervous tissue. J Neurosci Meth 37: 173–182

Young ST, Porrino LJ, Iadarola MJ (1991) Cocaine induces striatal c-fos-immunoreactive proteins via dopaminergic D1 receptors. Proc Natl Acad Sci USA 88: 1291–1295

Zamboni I, DeMartino C (1967) Buffered picric acid formaldehyde: a new rapid fixative for electron microscopy. J Cell Biol 25: 148A

Zhang P, Hirsch EC, Damier P, Duyckaerts C, Javoy-Agid F (1992) C-fos protein-like immunoreactivity: distribution in the human brain and over-expression in the hippocampus of patients with Alzheimer's disease. Neurosci 46: 9–21

**Authors' address:** Dr. Alois Saria, Division of Neurochemistry, Department of Psychiatry, Anichstraße 35, A-6020 Innsbruck, Austria,
Fax +43-512-504-3716; E-mail: alois.saria@uibk.ac.at

Received August 31, 1999