Journal of Neuroscience Research 45:542–548 (1996)

Induced Expression of Neurotrophins in Transgenic Mice Overexpressing Ornithine Decarboxylase and Overproducing Putrescine

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Transgenic mice overexpressing ornithine decarboxylase (ODC) were recently generated (Halmekytö et al.: Biochem Biophys Res Commun 180:262–267, 1991). The dramatic ODC overexpression resulted in a very high accumulation of the polyamine putrescine in the brain. As elevated polyamine levels in the brain are believed to be associated with neuronal damage, we studied whether enhanced putrescine accumulation in the brain of these mice affects the expression of neurotrophins and their high affinity receptors. Northern blot analysis indicated that mRNA levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) were significantly elevated about 1.5-fold in the hippocampus of ODC transgenic mice as compared with control animals. The levels of BDNF, NGF and NT-3 mRNA were also elevated in the kidneys of the transgenic mice. In eight other tissues examined there were no significant differences. The expression pattern of BDNF mRNA and of trkB and trkC (high affinity receptors for BDNF and NT-3 respectively) immunoreactivity in the hippocampal formation did not reveal significant differences. The induction of the expression of neurotrophins could belong to neuroprotective measures triggered by ODC overexpression.

Key words: hippocampus, neurotrophins, trk receptors, ornithine decarboxylase, polyamines, putrescine, transgenic mice

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17) (ODC) catalyzes the formation of putrescine from ornithine, the first step in the biosynthesis of polyamines. A rapid and transient elevation in polyamine biosynthesis not only accompanies cell division, but is also a characteristic of quiescent cells such as neurons responding to strong physiological or traumatic stimuli (Paschen, 1992). Transgenic mice overexpressing the human ODC gene were recently generated (Halmekytö et al., 1991a). These mice displayed a 50- to 70-fold higher ODC activity in their brains than their non-transgenic littermates. Due to ODC overexpression, the transgenic mice had a high putrescine level (>60 μmol/g of tissue) in the brain. The concentrations of the higher polyamines spermidine and spermine were not significantly different from control values (Halmekytö et al., 1991c; Kauppinen et al., 1992). It was shown that the transgenic mice had significantly impaired spatial learning and memory in the Morris water maze test and had an elevated seizure threshold to chemical and electrical stimuli (Halonen et al., 1993). As elevated polyamine levels in the brain are believed to be associated with neuronal damage, we investigated whether enhanced putrescine accumulation in the brain of these mice affects the expression of neurotrophins and their high affinity receptors. Hypotheses that polyamine levels can have effects on neurotrophin expression have been made earlier (Gilad et al., 1989); however, few experimental data directly address this question. Transgenic mice overexpressing ODC provide a unique model for studies of the effects of elevated levels of putrescine inside the cells in vivo.

Members of the neurotrophin family, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), are structurally related neurotrophic proteins which induce

Received October 5, 1995; revised March 14, 1996; accepted March 14, 1996.

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the differentiation of neuronal cells, rescue them from naturally occurring death, and trigger neuronal regeneration. In the adult brain, the highest levels of NGF, BDNF and NT-3 mRNAs are found within the hippocampal formation (Lindsay et al., 1994). Neurotrophins affect the responsive neurons by interacting with specific cell surface receptors which are divided into two classes according to their binding affinity for the ligand: high and low affinity receptors. High affinity receptors are members of the tyrosine kinase family encoded by trk proto-oncogenes. The main receptor for NGF is trkA, that for BDNF is trkB and that for NT-3 is trkC; however, "cross-binding" also occurs (Barbacid, 1994). All neurotrophins bind with low affinity to another type of receptor usually referred to as the low affinity NGF receptor (LANR) because it was initially discovered for its ability to bind NGF (Chao, 1994).

In this report, we describe that BDNF, NGF and NT-3 mRNA levels are elevated in the hippocampi and kidneys of ODC overexpressing transgenic mice as revealed by Northern analysis. Likewise, immunohistochemistry was used to show the distribution of the high affinity trkB and trkC neurotrophin receptor proteins in the brains of transgenic and control mice.

MATERIALS AND METHODS

Materials, Animals and Tissues

trkB(794) (cat. no. sc-12) and trkC(798) (cat. no. sc-117) rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were obtained from Sigma (St. Louis, MO). The transgenic mice used in this study were adult males of the K2 (24 gene copies) and K14 (six gene copies) lines harbouring a functional human ornithine decarboxylase gene (Halmekytö et al., 1991a). Syngenic littermates served as controls. The metabolism of polyamines in the animals has been described in detail (Halmekytö et al., 1991b).

RNA Preparation and Northern Blot Analysis

The dentate gyrus and hilar regions as well as the rest of the hippocampus were dissected from adult mice. All tissue samples were frozen at −70°C until use. Total RNA from tissue was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Twenty micrograms of total RNA was subjected to electrophoresis on a 1.2% formaldehyde agarose gel, and then transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL). Following transfer, membranes were fixed by UV irradiation, prehybridized, and hybridized with ^{32}P-labelled neurotrophin cDNA probes at 65°C in 1 M NaCl, 1% SDS, 10% dextran sulphate. The membranes were washed and exposed to Kodak XT films at −80°C for about 2 weeks. The amount of cellular neurotrophin mRNA was quantified by densitometric scanning (Hewlett Packard ScanJet Plus) of the autoradiograms and corrected for α-actin mRNA quantity.

The cDNA probes, a rat 187 bp NT-3 fragment, a 299 bp rat BDNF cDNA fragment and the rat NGF 434 bp cDNA fragment have been described earlier (Pirvola et al., 1992).

Immunostaining and In Situ Hybridization

For immunohistochemistry studies, 10 mice (five transgenic and five normal) were perfused intracardially under deep anaesthesia (chlornembutal, 60 mg/ml, 0.3 ml/100 g i.p.), first with physiological saline (3 min) and then with fixative (4% paraformaldehyde, 0.05% glutaraldehyde, 0.2% picric acid) for 30 min. After fixation the brains were dissected and 60 μm thick coronal sections at the level of the dorsal hippocampus were cut on a Vibratome.

Free-floating brain sections were thoroughly washed in 0.1 M phosphate buffer (PB), pH 7.4, then in 50 mM Tris-buffered saline (TBS), pH 7.4. After repeated washes the sections were incubated first in 10% normal goat serum (NGS) in TBS for 40 min and then in 1% NGS in TBS for 15 min. This was followed by incubation in rabbit anti-trkB (diluted 1:50) or anti-trkC antiserum (diluted 1:50) for 24 hr at room temperature. Then the sections were washed with 1% NGS in TBS and incubated with biotinylated goat anti-rabbit IgG (diluted 1:50) overnight at 4°C. After washing with 1% NGS in TBS, the antibody-antigen complex was visualized using the avidin-biotinylated horseradish peroxidase complex of a commercially available kit (Vector Laboratories, Burlingame, CA). The peroxidase reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Then the sections were dried on gelatin-coated slides, dehydrated, and coverslipped in Depex (Serva, Heidelberg, FRG) for light microscopy.

Preparation of the complementary RNA BDNF probe and in situ hybridization was performed as described by Wilkinson and Green (1990) with modifications (Pirvola et al., 1992).

RESULTS

We studied the expression of the neurotrophic factors BDNF, NGF and NT-3 and their high affinity receptors trkB and trkC in the hippocampi of adult ODC overexpressing mice having a very high putrescine content in their brains, approximately a 50-fold increase compared with control animal brains (Halonen et al., 1993). For this purpose, we performed Northern blots to measure neurotrophin mRNA levels in the hippocampus of these mice. The hippocampus was chosen as being the

main site of high level neurotrophin expression in the brain.

Northern blots demonstrated that BDNF, NGF and NT-3 mRNA levels were 1.3–1.6-fold higher in the hippocampi of ODC overexpressing transgenic mice as compared with control animals. In two different transgenic mouse lines that had different levels of putrescine there was more neurotrophin mRNA in the line K2 having more putrescine (Figs. 1, 2). To investigate whether the effect is brain specific, some other tissues were examined. In the lung, heart, skeletal muscle, skin, spleen, liver, thymus and testis there were no significant differences in NGF (Fig. 2), BDNF and NT-3 mRNA expression (data not shown). However, in the kidney tissue of the transgenic mice there was an increase in the expression of BDNF, NGF and NT-3 mRNA, as compared with control mice. In non-transgenic mice the mRNA for BDNF and NT-3 was hardly detectable (Figs. 1, 3).

To examine where in the hippocampus the changes in the expression of neurotrophins take place, we performed in situ hybridization with a BDNF mRNA-specific probe in a series of coronal brain sections of 4-week-old mice. When the section was hybridized to the BDNF cRNA probe, cell bodies of those neurons that form the pyramidal cell layer of the hippocampus and the granule cells of the dentate gyrus showed expression of BDNF transcripts. However, the expression patterns were similar for transgenic and control mice (data not shown).

Next, we investigated the expression of trkB and trkC proteins in the brains of the ODC overexpressing and control mice. We did not analyze the level of the high affinity receptor for NGF-trkA, since in the hippocampus its mRNA level is below the detection limit of in situ hybridization (Merlio et al., 1993), and specific trkA antibodies were not available. trkB- and trkC-containing cells were visualized immunohistochemically in the rat dorsal hippocampal formation. trkB- and trkC-like immunoreactivity was detected mainly in the principal cells of the dentate gyrus and the hippocampus proper, but some nonprincipal cells, especially in the hilus, were also labeled (Fig. 4). Immunohistochemistry demonstrated that trkB-and trkC-like immunoreactivity is similarly distributed in the pyramidal cells of the hippocampus proper and the granule cells of the dentate gyrus of ODC transgenic and control mice. trkB-positive nonpyramidal cells in the stratum radiatum of the ODC overexpressing mice showed somewhat stronger trkB-like immunoreactivity compared to controls (Fig. 4A, B, E).

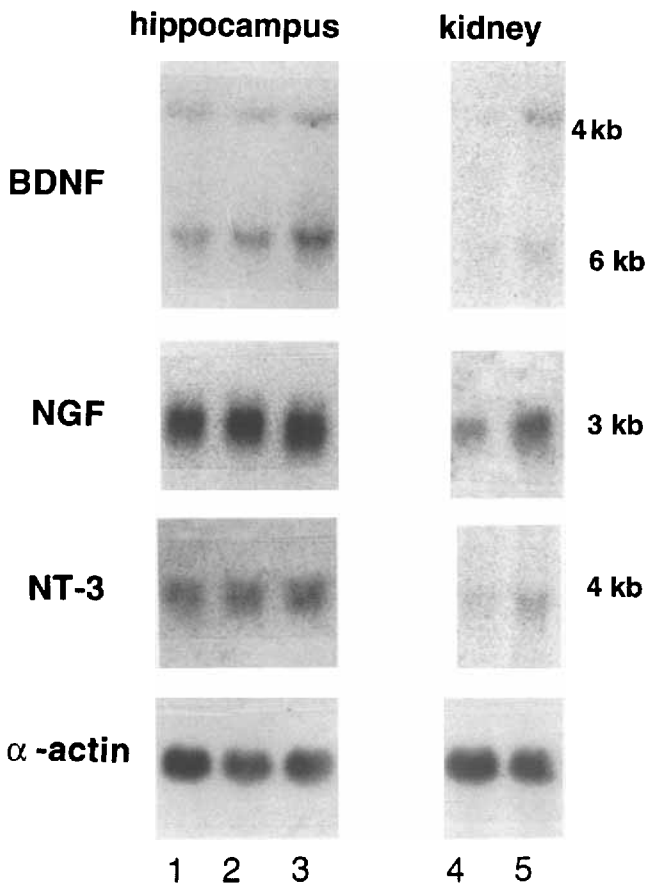

Fig. 1. Northern analysis of neurotrophins mRNA expression in the hippocampus and kidney of transgenic ODC overexpressing and control mice. Lanes 1, 4, control mouse; lane 2, transgenic mouse line K14; lanes 3, 5, transgenic mouse line K2. K2 line has higher putrescine levels than K14 line. The same filter was first hybridized with the BDNF probe and then after washing with other probes. On the right neurotrophins mRNA sizes are shown; BDNF has two transcripts.

DISCUSSION

The aim of this study was to determine whether there is crosstalk between putrescine and neurotrophins in the brain, using transgenic mice with high putrescine levels in their brains due to ODC overexpression. The influence of NGF on ODC and putrescine levels has been known for a long time—ODC activity has been shown to be markedly stimulated by NGF in rat brain (Hendry and Bonyhady, 1980), rat superior cervical ganglia (MacDonnell et al., 1977) and rat pheochromocytoma PC12 cells (Hatanaka et al., 1978; Volonté and Greene, 1990). On the contrary, it has also been shown that injection of a mixture of polyamines induced elevated levels of the NGF protein in the iris and the submaxillary salivary gland (Gilad et al., 1989); however, data on the possible influence of putrescine on neurotrophins in the brain were lacking. In this report we demonstrate that in transgenic mice with very high putrescine contents in their brains due to ODC overexpression, NGF, BDNF and

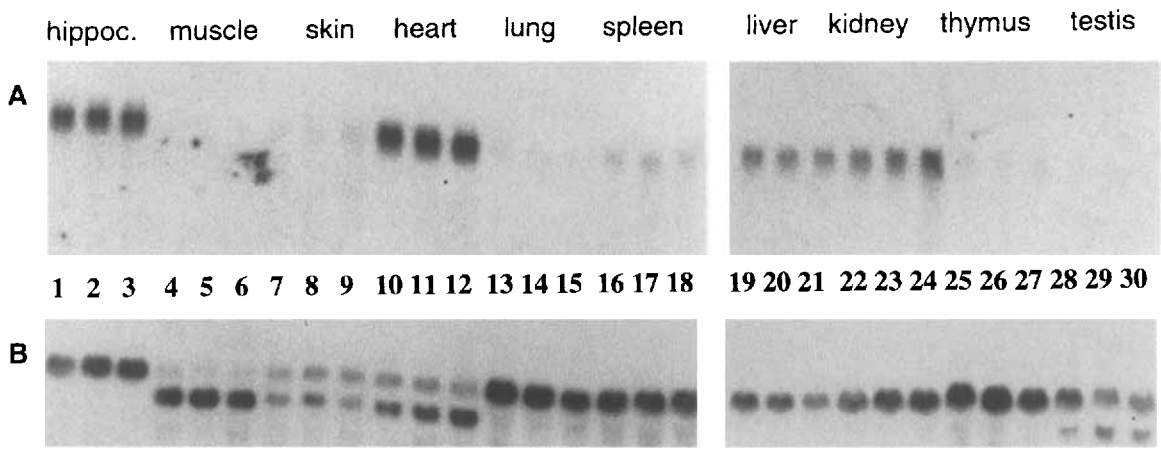

Fig. 2. **A:** NGF mRNA levels in different tissues of ODC overexpressing and control mice. Autoradiograph of RNA blot. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, control mice; lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, transgenic mice line K14; lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, transgenic mice line K2. Lanes 1–3, NGF mRNA levels in hippocampus; 4–6, in skeletal muscle; 7–9, skin; 10–12, heart; 13–15, lung; 16–18, spleen; 19–21, liver; 22–24, kidney; 25–27, thymus; 28–30, testis. Small, but significant (see Fig. 3) increases in NGF mRNA levels in mouse line K2 with high levels of putrescine compared to control mouse are seen in the hippocampus and kidney. **B:** The same filters were rehybridized with α-actin cDNA probe. α-actin has different length transcripts in different tissues.

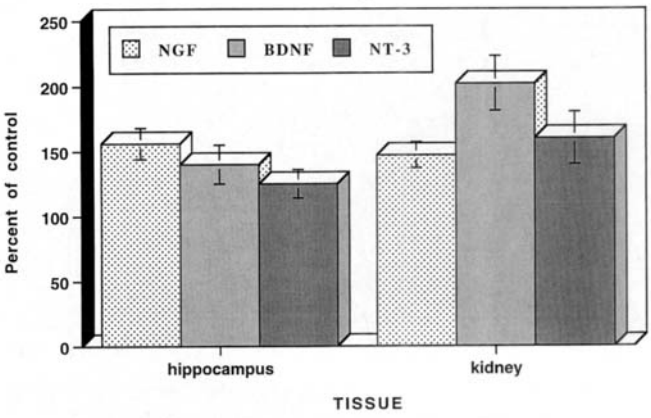

Fig. 3. mRNA expression levels of neurotrophins in the hippocampus and kidney of ODC overexpressing mice are higher as compared to control mice. Transgenic mice K2 (n = 7) and their syngenic littermates (n = 7) were used for the analysis. NGF, BDNF and NT-3 mRNA content was determined as described in Materials and Methods. Data, expressed as percentage of control (nontransgenic littermates), are reported as the mean ± SEM (n = 7). Results were analyzed with unpaired *t*-tests, $P < 0.05$ for all the data.

NT-3 mRNA levels were significantly elevated by approximately 1.5-fold in the hippocampus and 1.5–2-fold in the kidney, but not in other tissues.

What type of mechanism could account for the induction of neurotrophins in ODC overexpressing mice? One possibility is that this effect of increased neurotrophin levels in the hippocampus of mice with high brain putrescine contents could be mediated by the NMDA receptor. There is evidence for a polyamine binding or modulatory site on the NMDA receptor complex (Durand et al., 1993; Traynelis et al., 1995) while NMDA receptor has been shown to regulate NGF and BDNF mRNA levels in the hippocampal neurons (Zafra et al., 1990, 1991). Halonen et al. (1993) suggested the possible role of NMDA receptor in the memory deficiency of ODC overexpressing mice. The data about the influence of different polyamines on the NMDA receptor are rather contradictive: both agonism and antagonism have been observed in different brain regions and in different developmental stages (for review see Rock and Macdonald, 1995). Other possibilities include Ca^{2+} channels (Alvarez Maubecin et al., 1995) and phosphoinositide pathways (Periyasamy et al., 1994) that have been shown to be regulated by polyamines. These three pathways affect gene expression but further experiments are necessary to discriminate their role in the polyamine regulation of neurotrophins expression.

There are two at least partly contradicting points of view on the functional significance of high putrescine levels in brain. One point of view is that commonly observed increase in ODC activity and the resulting massive increase in brain putrescine is a cause for the neuronal damage (Paschen et al., 1988a,b). Role for putrescine in the neuronal necrosis can be deduced only indirectly from 1) the close relationship between putrescine accumulation and the extent of necrosis, 2) the known activities of putrescine, and 3) the observation that pharmacological prevention of ischemia-induced neuronal necrosis also reduces or even inhibits the posti-

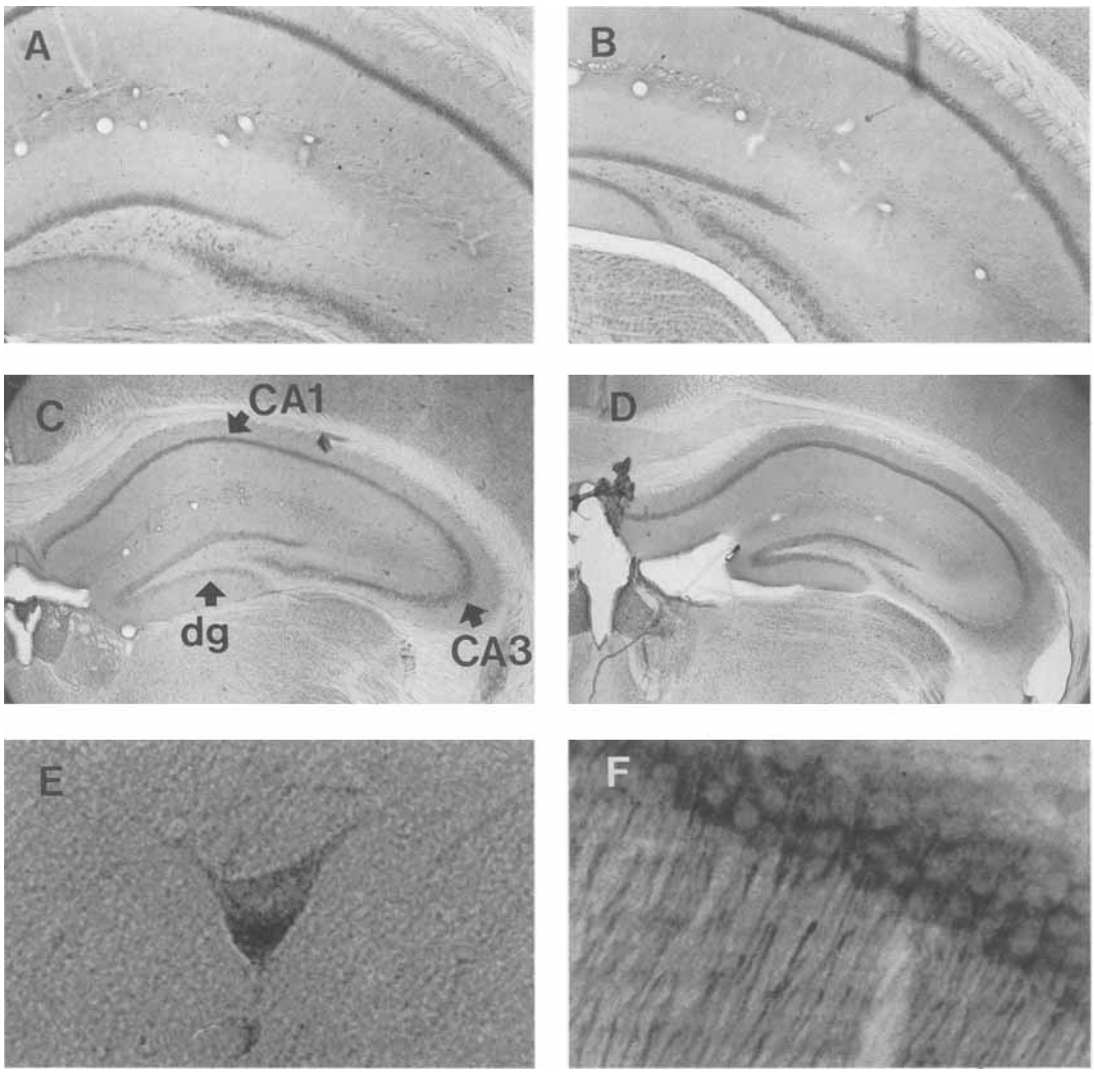

Fig. 4. Distribution of trkB- and trkC-like immunoreactivity in the hippocampus of control (**B,D**) and ODC overexpressing (**A,C,E,F**) transgenic mice. Light microphotographs of a 60 μm thick hippocampal sections showing labeling of cells in dorsal hippocampus and dentate gyrus with antisera to trkB (A,B) and trkC (C,D). E: Higher magnification from the section A shows a trkB-positive nonpyramidal cell in the stratum radiatum of ODC overexpressing transgenic K2 mouse line and (F) in the hippocampal CA1 region. Abbreviations: dg, dentate gyrus; CA1 and CA3, pyramidal cell layers CA1 and CA3 of the hippocampus.

schemic overshoot in putrescine formation (Paschen, 1992). Up to now, there is no direct proof of the involvement of putrescine in cell damage. On the contrary, some reports indicate that increase in the putrescine content associated with neuron damage is a neuroprotective measure rather than a cause of the damage (Dornay et al., 1986; Gilad et al., 1988a,b, 1991; Halonen et al., 1993). Activation of polyamine metabolism may be viewed as a physiological process for repair or regeneration of metabolically stressed neurons and the enhanced expression of neurotrophins may complement this process, as neurotrophins have been shown to attenuate the pathological death of neurons induced by different insults (for review see Isackson, 1995). In line with this view are likewise the recent finding indicating that a life-long overexpression of ODC in transgenic mice does not lead to neuronal degeneration (Alhonen et al., 1995) and a recent work that polyamines promote regeneration of injured axons of cultured rat hippocampal neurons (Chu et al., 1995).

Hippocampal neurons are expressing both neurotrophins and trkB and trkC but not trkA receptors (Kokaia et al., 1993). After brain insults (seizures, ischemia, hypoglycemic coma) NGF and BDNF are induced prominently (more than 10-fold in the case of seizures, for

review see Isackson, 1995), while trkB receptor is induced twofold to threefold and there is no change in trkC mRNA and protein. trkA is not expressed at significant levels in the hippocampus (Merlio et al., 1993). Along induction of several other brain activity dependent genes (Nedivi et al., 1993), these insults are accompanied by elevated putrescine levels due to ODC induction (Paschen, 1992). In ODC overexpressing mice compared with control mice we did not detect significant changes in trkB- and trkC-like immunoreactivity in principal cells of hippocampus. Regulation of neurotrophin and trk mRNAs by polyamines shows similarities to their regulation in brain insults, i.e. neurotrophin induction is more prominent than trk induction. In ODC transgenic mice, however, the steady state elevation of neurotrophins is rather modest (about 50%), although statistically significant, and we see no trk elevation. According to the target field theory neurotrophins are produced in rate limiting amounts. It may imply that even relatively small changes in the neurotrophin levels have significant biological effects on target neurons. The physiological role of the effect of putrescin to enhance neurotrophin expression could be related to increased neuroprotectivity. Applying this transgenic mice model we need to be careful by extending the results to polyamines other than putrescin, because spermin and spermidin levels were practically not elevated (Kauppinen et al., 1992).

All four neurotrophins are expressed in developing and adult mouse and rat kidney and recently it has been demonstrated that in the developing rat kidney NT-3 stimulates kidney differentiation and acts as a survival factor for renal neurons (Karavanov et al., 1995). However, the roles of neurotrophins in the adult kidney remain obscure, as well as their elevated levels in the kidney of ODC overexpressing transgenic mice.

ACKNOWLEDGMENTS

We are grateful to Dr. Toivo Halonen for helpful discussions, Anna-Lisa Gidlund for technical assistance and Dr. Urmas Arumäe for comments on the manuscript.

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