METHODS

Development and Characterization of Thy*IκBα-SI Transgenic Mice

S. V. Gushchina, G. Michael*, O. V. Volkova, and C. B. Magoulas *

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We developed and characterized a new transgenic model where NF-κB activity is inhibited only in mature neurons. Transgenic mouse strain Thy*IκBα-SI was created using trans-dominant super inhibitor NF-κB (IκBα-SI), which is a multimutant form of IκBα inhibitory protein cloned into specific neutral Thy-1.2 cassette. Detailed molecular analysis showed that the transgene and its product (IκBα-SI protein) are expressed in the nervous system of transgenic mice. *In situ* hybridization showed that Thy*IκBα-SI in the nervous system is expressed exclusively in neurons. The developed model provides wide opportunities for studying functional role of NF-κB in mature neurons in the central and peripheral nervous system *in vivo*.

Key Words: *neuron; NF-κB; trans-dominant super inhibitor NF-κB; transgenic mice*

Nuclear transcriptional factor NF-κB is a family of proteins forming active dimers in response to various stimuli and regulating expression of some genes, including cytokine, chemokine, and growth factor genes. Five principal members of NF-κB family are described in mammals: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel; they are characterized by the presence of Rel-homology-domain, RHD [1]. Inactive form of NF-κB is hold in the cytoplasm by protein IκBα from the family of inhibitory kappa B proteins. Relevant stimulus induces phosphorylation of IκB-protein by specific kinase (IKKs) followed by degradation of the inhibitory protein and release of NF-κB, which is transported to the nucleus and activates dependent genes. Since the discovert of the role of NF-κB as apoptosis mediator in immune system cells, this transcriptional factor was also extensively studied in CNS. Its activity in CNS is associated with the processes related to the development, plasticity, neuronal degeneration, and trauma [9]. However, its role in survival of mature neurons in CNS is ambiguous [8]. Activated NF-κB was found to promote neuronal death in the brain under conditions of experimental ischemia [6], whereas specific inactivation of NF-κB in forebrain neurons results in loss of neuroprotection [5].

NF-κB was studied in mature neurons of spinal ganglia to reveal its potential neuroprotective role after peripheral nerve injury [3,4]. Mature sensory neurons are relatively resistant to apoptosis induced by cutting of the peripheral nerve [7]. By contrast, in embryonic or early postnatal periods, neuronal death after trauma develops much more rapidly [14]. Neuroprotective role of NF-κB in mature sensory neurons was shown in experiments on the culture of spinal cord neurons *in vitro* [4]. However, the role of NF-κB in survival of these neurons after peripheral nerve injury *in vivo* remains unclear.

Department of Histology and Embryology, Pediatric Faculty, Russian State Medical University, Moscow, Russia; *Neuroscience Centre, Institute of Cell and Molecular Science, Queen Mary's School of Medicine and Dentistry, University of London, Great Britan. ***Address for correspondence:*** sguschin@mail.ru. S. V. Gushchina

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The objective of this work was to develop and characterize transgenic animal model with modified activity of transcriptional factor NF-κB exclusively in mature neurons *in vivo*.

MATERIALS AND METHODS

To create transgene Thy*IκBα-SI mouse strain we used potent trans-dominant inhibitor NF-κB (IκBα-SI) [13], a multimutant form of inhibitory IκBα protein (Fig. 1, *a*). IκBα-SI was inserted into XhoI site of specific neutral Thy-1.2 cassette [2] ensuring expression of the transgene exclusively in mature neurons of transgenic mice (tg). IκBα-IR-coding site was amplified from IκBαRR plasmid [13] by direct PCR inserted flanking recognition site for restrictase SalI to XhoI-site of Thy-1.2 cassette. The following primers were used for PCR: direct: 5'-gacgcgtcgacgcggccgccaccATGGACTACCCCTACGACGTCCCCGACTAC.3', reverse – 5'-gacgcgtcgacgcggccgcTCATAACGTCAGACGCTGGCCTCCAAACAC.3' (capital letters show sequence complementary to the coding site of mutant IκBα form, underlined letters show recognition site for SalI restrictase , bold letters show recognition site for NotI restrictase). Patinum *Pfx* DNA Polymerase (Invitrogen) was used in the reaction. PCR regimen: 30 sec at 94°C, 30 sec at 50°C, 3 min at 72°C, 30 cycles. Amplified DNA fragment was purified using kit Qiagen-II agarose gel extraction method, cleaved using SalI-endonuclease, and inserted into complementary XhoI-site of Thy-1.2 cassette.

The resultant construct Thy*IκBα-SI was used for creation of transgenic animals by pronuclear microinjection of the genetic construct into the zygote of hybrid strain CBA×C57Bl/10. Seven positive animals were revealed in F_1 generation, and then they were crossed with CBA mice. Three transgenic mouse strains were obtained and registered in FESA (Frozen Embryo & Sperm Archive, MRC Harwell) as Tg(Thy-mutIκB)74-3, -5, and -7.

Transgenic animals were genetically typed using PCR, which simultaneously revealed both transgene and its endogenous homolog (Fig. 1, *b*). Genomic DNA was extracted from the sample of the ear using kit Direct PCR Lysis Reagent (Viagen Biotech, Inc.) in accordance with manufacturer protocol, and amplified using PCR (Thermo-start High Performance PCR Master Mix, ABgene; primers: 5'-TTGCCTGTGAGCAGGGCTGCCT-3' (Pr-1, direct) и 5'-GTCAGCTGGCCCAGCTGCTGCT-3' (Pr-2, reverse); heating regimen: 95°C for 15 min and then 30 cycles: 30 sec at 95°C, 30 sec 60°C, and 90 sec at 72°C.

The expression of IκBα-SI transgene was revealed in various tissues by RT-PCR (reverse transcription polymerase chain reaction). Total RNA was extracted using Trisol method (Life Technologies, GibcoBRL) according to manufacturer protocol. Total RNA (25 ng) was subjected to reverse transcription using ImPromII Reverse Transcriptase according to Promega protocol. The reaction mixture was supplemented with the primers specific for human IκBα-transgene sequence (5'-GGGAGGGAGTCAGCTGACCG-3' (direct) and 5'-AGGGCTGCCTGGCCAGCTTG-3' (reverse), PCR regimen: 15 sec at 95°C, 30 sec at 60°C, and 1 min at 72°C, 30 cycles. GAPDH gene (5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' (direct) and 5'-GCTAAGCAGTTGGTGGTGAC-3' (reverse) served as positive control.

Expression of IκBα-SI transgene was also determined by *in situ* hybridization [11]. Oligonucleotide probe 5'-AGGCGTAGTCGGGGACGTCGTAGGGGTAGTCCAT-3' was generated to be complementary to HA (hemagglutinin) epitope tag (HA epitope tag) of IκBα-SI transgene.

Expression of IκBα-SI protein was determined in spinal cord extracts by Western-blotting [13] using anti-IκBα antibodies (C21, Santa Cruz) in dilution 1:100.

RESULTS

For evaluation of functional role of NF-κB in mature sensory neurons *in vivo* we created transgenic Thy*IκBα-SI mice; neurons of these mice express super inhibitor NF-κB — IκBα-SI. Mouse Thy-1.2-transgenic cassette (Fig. 1, *a*) includes *cis*-acting element for neuron specific expression after 8-day postnatal period [2]. IκBα-SI is a mutant form of IκBα in which animo acid series susceptible for ubiquitination, phosphorylation, and tyrosine phosphorylation signals is replaced with non-susceptible amino acids (Fig. 1, *a*). This form of the protein is not prone to degradation and acts as a super repressor of NF-κB activity by restraining and isolating NF-κB–RelA complexes in the cytoplasm and preventing their binding with DNA and transcription activation [13]. Transgenic mice were revealed using PCR with a pair of primers detecting positive and negative animals (Fig. 1, *b*). These primers amplified the fragment of 733 nucleotides of mouse endogenous genomic IκBα-locus containing exons III and IV of coding sites and introns. The same primers corresponded to coding site of human IκBα-SI-transgene, in which introns were deleted, and therefore amplified fragment in transgenic mice was shorter (369 n.p.).

Molecular analysis of generated transgenic Tg(Thy-mutIκB)74-3 animals was performed to determine whether the transgene and its product (IκBα-SI protein) are expressed in the nervous system, particularly in mature neurons of the spinal ganglia.

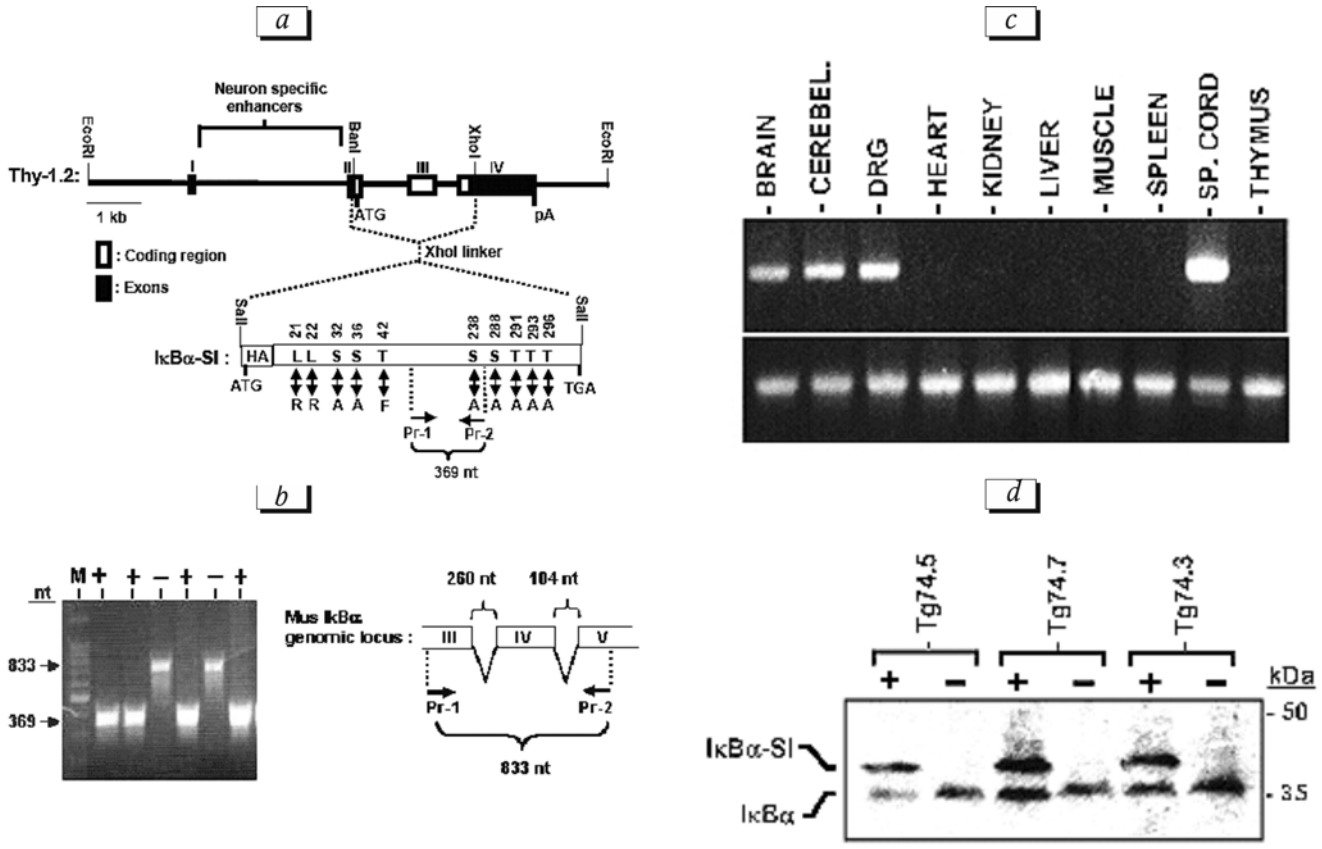

Fig. 1. Scheme of genetic construction Thy*IκBα-SI (*a*) and analysis of obtained transgenic mice (*b*-*e*). *a*: IκBα-SI, super inhibitor of NFk-B. Double arrows show amino acids, which are replaced in mutant IκBα form. HA-hemagglutinin tag; *b*: Pr-1 and Pr-2 are primers amplifying transgene site and mouse genomic IκBα; *c*: IκBα-SI expression in various tissues of transgenic animals detected by RT-PCR. Lower panel shows expression of control gene GAPDH in the same tissue for assessment of equal amount of applied RNA; *d*: expression of transgene product, IκBα-SI protein, in extracts from spinal cords of three transgenic strains detected by Western-blotting. IκBα is endogenous mouse protein IκBα.

First, transgene expression in various tissues was studied by RT-PCR. Transgene was expressed only in the nervous system (brain, cerebellum, spinal cord and spinal ganglia; Fig. 1, *c*).

Then, during the Western blot analysis of the expression of the product of inserted transgene in spinal cord extracts using IκBα-specific antibodies [13], the protein corresponding by size to IκBα-SI was detected in spinal cord extracts obtained from three transgenic strains (Fig. 1, *d*), which demonstrates that these animals carry actively expressed transgene.

Western-blot and RT-PCR used to confirm transgene expression were performed on the homogenates of the whole organs of transgenic animals (brain, spinal cord, spinal ganglion) and did not allow detection whether this transgene is expressed exclusively in neurons or also in other, non-neuronal cells. For this purpose, *in situ* hybridization was performed on cryostat sections of organs from transgenic animals of two strains Tg(Thy-mutIκB)74-3 and -5. This method allows localization of mRNA in cell cytoplasm and allows obtaining clear morphological picture of the distribution of transgene product expression (mRNA) in cell populations. Cryostat sections of the nervous tissue obtained from transgenic animals (brain, spinal cord, and spinal ganglia) were subjected to hybridization with ^{35}S-dATP radioactively labeled oligonucleotide probe specific to the transgene. Due to peculiarities of the transgene construction, these results are able to characterize transcriptional activity of the transgene. *In situ* hybridization showed that both strains exhibit wide transgene expression in neurons, but with individual peculiarities. Strain 5 showed high transgene expression in limited number of neurons. Appreciable concentration of silver granules was detected in some spinal cord motoneurons (Fig. 2, *a*, *b*), in population of large neurons of spinal ganglia (Fig. 2, *c*, *d*), in CA1 and CA3 hippocampal areas and dentate gyrus, and in few neurons of inner layer of the cortex (Fig. 2, *e*, *f*). In over neurons, expression was weak or even absent.

Strain 3 exhibited high transgene expression in all types of neurons with approximately equal intensity. Silver granules were present in all neurons of the brain

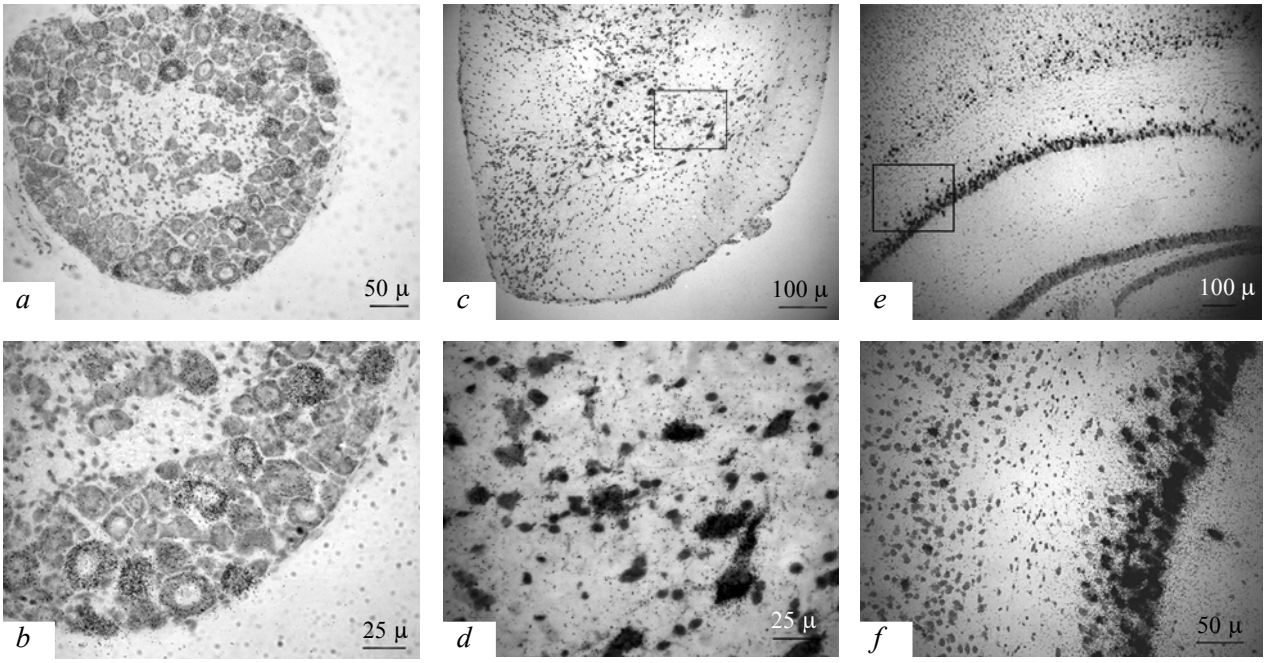

Fig. 2. *In situ* hybridization on the spinal cord (*a*, *b*), spinal ganglion (*c*, *d*), and brain (*e*, *f*) sections from transgenic Tg(Thy-mutIκB)74-5 mice. *b*, *d*, *f*: sections with the neurons containing silver granules in the cytoplasm at higher magnifications. Substantial concentration of the silver granules in limited group of neurons is seen. Many neurons did not exhibit transgene expression.

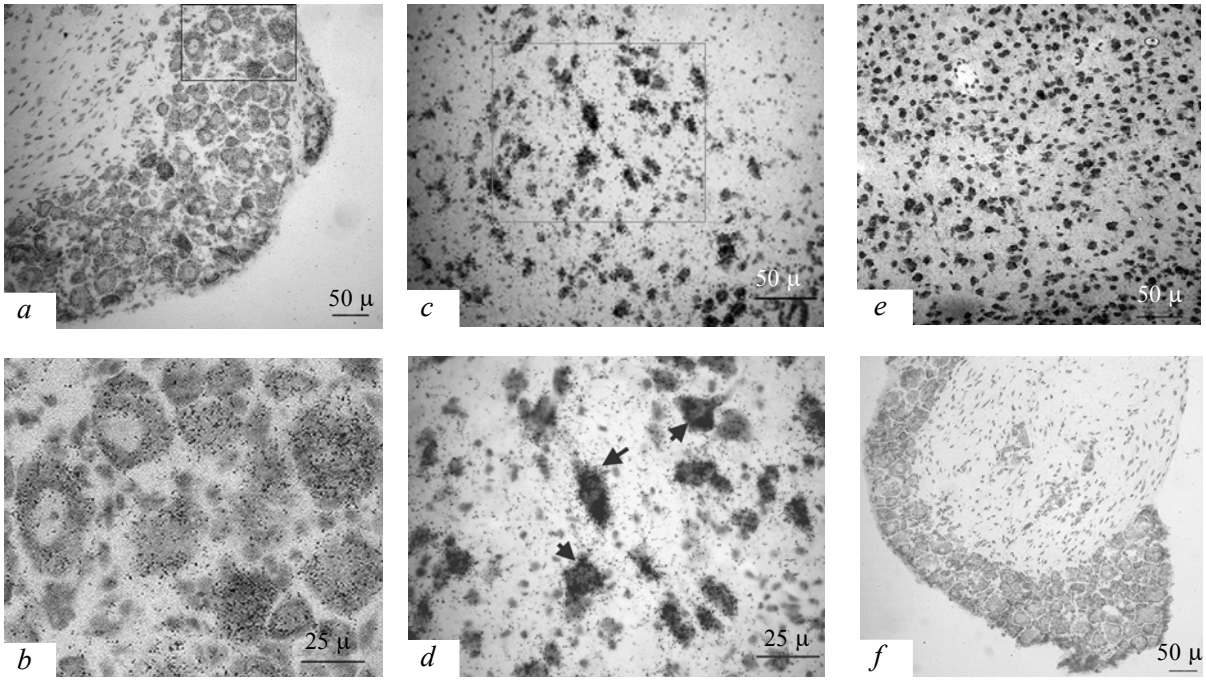

Fig. 3. *In situ* hybridization on the spinal cord (*a*, *b*), spinal ganglion (*c*, *d*), and brain (*e*) sections from transgenic Tg(Thy-mutIκB)74-3 mice. *b*, *d*: sections with the neurons containing silver granules in the cytoplasm (arrows) at higher magnifications. Concentration of silver granules in all groups of neurons is seen; *f*: *in situ* hybridization of the spinal cord sections from wild type mice demonstrates uniformly distributed silver granules not associated with cells.

(Fig. 3, *e*), spinal cord (Fig. 3, *a*, *b*), and in all neuronal populations of the spinal ganglion (Fig. 3, *c*, *d*).

Of particular importance is the fact that no non-neuronal transgene expression was found in the nervous system (Figs. 2 and 3). Negative samples included glial cells as a part of peripheral nerve and white matter cells of CNS. It proves that Thy*IκBα-SI transgene expression is highly specific and limited to neurons.

As a control, *in situ* hybridization was performed on tissue samples obtained from wild type mice (wt). In this case, only low radioactive background appeared on preparations and silver granules were distributed uniformly and were not associated with cells, but covered homogeneously all the sample (Fig. 3, *f*).

The role of NF-κB in processes of neuronal survival and death is still discussed [10]. This can be explained by the fact that it is difficult to differentiate *in vivo* two non-mutually exclusive potential mechanisms of NF-κB action: indirect effects on neurons mediated by NF-κB activation in glial cells and direct NF-κB effects in neurons. In available knock-out mouse models there is no specification in terms of cell types, what does not allow differentiation of NF-κB role in glial cells and neurons [12]. Hence, we generated transgenic mice, in which NF-κB activity was inhibited exclusively in mature neurons. Up to date, this is the only transgenic model, where NF-κB activity is purposely inhibited exclusively in mature neurons. To develop this model, we used mutant form of NF-κB inhibitor, IκBα-SI, which is not subjected to degradation and therefore acts as a super repressor of NF-κB activity restraining and isolating NF-κB–RelA complex in the cytoplasm and preventing its binding with DNA and transcription activation. The results showed that gene of the mutant form of NF-κB inhibitor is successfully expressed exclusively in neurons of transgenic mice. The advantage of our model is that Thy-1.2 promoter controlling the gene of super inhibitor NF-κB is strictly specifically activated in mature neurons of transgenic mice on day 8 of postnatal development. Thus, the use of this mouse strain for experimental modeling of trauma, stress, and etc. provides wide opportunities for studying the protective role of NF-κB in mature neurons both in CNS and in the peripheral nervous system.

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