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Genes for neurotrophic factors and their receptors: structure and regulation

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Abstract. Neurotrophins and their receptors have attracted much interest during the last two decades. Although the mode of action of molecules of the neurotrophin system has been studied extensively, information on molecular mechanisms governing their expression is mosaic and incomplete. This review attempts to summarize the data available on gene structure and transcriptional regulation of neurotrophins and their receptors, and outlines perspectives for the future in this field.

Key words. Neurotrophin; receptor; tyrosine kinase; gene regulation; transcription.

Introduction

During the last two decades, research in the field of neurotrophic factors has provided a vast amount of data on how they function, provide support and organize neuronal networks during development, but also participate in maintaining nervous system function. Much is now known about the physiology of neurotrophic action. However the information necessary to understand exhaustively the genetic networks underlying the epigenetic realization of the trophic and tropic actions of these factors in the temporospatial window of ontogenesis is mosaic or almost lacking. Only a few among the many scientific publications dedicated to neurotrophins and their receptors elucidate molecular aspects of their gene expression.

The beginning of the new millennium provides new possibilities and challenges to resolve these problems. The results of the HUGO project together with the rapid collection of data on the genome organization of major model organisms used for neurobiological research are going to add a new 'genomic' dimension to our view of the problems of neurotrophism. Comparison of the structural features of genes encoding neurotrophic factors and their receptors from different organisms integrated with data on the molecular mechanisms of gene regulation from other fields of biomedical science and physiological data on neurotrophic factor action and known intracellular signal transduction pathways will open up new 'postgenomic' approaches that will complete the picture.

Neurotrophic properties have been documented for many different molecules belonging to various classes of biologically active compounds. This review will be limited to the 'classical' neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5), and their receptors $p75^{NTR}$, TrkA, TrkB, and TrkC. First, an overview of the known information on the structure of the genes encoding these molecules will be presented. Then follows an attempt to collate and systematize the data elucidating possible molecular mechanisms involved in the regulation of these genes at the level of transcription. Finally, some possible developments and promising implications of the field are presented in the context of their importance to basic and applied biomedicine.

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Neurotrophin genes

All genes encoding neurotrophins have a basically similar structure. A single discontinuous exon contains all the information for encoding the prepropeptide. The coding exon is preceded by several noncoding exons that are subject to alternative splicing and give rise to alternative 5′-untranslated regions (UTRs) of mRNA. Multiple promoters have been demonstrated for all neurotrophins. The alternative promoters flank different upstream exons.

The BDNF gene contains four upstream exons, and the NT-3 gene two upstream exons all flanked by promoters [1, 2]. The usage of different promoters gives rise to mRNAs that contain only one of the upstream noncoding exons spliced to the coding exon. Upstream exons of BDNF and NT-3 genes do not contain a splicing acceptor site thus preventing the splicing of the more upstream exons to those located proximally to the coding exon. A very efficient acceptor site at the last exon perhaps also facilitates effective splicing of different upstream exons to the coding one.

NGF and NT-4 genes have similar features; however, their pre-mRNA splicing is more complex than in BDNF and NT-3 genes. The rat NT-4 gene has two upstream exons [3] and the human NGF gene, three upstream exons [4]. Transcription initiation activity of the NT-4 gene has been demonstrated for genomic sequences proximal to both exons. In the NGF gene, promoter activity has been linked to the first and the third exon. Transcripts resulting from the usage of distal promoters also contain more proximal exons. This demonstrates the presence of a splicing acceptor site overlapping with promoter sequence. NT-4 mRNAs contain either exon II and a coding exon III (product from the promoter flanking exon II) or both exons II and I linked to the coding exon III. The NGF gene has the most complex splicing pattern. The majority of transcripts are transcribed from the distal promoter and contain exon I, while the other noncoding exons are combined by alternative splicing and give rise to a variety of mRNAs with different 5′-UTRs. A small number of transcripts are initiated from a promoter linked to exon III and contain only sequences corresponding to that exon and the coding exon. In the rat NGF gene, several more noncoding exons have been described, generating an even more complex pattern of mRNAs [T. Timmusk and M. Metsis, unpublished data].

Our picture of the various noncoding exons of neurotrophin genes may still be incomplete, and the information on multiple promoters is limited. Detailed analysis of transcriptional units has been complicated by very low amounts of neurotrophin mRNA. Future analysis of neurotrophin genes, aided by complete genome sequences, will likely reveal more information to complete the picture.

We still have no satisfactory answer as to why the same neurotrophic factor protein is encoded by different mRNAs with different 5′-UTRs and why their genes give rise to different mRNA species. Different promoters in the BDNF gene have specific developmental patters of expression and differential activation capacities in response to the activation of the glutamatergic system. In the NT-3 gene, only one promoter is responsible for the regulation of transcriptional activity by thyroid hormone. mRNA isoforms with different 5′-UTRs may have specific translational activity, but in the case of BDNF, the experimental data do not support this idea [5]. We can only speculate that with separate promoter usage, the specific 5′-UTRs could be *ad hoc* by-products. Since NGF is the most prominent target-derived neurotrophin for the peripheral nervous system, expressed in most tissues, the variety of 5′-UTRs could play a role in stabilizing the mRNA and facilitating translation in a tissue- and ontogenetic stage-specific manner.

Molecular mechanisms of neurotrophin gene transcription regulation

Despite the well-described regulation of mRNA levels for all neurotrophins, the molecular mechanisms regulating the genes encoding them are mostly unknown or limited to crude mapping of genomic regions conferring the regulation.

Regulation of the NGF gene

An AP-1 site in the first intron of the NGF gene has been demonstrated to bind the c-fos transcription factor. Lesion of the sciatic nerve causes a rapid increase in c-fos and c-jun mRNA followed by an increase in NGF mRNA about 2 h later. In primary cultures of fibroblasts from transgenic mice carrying an exogenous c-fos gene under the control of a metallothionin promoter, $CdCl_2$ evoked a rapid increase in exogenous c-fos mRNA immediately followed by an increase in endogenous c-jun mRNA, with a slight delay before an increase in NGF mRNA [6]. Those experiments demonstrate clearly the possibile engagement of this particular AP-1 site in c-fos regulation of mouse NGF gene expression. However, analysis of the rat NGF gene sequence did not reveal a similar transcription factor-binding site [T. Timmusk and M. Metsis, unpublished data].

Addition of vitamin D3, phorbol myristate, and horse serum to serum-free cultures of fibroblasts and other cell lines [7] rapidly induces NGF mRNA and NGF protein production. In ROS 17/2.8 osteosarcoma cells transfected with the NGF-hGH reporter plasmids, the AP-1 site in the first intron was suggested to be involved in transcriptional regulation. Since the vitamin D3 receptor does not bind to the AP-1 site may bind the c-fos/c-jun complex via an as yet unidentified mechanism [8]. Although ex-

perimental data are lacking, the same site has been proposed to be involved in the regulation of NGF gene transcription by phorbole myristate and other protein kinase C-activating agents [9].

However, taking into account that the NGF gene has a second promoter proximal to exon III, the regulation of the NGF gene by these very different agents, resulting in similar time courses and elevation rates, could be much more complex. The second promoter is also strongly induced by all three agents (vitamin D3, phorbole myristate, and horse serum) [10]. In the absence of more detailed information, we can only suggest that the regulation could be cell type specific, or that all agents regulate both promoters in parallel.

Attempts to use NGF promoter constructs to target transgenes in mice were not successful until recently. Detailed analysis of rat NGF gene structure provided information to overcome the problem. Combination of regions flanking the first exon with genomic sequences surrounding the third exon allowed construction of a transgene that conferred recapitulation of the endogenous NGF gene expression pattern and regulation by a reporter gene [T. Timmusk and M.Metsis, unpublished data]. Further detailed analysis of regions used in the transgene will hopefully provide information on genomic elements governing tissue-specific expression of the NGF gene.

BDNF gene regulation

Molecular mechanisms of BDNF gene regulation have been studied more extensively than for other neurotrophins. Following the detailed description of BDNF gene structure, many attempts have been made using various experimental paradigms to localize structural elements of the gene governing mRNA regulation in vivo or in vitro.

Using a transgenic approach, large regions in the BDNF gene have been identified that facilitate its tissue-specific expression by separate promoters. Regions involved in BDNF gene regulation by activation of neuronal glutamate receptors have been established. A 5-kb region of promoter III is ivolved in BDNF gene induction after sciatic nerve transection [11].

The first intron of the BDNF gene contains a neuron-restrictive silencer element (NRSE)-type regulatory sequence [2]. Mutations in the NRSE did not affect the expression pattern of the BDNF transgene, excluding the possibility that it plays a role in tissue-specific expression of the BDNF gene. Furthermore, no ectopic expression of BDNF in nonneuronal tissues was detected. However, alterations in the NRSE affected expression levels of promoters II and I that are located nearby. In the brain, mutation of the NRSE modulated the responsiveness of BDNF promoters I and II to activation of the glutamatergic system [12]. A more detailed search using in vitro and cell/tissue culture methods has provided some additional data on the possible genomic sequence elements involved. Short regions flanking promoters I, II, and III in the BDNF gene have been localized as putative calcium-responsive regions in glioma C6 cultures [13]. Most understood are the molecular mechanisms governing promoter III regulation. Recently, two groups studied in detail regulation of BDNF promoter III triggered by Ca^{2+} influx. Two different regulatory sequences involved in the activation of transcription were localized in the proximal promoter region. One is a novel calcium response element required for calcium-dependent BDNF expression in both embryonic and postnatal cortical neurons. The second element matches the consensus sequence of a cAMP response element (CRE) and is required for transactivation of the promoter in postnatal but not in embryonic neurons. The CRE-dependent component appears to be mediated by CREB activated by CaM kinase IV, but not by CaM kinase II [14, 15].

CaM kinase II has been demonstrated to specifically mediate the BDNF promoter IV activation by Ca^{2+} influx. Two nuclear isoforms of CaM kinase II (delta3 and alphaB) were demonstrated in transient transfection and overexpression experiments to activate specifically only promoter IV, whereas Ca^{2+} influx induced by ionofore Bay K 8644 activated both promoters III and IV [16].

These are the first results to specify the molecular mechanisms that lead to specific promoter activation in response to different pharmacological treatments involving Ca^{2+} influx. Differential cell-specific activation of different BDNF promoters *via* activation of the glutamatergic system [17] most likely reflects involvement of specific kinase pathways in the cells.

NT-3 gene regulation

Relatively little is known about the transcriptional regulation of the NT-3 gene. Two promoter regions have been mapped. Specific activation by tri-iodothyronine (T3) of the second promoter has been mapped in vivo. Nuclear run-on experiments demonstrate that the effect of T3 on NT-3 mRNA is caused by transcription enhancement; however, no specific regulatory element has been identified. By transient transfection assays, strong silencing activity has been found in the upstream distal section of part of the NT-3 gene flanking the promoters [1].

NT-4 gene regulation

No detailed information is available for NT-4 gene transcriptional regulation. However, transgenic mapping has suggested that only a relatively short, 1.5-kb flanking genomic region is required to recapitulate specific expression of the NT-4 gene. This region, however, perhaps does not contain all the restrictive information, because its use results in very high superfluous expres-

sion in kidney and, in some transgenic lines, also in liver. The short 1.5-kb promoter region provides the only prominent regulation of the NT-4 gene by electrical stimulation of muscle tissue [3].

Neurotrophin receptor genes

Neurotrophic factors employ the specific Trk family tyrosine kinase receptors TrkA, TrkB and TrkC for NGF, BDNF/NT-4/5, and NT-3, respectively. In addition, all neurotrophins bind to the common neurotrophin receptor p75 ($p75^{NTR}$) previously referred to as NGF receptor (NGFR) or low-affinity NGF receptor (LNGFR).

Trk receptors

A detailed genomic organization is known for TrkC and TrkA receptors, while partial characterization of TrkB receptors covers the genomic region encoding the extracellular part of the receptor. The TrkA gene spans 23 kb [18] and the TrkC gene is suggested to reach at least 80 kb in size [19]. Based on the available data, all three genes have a similar structure. The extracellular part of the receptors is encoded by nine (TrkC and TrkA) [18, 19] or ten (TrkB) [20] exons. For all receptors, there is an alternatively spliced form of the extracellular part, in which the last exon located adjacent to the transmembrane domain coding region is skipped. This exon encodes eight or nine amino acids and is an important structural element that determines the interaction specificity of receptors with their ligands [20, 21].

Promoter regions for all the Trk receptors have been sequenced. In silico analysis has revealed multiple putative transcription factor-binding sites [19, 22–24]; however, in vitro evaluation and in vivo verification of their role remain to be performed. The only experimental data available concern the proximal region of the TrkA promoter [22]. It contains a 13-nucleotide regulatory element essential for cell type-specific activity of the TrkA promoter in transient assays and binds a protein complex containing the SP1 transcription factor [23]. All Trk gene promoters are TATA-less and relatively GC rich. They have significant sequence similarity [24]. Surprisingly, analysis of the TrkA locus demonstrated that the gene encoding the insulin receptor-related receptor (IRR) is located just 1.6 kb upstream of the TrkA gene and is transcribed in the opposite direction, suggesting shared regulatory elements that partially explain their coordinated regulation [23]. For the TrkB gene, two alternative promoters have been described, but their biological significance or alternative expression capacity have not been elucidated [24].

Neurotrophin receptor p75

$p75^{NTR}$ was first cloned using gene transfer and detection with monoclonal antibodies raised against melanoma cells expressing very high levels of $p75^{NTR}$ [25]. Comparison of the human $p75^{NTR}$ gene structure [26] with a partially characterized rat $p75^{NTR}$ gene (M. Metsis, unpublished data) demonstrates a high level of similarity. The $p75^{NTR}$ gene contains six exons, and spans approximately 23 kb. The promoter is TATA-less and GC rich, with only one major transcription start site [26]. Comparison of human, rat and mouse 1.8-kb promoter region sequences proximal to the start codon of the $p75^{NTR}$ promoter reveals over 80% identity. Surprisingly, the identity disappears sharply upstream of that region, dropping to 40–50%: large blocks of identical sequence there are sparse-homology stretches, about 20 bp in length.

The $p75^{NTR}$ gene has been studied relatively extensively, due to several available experimental paradigms where the $p75^{NTR}$ protein and mRNA levels are strongly regulated. The best-known paradigms are the regulation of $p75^{NTR}$ gene expression by NGF in PC12 cells [27] and in Schwann cells after sciatic nerve lesion [28–30]. Developmentally regulated expression of $p75^{NTR}$ with a specific spatial and temporal pattern has also suggested participation of specific transcription factors [reviewed in ref. 31].

Intensive studies using transgenic technology have provided significant information on the presence of specific regulatory elements in the $p75^{NTR}$ gene. A transgene containing the entire $p75^{NTR}$ gene – 8 kb of 5′-flanking sequence, all exons and introns, and 7.5 kb of the 3′-flanking region – recapitulates endogenous patterns of $p75^{NTR}$ expression [32]. Data based on the use of promoter regions alone in transgenic animals have been controversial. 4 kb of human $p75^{NTR}$ promoter sequence has been reported to recapitulate mesenchymal and peripheral nervous system expression, to confer up-regulation of $p75^{NTR}$ expression in lesioned sciatic nerve, but not to support expression in the central nervous system [33]. In another report only 470 bp of mouse promoter sequence were shown to drive brain expression, while 8.4 kb has been reported not to support expression in the Schwann cells of the sciatic nerve [34]. One could speculate that the discrepancy is caused by differences in species (human versus mouse promoter); however, differences could also result from the different reporter genes used.

More detailed analysis of the $p75^{NTR}$ promoter has identified regions capable of mediating the promoter regulation by retinoic acid [35] described earlier in tissue culture experiments [36, 37]. Specific areas of the promoter confer activation of transcription by vitamin D3 [38] and testosterone [35]; however, no specific regulatory elements have been found. A non DNA-binding mode of regulation $p75^{NTR}$ promoter by the androgen receptor has been suggested [39].

The above-mentioned regulation of $p75^{NTR}$ gene activity in Schwann cells may be the mediated by zinc finger transcription factor Zif268. Zif268 expression is rapidly induced by nerve transection in vivo. The expression pattern of $p75^{NTR}$ in Schwann cells after nerve injury is closely correlated with the Zif268 expression profile. In transient transfection assays, Zif268 transactivates the $p75^{NTR}$ promoter [40].

A search for specific regulatory elements participating in developmental regulation of $p75^{NTR}$ expression revealed E-box-like elements in the $p75^{NTR}$ promoter. A conserved E-box in the proximal promoter specifically binds the ME1 transcription factor [31], repressing promoter activity. A reciprocal pattern of ME1 and $p75^{NTR}$ expression in early embryo suggests biological role for that interaction. The same E-box may be involved in other regulations since NeuroD and a related bHLH neurogenic transcription factor are capable to reverse the ME1 repression and activate $p75^{NTR}$ gene transcription (T. Neuman, unpublished data).

A more distally located cluster of two E-boxes confers cell type-specific regulatory capacity on the $p75^{NTR}$ promoter [41]. The regulatory cluster alone in heterologous context inhibits the transcription of the $p75^{NTR}$ gene in neuronal cells. In the presence of more upstream promoter sequences, the element, however, becomes a strong activator of transcription. The E-box cluster is perhaps a negative part of a more sophisticated regulatory machinery.

E-box-like elements in the promoter could be involved in strong down-regulation of the $p75^{NTR}$ promoter during myoblast differentiation [42]. A role for bHLH transcription factors in myoblast differentiation is well known; however, the involvement of biologically active E-box sequences remains to be clarified.

Perspectives

This review has demonstrated how little we know about the molecular biology of the genes of neurotrophins and their receptors. One of the reasons is the scarcity of tissue culture models for recapitulating in vivo situations. The nervous system is extremely complex and many characteristics of particular cells are exhibited only in a tissue context. The same gene can be regulated very precisely and differentially in different cells, depending on the surrounding microenvironment, signals from other parts of the organism, overall physiological status, age, and so on. In addition to these objective obstacles, interest in using neurotrophins as therapeutic agents has boosted research on their physiology, while undermining the research on 'genomic problems'. Nevertheless, a large amount of experimental data on the regulation of mRNA levels of molecules of theneurotrophin system has been gathered, although in most cases, we do not know if the data reflect regulation at the transcriptional level. The use of very different experimental systems has created a situation where different reports are seemingly contradictory. Any attempts to fuse into a cohesive logical framework the information presented in approximately 10,000 reports dedicated to the physiology and molecular biology of the neurotrophic system are most likely ill-fated.

However, we need to understand the details of the molecular biology of the neurotrophin system and integrate them with the physiology of neurotrophins for two major reasons. First, fundamental knowledge on nervous system development (in which neurotrophins certainly hold key positions) and maintenance provides possibilities for predicting therapeutic approaches in the treatment of psychiatric and neurological disorders. Neurotrophic actions are involved in the rearrangement of neural circuits during learning processes. Second, development of new 'genomic' drugs that regulate specific genes at the transcriptional level may provide an alternative to delivery of therapeutic biomolecules produced *ex corpora*. In this way, the internal resources of an organism can be mobilized to cure the diseases. Cell and gene therapy require information on the transcriptional regulation of genes of the neurotrophin system. The best synthetic promoter driving expression of therapeutic biomolecules for gene therapy in the nervous system may well be one whose construction is based upon regulatory blocks derived from the promoters of molecules that function in the nervous system.

Three main paradigms of regulation of neurotrophin system genes are obvious candidates requiring resolution at the molecular level: regulation of transcription by neural activity in neurons and target tissues, regulation of transcription by the ligands of nuclear hormone receptors, and regulation in glial cells by contacts with neurites and axons.

Striking regulation of BDNF and TrkB mRNA levels in neurons in response to, e.g., trauma, epileptic seizures, activation of the glutamatergic system, and hypoxia have been described [reviewed in ref. 43]. Most of these physiological paradigms involve Ca^{2+}/calmodulin pathway activation. Systematic analysis of putative genomic regions binding transcription factors known to be activated directly or by cross-talk with other secondary messenger systems should reveal the molecular mechanisms involved. In addition, comparison with other promoters regulated by Ca^{2+} influx may suggest new solutions. In the case of BDNF, some success has been achieved, but the picture is not yet complete.

The ligands of nuclear hormone receptors are possible prototypes for 'genomic' drugs. The up-regulation of TrkB mRNA levels in neuroblastoma cells by nanomolar concentrations of retinoic acid is perhaps one of the most interesting and intriguing issues in gene regulation in the

neurotrophin system. Such regulation has also been demonstrated for primary cultures of sympathetic neurons of superior cervical ganglion, suggesting a role in normal development [44]. Increased levels of TrkB expression have been suggested to enhance the auto/paracrine loop leading to neuroblastoma cell differentiation. Neuroblastoma, particularly medulloblastoma, is well-known as an early childhood malignant tumor. Retinoic acid treatment has been suggested as a possible therapeutic approach for patients with a suitable type of tumor [45]. Dissection of the molecular mechanisms of retinoic acid action and determination of the specific receptor type involved may suggest new drugs, analogues of retinoic acid, for the treatment of tumors in children.

Estrogen modulates the expression of neurotrophins and their receptors [reviewed in ref. 46]. In vivo, BDNF mRNA is rapidly up-regulated in the cerebral cortex and the olfactory bulb of ovariectomized animals exposed to estrogen [47]. Given the widespread use of estrogen substitution therapy among the postmenopausal female population in Western society, understanding the pharmacological implications and the molecular mechanisms of trophic interactions in the nervous system is necessary. Vitamin D3 is one candidate drug for the regulation of neurotrophins, particularly the regulation of NGF in target tissues. In addition, vitamin D3 regulates NT-3 and NT-4 expression in glial cells [48], and $p75^{NTR}$ expression [38]. Thus, the drug could modulate trophic support by altering neurotrophin production. It could also act as an anticancer agent by inducing apoptosis of cells via increased $p75^{NTR}$ expression. Unraveling the molecular mechanisms of vitamin D3 action and its cell and tissue type specificity may be of pharmacological interest.

All molecules of the neurotrophic system are regulated in the classical sciatic nerve crush or transection paradigm. Every molecule has its own specific temporospatial pattern in injured nerves after trauma and during healing [49, 50]. Similar effects have also been detected in glial cells of the central nervous system [51]. Increased expression of neurotrophins and their recptors in glia could result in auto/paracrine induction of glial cell proliferation and may be one of the events leading to scar tissue formation that prevents regeneration in the brain after trauma. Understanding the molecular mechanisms governing the events at the genomic level would provide us with valuable information for developing of new therapeutic strategies to cure peripheral nerve trauma, to support injured neurons until contact with target tissue is re-established, and to assist neurons during regeneration.

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