Distinct Permissive Pathways Mediate the Effects of Nerve Growth Factor and Lithium on Neurotensin/Neuromedin N Gene Expression in PC12 Cells: A Thesis

Bryant Paul Bullock

University of Massachusetts Medical School

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DISTINCT PERMISSIVE PATHWAYS MEDIATE THE EFFECTS
OF NERVE GROWTH FACTOR AND LITHIUM ON
NEUROTENSIN/NEUROMEDIN N GENE EXPRESSION IN PC12 CELLS

A Thesis Presented
by
Bryant Paul Bullock

Submitted to the Faculty of the
University of Massachusetts
Graduate School of Biomedical Sciences, Worcester
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MOLECULAR GENETICS AND MICROBIOLOGY

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Approved as to style and content by:

------------------------------------------
Allan Jacobson, Chairman of Committee

------------------------------------------
Louis DeGennaro, Member of Committee

------------------------------------------
Richard E. Baker, Member of Committee

------------------------------------------
Steven Treistman, Member of Committee

------------------------------------------
Michael Comb, Member of Committee

------------------------------------------
Paul R. Dobner, Thesis Advisor

------------------------------------------
Thomas Miller, Dean of Graduate
School of Biomedical Sciences
This thesis is dedicated to
Caroline, Kimberly, Christopher
and most importantly, Laurie.
Your love made it all worth while.
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accomplishments of this thesis are minor in comparison to the happiness you have given me. To Laurie, who more than anyone else has had to endure the hardships during the last six years, I am extremely grateful for your patience and love. I Will Love You Forever.
This thesis examines the effects of nerve growth factor (NGF) and lithium on the regulation of neurotensin/neuromedin N (NT/N) gene expression in PC12 pheochromocytoma cells. In PC12 cells, the expression of the rat NT/N gene is strictly dependent on simultaneous exposure to combinations of NGF, glucocorticoids, activators of adenylate cyclase, and lithium. Transient transfection experiments indicated that a consensus AP-1 site located within the NT/N promoter is the principal target of NGF and lithium action. NGF rapidly, but transiently, induces the expression of several AP-1 genes in PC12 cells, suggesting that the effect of NGF on NT/N gene expression results from increased AP-1 activity. These results led to the prediction that the induction of NT/N gene expression should be rapid, transient and dependent on *de novo* protein synthesis. These experiments also suggested that the NT/N gene is principally regulated through the initiation of transcription. However, post-transcriptional mechanisms may also be involved. Experiments in this thesis were designed to examine the regulatory mechanisms responsible for increased NT production in PC12 cells when treated with different inducer combinations and whether AP-1 factors could act as mediators in responses to NGF and lithium.

Results described in this thesis indicate that the principal mechanism by which NGF and lithium regulate NT biosynthesis is by activating NT/N gene transcription. Comparison of NT/N mRNA, pro NT/N synthetic rates, proNT/N proteins and mature NT levels in induced PC12 cells, demonstrated that NGF and lithium had no effect on the translation of NT/N mRNA and had only a modest
effect on post-translational processing. Nuclear run-on assays showed that NT/N transcription is transiently activated in maximally induced cells. A rapid RNase protection assay was developed to examine both the kinetics of NT/N gene activation and whether activation requires newly synthesized proteins. Quantitation of nuclear NT/N precursor RNA, using a probe spanning the junction between exon one and intron one, provides a sensitive measure of NT/N gene activity and by several criteria provides an accurate measure of NT/N transcription. When either NGF or lithium was combined with dexamethasone and forskolin, nuclear NT/N precursor RNA transiently accumulated, although each inducer displayed different kinetics, rapid and delayed, respectively. De novo protein synthesis was not required for activating NT/N transcription when NGF was used as the permissive agent, although newly synthesized proteins seem to be needed for subsequent down-regulation. The response to lithium displayed a marked requirement for new protein synthesis, consistent with the involvement of newly synthesized AP-1 factors. RNA blot analysis showed that lithium either alone or in combination with dexamethasone and forskolin induced c-jun and fra-1 gene expression with delayed kinetics, consistent with c-Jun/Fra-1 complexes mediating the effects of lithium on NT/N gene transcription. The pathway identified by lithium does not activate or require protein kinase C. This pathway is also active in neuronally-differentiated PC12 cells suggesting that it could be involved in the regulation of NT/N gene expression in the intact nervous system. These results and order of addition experiments demonstrate that NGF and lithium activate distinct pathways required for NT/N gene induction.
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CHAPTER I

INTRODUCTION
Overview. The neuropeptide, neurotensin (NT), has been implicated in diverse functions in the CNS, including control of the estrous cycle and modulation of dopaminergic pathways (Ervin and Nemeroff 1988). NT and a related neuropeptide, neuromedin N, are encoded by a common gene (NT/N gene) (Dobner et al. 1987; Kislauskis et al. 1988) which is expressed throughout the limbic regions of the rat central nervous system (CNS) in a complex pattern (Alexander et al. 1989b). Several lines of evidence indicate that endocrine hormones and neurotransmitters play key region-specific roles in the generation of this pattern. For example, estrogen and the antipsychotic drug, haloperidol, have striking regulatory effects on NT/N gene expression in the medial preoptic area (Alexander et al. 1989b; Alexander et al. 1991) and the striatum (Merchant et al. 1992; Merchant et al. 1991), respectively, suggesting that diverse environmental stimuli are required to establish the complex pattern of NT/N gene expression observed in the adult rat CNS.

PC12 cells, which differentiate neuronally in response to nerve growth factor (NGF) (Greene and Tischler 1976), have provided insights into the pathways through which neurotrophic factors and neurotransmitters control gene expression. Among the earliest transcriptional responses to NGF in PC12 cells, is the rapid induction of the c-fos and c-jun proto-oncogenes (Sheng and Greenberg 1990). These genes encode components of the AP-1 family of transcription factors that recognize a common cis-regulatory sequence within the promoters of many genes, including NT/N (Kislauskis and Dobner 1990; Morgan and Curran 1991; Sheng and Greenberg 1990). A variety of physiological and pharmacological stimuli induce AP-1 gene and protein expression in the central nervous system, indicating that AP-1 complexes may
play an important role in controlling neuronal gene expression (Morgan and Curran 1991). The identification of functional AP-1-related sequences required for the expression of genes encoding neurotransmitter biosynthetic enzymes (Gizang-Ginsberg and Ziff 1990) and neuropeptides (Kislauskis and Dobner 1990; Kobierski et al. 1991; Sonnenberg et al. 1989c) suggest that these genes comprise an important class of neuronal AP-1 target genes. PC12 cells provide a valuable model system for understanding how AP-1 regulates the expression of this class of target genes.

The AP-1 site is closely related to the cyclic AMP response element or CRE (Montminy et al. 1986). The transcriptional activation function of the major CRE-binding protein in PC12 cells, CREB, is regulated in response to cAMP through phosphorylation by protein kinase A (Gonzalez and Montminy 1989). However, similar to the AP-1 site, the CRE is recognized by a family of DNA binding proteins that are structurally related to the AP-1 family (Habener 1990). Specific AP-1 family proteins can also form heterodimers with CREB family proteins that bind the CRE with high affinity (Benbrook and Jones 1990; Hai and Curran 1991; Ivashkiv et al. 1990). Since diverse stimuli induce AP-1 genes in a wide variety of cells and the cAMP pathway is present in most cell types, a major question concerning the regulation of target genes by these two pathways is how specificity is generated. The analysis of the proenkephalin (Comb et al. 1989; Kobierski et al. 1991) and NT/N (Kislauskis and Dobner 1990; Kislauskis and Dobner 1992) genes has provided evidence that functional interdependence between AP-1-related, CRE-related, and other cis-regulatory sequences may play an important role in determining which target genes are activated through the AP-1 and cAMP pathways.
NT/N gene expression in PC12 cells is subject to synergistic regulation by combinations of NGF, glucocorticoids, and adenylate cyclase activators (Dobner et al. 1988). NGF is required for responses to glucocorticoids and forskolin suggesting that it acts to permit NT/N gene expression. Lithium, which perturbs phosphatidylinositol signal transduction pathways (Berridge et al. 1989) and blocks at least some NGF effects in PC12 cells (Burstein et al. 1985), is able to substitute for NGF in this permissive role (Dobner et al. 1988). Increases in NT/N mRNA were not always paralleled by increases in intracellular NT peptide, suggesting that post-transcriptional mechanisms may also be regulated by NGF and lithium (Dobner et al. 1988). A rat NT/N gene promoter fragment confers the regulatory properties exhibited by the endogenous gene to reporter genes and contains a consensus AP-1 site, two CRE-related sequences, and a glucocorticoid response element (GRE) which are required for appropriate regulation (Kislauskis and Dobner 1990). Alterations in the CRE and GRE motifs selectively affect responses to inducer combinations that include forskolin or dexamethasone, respectively, whereas the AP-1 site is likely to mediate the permissive effects of NGF and lithium (Kislauskis and Dobner 1990). These results indicate that the consensus AP-1 site plays a pivotal role in the transcriptional regulation of the NT/N gene and leads to the prediction that NT/N gene activation in PC12 cells will be both transient and require ongoing protein synthesis, since NGF and other stimuli transiently induce AP-1 expression in these cells (Sheng and Greenberg 1990). Experiments described in this thesis were designed to test whether the permissive responses of NGF and lithium on NT/N gene transcription is mediated by the induction of AP-1 factors.
Neurotensin Neurotensin, a thirteen amino acid peptide, was originally isolated from extracts of bovine hypothalamus based on its potent hypotensive effects (Carraway and Leeman 1973; Leeman and Carraway 1982). NT is a member of a family of related peptides, all having similar biological effects, that possess as the major determinant of biological activity common carboxy-terminal sequences (Carraway and Reinecke 1984). NT/N mRNA and NT-like immunoreactivity are expressed most abundantly in the adult rat in the gastrointestinal (GI) tract and are widely distributed in the CNS, most significantly in the forebrain and limbic system (Carraway and Leeman 1976). In mammals, NT immunoreactive cells are scattered in the epithelium of the jejunum-ileum where NT seems to play a role in the regulation of fat metabolism (Ferris et al. 1985). In the rat CNS, NT has been implicated in regulating distinct systems. For instance, within the anterior medial preoptic nucleus, NT/N mRNA levels are induced in response to estrogen treatment in ovariectomized rats suggesting that NT neurons mediate stimulatory effects of ovarian steroids on GnRH secretion thus modulating the pre-ovulatory surge (Alexander et al. 1989a). NT also co-localizes to dopamine containing neurons within rodent brains (Nemeroff et al. 1983) and exerts behavioral properties that resemble those of neuroleptics, antipsychotic drugs whose actions correlate with dopamine D2 receptor antagonism (Merchant et al. 1991; Nemeroff and Cain 1985). Treatment with the dopamine antagonist, haloperidol, results in the rapid transient induction of both nuclear NT/N precursor RNA and NT/N mRNA within the rat striatum (Merchant et al. 1992). These results suggest that NT/N gene expression is specifically localized within
the rat CNS and that distinct environmental inputs are responsible for the establishment of the complex pattern of expression.

Characterization of the NT/N gene and cloned cDNAs has provided clues on how diverse environmental inputs regulate the specific pattern of expression in the adult rat. Rat NT is synthesized from a precursor peptide (preproNT/N), of 169 amino acids, that also codes for a related peptide, neuromedin N (Dobner et al. 1987). Sequences are highly conserved between canine, bovine, human and rat preproNT/N, which all exhibit a similar prohormone structure (Kislauskis et al. 1988, and unpublished results). The identification of sequences that are similar to neuromedin N (NN) and that are N-terminally flanked by dibasic amino acids suggests that an unidentified peptide may be synthesized along with NT and NN. Typical of other secretory peptides (Mains et al. 1983), the NT/N precursor protein contains a consensus signal sequence at the amino-terminus. The preprohormone structure of NT/N indicates that intracellular NT content may be regulated at different levels, such as initiation of transcription, mRNA splicing, and peptide processing.

The distribution of NT/N mRNA to regions of the CNS that also express NT-like immunoreactivity suggest that the induction of transcription may be the major mechanism for establishing tissue-specific localization. However, in the rat hippocampus and subiculum the detection of abundant NT/N mRNA where NT-like immunoreactivity was not observed indicated that translational or post-translational mechanisms may also regulate NT/N biosynthesis within the CNS (Alexander et al. 1989b). Several other studies have also shown that there is a difference in the regional distribution of intracellular NT, NN, and extended forms of NT and NN in the mouse (Shaw et al. 1990b), dog (Carraway
and Mitra 1990; Carraway et al. 1991), and rat (Carraway et al. 1991; Sato et al. 1990), indicating that alterations in post-translational processing contribute to the tissue specific distribution of NT and the related peptides. Several examples of differentially processed neuropeptides that are located within different regions of the CNS have been documented, including preprotachykinin, preproopiomelanocorticotropicin, and proenkelphalin (Douglass et al. 1984). Therefore, the regulation of NT/N gene transcription and peptide processing may be responsible for the tissue specific localization of NT/N mRNA and NT peptides within the CNS. Distinct environmental inputs which regulate transcription, peptide processing, or other NT biosynthetic pathways are likely to be necessary for the establishment of the this complex pattern of distribution.

**PC12 cells** An excellent model for studying environmentally induced changes in neuron-specific gene expression is the PC12 cell line. Isolated from a rat pheochromocytoma, PC12 cells are thought to be representative of progenitor cells of the sympathoadrenal lineage (Greene and Tischler 1976; Greene and Tischler 1982). Sympathoadrenal precursor cells are derived from the neural crest, a transient structure originating on the apical surface of the neural tube. During embryogenesis, these cells migrate to form the peripheral nervous system along with several non-neuronal cell types, including adrenal chromaffin cells (LeDouarin 1982). Several studies have shown that differentiation of the sympathoadrenal progenitors is dictated by the environment to which they migrate (Anderson 1989; LeDouarin 1980). Two such environmental determinants play both permissive and instructive roles
in these phenotypic decisions: glucocorticoids, secreted from the adrenal cortex, promote the differentiation of adrenal chromaffin cells; whereas NGF, secreted from target tissues, causes neuronal differentiation (Anderson and Axel 1986). Like sympathoadrenal precursor cells, unstimulated PC12 cells resemble adrenal chromaffin cells. Furthermore, when exposed to NGF, they cease to divide, and differentiate into cells that have all the characteristics of sympathetic neurons (Greene and Tischler 1976; Greene and Tischler 1982). Both NGF and glucocorticoids can alter the expression of many neuron-specific genes in PC12 cells, including the tyrosine hydroxylase (Gizang-Ginsberg and Ziff 1990) and NT/N genes (Dobner et al. 1988). Thus, PC12 cells have become important in understanding the mechanisms by which environmental factors, such as NGF, hormones, and neurotransmitters, interact to induce gene expression.

**AP-1 transcription factors** In PC12 cells, NGF induces a class of "cellular immediate early genes" (IEGs) whose protein products are important in mediating the response of extracellular stimuli to changes in gene expression. Many of these genes, which include the *fos/jun* family of proto-oncogenes, code for transcription factors that preferentially bind as heterodimers to AP-1 *cis*-regulatory sequences (5'-TGA G/C TCA-3') on specific target genes (Herschman 1991). This family includes c-*fos*, at least three *fos*-related genes (*fos* B, *fra*-1, *fra*-2), c-*jun*, and at least two *jun*-related genes (*jun*-B, *jun*-D). AP-1 factors are phosphoproteins comprised of at least two functional domains: a transactivation domain and a bZIP domain (Curran and Franza 1988; Curran et al. 1987). The bZIP domain is comprised of a region of basic amino acids
along with a leucine zipper. An extensive number of studies have concluded the following about the bZIP domain: 1) DNA recognition specificity resides in the basic region; 2) the leucine zipper is responsible for dimerization which is required for binding to the DNA element; 3) heterodimerization of different AP-1 factors allows for variability in activation potential 4) there is a dimerization code which assures that only certain combinations of leucine zipper containing proteins can form stable dimers (Abate et al. 1991; Abate et al. 1990; Kouzarides and Ziff 1988; Ransone et al. 1989; Sassone-Corsi et al. 1988b). Phosphorylation of AP-1 proteins within the transactivation and bZIP domains can affect both DNA binding and transcriptional activation (Binetruy et al. 1991; Boyle et al. 1991). The presence of these domains in AP-1 factors thus allows for two different modes of regulation, phosphorylation and dimerization.

Cellular IEGs are rapidly and transiently transcribed in PC12 cells in response to extracellular stimuli in a protein synthesis independent manner (Greenberg et al. 1986; Morgan and Curran 1989). For example, transcriptional activation of c-fos occurs within 5 minutes of NGF treatment in PC12 cells, and continues for 15-20 minutes (Greenberg et al. 1985). c-fos mRNA accumulates and reaches peak values at 30-45 minutes post-stimulation; thereafter it declines with a relatively short half-life of approximately 12 minutes (Morgan and Curran 1991). Synthesis of c-fos protein follows mRNA expression and it is turned over with a half-life of about 2 hours (Morgan and Curran 1991). A model of how environmental stimuli can regulate gene transcription, by inducing AP-1 factors, is depicted in Figure 1-1. Ligands binding to cognate receptors act through signal transduction pathways to induce AP-1 gene
Figure 1-1. Model for the regulation of target gene expression by the induction of AP-1 factors. Ligands (L) binding to receptors (R) activate signal transduction pathways (step 1) that induce AP-1 gene transcription (step 2). AP-1 mRNAs are processed and transported into the cytoplasm (step 3) where they are translated (step 4). Newly translated AP-1 proteins are modified and translocated into the nucleus (step 5). These factors bind to the AP-1 site within the promoter of a target gene as dimers (step 6) and activate transcription (step 7). Each gene is depicted with a regulatory region (dark shaded boxes) and a transcribed region (open boxes). mRNAs are shown as solid heavy lines followed by poly A tracts. The AP-1 protein domains are shown: N-terminal region (light shaded boxes), DNA binding (horizontal stripes) and leucine zipper region (diagonal stripes). Solid thin line represents the cellular membrane while the double thin line represents the nuclear membrane.
transcription. AP-1 mRNAs are processed and transported to the cytoplasm where they are translated. Newly translated proteins are modified, translocated into the nucleus and bind to AP-1 sites either as homo- or heterodimers thereby activating transcription of target genes. Extracellular stimuli could regulate the synthesis or activity of AP-1 proteins at any one of these steps, although induction of AP-1 gene transcription or the post-translational modification of AP-1 proteins are the most likely mechanisms. Subsequent attenuation of target gene transcription is normally achieved through autorepression of AP-1 genes coupled with the unstable nature of both AP-1 mRNA and proteins (Sassone-Corsi et al. 1988c). However, inactivation can also be achieved by inducing AP-1 factors that negatively regulate transcription (Chiu et al. 1989). Following stimulation, specific AP-1 genes are expressed with varying kinetics (Bartel et al. 1989; Sonnenberg et al. 1989a), suggesting that there is a temporal alteration in the composition of the AP-1 complex. The relative levels of active or inactive AP-1 factors, at any given time after stimulation, could mediate the response to neuropeptide gene transcription.

Induction of AP-1 genes during stimulation of the intact nervous system supports the idea that these genes are important regulators of neuronal gene expression in vivo. A rapid and transient induction of c-fos, fra-1 and c-jun genes occurs in neurons of the rodent brain in response to various stimuli, including drug-induced seizures (Morgan et al. 1987), electrical stimulation (Sagar et al. 1988) and peripheral sensory stimulation (Naranjo et al. 1991). Several neuropeptide genes, including the proenkephalin (Sonnenberg et al. 1989a) and prodynorphin genes (Naranjo et al. 1991), are rapidly and
transiently expressed in parallel with AP-1 genes following stimulation, although with slightly delayed kinetics. Even though this is only correlative evidence, these experiments strongly suggest that the induction of AP-1 complexes are involved the control of neuropeptide gene expression. Therefore, cellular IEGs, especially AP-1 binding factors, may function as third messenger mediators of long-term responses to environmental factors within the nervous system.

Neurotensin regulation in PC12 cells The localization of NT to cells of the cat adrenal gland (Terenghi et al. 1983) suggested that NT might also be expressed in PC12 cells and could be used as a model system to study how environmental factors regulate neurotensin gene expression (Tischler et al. 1982). NT-like immunoreactivity was detected in PC12 cells, but only after induction with combinations of NGF, forskolin, and the synthetic glucocorticoid, dexamethasone (Tischler et al. 1986). Naive cells were devoid of NT, as were cells treated with individual inducers. Dexamethasone and forskolin only affected NT levels when used in combination with NGF, indicating that NGF acts permissively to facilitate the actions of both dexamethasone and forskolin. Surprisingly, lithium chloride could substitute for NGF in its permissive role (Dobner et al. 1988), even though it has been shown to antagonize the effects of NGF on neurite outgrowth in PC12 cells (Burstein et al. 1985). Lithium alone had no effect on NT expression in PC12 cells, whereas it acted synergistically with NGF, dexamethasone and forskolin to increase NT content (Dobner et al. 1988). Therefore, NT expression in PC12 cells is strictly dependent on combined treatment with multiple inducers.
In most cases, increases in the levels of NT/N mRNA in response to combinations of NGF, lithium, dexamethasone, and forskolin paralleled increases in intracellular NT content indicating that the major mechanism for regulating NT levels in PC12 cells is through the activation of NT/N gene transcription (Dobner et al. 1988). A promoter fragment encompassing sequences between -216 and +56 confers the regulatory properties exhibited by the endogenous gene to reporter genes, indicating that NT/N gene expression is regulated principally at the transcriptional level (Kislauskis and Dobner 1990). Detailed mutational analysis of this region has provided evidence that a consensus AP-1 site, two CRE-related sequences, and a glucocorticoid response element (GRE) are required for appropriate regulation. Alterations in the CRE and GRE motifs selectively affected responses to inducer combinations that include forskolin and dexamethasone, respectively (Kislauskis and Dobner 1990). Deletion or mutation of the AP-1 element reduced responses to all inducer combinations by about an order of magnitude, suggesting that the AP-1 site mediates the permissive effects of NGF and lithium (Kislauskis and Dobner 1990). The selective activation of CAT fusion genes driven by multimerized consensus AP-1 sites in response to either NGF or lithium in PC12 cells also supports this view (Dobner et al. 1992). These results indicate that AP-1 factors mediate the permissive actions of NGF and lithium.

A model for the transcriptional regulation of the NT/N gene is shown in Figure 1-2. NGF or lithium, acting through signal transduction pathways, initiate the binding of Fos/Jun complexes to the AP-1 site. CREB-like proteins constitutively bound to the distal CRE are activated by forskolin, presumably
Figure 1-2 Model for the cooperative induction of the NT/N gene. NGF, lithium, dexamethasone, and forskolin, acting via signal transduction pathways (arrows), induce NT/N gene expression through the interaction of DNA binding proteins with the NT/N promoter. NGF and lithium act through Fos/Jun dimers (striped/open circles) that bind to the distal AP-1 site. Forskolin activates the cAMP signalling pathway to induce the phosphorylation of CREB (open boxes) bound to the distal CRE. Dexamethasone stimulates translocation of GC receptors to the nucleus (shaded boxes) which then bind to the GRE. It is unclear whether Fos/Jun complexes or CREB interact with the proximal CRE/AP-1 site. The multifactor complex cooperatively interacts with basal transcription factors (BTFs, shaded circle) bound at the TATA box and enhances the initiation of RNA polymerase (RNA pol, shaded circle), thus activating transcription of the NT/N gene.
through phosphorylation by protein kinase A (PKA). Activated glucocorticoid receptors bind to the GRE. This multifactor complex then interacts with the basal transcription machinery resulting in an increase in NT/N transcription. It is unclear which signalling pathway interacts with the proximal CRE/AP-1 site, since it is composed of sequences that resemble both an AP-1 site and CRE (Kislauskis and Dobner 1990). Interestingly, the CRE differs from the AP-1 site by the insertion of a single base-pair (Montminy et al. 1986). Recent gel shift and DNase footprinting experiments demonstrated that both CREB and various AP-1 complexes interact with this dual element, in vitro (G. McNeil and P. Dobner, unpublished results). The interaction of proteins bound to these mutually dependent regulatory elements allow for the synergistic activation of NT/N transcription. NT/N gene expression in PC12 cells appears to be an excellent experimental model to study how the coordination of multiple environmental stimuli regulate neuropeptide biosynthesis, especially changes in gene transcription.

Even though increases in NT/N mRNA levels seem to be the major response to these inducers, post-transcriptional mechanisms may also control intracellular NT content (Dobner et al. 1988). In cells treated with lithium, dexamethasone, and forskolin, the level of NT/N mRNA increases 348-fold while intracellular NT increases just 4-fold as compared with cells exposed to only the latter two inducers (Dobner et al. 1988). In contrast, treatment of PC12 cells with NGF, dexamethasone, forskolin, and lithium results in only a 2.5-fold increase in NT/N mRNA whereas a 24-fold increase in intracellular NT was observed when compared to with cells treated with only the latter three inducers (Dobner et al. 1988). These results indicate that NGF and lithium can
influence either the processing events required for the generation of the mature NT peptide or steps involved in the intracellular compartmentalization of NT.

**Signal transduction**

*Nerve growth factor* - NGF is the prototype of a family of "neurotrophic factors", that are necessary for the function and survival of both peripheral and central neurons (Barde 1989; Thoenen and Edgar 1985; Walicke 1989). NGF is secreted by target tissues, where it is taken up by nerve terminals and retrogradely transported to the neuronal soma to regulate cellular processes (Levi-Montalcini 1987). The receptors for NGF have not been fully defined, although two forms, low and high affinity, have been identified (Vale and Shooter 1985). Characterization of the proto-oncogene trk suggests that it may be the homolog of the NGF receptor. Trk protein binds NGF with high affinity and both trk mRNA and protein are expressed mainly within the peripheral nervous system (Martin-Zanca et al. 1989). A homologous receptor TrkB, which preferentially binds two other neurotrophic factors, BDNF and neurotrophin-3, is strongly expressed in the cerebral cortex and hippocampus, but barely detectable in the peripheral nervous system (Soppet et al. 1991; Squinto et al. 1991). These observations indicate that various neurotrophic factors act in a tissue-specific manner within the nervous system. Trk, which like the isolated high affinity NGF receptor from PC12 cells, has tyrosine kinase activity, suggesting that NGF may act through a second messenger pathway involving multiple phosphorylation steps. As of yet, the actual substrates of the Trk tyrosine kinase have not been identified, although two likely candidates are phospholipase C-δ and MAP kinases (Boulton et al. 1991; Chan et al. 1989). NGF
stimulates phosphorylation of phospholipase C-δ on serine and tyrosine residues (Kim et al. 1991), potentially activating the phosphatidylinositol signal transduction pathway (Chan et al. 1989). NGF has also been shown to activate MAP2 kinases, otherwise known as extracellular signal-regulated kinases (ERKs), by phosphorylation on both tyrosine and serine/threonine residues (Boulton et al. 1991). Since Trk tyrosine kinase appears to trigger not one but multiple signal transduction pathways, NGF signals may diverge into several independent pathways (Ross 1991). NGF binds to cognate receptors, thereby initiating second messenger pathways leading to the phosphorylation of substrates important for both short and long-term changes in neuronal plasticity.

**Forskolin** - The cAMP second messenger signal transduction cascade has been well characterized (Sutherland 1972). Ligands activate adenylate cyclase through receptor-coupled G-proteins. Forskolin, a lipid soluble molecule extracted from the perennial aromatic herb, *Coleus forskohlii*, directly activates the catalytic subunit of adenylate cyclase (Seamon and Daly 1981). Adenylate cyclase converts ATP into cAMP which acts as an intracellular ligand to liberate the regulatory subunit of protein kinase A (PKA). The activated PKA subunit phosphorylates a wide range of substrates, including proteins that influence the activation of gene transcription (Gizang-Ginsberg and Ziff 1990; Kislauskis and Dobner 1990; Kobierski et al. 1991). cAMP's effect on transcription is mediated by various members of a family of CREB-like DNA binding proteins. These factors bind, either as monomers or dimers, to a common consensus sequence (5'-TGACGTCA-3') which acts like a classical enhancer (Habener 1990). cAMP response elements (CRE) are highly
conserved among several cAMP-inducible promoters (Montminy et al. 1990),
including the NT/N gene (Kislauskis and Dobner 1990), and have been shown

to be both necessary and sufficient for responsiveness to cAMP signalling
(Montminy et al. 1990). CREB is a 43 kDa phosphoprotein, which similar to AP-1
factors, is subdivided into two domains: a transactivation domain and a bZIP
domain. PKA phosphorylates CREB within a phosphorylation region (P-box) of
the amino-terminal transactivation domain which is necessary for activation
(Habener 1990; Lee et al. 1990; Yamamoto et al. 1988). However,
phosphorylation by PKA was not sufficient to activate transcription (Merino
et al. 1989) thus additional phosphorylations by PKC or caseine kinase II may
be necessary for full activation (Lee et al. 1990). Downstream of the
transactivation domain lies the bZIP domain, consisting of the basic amino acid
DNA binding domain and a carboxy-terminal leucine zipper (Habener 1990;
Montminy et al. 1990). The identification of the leucine zipper suggests that
various members of the leucine zipper family, such as CREB, CRE-BP1, ATFs or
AP-1 factors, can heterodimerize (Benbrook and Jones 1990). Similar to the AP-
1 factors, CREB can be regulated by two different modes, phosphorylation and
dimerization. CREB is the major CRE binding protein in PC12 cells (Montminy
and Bilezikjian 1987) and is likely to mediate the transcriptional effects of
forskolin on the NT/N gene. Recent in vitro DNA binding and footprint assays
have demonstrated that bacterially produced CREB binds in vitro to both the
proximal and distal CRE within the NT/N promoter (G. McNeil and P. Dobner,
unpublished results).

Lithium - Lithium influences components of two well characterized
signal transduction pathways, the coupling between receptors and G-proteins
and perturbations of the phosphatidylinositol (PI) signalling pathway. Experiments which analyzed the effects of lithium on the adrenergic and cholinergic stimulation of GTP binding in rat cortex indicated that lithium competes with magnesium ions. These ions are essential for the binding of GTP to G-proteins thus lithium can interfere with G-protein linked signal transduction pathways, such as the cAMP signalling cascade (Avissar et al. 1988). Lithium also has a profound affect on the PI signalling pathway. Figure 1-3 diagrams the metabolic events that occur during signalling through this pathway. Ligand stimulated receptors activate phospholipase C which hydrolyzes phosphatidylinositol (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) (Berridge and Irvine 1984; Nishizuka 1984). DAG is a strong activator of protein kinase C (Huang 1989; Nishizuka 1984). IP3, specifically I(1,4,5)P3, mobilizes calcium from the endoplasmic reticulum (Berridge and Irvine 1984), which greatly influences cellular processes, such as activating calcium/calmodulin kinases (Griffith and Schulman 1988). Protein kinase C (PKC) and calcium/calmodulin kinase both potently alter gene expression (Chiu et al. 1987; Sheng et al. 1991; Van Nguyen et al. 1990) and are thus important mediators for translating extracellular stimuli into transcriptional responses. Lithium acts as an uncompetitive inhibitor of mono- and polyphosphatases, which are important for recycling inositol phosphate metabolites back into free inositol. This recycling is thought to be necessary for the regeneration of the signalling pathway (Berridge et al. 1989; Nahorski et al. 1991), especially where dietary inositol is unavailable. The inhibition of these phosphatases causes the accumulation of mostly inactive metabolites, although in GH3 cells there is an accumulation of DAG after treatment with
Lithium perturbs the phosphatidylinositol signal transduction pathway. A ligand (L) binding to its cognate receptor (R) activates phospholipase C (PLC). This enzyme converts phosphatidylinositol diphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). These metabolites strongly activate signal transduction cascades (shown as broken arrows), DAG through the activation of protein kinase C (PKC) or IP3 by the mobilization of calcium (Ca++) and subsequent activation of calcium/calmodulin kinases (CCK). Both these pathways can lead to alterations in gene expression. The regeneration of the signalling system (thin arrows) involves the synthesis of phosphatidylinositol (PI) from free inositol, generated from dephosphorylation of the inositol phosphate metabolites (IP4, IP3, IP2 and IP1), and phosphatidic acid (PA) derived from the metabolism of DAG. Lithium inhibits monophosphatases (thick arrows) needed to regenerate free inositol. In an environment where inositol uptake is limited, this could lead to a depletion of PIP2 and an accumulation of the biologically active metabolite, DAG.
lithium (Drummond and Raeburn 1984). These observations raised the possibility that lithium influences NT/N gene expression as a result of its effect on the PI signal transduction pathway.

Dexamethasone - Dexamethasone is a potent synthetic glucocorticoid that mimics the action of cortisol and other corticosteroids (Norris 1980). Glucocorticoids (GCs), like other steroid hormones, are derived from cholesterol and released into the circulatory system by endocrine organs. The lipid-soluble nature of GCs allow them to diffuse through target cell membranes where they interact with intracellular receptors. GC initiates the release of the active receptor from a cytoplasmic complex, the GC receptor associated with the 90 kd heat shock protein (hsp90). The activated GC receptor translocates across the nuclear membrane and binds as a dimer to cis-acting elements on target genes to activate transcription. DNA sequences (GREs) responsible for regulation by GC hormones are found in many genes (Evans and Arriza 1989; Yamamoto 1985) and share elements of a 15-mer consensus sequence (5'-GGTACAnnnTGTTCT-3') (Beato 1989). Structural analysis has demonstrated that the GC receptor is a member of a superfamily of related receptors that share a common mode of action (Evans 1988) and are similarly organized into different functional domains (Beato 1989). Invariant cysteine residues that form zinc fingers are found within the central domain (Freedman et al. 1988) and are responsible for the specific binding to the GRE (Hollenberg 1987). Immediately adjacent to the zipper region is a short sequence that is responsible for intranuclear localization (Picard and Yamamoto 1987) and the interaction with hsp90 (Pratt et al. 1988). The carboxy-terminal domain contains residues important for ligand binding,
along with those needed for dimerization, which may be a prerequisite for efficient trans-activation (Kumar and Chambon 1988). Sequences located within amino-terminal (tau 1) and the carboxy-terminal ligand binding regions (tau 2) and are both necessary and sufficient for trans-activation (Giguere et al. 1986). The tau sequences are rich in acidic residues (Hollenberg and Evans 1988) and, thus may have certain properties in common with acidic yeast activator sequences (Sigler 1988). The amino-terminal domain, which shows the greatest variability between receptors, seems to play a modulatory role in transactivation (Beato 1989). By functioning as ligand-dependent transcription factors, GC receptors play a key role in hormone signal transduction within the nervous system.

**Transcriptional Synergy** One of the unique features of NT/N gene induction in PC12 cells is the high degree of synergy observed between different environmental stimuli. NGF and lithium act cooperatively with forskolin and dexamethasone to enhance NT/N gene expression in PC12 cells (Dobner, et al. 1988; Tischler et al. 1986). Promoter analysis has indicated that different cis-regulatory elements mediate responses to the permissive agents NGF and lithium, activators of adenylate cyclase, and glucocorticoids, and that the functional interdependence of these elements account for the synergistic action of these inducers (Kislauskis and Dobner 1990; Kislauskis and Dobner 1992). Synergistic interaction between transcription factors bound to distinct regulatory sites have been documented (Jantzen et al. 1987; Sabol and Higuchi 1990; Schule et al. 1988a; Schule et al. 1988b). It is unclear how synergy is generated between the regulatory elements within the NT/N promoter. One
possibility is that cooperativity of binding between transcription factors can occur, of which several examples have been described, such as the glucocorticoid receptor synergizing with NF1, C/EBP and SP1 transcription factors (Schule et al. 1988a; Strahle et al. 1988). Interestingly, the degree of synergism is inversely related to the strength of the GRE (Schule et al. 1988a). This mechanism may be relevant to NT/N gene expression, since GRE and CRE sequences, within the NT/N promoter, deviate from consensus and thus may be weak binding sites. Another possibility is that the combined activation potential of the transcriptional complex, bound to the promoter, is greater than the sum of each individual factor. Experiments with GAL4 derivatives transfected into mammalian cells have suggested that this second possibility is likely to occur in eukaryotic cells (Carey et al. 1990). Cooperativity between different transcription factors, bound to adjacent AP-1, CRE and GRE sites in the NT/N promoter, controlled by distinct signalling pathways, could account for the highly synergistic induction of NT/N gene expression in PC12 cells.

Cooperative interactions of multiple signal transduction pathways could occur through a single cis-regulatory element. This model may be particularly important in the synergistic induction of the NT/N gene by NGF and lithium, in combination with dexamethasone and forskolin. Transient transfection assays have demonstrated that both NGF and lithium activate multimerized consensus AP-1 sites linked to reporter genes (Dobner et al. 1992). Even though these experiments showed that the effects of NGF and lithium were additive, the observed synergy between NGF and lithium on endogenous NT/N gene expression in PC12 cells could occur through the distal AP-1 site, which may act differently in the context of the NT/N promoter. As shown in figure 1-
NGF and lithium could interact at the distal AP-1 site utilizing either common (A) or separate (B) signalling components. An example in support of the first possibility is the activation of the human α-gonadotropin gene (hCG) by both cAMP and phorbol esters (Hoeffler et al. 1989). Both PKA and PKC are involved in activating the hCG gene through an AP-1 site, known in this gene as a TPA-response element (TRE). The function of either kinase alone results in a moderate level of activity, whereas both of the stimulated kinases synergistically activate transcription (Hoeffler et al. 1989). An example of the second case is the induction of both Fos and Jun proteins by various stimuli. Jun homodimers have minimal effects on AP-1 containing promoters whereas the Fos/Jun heterodimers strongly activate gene transcription (Chiu et al. 1988; Sassone-Corsi et al. 1988b). Determining whether NGF and lithium interact by similar or distinct signalling pathways to influence NT/N gene transcription, is one of the major questions addressed in this thesis.

The signalling pathways utilized by NGF and lithium could act synergistically through the induction of AP-1 factors. A likely mechanism by which signalling pathways act on a single AP-1 site is through activation of different leucine-zipper factors. Members of the Jun family can form heterodimers with members of the Fos family. While these heterodimers are all capable of binding to AP-1 sites (Cohen et al. 1989; Halazonetis et al. 1988; Nakabeppu et al. 1988) some of them form either active (Chiu et al. 1988; Sassone-Corsi et al. 1988b) or inactive transcriptional complexes (Chiu et al. 1989; Schutte et al. 1989). The ability of AP-1 and CRE-binding proteins to heterodimerize thus altering both DNA binding and transactivation provides another mechanism for synergistic interactions at this site. c-Jun has been
Figure 1-4 Models for the synergistic interaction of NGF and lithium at the distal AP-1 site. A. Lithium (Li) and NGF could interact through a common signalling pathway to induce multiple AP-1 factors. These proteins would heterodimerize and bind to the AP-1 site. B. Lithium and NGF could act via separate signalling pathways to induce different AP-1 factors which would heterodimerize and bind to the AP-1 site.
shown to heterodimerize with CRE-BP1 and the resulting complex has the binding specificity of a CRE-binding protein (Hai and Curran 1991; Hurst et al. 1990; Ivashkiv et al. 1990). The formation of dimers from factors induced by distinct signal transduction pathways expands the repertoire of sequence-specific DNA binding complexes.

Post-translational modifications of induced or pre-existing AP-1 factors could also account for the synergistic action of NGF and lithium on the AP-1 site. Since many AP-1 and other leucine zipper factors are highly phosphorylated, this model seems likely (Angel and Karin 1991; Habener 1990). A good example of how multiple phosphorylations can synergistically activate transcription factors is shown with the CRE-binding protein, CREB. Within the transactivation domain there are consensus sequences for PKA, PKC, and casein kinase II (Gonzalez et al. 1989). Phosphorylation by PKA of CREB at serine-119/131 is required for transactivation, although it does not seem to be sufficient, suggesting that other phosphorylations are required for activation (Lee et al. 1990). This serine residue is also phosphorylated by calmodulin-dependent kinases, indicating that distinct phosphorylation cascades can influence the activity of CREB, at the same phosphorylation site (Sheng et al. 1991). Phosphorylation of CREB by PKC is required for efficient dimerization (Yamamoto et al. 1988). Taken, together these results indicate that the activation of CREB depends on multiple phosphorylations, that result from the activation of distinct signalling pathways. In further support of this conclusion, proenkephalin gene expression is synergistically activated by membrane depolarization and activation of adenylate cyclase by independent pathways, which converge on a single CRE within the proenkephalin
promoter (Van Nguyen et al. 1990). Therefore, the post-translational modification of transcription factors plays an integral role in developing transcriptional synergy.

**Experimental Approach** This thesis focuses on the role that NGF and lithium play in regulating NT/N gene expression and the biosynthesis of the NT peptide in PC12 cells. Experimental evidence strongly supports the role of these two permissive agents, in combination with dexamethasone and forskolin, in activating NT/N transcription, however they may also be involved in regulating translational or post-translational events (Dobner et al. 1988; Kislauskis and Dobner 1990). Two questions were initially proposed: 1) do combinations of these inducers influence the translation of NT/N mRNA or processing of proNT/N protein and 2) do they activate NT/N transcription. Analysis of proNT/N protein synthesis in metabolically-labeled PC12 cells, utilizing antiserum raised against a fusion protein containing preproNT/N sequences, showed that NGF and lithium do not influence the translation of NT/N mRNA, whereas lithium modestly altered the proteolytic processing of proNT/N. Nuclear run-on transcription assays demonstrated that NT/N gene transcription is transiently activated in maximally stimulated PC12 cells, although at low levels. A RNase protection assay, used as an indirect assay for transcription, showed a transient activation of nuclear precursor NT/N RNA which closely paralleled the kinetics of activation of nuclear run-on transcripts. Taken together, these results confirm that NGF and lithium, in combination with dexamethasone and forskolin, primarily act by inducing NT/N gene transcription.
The kinetics of precursor NT/N RNA in maximally treated cells are consistent with the induction of AP-1 factors mediating the response to NGF and lithium. Previous experiments indicated that the AP-1 site, located within the NT/N promoter, is necessary for the response to both NGF and lithium (Kislauskis and Dobner 1990). Since NGF and activity of other stimuli rapidly induce AP-1 gene and protein expression in PC12 cells, NT/N gene expression should be dependent on de novo AP-1 protein synthesis. Pre-treating maximally induced PC12 cells with protein synthesis inhibitors showed a marked attenuation in NT/N gene expression consistent with the involvement of transiently expressed AP-1 complexes. Surprisingly, protein synthesis inhibitors did not attenuate the response to NGF, dexamethasone and forskolin, suggesting that post-translational modification of pre-existing AP-1 factors may be mediating the response to NGF. The requirement of de novo protein synthesis on the induction of NT/N gene expression in PC12 cells treated with lithium, dexamethasone and forskolin suggested that AP-1 factors could be mediating the response to lithium. RNA blot analysis of PC12 cells induced by lithium alone or in combination with dexamethasone and forskolin indicated that c-Jun/Fra-1 complexes may be mediating the response to forskolin.

When either NGF or lithium is used as the permissive inducer, two different kinetic profiles were observed, rapid and delayed, respectively. These results together with the observation that NGF and lithium each display distinct requirements for ongoing protein synthesis suggested that each is acting through a distinct signalling pathway. Order of addition experiments were done to confirm this conclusion. Long-term differentiation studies indicated that the pathways identified by NGF and lithium are available in
neurons. Finally, experiments were done to assess whether PKC activity is necessary for the activation by lithium. Both in vitro PKC activity studies and protein kinase C down-regulation experiments demonstrated that PKC activity did not mediate the response to lithium. Together these experiments show that NGF and lithium act through distinct signalling pathways which may be available in neurons, although the response to both agents does not require the activation of PKC. They also suggest that potentially novel modes of AP-1 regulation underlie the synergistic activation of NT/N gene expression in PC12 cells.
CHAPTER II

TRANSCRIPTIONAL ACTIVATION OF THE
NEUROTENSIN/NEUROMEDIN N GENE IN PC12 CELLS
The expression of the NT/N gene is synergistically induced in PC12 cells by combinations of NGF, lithium, glucocorticoids, and activators of adenylate cyclase (Dobner et al. 1988). Several lines of evidence have indicated that these agents mainly act by increasing transcription of the NT/N gene (Dobner et al. 1992; Kislauskis and Dobner 1990). However, in some induction conditions, particularly those that include NGF and lithium, the relative levels of NT peptide did not always parallel that of NT/N mRNA, suggesting that translational or post-translational mechanisms may also be involved (Dobner et al. 1988). The first set of experiments were designed to examine whether these inducers regulate the induction of NT in PC12 cells at the level of transcription, translation of NT/N mRNA or the processing of proNT/N.

Previously described experiments indicated that an AP-1 cis-regulatory element within the promoter of the NT/N gene mediates the response to NGF and lithium, suggesting the NT/N promoter is a target for AP-1 complexes (Dobner et al. 1992; Kislauskis and Dobner 1990). This leads to the prediction that the response of NGF and lithium, in combination with dexamethasone and forskolin, on NT/N transcription will be rapid and transient, consistent with newly synthesized AP-1 factors mediating the effect. Detailed kinetic analysis of nuclear NT/N precursor RNA, detected by RNase protection, was performed to test this prediction. Results presented in this chapter show that combinations of these inducers primarily regulate NT/N gene expression through the activation of transcription, and that the gene is activated with different kinetics when either NGF or lithium is used as the permissive inducer.
Production of antiserum to the NT/N precursor protein. To examine the potential effects of NGF and lithium on translation or processing of the NT/N precursor peptide (proNT/N) an immunoprecipitation assay was developed using rabbit antiserum raised against a bacterially produced fusion protein containing rat proNT/N sequences. Antibodies specifically generated against the precursor protein were needed, since anti-NT peptide antibodies were ineffective in detecting proNT/N (Robert Carraway, personal communication). To generate antiserum specific for the NT/N precursor peptide, rabbits were immunized with a fusion protein, TrpE-NT/N. The Pvu II - Bam HI fragment of the rat NT/N cDNA clone (rbNT), containing nearly the entire coding region (lacking the first 7 amino acids) along with 200 nucleotides of 3' untranslated region, was subcloned in-frame into the Sma I site of pATH 2 to produce pATH-rbNT (figure 2-1A). pATH2 contains the E. coli tryptophan promoter and operator, from which heterologous proteins linked to the trp ΔLE 1413 protein can be efficiently synthesized (Kleid et al. 1981). The LE segment, containing the trp leader (L) peptide along with a third of the trp E protein, is 190 amino acids in length (approximately 39 kd). Fusion proteins derived from pATH2 constructs are insoluble inside bacteria, thus protecting them from proteolytic degradation. This also greatly aids in purification of the fusion protein products, since they can be recovered in the cellular debris of lysed E. coli. After transformation of pATH-rbNT into E. coli, induction by indoleacrylic acid resulted in the production of large amounts (~1mg/100 ml media) of TrpE-NT/N with the expected molecular weight of 56 kd.
Figure 2-1 NT/N sequences and plasmid constructions used for producing antibodies to the NT/N precursor protein. A. The rat cDNA (open boxes) was ligated to either a fragment of the bacterial trpE gene (diagonal lines) in pATH-rbNT, or to a fragment coding for the first seven residues of canine NT/N (grey box) in pGEM-crhNT. Thick lines represent 3' untranslated region and thin lines are 5' untranslated and vector sequences. The restriction sites used for the ligations are indicated. Transcription of pGEM-crhNT with T7 RNA polymerase produces sense RNA used for in vitro translation of full length NT/N precursor protein. B. A Coomassie stained SDS/polyacrylamide (10%) gel that shows full length TrpE-rbNT (56 kd) induced in HB101 bacterial cells. Protein markers (M) are shown, with sizes denoted on the left in kilodaltons (kd). Proteins extracted from cells left untreated (-) or induced with 50 μg/ml of indoleacrylic acid (+) are shown. The amount of TrpE-rbNT was quantitated by comparison with BSA (68 kd) standards (20, 10, 4 μg respectively) (figure 2-1B).
A

**pATH-rbNT**

- Sma I/Pvu II
- **Trp**
- Rat NTIN cDNA
- Bam HI

**pGEM-crhNT**

- Met
- Dog NTIN
- Rat NTIN cDNA
- Hind III/Pvu II
- Eco RI

- T7 RNA polymerase (sense strand)

B

**TrpE-rbNT**

- BSA

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**TrpE rbNT**
The insoluble proteins were resolved by SDS-PAGE and the appropriate band was excised. The gel slice was homogenized with Freund’s adjuvant and approximately 300 µg were injected subcutaneously into each of two New Zealand white rabbits. This was followed by a boost of approximately 150 µg per rabbit, two weeks later. Blood was collected, serum prepared and analyzed in an immunoprecipitation assay.

Specific antibodies were titered by an immunoprecipitation assay utilizing in vitro translated proteins produced from synthetic NT/N RNA. To obtain synthetic RNA coding for the full length NT/N precursor peptide, the Pvu II- Eco RI fragment of rbNT was ligated to the Hind III - Pvu II fragment of the canine NT/N cDNA and subcloned into the Hind III - Eco RI site of pGEM4 to produce pGEM-crhNT (figure 2-2A). This construction was necessary since the rbNT cDNA clone did not contain an in-frame initiator methionine codon. The hybrid protein contains 162 amino acids of rat preproNT/N along with the first 8 residues of canine preproNT/N, of which only 4 are unique to the dog. Synthetic crhNT RNA, transcribed with T7 RNA polymerase, was translated in rabbit reticulocyte lysates, and 35S-labeled proteins were immunoprecipitated using formalin-fixed Staphylococcus aureus (Staph A) and specific antiserum. Antiserum from Rb 3 was shown to have a good titer; 1:400 dilution precipitates nearly all the input protein. This antiserum was specific for NT/N since no protein product was precipitated from in vitro translations of testis RNA. Antiserum Rb 3 was used to detect precursor NT/N protein in metabolically labeled cells to examine the influence of NGF and lithium on the translation of NT/N mRNA and proteolytic processing of proNT/N.
Metabolic labeling of NT/N precursor proteins in PC12 cells. To follow the appearance of newly synthesized NT/N precursor proteins in induced PC12 cells, proper pulse-labeling conditions were established. Continuous labeling experiments using ~5 X 10⁶ PC12 cells in 1 ml of labeling medium demonstrated that between 25 and 30 percent of the total ³H-leucine (20 μCi) was linearly incorporated into acid insoluble material within one hour. In some cases, both ³H-leucine (20 μCi) and ³⁵S-methionine (60 μCi) were used to enhance detection of the NT precursor protein.

ProNT/N synthetic rates were determined by immunoprecipitation of *in vivo*-labeled PC12 cell lysates with antiserum Rb 3. PC12 cells were treated with NGF, lithium, dexamethasone, and forskolin for 24 hours, to induce NT/N mRNA to maximal levels (Dobner et al. 1988), and then labeled for various times with ³H-leucine. Cytoplasmic extracts were immunoprecipitated with antiserum Rb 3 and resolved on SDS/PAGE. Figure 2-2 is an autoradiogram from an experiment showing that the proNT/N accumulates linearly for at least 30 minutes. Size-comparison with ³⁵S-labeled protein markers established that the molecular weight of the immunoprecipitated product is approximately 17 kd (the 19.7 kd band is full-length preproNT) which corresponds to the predicted molecular weight of proNT/N after removal of the 23 amino-acid signal peptide. These experiments established conditions for determining the relative synthetic rate of proNT/N in cells treated with different combinations of inducers.
Figure 2-2  NT/N precursor peptide is synthesized in induced PC12 cells. PC12 cells were treated with a combination of NGF (100 ng/ml), LiCl (20 mM), dexamethasone (1 μM), and forskolin (1 μM) for 24 hours. Cells were then washed with leucine-free DMEM, containing dialyzed serum (10%horse and 5%fetal bovine) and inducers. Following the wash, the cells were resuspended in the same media and incubated for 30 minutes at 37 °C. Following the addition of 3H-leucine (20 μCi) at the indicated times, cellular extracts were prepared and immunoprecipitated with either preimmune serum (P) or antiserum Rb 3 (I). The immunoprecipitated products were resolved on a SDS/polyacrylamide gel (15%) and treated with standard fluorographic techniques. Truncated crhNT RNA was translated in vitro with 35S-methionine and the precipitated proteins were run as molecular weight markers. The 19.7 kd protein represents full-length crhNT.
Minutes M 0 5 10 15 20 30 30
Antiserum I I I I I I I P

19.7
14.0
7.1

Pro NT/N
NGF and lithium do not regulate NT/N translation. To test whether NGF or lithium alter the translation of the NT/N mRNA, the above immunoprecipitation assay was used to detect proNT/N in PC12 cells that were induced for 24 hours with various combinations of NGF, lithium, dexamethasone, and forskolin. Induced cells were labeled with both $^3$H-leucine and $^{35}$S-methionine for 30 minutes and proNT/N was assayed as described above (figure 2-2). In cells treated with NGF, lithium, dexamethasone, and forskolin, the relative proNT/N synthetic rates increased 1.6 fold as compared to cells treated with the latter three inducers, whereas there was a 17.2-fold increase when compared to cells treated with NGF, dexamethasone, and forskolin (figure 2-3). These increases are comparable to increases in NT/N mRNA levels in similarly treated PC12 cells (Table 2-1 and Dobner et al. 1988), suggesting that neither NGF nor lithium influences the translation of NT/N mRNA.

Pulse-chase experiments were done next to examine the effect of inducers on proNT/N processing. The initial objective was to identify processing pathways by analysis of proteolytic intermediates. PC12 cells were treated for 24 hours with NGF and/or lithium, dexamethasone, and forskolin, and subsequently labeled for 30 minutes with $^3$H-leucine. Cells were washed, resuspended in medium containing 10 mM unlabeled leucine and the appropriate inducers, and incubated for an additional 2, 4, and 6 hours. Cells were harvested and cell extracts were analyzed by immunoprecipitation with antiserum Rb 3. Unfortunately, proteolytic intermediates were not detected by 15% SDS/PAGE, which could only resolve proteins down to 5 kd. Therefore, specific processing pathways could not be identified.
Figure 2-3. Synthesis of the NT/N precursor protein in PC12 cells treated with various combinations of inducers. PC12 cells were treated with the indicated combinations of NGF (N), lithium (L), dexamethasone (D) and forskolin (F) for 24 hours or left uninduced (NA). Inducer concentrations were as in figure 2-2. Cells were washed and incubated for 30 minutes in leucine/methionine-free DMEM supplemented with dialyzed serum and appropriate inducers. Following addition of $^3$H-leucine (20 μCi) and $^{35}$S-methionine (60 μCi) for an additional 30 minutes, cellular extracts were prepared and immunoprecipitated with either preimmune serum (P) or Rb 3 antiserum (I). The immunoprecipitated products were resolved by SDS/PAGE (15%) and processed as described in figure 2-2. The 19.7 kd band represents full length crhNT.
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<th>NA</th>
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<tbody>
<tr>
<td>Antiserum</td>
<td>M</td>
<td>P</td>
<td>I</td>
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</table>

- 19.7
- 14.0

Pro NT/N
Gross alterations in proteolytic processing could affect the rate of disappearance of immunologically-detectable proNT/N. The half-lives for the disappearance of immunoprecipitable proNT/N, as determined by pulse-chase experiments in similarly induced cells, were essentially the same: 2.62 hours for cells treated with NGF, dexamethasone, and forskolin, 2.32 hours for cells treated with lithium, dexamethasone, and forskolin, and 1.97 hours for maximally induced cells (data not shown). These results suggest that gross alterations in proNT/N proteolytic processing or degradation do not occur. An alternative approach to determine whether these inducers influence proNT/N protein processing is to compare the levels of mature NT peptide with proNT/N synthetic rates.

Mature neurotensin was quantitated in PC12 cells by a radioimmunoassay utilizing N- or C-terminally directed anti-NT antibodies; BSA-8 and HC-8, respectively (Carraway 1979; Carraway, Mitra et al. 1991). Both of these antibodies require 8 residues for full reaction. The antisera are highly specific, since there is minimal cross-reaction with neuromedin N and unrelated peptides (Carraway 1979). The usefulness of this assay for specific quantitation of neurotensin, its metabolites, and processing intermediates has been firmly established (Carraway 1979; Carraway et al. 1991). To determine the amount of NT/N precursor proteins, PC12 cell extracts were first treated with pepsin to release immunoreactive NT which was then detected using the HC-8 antiserum. NT/N precursor levels were calculated by comparing the levels of immunoreactive NT before and after pepsin treatment.

Table 2-1 shows results of an experiment in which NT/N mRNA levels, proNT/N synthetic rates, NT/N precursor levels, and mature NT levels were
Table 2-1. NT/N mRNA levels, NT/N precursor peptide synthetic rates, and levels of processed neurotensin peptides in PC12 cells treated with different combinations of inducers. PC12 cells were treated with the indicated combinations of NGF (N, 100 ng/ml), lithium (L, 10 mM), dexamethasone (D, 1 μM), and forskolin (F, 1μM) for 96 hours. NT/N mRNA levels were determined by RNase protection assay using the pGEM-rNT4 probe (see Materials and Methods). Quantitation of NT/N mRNA levels was done essentially as in figure 2-6. The synthetic rates of NT/N precursor peptide (ProNT/N) were determined by immunoprecipitation of metabolically labeled PC12 cell extracts using Rb 3 antiserum (see Materials and Methods). C-and N-terminally processed neurotensin peptides were detected by a radioimmunoassay using HC-8 and BSA-8 antibodies, respectively. NT/N precursor protein levels were determined by comparing the levels of N-terminally detected NT in pepsin treated vs untreated extracts. The amounts were calculated using NT standards and are expressed as fmolc/10^6 cells. Pertinent values are also expressed as a fold increase over NDF or LDF.
<table>
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<th>Conditions</th>
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<td>Fold Increase over LDF</td>
<td>fmole/10^6 cells</td>
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<tr>
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Table 2-1
compared. In cells treated with all four inducers, the relative levels of NT/N mRNA, proNT/N synthetic rates, and all NT peptides species increased in a similar manner when compared to cells treated with lithium, dexamethasone, and forskolin (~2.3 fold), indicating that NGF does not alter translation or processing of proNT/N. Similarly, treatment of PC12 cells with NGF, lithium, dexamethasone, and forskolin resulted in a 31.2-fold increase in NT/N mRNA and a 21.3-fold increase in proNT/N synthetic rates as compared to cells treated with NGF, dexamethasone, and forskolin, confirming that lithium does not affect translation of NT/N mRNA. However, lithium appears to slightly inhibit the production of mature NT as revealed using the C- and N-terminally directed antibodies. In maximally treated cells, HC-8 antibodies detected a 7.5 fold increase in C-terminal NT, whereas only a 3.6 fold increase in N-terminal NT was observed with BSA-8 antiserum, over cells treated with NGF, dexamethasone, and forskolin. Interestingly, comparison of the levels of NT/N precursor and mature NT suggests that NT exists predominantly in extended or precursor forms in induced PC12 cells. In summary, these results indicate that neither NGF nor lithium regulate the translation of NT/N mRNA. However, lithium may have a inhibitory effect on the processing of proNT/N to yield mature NT. Differences in PC12 cell lines or experimental conditions may account for the discrepancies from previous results which indicated that NGF stimulated the production of mature NT (Dobner et al. 1988). The results presented here indicate that NT production in PC12 cells is regulated principally at the level of NT/N mRNA accumulation.
Transcriptional Activation of the NT/N Gene in PC12 Cells

Rat NT/N genomic clones. The isolation and characterization of the rat gene was instrumental in analyzing the transcriptional regulation of NT/N gene and defining signalling pathways activated by NGF and lithium. This gene was utilized for two purposes: 1) fragments of the total rat gene were used to optimize the detection of nuclear run-on transcripts, 2) antisense RNA probes that span the first exon/intron junction of the rat gene were used to quantitate NT/N mRNA and unspliced transcripts in induced PC12 cells by an RNase protection assay.

A genomic library was constructed to isolate the full length rat gene. High molecular weight DNA was isolated from rat testes, partially digested with Sau 3A, fractionated on a sucrose gradient and DNA in the 10-20 kb size range was pooled for ligation into Bam Hi digested λ EMBL4 phage arms. Ligated DNA was packaged in vitro to yield approximately 5.5 x 10^6 pfu/μg of ligated DNA. Approximately 3.6 x 10^5 independent recombinants were plated and screened using a ^32^P-labeled probe derived from the NT 8A canine NT/N cDNA clone (Dobner et al. 1987). Three positive clones, rNT18, rNT19, and rNT23 were identified and plaque purified. Southern blot analysis using the canine cDNA probe indicated that rNT19 contained the entire rat NT/N precursor gene (Kislauskis et al. 1988).

Detailed analysis of rNT19 revealed that the rat NT/N gene spans approximately 10.2 kb of DNA. Comparison with heterologous canine and bovine cDNAs identified the positions of three intervening sequences which divide the mRNA sequence into four exons (figure 2-4). The sequences at
Figure 2-4  Structure of the rat neurotensin/neuromedin N gene.

The structure of the rat genomic clone (rNT19) and NT/N mRNA. In the rat gene (upper), black boxes denote exons and thin lines denote either introns or flanking sequences. Eco RI (E) and Hind III (H) restriction sites are indicated. The structure of the gene is projected onto a schematic representation of rat NT/N mRNA (lower). Boxes represent coding sequences, and known functional domains are indicated by diamonds, signal sequence; horizontal lines, neuromedin N-like; diagonal lines, neuromedin N; shaded, neurotensin; and open, regions of unknown function. Two different polyadenylation sites (polyA) are indicated.
Rat Neurotensin Gene

500 bp

H H H H

EE H

50 bp

Poly A Poly A

Rat NT/N mRNA
exons and introns junctions conform to consensus splice donor and acceptor sequences (Padgett et al. 1986). Exon 1 encodes a putative signal peptide (amino acids 1-23). The neurotensin and neuromedin N coding domains are located in tandem on exon 4 (amino acids 120-169), separated only by Lys-Arg residues. Exon 3 codes for amino acids 45-119 and contains a sequence similar to neuromedin N that is preceded by paired basic amino acids, suggesting that the preproNT/N may be processed to yield additional peptides. Exon 2 which encodes amino acids 24-44, contains no obvious functional domains.

**Induction of NT/N gene transcription in maximally stimulated PC12 cells.**

Treatment of PC12 cells with a combination of NGF, lithium, dexamethasone, and forskolin, produces a rapid increase in NT/N gene expression (Dobner 1988). Nuclear run-on transcription assays were performed to test directly whether these inducers alter mRNA levels by increasing the transcriptional rate of the NT/N gene. At various times following treatment with all four inducers, PC12 nuclei were isolated, nascent transcripts were labeled with $^{32}$P-UTP, and specific transcripts were detected by hybridization to plasmid DNA bound to nylon membranes. Figure 2-5A shows an autoradiogram of run-on transcripts hybridized to membranes containing rat NT/N cDNA, a constitutive positive control (α-tubulin), two inducible positive controls (c-fos and c-jun), and pGEM-4 vector DNA as a negative control. NT/N transcripts were induced rapidly, within 2 hours, and expression was transient, reaching maximum levels at 4 hours. Figure 2-5B shows the results of an RNase protection assay in which NT/N mRNA was detected in the cytoplasmic fractions, confirming that induction resulted in increased mRNA levels in this experiment. However, the
Figure 2-5. Activation of NT/N gene transcription in maximally induced PC12 cells. A. PC12 cells were treated with a combination of NGF, lithium, dexamethasone and forskolin (concentrations as in figure 2-2). Nuclei were isolated from ~1x10^8 cells at the indicated times, labeled with ^32P-UTP, RNA was isolated and hybridized to filters containing 5 µg of the following plasmids; rat NT/N cDNA (rbNT), mouse α-tubulin (MAT α1), c-fos (pGEMfos3), mouse c-jun (JAC1.1) and pGEM 4 (see Materials and Methods). The high stringency wash was in 0.1 X SSC, 0.1% SDS and 0.1% sodium pyrophosphate at 60 °C. Run-on transcripts were visualized by autoradiography with an intensifying screen (2 week exposure). B. NT/N mRNA was analyzed by hybridizing the above cytoplasmic RNAs (10 µg) with ^32P-antisense E11-NT riboprobe (see figure 2-6) followed by digestion with RNase T1 and nuclease P1. The protected products resulting from hybridization to NT/N mRNA (169) or internal standard RNA (104) are indicated (see figure 2-6).
### A

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### B

- 169
- 104

0 0.5 1 1.5 2 3 4 6 8 12
level of NT/N gene transcription was low when compared to c-fos and c-jun. This low level indicated that the nuclear run-on assay could not be used to analyze NT/N transcription in PC12 cells treated with less effective inducer combinations. Attempts to increase the signal, such as hybridization to larger segments of the rat gene, were unsuccessful. Therefore, even though nuclear run-on experiments demonstrated that the NT/N gene was transcriptionally activated by a combination of all four inducers, an alternative transcriptional assay was needed to analyze NT/N gene expression in cells treated with different inducer combinations.

NT/N precursor RNA is transiently induced in the nuclei of PC12 cells.

To examine the kinetics of NT/N gene activation in detail, a RNase protection assay was developed to monitor the appearance of NT/N nuclear precursor RNA using an antisense probe (E11-NT) that spans the junction between exon one and intron one (see figure 2-6C). PC12 cells were treated with a combination of NGF, lithium, forskolin, and dexamethasone for the indicated times, and aliquots of nuclear and cytoplasmic RNA were analyzed using a $^{32}$P-labeled E11-NT probe (figure 2-6A and B). NT/N precursor RNAs containing intron one (325 b protection product) were clearly detectable after 2 hours, increased to maximum levels within 3-4 hours, and subsequently decreased in abundance over the next 8-9 hours. Spliced nuclear transcripts lacking intron one (169 b protection product) appeared coincidently with intron-containing transcripts, but accumulated with somewhat slower kinetics. Cytoplasmic NT/N mRNA (169 b protection product) first became detectable after 2 hours and accumulated throughout the 12 hour interval examined. These results indicate
Figure 2-6. RNase protection assay to monitor NT/N gene expression in PC12 cells. A. PC12 cells were treated with a combination of NGF, LiCl, dexamethasone, and forskolin (concentrations as in figure 2-2), harvested at the indicated time points (~5 x 10^7 cells/time point), and both nuclear and cytoplasmic RNA were prepared using the LiCl-urea extraction protocol. A 32p-labeled antisense riboprobe (E11-NT) spanning the first splice junction was used to detect precursor RNA containing intron one and processed RNA lacking intron one after hybridization to 10 μg of either nuclear or cytoplasmic RNA. Protection products were resolved on a sequencing gel and visualized by autoradiography (9 days without an intensifying screen). The protection products resulting from hybridization with either precursor (325 b) or spliced (169 b) NT/N transcripts are indicated. Hybridization with a sense RNA standard, added prior to RNA extraction, resulted in a 104 b protection product (internal control). B. The 169 b and 325 b protected products were quantitated by direct scintillation counting using a Betagen model 603 blot analyzer. The levels of internal control RNA signal were used to correct for small variations in sample recovery. Levels of either processed nuclear (squares) and cytoplasmic (triangles) NT/N RNA, or precursor (circles) NT/N transcripts are plotted using the left and right ordinate scales, respectively. C. Schematic of the E11-NT probe. A 489 bp Eco RI/Dra I DNA fragment of the rat NT/N gene was subcloned into pGEM-4 digested with Eco RI and Dra I. 32p-labeled antisense probe (E11-NT) was transcribed using T7 RNA polymerase following linearization with Eco RI. Sense strand RNA was transcribed with SP6 RNA polymerase after linearization with Rsa I. The position of exon one (striped box), intron 1 (open box), and 5' flanking sequences (thick line) are
indicated. The sizes of the probe and expected protection products are indicated.
A  

Nuclear  Cytoplasmic

NA 1 2 3 4 6 8 12 M NA 1 2 3 4 6 8 12

325  

169  

104  

B  

Hours  

SP6 (sense)  

Eco R1  

Probe  

Internal  

Control  

C  

pGEM E1I-NT  

SP6 (sense)  

CAP  

Eco R1  

Dra 1  

T7 (antisense)  

Probe  

Unspliced  489 b  

Spliced  169 b  

Internal Control  104 b
that the NT/N gene is transiently activated in maximally stimulated PC12 cells over about a 10 hour interval.

The results presented in figure 2-6 demonstrate that the RNase protection assay provides a sensitive method for quantitating NT/N nuclear precursor RNAs containing intron one. Three lines of evidence strongly indicate that increases in the levels of NT/N precursor RNA are principally the result of transcriptional regulation and not changes in precursor stability or processing. First, the kinetics of appearance of the 325 b product is similar to NT/N transcripts observed in nuclear run-on assays. Second, both CAT and luciferase reporter genes fused to rat NT/N gene cis-regulatory sequences are regulated nearly identically to the endogenous NT/N gene when transiently expressed in PC12 cells (Kislauskis and Dobner 1990). Third, the luciferase fusion gene is transiently induced in maximally stimulated PC12 cells and the time course of luciferase expression closely parallels the induction of intron one-containing NT/N precursor RNAs (Kislauskis and Dobner 1992). Since the luciferase fusion gene contains only a limited amount of rat NT/N sequences (-216 to +56), the transient induction of luciferase activity is likely due to the transient transcriptional activation of the reporter gene and the instability of both luciferase mRNA and protein in PC12 cells. Thus, the levels of intron one-containing NT/N nuclear precursor RNAs appear to accurately reflect the transcriptional status of the NT/N gene.
Different activation kinetics when NGF or lithium is used as the permissive inducer. The kinetics of NT/N gene activation in PC12 cells treated with either NGF, lithium, or both agents, in combination with forskolin and dexamethasone was examined next, using the RNase protection assay to monitor the appearance NT/N nuclear precursor RNA (figure 2-7). In cells treated with NGF, dexamethasone, and forskolin, intron one-containing transcripts were rapidly induced within 1 hour, reached maximum levels after 2 hours, and declined below the limit of detection by 8 hours (figure 2-7). Intron-one containing transcripts were induced for a more prolonged period in PC12 cells treated with lithium, dexamethasone, and forskolin. Levels of intron one-containing transcripts reached maximum levels by 4 hours and subsequently declined to a low, steady-state level after 12 hours, similar to the response observed in cells treated with all four inducers (figure 2-6). NT/N transcripts were not detected in cells treated with forskolin and dexamethasone (data not shown), consistent with previous evidence that the permissive inducers, NGF or lithium, are required for activation (Dobner, Tischler et al. 1988; Kislauskis and Dobner 1990). The distinct induction kinetics observed when cells were stimulated with combinations of either NGF or lithium, forskolin, and dexamethasone provided a preliminary indication that the responses involved distinct signalling mechanisms.
Figure 2-7. Differential time course of NT/N gene activation when either NGF or lithium is used as the permissive agent. A. PC12 cells (~ 5x10^7 cells/time point) were treated with all four inducers (NLDF), NGF, dexamethasone, and forskolin (NDF), or lithium, dexamethasone, and forskolin (LDF) at the concentrations shown in figure 2-2. At the indicated times, nuclear RNA was isolated and 20 μg analyzed in an RNase protection assay using the ElI-NT probe as described in figure 2-6. The protection products resulting from hybridization with either precursor (325 b) or spliced (169 b) NT/N transcripts are indicated. Hybridization with internal control RNA resulted in a 104 b protection product. A control reaction was performed by hybridizing the probe with 10 μg yeast RNA (lane Y). Radiolabeled RNA markers are shown (M). B. A graph representing three independent experiments. PC12 cells were treated with either NGF (open circles), lithium (open triangles), or both inducers (solid circles), in combination with dexamethasone and forskolin, for the indicated times, and levels of intron one-containing NT/N nuclear precursor RNAs were quantitated as in figure 2-6. The levels of 325 b protection product (cpm of 325 b protection product/μg input total nuclear RNA) are plotted using the lefthand scale for maximally induced cells and the righthand scale for cells treated with either NGF or lithium, in combination with dexamethasone and forskolin. Error bars represent the standard error of the mean for the 2, 4, and 6 hour time points (n=3) or the range for the 1 and 8 hour time point (n=2).
As discussed above, the RNase protection assay appears to accurately reflect the transcriptional status of the NT/N gene. Quantitative analysis of intron one-containing nuclear RNAs, and both nuclear and cytoplasmic RNAs lacking intron one has provided an indication that there are no major differences in either the splicing of intron one, or the transport of processed NT/N RNA to the cytoplasm in cells treated with different combinations of inducers. The 169 b protected fragment represents the processed NT/N RNA, at least at the level of splicing of intron 1. Greater than 90% of the NT/N RNA, after correcting for differences in protected fragment sizes, is spliced at the intron one junction and rapidly accumulates in the nucleus (figure 2-6). Processed NT/N RNA appeared in the cytoplasm following the appearance of nuclear NT/N RNA. In other experiments in which processed NT/N was assayed at more frequent intervals (data not shown), the appearance of NT/N RNA in the cytoplasm lagged behind that of nuclear processed NT/N RNA by approximately 30 minutes. At later time points, i.e., 12 hours, the cytoplasmic fraction is about 75% of the total NT/N RNA. These results are consistent with the NT/N primary transcript being rapidly spliced, followed by transport of mature NT/N mRNA to the cytoplasm within about 30 minutes.

To assess the impact of these inducers on splicing of intron one, processed NT/N RNA was analyzed in cells induced with NGF and/or lithium in combination with dexamethasone and forskolin by RNase protection with E11-NT as a probe. The processed NT/N RNA in the nucleus of cells treated with NGF, dexamethasone and forskolin showed a kinetic pattern similar to that of intron
one-containing transcripts; rapidly appearing at 1 hour, peaking at 2 hours and declining to minimal levels by 12 hours (figure 2-7A). Similarly, in cells treated with lithium, dexamethasone, and forskolin, processed NT/N RNA was first observed at 2 hours, reaching maximum levels by 6 hours and returning to a non-zero basal level by 12 hours (figure 2-7A). No differences were observed in the ratio of precursor NT/N RNA to processed NT/N RNA (intron one spliced out) in the nucleus at the time of peak precursor NT/N RNA expression in cells treated with different inducer combinations (figure 2-8A). In all three conditions the processed RNA was between 90-95% of the total NT/N RNA in the nucleus. Together, these results indicate that NGF and lithium have no major effect on splicing of the NT/N RNA transcript, at least at the intron 1 junction, although differential splicing at the other exon-intron junctions cannot be ruled out.

Similar studies were done to analyze the effect of NGF and lithium, in combination with dexamethasone and forskolin, on the accumulation of cytoplasmic NT/N mRNA. In all cases, the delayed appearance of NT/N mRNA in the cytoplasm was similar to that of the appearance of nuclear processed NT/N RNA. In cells treated with NGF, dexamethasone, and forskolin, cytoplasmic NT/N mRNA accumulated rapidly, first appearing at about 2 hours, and reaching maximum levels by 4 hours (data not shown). In contrast, cytoplasmic NT/N mRNA first appeared 4-6 hours post-induction in cells treated with lithium, dexamethasone, and forskolin and accumulated continuously for up to 12 hours (data not shown). These results suggest that the spliced NT/N RNA is being efficiently transported out of the nucleus and that NGF and lithium do not alter the expected pattern of accumulation within
Figure 2-8. NGF and lithium do not alter NT/N RNA splicing or transport into the cytoplasm. A. A histogram showing the percent of total nuclear RNA that is processed (spliced at the exon 1-intron 1 junction) in PC12 cells treated with all four inducers (NLDF), NGF, dexamethasone, and forskolin (NDF), or lithium, dexamethasone, and forskolin (LDF) for 4, 2, and 6 hours, respectively. The number of experiments used to determine these values is shown in the open boxes. The error bars represent the standard error of the mean (SEM). Missing error bars indicate that the SEM was extremely small. B. A histogram showing the percent of total processed NT/N RNA that is found in the cytoplasm of PC12 cells treated with NLDF, NDF, or LDF for 12 hours. The number of experiments used to determine these values is shown in the open boxes. The error bars represent the SEM as described in A.
A

![Bar chart A](chart.png)

B

![Bar chart B](chart.png)
the cytoplasm. The levels of processed NT/N RNA in the nucleus were compared with levels in the cytoplasm to support this conclusion (figure 2-8B). For all three conditions, approximately 75% of the total processed NT/N RNA was found in the cytoplasm, after 12 hours of induction. Furthermore, the levels of processed NT/N RNA in the cytoplasm, at twelve hours, mimics NT/N mRNA in similarly induced cells, detected by using an exon four probe (Table 2-1). Taken together, these results indicate that NGF and lithium do not alter the splicing of the NT/N primary transcript or mechanisms controlling the accumulation of NT/N mRNA within the cytoplasm, such as transport.

The appearance of the two extra protected fragments, approximately 190 and 196 nucleotides in length was unexpected (figure 2-9). One possible explanation for the appearance of these bands is that alternate transcriptional start sites are being used resulting in NT/N mRNA that is extended 5' of the defined CAP site. Another possibility is that alternative splicing produces NT/N RNA with extra intron sequences. An RNase protection assay utilizing truncated EII-NT probes (figure 2-9A) was used to distinguish between these two possibilities. The Hae III probe is truncated just 3' of the predicted alternative start sites while the Ava II probe is truncated within exon 1. If alternative start sites are utilized, then only one fragment should be observed, whereas if alternative splicing occurs, then all three of these fragments will be detected by both probes. As shown in figure 2-8 B, three processed fragments were detected utilizing each of the probes. This suggests that the NT/N primary transcript is differentially spliced at the junction between exon 1 and intron 1, even though the possibility that the extended protection products are artifacts resulting from the probe cannot be ruled out.
Figure 2-9. NT/N mRNA is differentially spliced at the exon 1-intron 1 junction. A. Probes used to identify the extended processed NT/N RNA fragments. pGEM E11-NT was linearized at Eco RI, Hae III, or Ava II sites and transcribed with T7 RNA polymerase as described in figure 6. The total probe length (probe), precursor NT/N fragment (unspliced), and processed NT/N fragment (spliced) are shown for each probe. The predicted extension sites are indicated by arrows, either 5' of start site (A) or 3' of exon 1-intron 1 junction (B). B. RNase protection gel of 10 μg of nuclear (N) and cytoplasmic (C) RNA from fully induced (NLDF) PC12 cells. 10 μg of poly A+ and A− fractions of cytoplasmic RNA were also tested. Undigested probes are shown; Eco RI (E), Hae III (H), or Ava II (A). The unspliced (325 or 254) and spliced (169 or 95) protected fragments are indicated. Fragment sizes were determined from a standard curve of the radiolabeled RNA markers (M). C. The nucleotide and predicted amino acid sequence of the area surrounding the exon 1-intron 1 junction. The authentic (1) and predicted (2, 3) spliced donor sites are indicated.
A

Eco R1

T7 (antisense)

Probe

Eco RI

Probe

Unspliced

489 b

Spliced

325 b

Hae III

Probe

Unspliced

382 b

Spliced

169 b

Ava II

Probe

Unspliced

298 b

Spliced

258 b

B

Gly Lys Leu Ala Pro Leu TERM

CTG TGC TCA CAT AAG CTC GCA CCT TGA AG TGA GTG TTT

1

2

3
Inspection of the sequences surrounding the exon1-intron 1 boundary (figure 2-9C) identified two potential splice donor sites (sites 2+3) that could generate transcripts that would yield these 190 b and 194 b protection fragments. Splice donor sites 2 and 3 are 66 and 44 percent identical, respectively, to the consensus splice donor site. However, both of these are located 3' to an in-frame termination codon, indicating that neither of these splicing events would produce functional peptides. Interestingly, these alternatively spliced NT/N RNAs were not isolated as poly A⁺ RNA. Further experiments need to be done to determine if the lack of polyadenylation correlates with the production of the putative alternatively spliced RNAs.

In summary, the results presented in this chapter have shown that the NT/N gene is primarily regulated at the level of transcription, and that alterations in peptide processing playing a minor role in controlling NT production in PC12 cells. NGF and lithium in combination with dexamethasone and forskolin rapidly induce NT/N gene expression, although with distinct kinetics. The kinetics of activation are consistent with a role for AP-1 complexes in controlling NT/N gene expression leading to the prediction that de novo synthesis of AP-1 proteins is required for NT/N gene activation.
CHAPTER III

PROTEIN SYNTHESIS INHIBITORS HAVE DIFFERENT EFFECTS ON NT/N GENE TRANSCRIPTION
AP-1 genes encode transcription factors that couple extracellular stimuli to alterations in target gene expression. AP-1 factors interact with cis-acting elements located within regulatory regions of target genes thereby altering the transcriptional state of these genes (Sheng and Greenberg 1990; Curran and Morgan 1987). NGF rapidly induces the expression of several proto-oncogenes, including those that encode AP-1 factors (Sheng and Greenberg 1990). Several lines of evidence have implicated AP-1 complexes as mediators of the permissive effects of NGF and lithium on NT/N gene transcription: 1) the NT/N promoter contains a functional AP-1 site (Kislauskis and Dobner 1992), 2) as shown in the previous chapter, the NT/N gene is transiently activated with kinetics consistent with the induction of AP-1 genes (Bartel et al. 1989), 3) NGF and lithium selectively activated reporter genes driven by consensus AP-1 sites (Dobner et al. 1992) and, 4) various combinations of bacterially produced AP-1 factors bind as heterodimers to the NT/N AP-1 site, in vitro (G. McNeil and P. Dobner, unpublished results). Experiments described in this chapter were designed to determine whether de novo protein synthesis is required for the transient activation of the NT/N gene. Such a requirement would be consistent with a role for induced AP-1 factors in the activation of NT/N transcription. PC12 cells were pre-treated with protein synthesis inhibitors prior to treatment with different combinations of NGF, lithium, dexamethasone and forskolin, and levels of intron one-containing transcripts were quantitated to assess transcriptional changes. The results from experiments reported in this chapter demonstrate that the permissive effect of NGF and lithium on NT/N gene expression in PC12 cells involves both the
induction and post-translational modification of signalling proteins, presumably AP-1 factors.

Cycloheximide and anisomycin efficiently inhibit protein synthesis in PC12 cells. To assess the requirement for newly synthesized proteins in regulating NT/N gene expression, PC12 cells were treated with the protein synthesis inhibitors, cycloheximide and anisomycin. Both these drugs inhibit protein synthesis, to greater than 95%, in eukaryotic cells without seriously affecting RNA synthesis (McKnight 1978; Jimenez and Vazquez 1979). Anisomycin binds to both the acceptor and donor sites of 60 S ribosomal subunits thus inhibiting peptide bond formation (Jimenez and Vazquez 1979). Cycloheximide blocks translocation of peptidyl-tRNA from the ribosomal acceptor site to the donor site (Gale et al. 1981). To assess the efficiency of these drugs, PC12 cells were treated with either anisomycin (50 μM) or cycloheximide (10 μg/ml) for various times and protein synthesis, RNA synthesis, and viability were quantitated. Protein synthesis inhibition was examined by comparing the incorporation of 35S-methionine into trichloroacetic acid-precipitable proteins, in treated and untreated cells. As shown in figure 3-1, both drugs inhibited protein synthesis to maximum levels within 2 hours. Greater than 97% inhibition was achieved with anisomycin whereas approximately 95% inhibition was observed with cycloheximide. These levels of protein synthesis were sustained throughout the twelve hour course of the experiment and are consistent with results reported by Greenberg et al., who showed that anisomycin was a more stringent inhibitor of protein synthesis in PC12 cells (Greenberg et al. 1986). Further experiments
Figure 3-1. Protein synthesis in PC12 cells is efficiently inhibited by anisomycin and cycloheximide. PC12 cells were treated with either 50 μM anisomycin (solid symbols and broken lines) or 10 μg/ml cycloheximide (open symbols and solid lines). Cells were harvested at various times and protein synthesis (circles), RNA synthesis (squares) and cell viability (triangles) were determined as described in Material and Methods. The values are represented as percent of untreated cells at each time point.
showed that anisomycin was just as effective within 15 minutes of addition (data not shown). Incorporation of $^3$H-uridine into acid-precipitable material was used to examine the effects of the inhibitors on RNA synthesis. Acid precipitable counts were compared to total cellular counts to correct for differences in uridine uptake, since a marked inhibition of uridine uptake, by protein synthesis inhibitors, was previously demonstrated in chick oviduct explant cultures (McKnight 1978). Both anisomycin and cycloheximide reduced the levels of RNA synthesis, approximately 40%, within 4 hours of treatment, which was then sustained at that level for the duration of the experiment (figure 3-1). Neither agent affected cell viability, as determined by trypan blue exclusion, during the first 8 hours of treatment (figure 3-1), although a slight decrease in viability was observed after 12 hours. These results demonstrate that both drugs efficiently inhibit protein synthesis in PC12 cells, anisomycin being slightly more effective than cycloheximide, while only having a small effect on RNA synthesis and cell viability.

Anisomycin and cycloheximide attenuate NT/N gene activation in maximally stimulated cells To determine whether NT/N gene activation requires ongoing protein synthesis, PC12 cells were pre-treated with either cycloheximide (10 μg/ml) or anisomycin (50 μM) for 30 min followed by treatment with a combination of NGF, lithium, dexamethasone, and forskolin in the continued presence of the inhibitors. Cells were harvested at the indicated times after induction and intron one-containing transcripts were quantitated using the RNase protection assay. Figure 3-2A shows that treatment with either inhibitor markedly attenuated NT/N gene activation, anisomycin having a greater effect
Figure 3-2. Effects of protein synthesis inhibitors on NT/N gene activation.

A. PC12 cells were pre-treated with either cycloheximide (10 µg/ml, solid squares), anisomycin (50 µM, solid circles), or no inhibitor (open circles) for 30 min, prior to induction with a combination of NGF, lithium, dexamethasone, and forskolin. Cells were harvested at the indicated times following induction, nuclear RNA was isolated, and aliquots (25 µg) were used to quantitate levels of intron one-containing NT/N nuclear precursor RNAs (325 b product) as in figure 2-6. The graph represents a single experiment. B. Control PC12 cells (open circles) or cells that had been pre-treated with 50 µM anisomycin for 0.5 (solid circles) or 2 hr (solid squares) were treated with NGF, forskolin, and dexamethasone in the continued presence of anisomycin and harvested at the indicated times. Nuclear RNA (25 µg) was analyzed as described above. C. As in B, except control PC12 cells (open circles) or cells pre-treated with anisomycin for 30 min (solid circles) were subsequently stimulated with lithium, forskolin, and dexamethasone. D. Autoradiogram of a sequencing gel (8M urea/6%PAGE) used to quantitate levels of the 325 b protection product in the experiment depicted in panel B. The positions of the 325, 169 and 104 b protection products resulting from hybridization to NT/N nuclear RNAs containing or lacking intron one and sense RNA standard RNA, respectively, are indicated. Nuclear RNA from untreated cells (NA) or yeast RNA (Y) were also probed. The length of anisomycin pre-treatment (A 0.5, 30 min; A 2, 2 hr) and the induction times (hr) are indicated above the gel.
than cycloheximide. This is consistent with anisomycin being a more stringent inhibitor of protein synthesis than cycloheximide in PC12 cells. 

However, neither inhibitor completely abrogated expression, indicating that post-translational mechanisms are also involved in activating the NT/N gene within maximally stimulated cells, although the limited amount of residual protein synthesis may be sufficient for activation.

Anisomycin attenuates responses involving lithium, but not NGF. Similar experiments were performed in cells treated with either NGF or lithium, in combination with dexamethasone and forskolin (figures 3-2B-D). Surprisingly, anisomycin pre-treatment had no effect on the initial activation of NT/N gene expression in cells treated with a combination of NGF, dexamethasone, and forskolin, although anisomycin pre-treatment prolonged the appearance of intron one-containing transcripts in these cells (figures 3-2B and D). Longer pre-treatment with anisomycin (2 hr) had essentially the same effect on NT/N gene activation (figures 3-2B and D). These results indicate that newly synthesized proteins are not required for NT/N gene activation by NGF, forskolin, and dexamethasone, but are required for subsequent down-regulation. In contrast, new protein synthesis is required for the initial activation phase in cells treated with lithium, forskolin, and dexamethasone (figure 3-2C). The later stable phase of activation was not affected, even when the inhibitors were present for up to 48 hours (data not shown). These results taken together indicate that NGF and lithium influence NT/N gene expression through distinct permissive pathways, distinguished by their requirements for ongoing protein synthesis.
NGF and lithium induce AP-1 gene expression. The requirement for protein synthesis in the response to lithium on NT/N gene expression suggests that lithium may be acting through newly synthesized factors. To examine how lithium influences AP-1 activity, the expression of the seven characterized AP-1 genes was analyzed in PC12 cells treated with different inducer combinations (figure 3-6). RNA blot analysis demonstrated that NGF treatment resulted in the rapid, transient induction of c-fos, fra-1, c-jun, and jun-B gene expression as expected (Ito et al. 1990; Bartel et al. 1989), and had a similar effect on fos B gene expression. Treatment with NGF, forskolin, and dexamethasone enhanced c-fos, fos B and jun-B gene expression. NGF had no effect on jun-D and fra-2 gene expression under any circumstance. The c-jun, jun-D, fra-1, and fra-2 genes were expressed at detectable levels in unstimulated PC12 cells and thus post-translational regulation of these AP-1 proteins could be involved in the protein synthesis independent activation of NT/N gene expression in response to NGF, dexamethasone, and forskolin. Lithium had distinct effects on AP-1 gene expression either alone or in combination with dexamethasone and forskolin (figure 3-6). A particularly interesting effect was the delayed activation of c-jun and fra-1 gene expression in response to lithium. This selective effect opens the intriguing possibility that lithium influences NT/N gene expression as a consequence of the delayed induction of c-Jun/Fra-1 complexes.

Anisomycin and cycloheximide do not induce NT/N transcription.

The prolonged response to NGF, dexamethasone, and forskolin observed in anisomycin-treated cells suggests that newly synthesized inhibitory factors
Figure 3-3. Induction of AP-1 gene expression in PC12 cells. PC12 cells were treated with the indicated combinations of inducers and cells were harvested at the indicated times. Total RNA was extracted, and AP-1 gene expression was analyzed by RNA blotting using 32P-labeled probes prepared using the indicated AP-1 cDNA clones as described in Materials and Methods. The filters were washed and AP-1 mRNAs were visualized by autoradiography with an intensifying screen. Exposure times: c-fos, fos-B and jun-B, 24 hours; c-jun, 72 hours; jun-D, fra-1 and fra-2, 3 weeks. Inducer abbreviations are as follows NGF (N), lithium (L), dexamethasone (D) and forskolin (F). Inducer concentrations are as in figure 2-3.
may rapidly attenuate NT/N gene transcription. However, anisomycin could act by directly inducing the NT/N gene or potentiating the effects of dexamethasone and forskolin. Mahadevan and colleagues showed that anisomycin, at concentrations below those required to inhibit protein synthesis, was able to stimulate intracellular signalling resulting in the specific phosphorylation of chromatin and the induction of c-fos and c-jun mRNA in fibroblasts (Mahadevan and Edwards 1991). To rule out this possibility, cells were treated with either anisomycin (50 μM) or cycloheximide (10 μg/ml) alone or in combination with dexamethasone and forskolin. In all cases, neither drug influenced the levels of intron one-containing transcripts during a 12 hour period (data not shown). Therefore, these drugs do not induce NT/N gene expression alone or in combination with dexamethasone and forskolin.

To further support the role of protein synthesis inhibition in the perpetuation of the NGF phase in anisomycin treated cells, an experiment was performed by pre-treating PC12 cells with both anisomycin and cycloheximide for 30 minutes, at various concentrations, prior to induction with NGF, dexamethasone, and forskolin. Cells were harvested at 4 hours post-induction, and intron one-containing transcripts were analyzed by RNase protection. As shown in figure 3-4A, maximum levels of intron one-containing transcripts were observed in cells treated with 0.5 to 5.0 μM anisomycin and 0.1 to 1.0 μg/ml cycloheximide, concentrations that inhibited protein synthesis by 80-90% (figure 3-4B). No effect on the levels of intron one-containing transcripts was seen in cells treated with inhibitor concentrations that had little effect on protein synthesis (0.005 μM anisomycin and 0.001 μg/ml cycloheximide). Taken
Figure 3-4. Correlation between protein synthesis inhibition and prolonged activation of the NT/N gene by NGF, dexamethasone, and forskolin. A. RNase protection assay used to quantitate the levels of the 325 b protection product in an experiment in which PC12 cells were pre-treated for 30 minutes with various concentrations of either anisomycin or cycloheximide prior to induction for 4 hours with NGF, dexamethasone, and forskolin. The positions of the 325, 169 and 104 b protection products resulting from hybridization to NT/N nuclear RNAs containing or lacking intron one and sense RNA standards, respectively, are indicated. Nuclear RNA from untreated cells (NA) or yeast RNA (Y) were also probed. Concentrations of anisomycin (µM) and cycloheximide (µg/ml) are indicated above the gel. Radiolabeled RNA markers (M) are shown. B. A graph representing the levels of protein synthesis at various concentrations of anisomycin (µM, open circles) and cycloheximide (µg/ml, closed circles). Values on the y-axis are represented as percent of untreated cells.
together these results demonstrate that perpetuation of the response to NGF, dexamethasone, and forskolin in anisomycin-treated cells is not due to an inductive effect of the drug but is associated with the inhibition of protein synthesis.

**Anisomycin does not inhibit RNA splicing.** The prolonged response to NGF, dexamethasone, and forskolin could also be due to the inhibition of NT/N mRNA splicing. To rule out the possibility that this effect was due to a general inhibition of splicing in anisomycin-treated cells, α-tubulin transcripts were analyzed using a riboprobe (figure 3-5A) that spanned the junction between intron 3 and exon 4 of the λT14 α-tubulin gene (Lemischka and Sharp 1982). An RNase protection assay was performed using the same RNA samples as those in figure 3-2D and unspliced RNA (562 b protected fragment) was compared to spliced RNA (383 b protected fragment). However, other bands, approximately 470 and 300 nucleotides in length, were also detected when PC12 RNA was analyzed. The 470 b band seems to result from alternatively spliced α-tubulin RNA, since a near perfect consensus splice acceptor site is located within intron 3, 80 nucleotides 5' of the authentic acceptor site (Lemischka and Sharp 1982). A group of bands around 300 nucleotides probably represents identical sequences within genes coding for other rat α-tubulin isotypes, since this region is highly conserved between various mouse and human α-tubulin isotypes (Villasante et al. 1986). As shown in figure 3-4B, anisomycin treatment did not alter the relative abundance of α-tubulin nuclear RNAs containing or lacking intron 3 or the alternatively spliced products. Similar results were obtained from cells pre-treated with either anisomycin or
Figure 3-5. Anisomycin does not alter the splicing of α-tubulin mRNA.

A. A schematic of the rat α-tubulin gene (λT14) and riboprobe used to detect precursor and spliced α-tubulin mRNA. An Eco RI fragment containing intron 3 (I3) and part of exon 4 was subcloned into pGEM 4. Transcription with SP6 RNA polymerase after linearization with Hind III produces a 576 nucleotide antisense riboprobe. The expected protected fragments resulting from hybridization to intron three-containing (562 b) precursor RNA or spliced RNA lacking intron three (383 b). The predicted alternative splice acceptor site is denoted by an asterisk.

B. Autoradiogram showing a RNase protection experiment in which the α-tubulin probe was hybridized to the same RNAs (5 μg) used in the experiment shown in figure 2-3 D. The unspliced (562) and spliced (383) fragments are indicated. The 470 b band represents alternatively spliced mRNA, whereas homologous regions of other α-tubulin isotypes protect fragments of approximately 300 b (see text). Nuclear RNA from untreated cells (NA) or yeast RNA (Y) were also probed. The length of anisomycin pre-treatment (A 0.5, 30 min; A 2, 2 hr) and the induction times (hr) are indicated above the gel.
A  

Rat α-Tubulin Gene

B  

- A 0.5 A 2

Y NA 1 2 3 4 6 1 2 3 4 6 1 2 3 4 6

562
470
383
300
cycloheximide prior to treatment with lithium, dexamethasone, and forskolin or with all four inducers (data not shown). This result demonstrates that anisomycin treatment does not inhibit general splicing mechanisms. These results are consistent with those demonstrating that cycloheximide treatment of HeLa cells for up to 6 hr does not inhibit mRNA splicing (Zeitelin et al. 1987). Comparative analysis of the levels of precursor and spliced NT/N transcripts in treated PC12 cells reinforce these results. In all experiments analyzed, neither anisomycin nor cycloheximide alter the relative amounts of NT/N RNAs in the nucleus of cells induced with NGF and/or lithium in combination with dexamethasone and forskolin. Taken together, these results support the conclusion that anisomycin does not inhibit the splicing of the NT/N primary transcript, at least at the exon one-intron one junction.

**Differential effects of cycloheximide on NT/N gene expression.** To determine if the less stringent protein synthesis inhibitor had effects similar to anisomycin on NT/N gene expression, experiments were performed in PC12 cells treated with cycloheximide prior to induction with NGF or lithium in combination with dexamethasone and forskolin. Consistent with results obtained using anisomycin, cycloheximide did not attenuate responses to NGF, dexamethasone, and forskolin. Furthermore, pre-treatment with cycloheximide resulted in prolonged activation of NT/N gene expression after induction with a combination of these three inducers (data not shown). These results support the conclusion that newly synthesized proteins are not required for NT/N gene activation by NGF, forskolin, and dexamethasone, but are required for subsequent down-regulation.
Surprisingly, cycloheximide potentiated the response to lithium, dexamethasone, and forskolin. Figure 3-6 shows an experiment in which PC12 cells were treated with cycloheximide (10μg/ml) prior to induction with lithium, dexamethasone and forskolin. The levels of intron one-containing transcripts were higher in cells treated with cycloheximide than in untreated cells. It is unclear why cycloheximide had the opposite effect than anisomycin on the response to lithium, dexamethasone and forskolin, since it would be predicted that cycloheximide would attenuate this response similar to anisomycin, although not to the same degree. These results suggest that cycloheximide may be activating or inducing factors that increase NT/N gene expression. However, this cannot be a direct effect since cycloheximide had no effect on NT/N transcription alone or in combination with dexamethasone and forskolin (see above). One possible explanation that would account for these results relies on the fact that protein synthesis inhibitors cause a super-induction of immediate early gene expression, especially AP-1 genes (Ryder et al. 1988; Greenberg et al. 1986; Herschman 1991). If protein synthesis is not maximally inhibited, as in this case of cells treated with cycloheximide, the super-induced levels of AP-1 mRNA could be translated and higher than expected levels of AP-1 factors would be obtained. Identification of the AP-1 genes induced by lithium will be necessary before further experiments can be designed to address this discrepancy between anisomycin and cycloheximide.
Figure 3-6. Cycloheximide accentuates the response to lithium, dexamethasone and forskolin. Analysis of precursor NT/N RNA in an experiment where PC12 cells were treated with 10 μg/ml cycloheximide (closed circles) or left untreated (open circles) prior to induction with lithium, dexamethasone, and forskolin. At the indicated times, cells were harvested and the 325 protected fragment was quantitated by RNase protection as described in figure 2-6.
CHAPTER IV

NGF AND LITHIUM ACTIVATE DISTINCT SIGNALLING PATHWAYS REQUIRED FOR NT/N GENE EXPRESSION
The previous two chapters presented evidence that the permissive agents NGF and lithium, in combination with dexamethasone and forskolin, rapidly and transiently activate NT/N gene transcription, although exhibiting different kinetics and requirements for protein synthesis. The effect of these inducers are likely to be mediated through either the induction or modification of AP-1 factors. In this chapter three questions are addressed: 1) are separate or common signalling components mediating the effects of NGF and lithium, 2) are the signal transduction pathways activated by lithium available in NGF-differentiated PC12 cells, and 3) is the activation of protein kinase C (PKC) required for the induction of NT/N transcription? The results of experiments described in this chapter show that NGF and lithium activate distinct signalling pathways required for NT/N transcription in PC12 cells. The pathway identified by lithium is present in neuronally-differentiated PC12 cells and does not require PKC to influence NT/N gene expression.

NGF and lithium act through distinct signalling pathways. The transient induction of NT/N gene expression in response to different inducer combinations suggested that pre-treatment of PC12 cells with either NGF or lithium might block subsequent responses to inducer combinations and could be used to further distinguish the underlying pathways. For instance, pre-treatment with lithium could trigger the transient induction of a required activator protein(s), but would not activate NT/N gene expression in the absence of other inducers. The addition of forskolin and dexamethasone following lithium pre-treatment would also be ineffective due to the absence of the transiently expressed lithium-regulated component. Similarly, NGF pre-
treatment could induce the expression of a protein(s) involved in down-regulating NT/N gene expression blocking subsequent activation by a combination of NGF, forskolin, and dexamethasone. If the mechanisms of activation and inactivation are different for each inducer combination as is suggested by the anisomycin experiments described above, then pre-treatment with NGF would not be expected to block subsequent activation by lithium, forskolin, and dexamethasone and vice versa.

To examine the effects of pre-treatment on subsequent NT/N gene activation, PC12 cells were treated with either NGF or lithium for 12 hours prior to stimulation with different inducer combinations (figure 4-1). Initial experiments demonstrated that pre-treatment itself did not induce NT/N gene expression (data not shown). Pre-treatment with NGF blocked subsequent NT/N gene activation in response to combined treatment with NGF, forskolin, and dexamethasone, but did not block the response to lithium, forskolin, and dexamethasone. The reverse pattern was observed after lithium pre-treatment, indicating that distinct signalling components are utilized by NGF and lithium. Interestingly, both NGF and lithium pre-treatment affected the kinetics and magnitude of subsequent NT/N gene activation (figure 4-1). Pre-treatment with lithium for 12 hours prolonged subsequent NT/N gene activation by NGF, dexamethasone, and forskolin, implying that lithium influences the pathway controlling down-regulation of the response to NGF, forskolin, and dexamethasone, perhaps overriding it. In addition, lithium, dexamethasone, and forskolin significantly stimulated NT/N gene expression after NGF pre-treatment. These results indicate that NGF and lithium activate distinct signalling pathways required for NT/N gene expression, although there also
Figure 4-1  Effects of pre-treatment with either NGF or lithium for 12 hours on NT/N gene activation. PC12 cells were pre-treated with either NGF (N) or LiCl (L) for 12 hours, or left untreated (-), and subsequently induced with either NGF or lithium, in combination with dexamethasone and forskolin, for the indicated times. PC12 cells were also stimulated with a combination of dexamethasone and forskolin as a control. Nuclear RNA (25 μg) from each time point was analyzed using the RNase protection assay described in figure 2-6. Protected products were resolved on a sequencing gel and visualized by autoradiography (5 days) with an intensifying screen. The positions of the 325, 169, and 104 b protection products are indicated. Lanes containing 32P-labeled RNA markers and the products of a protection reaction in which 25 μg of yeast RNA was hybridized with the 32P-labeled E11-NT probe (lane Y) are shown. Pre-treatment and induction conditions are indicated above the autoradiogram using the following inducer abbreviations: NGF, N; lithium, L; forskolin, F; and dexamethasone, D. The inducers were used at the same concentrations as described in Fig. 2-6.
<table>
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<th>Induction Conditions</th>
<th>DF</th>
<th>NDF</th>
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<tr>
<td>Pre-treatment</td>
<td>-</td>
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<td></td>
<td>Y</td>
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- DF: Denotes the first condition.
- NDF: Denotes the second condition.
- LDF: Denotes the third condition.

Molecular markers: 325, 169, 104
appears to be some cross-talk between pathways.

The permissive agents NGF and lithium are active in NGF differentiated PC12 cells. Long-term NGF treatment causes PC12 cells to convert from an adrenal chromaffin-like to a sympathetic neuronal phenotype (Greene 1978; Greene and Tischler 1982). PC12 cells differentiated with NGF have been used extensively to investigate transcriptional control mechanisms involved in the expression of neuron-specific genes (Dobner et al. 1988; Gizang-Ginsberg and Ziff 1990; Kislauskis and Dobner 1990). If either NGF or lithium are active in neuronally-differentiated PC12 cells, then the signalling pathways mediating their effect are likely to be available in post-mitotic neurons and could be important for neuronal signalling.

The ability of lithium to cooperatively activate NT/N gene expression in PC12 cells that had been exposed to NGF for 12 hours (see figure 4-1) suggested that this pathway may be available in NGF-differentiated PC12 cells. To test this hypothesis, PC12 cells that were treated with NGF for 10 days or left untreated were subsequently stimulated with either lithium or NGF in combination with forskolin and dexamethasone and NT/N gene transcription was monitored by RNase protection. PC12 cells were treated with NGF every three days and fresh NGF was added 20 hr prior to treatment with the indicated inducer combinations. Consistent with the results obtained after 12 hr of NGF pre-treatment, the addition of a combination of lithium, forskolin, and dexamethasone more effectively stimulated NT/N gene expression in PC12 cells differentiated with NGF as compared to control cells (figure 4-2A). The level of expression in cells differentiated with NGF was as high as that observed in
Figure 4-2 NT/N gene induction in PC12 cells following long-term treatment with NGF. Either control PC12 cells or cells that had been treated with NGF (100 ng/ml) for 10 days were induced with either NGF or lithium in combination with dexamethasone and forskolin for the indicated times. The last addition of NGF in long-term NGF-treated cultures was 20 hr prior to induction. Nuclear RNA (25 μg) was analyzed using the RNase protection assay described in figure 2-6. A. Induction of intron one-containing NT/N precursor RNAs (325 b product) in either control cells (open circles) or NGF-differentiated cells (solid circles) in response to treatment with a combination of lithium, forskolin, and dexamethasone for the indicated times. B. Same as in A except control cells (open circles) or NGF-differentiated cells (closed circles) were treated with NGF, forskolin, and dexamethasone.
A

CPM/µg input RNA

Hours

B

CPM/µg input RNA

Hours
cells treated with all four inducers simultaneously (see figure 2-6). These results provide an indication that the pathway identified by lithium is present in neuronally-differentiated PC12 cells.

The effects of long-term NGF treatment on subsequent responses to either NGF, forskolin, and dexamethasone or just the latter two inducers were examined to determine whether NGF down-regulation persisted upon prolonged exposure to NGF. In contrast to cells pre-treated with NGF for just 12 hr, treatment with a combination of NGF, forskolin, and dexamethasone stimulated NT/N gene expression as effectively in long-term differentiated cells as in naive cells (figure 4-2B), although with somewhat delayed kinetics. Treatment with forskolin and dexamethasone following long-term exposure to NGF also stimulated NT/N gene expression, although somewhat less effectively than when fresh NGF was also added (data not shown). These results suggest that either the response to NGF, forskolin, and dexamethasone is only transiently down-regulated or that novel signalling components are induced after long-term exposure to NGF. The ability to induce NT/N gene expression in differentiated PC12 cells suggests that the pathways identified by both NGF and lithium may be important for the regulation of NT/N gene expression in neurons.

NGF and lithium in combination with dexamethasone and forskolin do not activate protein kinase C in PC12 cells. Lithium has well-characterized effects on the phosphatidylinositol (PI) pathway (Berridge et al. 1989) which could potentially account for its effects on NT/N gene expression, since the AP-1 site has been demonstrated to mediate at least some of the transcriptional effects of
phorbol esters (Lamph et al. 1988; Lee et al. 1987). Extracellular stimuli can alter the DNA binding or transcriptional activation of AP-1 factors through activation of PKC (Binetruy et al. 1991; Boyle et al. 1991). Phorbol esters strongly induce the activity and rapid translocation of PKC from the cytoplasm to the cellular membranes. However, PKC activity transiently declines to undetectable levels upon continued stimulation with phorbol esters (Matthies et al. 1987).

To examine whether induction is associated with changes in PKC activity, an *in vitro* phosphorylation assay was used to quantitate PKC levels in PC12 cell extracts. This assay is based upon the ability of PKC, in the presence of phosphatidylserine and calcium, to specifically phosphorylate histone H1 (Palfrey and Waseem 1985) and has been used previously to analyze PKC activity in PC12 cells (Damon et al. 1990; Matthies et al. 1987). In this experiment, PC12 cells were treated for 0, 1, 2, 4, 6 or 8 hours with all four inducers and fractionated into cytosolic and membrane extracts. These extracts were incubated with \(\gamma^{32}\)P-ATP and histone H1 either in the presence of phosphatidylserine and calcium to test for PKC specific phosphorylation or with EGTA as a non-specific control. The samples were run on a 15% SDS/polyacrylamide gel and exposed to X-ray film (figure 4-3). Even though the initial PKC activity was high, there was no further increase in PKC activity or in translocation to the membrane fraction during the 8 hour induction period. This result demonstrates that treatment with a combination of these inducers had no effect on the compartmentalization or activity of PKC during the interval examined.
Figure 4-3 PKC is not activated in fully induced PC12 cells. PC12 cells (~5 x 10^6 cells/time point) were treated with a combination of NGF, lithium, dexamethasone and forskolin. At the indicated time points (hours), cells were harvested and fractionated into cytosolic (cytosol) and membrane extracts. 5μg of protein were incubated with γ-^32P-ATP (500 μM) and histone H1 (200 μg/ml) either in the presence of phosphatidylserine (50 μg/ml) and CaCl2 (500 μM) to detect PKC specific phosphorylation (PS/Ca++, upper panel) or EGTA (500 μM) as a non-specific control (EGTA, lower panel). The products were resolved on a 15% SDS/polyacrylamide gel and exposed to X-ray film; the autoradiogram is shown (12 hour exposure). The positions of the phosphorylated histones are indicated by arrows.
Lithium activation does not require protein kinase C. To determine whether PKC is required for NT/N gene activation, PC12 cells were pre-treated with 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 hours in order to down-regulate PKC prior to the addition of inducers. Figure 4-4 shows an autoradiogram in which the levels of PKC were measured in PMA treated cells, using the above in vitro phosphorylation assay. This experiment demonstrated that exposure to 1 μM PMA for 24 hours resulted in the nearly complete loss (>95%) of PKC activity from PC12 cells, consistent with previous reports (Damon et al. 1990; Matthies et al. 1987). Furthermore, PKC activity was not restored by treatment with any of the inducer combinations tested. To test whether PKC was required for NT/N gene activation, PC12 cells were treated for 24 hours with 1 μM PMA or left untreated prior to induction with NGF and/or lithium, in combination with dexamethasone and forskolin. Detection of the intron one-containing transcripts by RNase protection showed (figure 4-5), unexpectedly, that phorbol ester pre-treatment potentiated NT/N transcriptional responses to all inducer combinations tested by approximately 5-fold, including responses involving lithium. These results demonstrate that PKC is not required for NT/N gene activation by combinations of lithium, NGF, forskolin, and dexamethasone. In fact, the pathways required for activation may be constitutively suppressed through a PKC-dependent mechanism.
Figure 4-4  PKC activity is down-regulated in phorbol ester pre-treated PC12 cells. PC12 cells (~1 x 10^7 cells/condition) were either pre-treated with 1 μM PMA (P) or left untreated (-) for 20 hours, and subsequently induced with either NGF and/or lithium in combination with dexamethasone and forskolin for the indicated times or left uninduced (NA). As a control, cells were also treated with 100 nM PMA (P). Induction times (hours) are indicated. PKC activity was assayed as in figure 3-5. The upper panel represents specific phosphorylation (PS/Ca++) while the bottom panel represents non-specific phosphorylation (EGTA). Induction conditions are indicated above the autoradiograph using the following inducer abbreviations: NGF, N; lithium, L; forskolin, F; and dexamethasone, D. The inducers were used at the same concentrations as described in Fig. 2-6.
### PS/Ca++

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Figure 4-5  Down-regulation of PKC does not inhibit NT/N gene induction.

PC12 cells were either pre-treated with 1 μM PMA for 20 hours or not pre-
treated, subsequently treated with the indicated inducer combinations, and
harvested at the indicated times. Nuclear RNA was prepared, and precursor and
spliced NT/N transcripts were quantitated using the RNase protection assay
described in figure 2-6. The positions of the protected products resulting from
hybridization to intron one-containing (325 b) and spliced transcripts (169 b),
as well as the 104 b protected product resulting from hybridization to sense
RNA standard, are indicated. Inducer abbreviations and concentrations are as
described in figure 4-3.
CHAPTER V

DISCUSSION
This thesis addresses the problem of how extracellular signals regulate NT/N gene expression in PC12 cells and defines pathways which may be important for controlling plastic changes within the adult CNS. Several lines of evidence indicate that environmental factors, such as growth factors, hormones and neurotransmitters play an essential role in the establishment of neuronal phenotypes during development of the peripheral (Anderson 1989; LeDouarin 1982) and central nervous system (Renfranz et al. 1991). Many neuronal genes are induced within the adult CNS in response to both physiological and pharmacological stimuli suggesting that extracellular signalling also regulates CNS plasticity (Merchant et al. 1992; Naranjo et al. 1991; Sonnenberg et al. 1989c). PC12 pheochromocytoma cells differentiate neuronally in response to NGF and respond to several neurotransmitters providing a useful model to study neuronal differentiation and plasticity (Greene and Tischler 1982). One of the earliest transcriptional responses to NGF in PC12 cells is the rapid induction of immediate early genes (IEGs), including several that encode AP-1 transcription factors (Morgan and Curran 1991). Certain neuronal genes contain consensus AP-1 sites within their promoters, suggesting that the induction of AP-1 factors may play a key role in the establishment of neuronal phenotypes both during development and in the adult nervous system (Kislauskis and Dobner 1990; Naranjo et al. 1991; Sonnenberg et al. 1989c).

Several neuropeptide genes that contain AP-1 sequences are expressed in the nervous system with kinetics that correlate with the expression of AP-1 genes (Naranjo et al. 1991; Sonnenberg et al. 1989c) but because of technical difficulties these transcription factors have not been shown to directly control
neuronal gene transcription, in vivo. Since AP-1 factors and putative target
genes are similarly expressed in PC12 cells, these cells are more amenable for
studying whether AP-1 complexes mediate the response of extracellular
stimuli to neuronal gene transcription. The neuropeptide, neurotensin, and
NT/N mRNA are induced in PC12 cells by combinations of environmental
factors, including NGF, glucocorticoids, activators of adenylate cyclase and
lithium salts (Dobner et al. 1988; Kislauskis and Dobner 1990). Previous
experiments have indicated that cis-regulatory elements located within the
NT/N promoter are necessary for the synergistic action of these inducers, with
a consensus AP-1 site required for the permissive effects of NGF and lithium
(Kislauskis and Dobner 1990). The NT/N promoter is unique in that it appears
to be composed entirely of inducible elements, which serve to integrate
multiple environmental stimuli into a unified transcriptional response. The
expression of the NT/N gene in PC12 cells thus provides a model to analyze
mechanisms by which environmental stimuli alter neuropeptide gene
expression, including those mediated by AP-1 factors.

Experiments described in Chapter 2 demonstrate that NT production in
PC12 cells is regulated principally at the transcriptional level by combinations
of inductive stimuli, although regulation of proNT/N protein processing may
also play a minor role. These results are consistent with transfection
experiments in which the 5' flanking region of the NT/N gene conferred the
regulatory properties exhibited by the endogenous gene to reporter genes
(Kislauskis and Dobner 1990). However, NT/N mRNA must be extremely stable,
since transient low level activation of NT/N transcription produced high
levels of NT/N mRNA (Figure 2-5). In support of this conclusion, NT/N mRNA
can be detected, by nuclease protection assays, without any reduction in steady state levels for up to 96 hours after induction (unpublished observations). The low level of expression of nuclear run-on transcripts made it necessary to develop an alternative transcriptional assay to analyze NT/N gene expression. Intron one-containing NT/N transcripts, detected in the nuclei of maximally induced PC12 cells, accumulated with similar kinetics to nuclear run-on transcripts, indicating that increases in NT/N precursor RNA represent changes in NT/N transcription. The detection of precursor RNA in the nucleus has been previously used to assay changes in transcription when nuclear run-on assays are impractical (Fremeau et al. 1989; Maurer 1981). The RNase protection assay described in this thesis provides a rapid, sensitive assay for the quantitation of NT/N precursor RNA in induced PC12 cells.

Analysis of NT/N precursor RNA in cells treated with either NGF or lithium as the permissive inducer showed that NT/N intron one-containing transcripts are induced with different kinetics in each case. In cells treated with NGF, dexamethasone, and forskolin, a rapid and transient increase in NT/N precursor RNA was observed, whereas a delayed response was seen when lithium was used as the permissive inducer. The kinetics of NT/N gene activation in cells treated with NGF, dexamethasone, and forskolin suggested that the induction of AP-1 factors may regulate NT/N transcription, since NGF rapidly induces AP-1 gene and protein expression (Curran and Morgan 1986; Sheng and Greenberg 1990). However, when PC12 cells were pre-treated with either anisomycin or cycloheximide prior to the induction with NGF, dexamethasone, and forskolin, the rapid transient increase in intron one-containing transcripts was not attenuated. Therefore, it is likely that post-
translational modification and not de novo synthesis of AP-1 factors is required for the permissive effect of NGF. Similar to the NT/N gene, cAMP-induction of proenkephalin gene expression in C6 glioma cells, acting through an AP-1-related element, is not inhibited by anisomycin (Kobierski et al. 1991), in support of this conclusion. Therefore, post-translational modification of constitutively expressed AP-1 factors may play an important role in rapid transcriptional responses of the NT/N gene to NGF, dexamethasone, and forskolin.

It is unclear why the induction of AP-1 factors, such as c-Fos and c-Jun, are not involved in the activation of NT/N transcription in response to NGF, dexamethasone, and forskolin. Three possibilities could account for this. First, nucleotide sequences would dictate which subset of AP-1 factors can interact with the AP-1 site. Recent observations by G. McNeil have shown by gel shift assays that certain AP-1 complexes do not interact with the distal AP-1 site (G. McNeil and P. Dobner unpublished results) supporting this possibility. Second, an inhibitory AP-1 factor, such as Jun-B, could be induced simultaneously thus antagonizing the action of c-Fos/c-Jun complexes (for discussion see below). Third, post-translational modification could result in the inactivation of induced AP-1 factors. In support of this third possibility, c-Fos is extensively modified in NGF-treated PC12 cells (Curran and Morgan 1986), and the activity of c-Jun has been shown to be inhibited by phosphorylation at certain sites (Boyle et al. 1991).

Post-translational modifications of transcription factors, especially phosphorylation, are an important link between signal transduction and regulation of gene expression (Bohmann 1990). Figure 5-1 diagrams a
Figure 5-1 A phosphorylation cascade activated by NGF and its influence on AP-1 factors. NGF binding to the NGF receptor activates a tyrosine kinase activity (1) which initiates the sequential phosphorylation of mitogen activation protein (MAP) kinases (2). An unidentified MAP kinase kinase, (MAPKKK) which is, either directly or indirectly, activated by NGF, phosphorylates serine and threonine residues on MAP kinase kinase (MAPKK). Active MAPKK subsequently activates MAP kinases (MAPK) by phosphorylations at tyrosine and threonine residues. MAPK induces the activation of c-Jun (3) through the phosphorylation of serine residues (63-73). Src and Ras also influence the activity of c-Jun presumably by phosphorylation of the same residues (4). Ras seems to be a component of the NGF pathway. The amino acid sequence similarity between the activation domains of c-Jun, Jun-D and Jun-B suggest that these AP-1 factors may also be regulated by this phosphorylation cascade or pathways involving Src and Ras.
NGF \rightarrow TRK \rightarrow MAPKKK \rightarrow MAPKK (inactive) \rightarrow MAPKK (active) \rightarrow MAPK (inactive) \rightarrow MAPK (active) 

\[ LLTSPDVGLLKLASPELERLV \]
\[ LLASPDGLLKLASPELERLI \]
\[ LKLASTEKERLI \]
potential phosphorylation cascade activated by NGF resulting in the trans-
activation of the AP-1 factor, c-Jun. 1) NGF binds to cognate receptors (TRK),
thereby inducing protein tyrosine kinase activity (Loeb et al. 1991; Ross 1991).
2) NGF initiates a phosphorylation cascade involving the mitogen activated
protein (MAP) kinases (Boulton et al. 1991; Gomez and Cohen 1991). Activation
of MAP kinases require both serine/threonine and tyrosine phosphorylations
and are the only known serine/threonine protein kinases that are activated
by tyrosine phosphorylation (Gomez and Cohen 1991). 3) Phosphorylation by
MAP kinases positively regulates the activity of c-Jun by specifically
phosphorylating serine residues (63 and 73) within the activation domain of c-
Jun (Pulverer et al. 1991; Smeal et al. 1991). 4) Similarly, the proto-oncogene
products, Ha-Ras and v-Src, also increase the transcriptional activity of c-Jun
through phosphorylations within the activation domain (Baichwal et al. 1991;
Binetruy et al. 1991). Both the ras and src oncogenes induce neuronal
differentiation in PC12 cells (Alema et al. 1985; Bar-Sagi and Feramisco 1985).
NGF-induced differentiation of PC12 cells is inhibited by injection of anti-ras
antibodies (Hagag et al. 1986). Taken together, these results suggest that Ras
and Src may be components of a NGF signalling pathway, although their exact
placement within the phosphorylation cascade has not been determined
(Hagag et al. 1986; Szeberenyi et al. 1990). Jun-D and Jun-B have similar
phosphorylation sites to that of c-Jun (Figure 5-1), thus other AP-1 factors
could be activated post-translationally by MAP kinases or the signalling
cascade initiated by the ras-like G-proteins or src-tyrosine kinase activity.
Jun-D is constitutively expressed in PC12 cells and is not regulated by any of
the factors which activate NT/N transcription (Figure 3-7 and de Groot et al.
1991; Ryder et al. 1989). Interestingly, Jun-D may be involved in the transient activation of proenkephalin gene expression through an element that resembles both an AP-1 site and a CRE (Kobierski et al. 1991). Transactivation by Jun-D in F9 cells requires co-expression of the catalytic subunit of PKA, although it is unclear whether Jun-D is phosphorylated by PKA (Kobierski et al. 1991). NGF could influence NT/N gene transcription through the post-translational modification of c-Jun, Jun-D or perhaps other constitutively expressed AP-1 factors.

Following the initial period of NT/N gene activation in cells treated with NGF, forskolin, and dexamethasone, transcription was rapidly extinguished by a mechanism that required newly synthesized proteins. Anisomycin treatment prolonged the expression of intron one-containing transcripts and this effect was not due to either a general inhibition of splicing or anisomycin effects on signal transduction (Mahadevan and Edwards 1991). These results suggest that NGF induces a transcriptional inhibitor which rapidly attenuates NT/N gene expression. One possibility would be that NGF is inducing an AP-1 factor, such as Jun-B, that acts as a negative regulator of transcription. The kinetics of expression of jun-B activation correlate well with the attenuation of NT/N gene expression (Figure 3-7 and Bartel et al. 1989). Jun-B has been shown to repress c-Jun activation of single AP-1 sites in co-transfected F9 embryonal carcinoma cells (Chiu et al. 1989), and to inhibit the transforming and trans-activating activities of c-Jun in primary rat embryonic cells (Schutte et al. 1989). Co-transfection experiments have demonstrated that Jun-B is a potent inhibitor of the PKA-dependent activation of proenkephalin transcription by Jun-D, suggesting that Jun-B is responsible for extinguishing transcription.
after the initial forskolin induction (Kobierski et al. 1991). The results reported in Chapter 3 are consistent with the notion that the rapid inactivation of NT/N transcription, following induction by NGF, dexamethasone and forskolin, may involve the de novo synthesis of an inhibitory AP-1 factor.

In contrast to the response to NGF, forskolin, and dexamethasone, responses involving lithium were markedly attenuated by anisomycin pre-treatment indicating that lithium action requires newly synthesized proteins. The importance of the AP-1 site for responses to inducer combinations involving lithium provides an indication that increased synthesis of AP-1 proteins is required for the response (Kislauskis and Dobner 1990). RNA blot analysis was used to examine the effects of lithium on the expression of the seven characterized AP-1 genes in PC12 cells. Lithium acted synergistically with dexamethasone and forskolin to increase c-fos and jun-B gene expression and activated c-jun, fra-1, and c-fos gene expression with delayed kinetics (> 4 hours), consistent with a role for the corresponding AP-1 proteins in NT/N gene activation. These results indicate that components of the lithium specific signalling pathway are interacting with the regulatory regions of these AP-1 genes. Analysis of the promoter regions of c-fos, c-jun, and fra-1 may be useful in identifying cis-regulatory elements required for activation and aid in defining the signalling pathway identified by lithium. The delayed kinetics of these responses may circumvent the potential negative effects of Jun-B, since the jun-B gene is transiently induced prior to the delayed activation of the c-jun and fra-1 genes in cells treated with lithium, dexamethasone and forskolin. In maximally stimulated cells, lithium could also override the
inhibition induced by NGF, since lithium pre-treatment potentiates transient activation of cells treated with NGF, dexamethasone and forskolin (figure 4-1). These results suggest that lithium-regulation of c-Jun/Fra-1 or other AP-1 heterodimer levels may underlie its transcriptional effects on both the NT/N gene and a CAT fusion gene controlled by multimerized AP-1 sites. However, the relatively modest effects of lithium on AP-1 gene expression leave open the possibility that the induction of novel AP-1 proteins or factors that modify AP-1 activity is involved in the response.

Lithium has been shown to influence signalling through the PI pathway (Berridge et al. 1989) and may have a more general effect on G protein-linked receptor signalling (Avissar et al. 1988). Lithium influences PI signalling by inhibiting mono- and polyphosphatases required for the regeneration of phosphatidylinositol in the absence of exogenous inositol (Berridge et al. 1989). The inhibition of these phosphatases causes the accumulation of mostly inactive phosphorylated forms of inositol, however, in GH3 cells there is an increase in levels of diacylglycerol (DAG) which is required for PKC activation (Drummond and Raeburn 1984). Lithium has been shown to potentiate cholinergic agonist activation of c-fos gene expression in PC12 cells (Kalasapudi et al. 1990) and TIS gene expression in cultured rat astrocytes (Arendard et al. 1989) and these effects may be mediated through PKC. However, experiments in chapter 4 demonstrated that the PKC pathway is not required for NT/N gene activation in PC12 cells. Interestingly, PKC down-regulation enhanced NT/N gene transcription, for all induction conditions tested, suggesting that PKC signalling may be important for tonically suppressing NT/N transcription. The constitutive activation of PKC in naive
and fully induced PC12 cells supports this conclusion (Figure 4-3). Coupled with previous observations indicating that Ca++ has only a modest effect on NT/N gene expression (Tischler et al. 1991), these results suggest that lithium's well-characterized effects on PI signalling are not responsible for its effects on NT/N gene transcription. Lithium also transcriptionally activates a CAT fusion gene construct controlled by multimerized AP-1 sites in PC12 cells and this response is essentially unaltered in protein kinase A-deficient PC12 cells (Dobner et al. 1992). These results taken together strongly indicate that lithium either affects signalling components downstream of these kinases or that lithium affects a novel signalling pathway. Therefore, PKC is not required for the permissive response to lithium but may be necessary for maintaining low level NT/N gene expression in unstimulated PC12 cells.

The signal transduction pathways which modulate NT/N gene transcription in PC12 cells may be important in maintaining NT/N gene expression within the CNS. NT/N mRNA-containing cells are discretely localized within the rat brain, predominantly within the limbic regions (Alexander et al. 1989b). Studies exploiting immunostaining or in situ hybridization of unstimulated brain sections have shown a significant anatomical specificity of c-fos and other IEGs (Sagar et al. 1988; Sonnenberg et al. 1989a). Therefore, the constitutive activation of signalling pathways utilizing AP-1 factors may be important for maintaining neuronal gene expression. This concept is clearly demonstrated in the rat visual cortex by the expression of the immediate early gene, zif268 (Worley et al. 1991). Constitutive levels of zif268 are maintained through visually induced synaptic activity.
These results suggest that IEG transcription factors play a role in maintaining normal brain physiology.

Many physiological and pharmacological induced events in the CNS correlate with the transient expression of AP-1 genes and proteins suggesting that AP-1 factors are important regulators of plastic changes within the CNS (Doucet et al. 1990; Morgan et al. 1987; Sonnenberg et al. 1989a). In one study, messenger RNAs encoding the AP-1 factors c-fos, c-jun, and junB were rapidly and transiently induced in rat hippocampus following chemically induced seizures (Sonnenberg et al. 1989c). Subsequent to the appearance of these transcripts, a transient burst of proenkephalin gene expression occurred in the same neurons, suggesting that the induction of AP-1 regulates proenkephalin gene expression in the hippocampus. The requirement of an AP-1-like regulatory element within the proenkephalin promoter supports this conclusion (Kobierski et al. 1991). In a follow-up experiment, agonists of the glutamate receptor, a major neurotransmitter receptor within the brain, increased c-fos and fra-1 gene expression and AP-1 DNA binding activity in the mouse hippocampus (Sonnenberg et al. 1989b), further supporting a role for AP-1 factors in mediating transcriptional responses to physiological stimuli. In another case, prodynorphin mRNA is expressed within the dorsal horn of the rat CNS after noxious stimulation with kinetics that are consistent with the expression of AP-1 target genes (Naranjo et al. 1991). The prodynorphin gene also contains a functional AP-1 site within its promoter (Naranjo et al. 1991). Taken together, these experiments suggest that IEGs, including those encoding AP-1 proteins, function as mediators of long-term transcriptional responses to environmental factors in neurons.
Recent experiments have indicated that regulation of NT/N gene expression in the rat brain requires the convergence of multiple signalling pathways, including those acting through AP-1 proteins. The use of an intron-specific probe for in situ hybridization has shown a rapid and transient increase in NT/N transcription (peaking between 3 to 7 hours) within the dorsolateral striatum of rats, after treatment with a single dose of the dopamine antagonist, haloperidol (Merchant et al. 1992). Haloperidol also transiently activates c-fos gene expression and increases c-Fos immunoreactivity in the striatum and nucleus accumbens (Miller 1990; Robertson and Fibiger 1992). Haloperidol is also likely to increase cAMP levels in responsive neurons by blocking dopamine D2 receptors which are negatively linked to adenylate cyclase (Onali et al. 1985). Consistent with our results in PC12 cells, haloperidol induction of the NT/N gene within the rat striatum and nucleus accumbens most likely depends upon convergent signalling pathways, presumably the phosphorylation of CRE-binding proteins by the adenylate cyclase/protein kinase A pathway cooperatively interacting with the transient induction of AP-1 factors. Different signals appear to be essential for NT/N gene expression in other discrete brain regions, particularly in the hypothalamus (Alexander et al. 1989b). Expression in the medial preoptic area in female rats is strikingly dependent on circulating estrogen (Alexander et al. 1989a) and varies throughout the estrous cycle implying that NT may be an important regulator of the pre-ovulatory surge in rats (Alexander et al. 1991). Similarly, colchicine and reserpine specifically induce NT/N gene expression in the paraventricular hypothalamus (Ceccatelli et al. 1991a). These responses may be stress-related,
perhaps indicating that corticosteroids regulate NT/N gene expression in the CNS (Ceccatelli et al. 1991a). In support of this conclusion, glucocorticoid receptor immunoreactivity is co-localized to NT containing cells within the rat brain (Cintra et al. 1991). Taken together, these results clearly indicate that different environmental stimuli play key region-specific roles in regulating NT/N gene expression in the CNS.

NGF and lithium stimulate distinct intracellular pathways that are required for NT/N gene expression in PC12 cells and that could be important for controlling the expression of the gene in the nervous system. NGF is a member of a family of structurally related neurotrophic factors that have recently been shown to exert their effects through related high affinity receptors encoded by the trk oncogene family (Loeb et al. 1991; Ross 1991). NGF and other members of this family are expressed in distinct patterns in the CNS (Ceccatelli et al. 1991b; Isackson et al. 1991) and could play a role in regulating neuronal gene expression, including the NT/N gene. Interestingly, NGF is synthesized in hippocampus (Ayer-LeLiever et al. 1988) and NGF mRNA is induced by limbic seizures within the same neurons (Gall and Isackson 1989). Like NGF, the pathways identified by lithium may also be important in the CNS. The demonstration that lithium can cooperatively activate NT/N gene expression after long-term NGF treatment of PC12 cells indicates that the pathway identified by lithium is active in neuronally-differentiated cells. The activation of this pathway by physiological ligands could thus play a role in the generation of the complex pattern of NT/N gene expression observed in the rat CNS. Results presented in this thesis suggest that one action of lithium within the brain could be to enhance the expression of AP-1 genes. This
pathway could then cooperate with other signalling pathways to alter transcriptional responses in neuronal cells elicited by growth factors, neurotransmitters or depolarization. Activation of c-fos expression by muscarinic agonists acting through the PKC signalling pathway in rat brains is enhanced by treatment with lithium (Weiner, 1991). Identification of the endogenous ligands that activate this pathway thus becomes paramount in understanding the effect of lithium on the physiology and long-term plastic changes within the CNS.

NT/N mRNA is localized to the same regions of the rat brain as NT-like immunoreactivity, with the exception of the CA1 region of the hippocampus and the subiculum (Alexander et al. 1989b). NT-like immunoreactivity has not been observed in these regions within the adult rat, even though abundant levels of authentic NT/N mRNA were detected by in situ hybridization (Alexander et al. 1989b). Similar discrepancies between mRNA abundance and immunostaining have been observed with other neuropeptides (Schiffmann and Vanderhaeghen 1991; Segerson et al. 1987). Four likely possibilities could explain this discrepancy: 1) the proNT/N peptide is alternatively processed into peptides other than NT, 2) the NT peptide is turned over rapidly, 3) the proNT/N protein is rapidly degraded after translation and 4) NT/N mRNA is not translated or is poorly translated. To determine if alternative processing of proNT/N could account for this discrepancy, an experiment was done to detect proNT/N peptides in these regions by immuno-histochemistry, using the Rb 3 antiserum. Surprisingly, proNT/N was not detected in either the subiculum or the CA1 region of the hippocampus (M. Alexander, B. Bullock and P. Dobner, unpublished results). However, proNT/N was detected in other areas that
contain both NT and NT/N mRNA, such as the arcuate nucleus, lateral septum, and paraventricular nucleus. Even though, further experiments need to be done to improve the sensitivity of these signals, these results suggest that NT/N mRNA may be regulated at the level of translation within the cells of the rat hippocampus and subiculum, thus providing for a novel mechanism to regulate neuropeptide gene expression in the CNS.

The region specific localization of different processed forms of precursor neuropeptides within the CNS has been well documented (Eiden 1987; Krause et al. 1990; Mains et al. 1983), although the elucidation of mechanisms by which differential processing occurs has been limited. Differential forms of processed proNT/N peptides have been observed within various tissues in several mammalian species, including the rat (Carraway et al. 1991; Shaw et al. 1990a). Experiments described in chapter 2 showed that lithium inhibits the formation of mature NT in PC12 cells (Table 2-1). Lithium could alter proNT/N processing by two possible mechanisms. 1) NT/N mRNA in lithium induced cells could be expressed at such high levels that the processing machinery is saturated and only a limited amount of peptide is being processed. 2) lithium is specifically inhibiting proteolytic enzymes needed to process proNT/N into mature peptide. Further studies will be needed to determine whether lithium may specifically regulate the proteolytic processing of mature NT peptide.

The observation that most proNT/N peptide is partially processed or unprocessed (> 80%) in PC12 cells suggests that an important mechanism for regulating NT synthesis may be through post-translational attenuation, a phenomena by which precursor peptide is inhibited from being fully
processed and accumulates in unprocessed non-bioactive forms (Rehfeld 1990). The mechanisms by which neuropeptide biosynthesis can be regulated in this manner are not well defined. Many post-translational processing events could be responsible for the inhibition of proteolytic cleavage, including phosphorylation, glycosylation, sulfonation and C-terminal amidation (Mains et al. 1983). Of particular interest is the inhibition of progastrin processing by phosphorylation. In human gastrinoma extracts, unprocessed progastrin was less likely to be phosphorylated when compared to the corresponding fully processed peptide, suggesting that phosphorylated progastrin might be more readily cleaved (Varro et al. 1990). Progastrin is phosphorylated at a serine residue within a consensus casein kinase site which overlaps the C-terminal proteolytic cleavage site (Dockray et al. 1987). Similarly, the proNT/N protein contains a consensus protein kinase A phosphorylation site (N-leu-lys-arg-ala-ser-tyr-C), which is located within its C-terminal cleavage site. Therefore, phosphorylation induced by forskolin, could alter the ability of proNT/N peptide to be processed. Experiments where high levels of precursor NT/N proteins are induced in either the presence or absence of forskolin will aid in understanding the role of the C-terminal phosphorylation and post-translational attenuation.

In summary, the experiments in this thesis show that the permissive agents NGF and lithium when combined with dexamethasone and forskolin induce NT/N transcription in PC12 cells through two distinct signalling pathways. Post-translational modification of constitutively expressed AP-1 factors may be required for the activation by NGF, dexamethasone, and forskolin, whereas, newly synthesized AP-1 proteins may subsequently down-
regulate NT/N gene expression. In contrast, lithium activates a permissive pathway which may result in the de novo synthesis of AP-1 complexes. Lithium's influence on NT/N gene expression does not require protein kinase C and thus is probably not acting through the phosphatidylinositol signal transduction pathway. NGF and lithium are active in neuronally-differentiated PC12 cells suggesting that these agents define signal transduction pathways that are important for regulating the NT/N gene in neurons.
Materials and Methods

Materials. Most restriction and other enzymes were purchased from Boehringer Mannheim Biochemicals; Sst I was from New England Biolabs; $^{32}$P-nucleotides, $^3$H-leucine and $^{35}$S-methionine were from Amersham Corp.; unlabeled nucleotides were from Pharmacia; anisomycin, cycloheximide, forskolin, nuclease P1, indolacrylic acid, Thiamine B1, lysozyme, histone H1, phorbol 12-myristate 13-acetate (PMA) and formalin fixed Staphylococcal aureus (Staph A) were all from Sigma; nerve growth factor (2.5 S) was from Bioproducts for Science Inc. (Indianapolis, IN); dexamethasone was a gift from Merck, Sharp and Dohme (West Point, PA); and lithium chloride, formamide, guanidinium thiocyanate and formaldehyde were from Fluka. In vitro packaging extract (Gigapack) was obtained from Vector Cloning Systems (San Diego, CA). Freund’s complete and incomplete adjuvant were purchased from DIFCO Laboratories; Rnase-free DNase I was from Worthington Biochemical Corporation (Freehold, NJ); and Zetabind nylon membranes from CUNO Laboratory Products (Meridan, CT). Recombinant plasmids for mouse c-jun, jun-B, and jun-D were obtained from the American Type Culture Collection. Additional AP-1 cDNA clones were generously provided by Drs. J. Bellasco and M. E. Greenberg (mouse c-fos), R. Bravo (mouse fos B), and M. Matsui (human fra 1, fra 2).

Cell Culture. PC12 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO) supplemented with 10% heat-inactivated horse serum (GIBCO), 5% fetal bovine serum (Sigma), and antibiotics (GIBCO) as described (Greene
and Tischler 1976). Inducers were added to the culture medium from concentrated stocks to final concentrations of 1 μM dexamethasone, 1 μM forskolin, 100 ng/ml nerve growth factor, and 20 mM lithium chloride. To inhibit protein synthesis, cells were pre-treated for 30 minutes (unless indicated otherwise) with either 50 μM anisomycin or 10 μg/ml cycloheximide (Greenberg et al. 1986). For protein kinase C (PKC) down-regulation experiments, PC12 cells were treated with PMA (1 μM) for 12 hours prior to induction as described (Damon et al. 1990; Matthies et al. 1987). PC12 cells were plated on collagen-coated tissue culture dishes for neuronal differentiation in response to long-term NGF treatment (Greene and Tischler 1976).

Isolation of the Rat NT/N Gene. A genomic library was constructed to isolate the rat gene (Kislauskis et al. 1988). High molecular weight DNA, greater than 20 kb in size, was extracted from rat testes as described (Bell et al. 1981). The DNA was partially digested with the restriction enzyme Sau 3A (0.004 units/μg DNA) for 1 hour at 37 °C and fractionated on a sucrose gradient (10-40%). Aliquots, corresponding to a size range of 10 to 20 kb, were pooled and ethanol precipitated. Size-selected DNA was ligated into EMBL 4 phage arms (Dente et al. 1985), digested with Bam HI, at a ratio of 2:1 (insert:arms), packaged into phage particles (Gigapack) and infected into E. Coli K802 bacterial cells as described (Sambrook et al. 1989). The titer of the library was determined to be approximately 5.5 X 10^6 pfu/ml of lysate. Approximately 3.6 X 10^5 independent recombinants were plated and screened using standard protocols (Sambrook et al. 1989). The library was probed with 32P-radiolabeled NT-8A, a canine NT/N
cDNA clone (Dobner et al. 1987), which was labeled by nick translation (Rigby et al. 1977) after linearization with Eco RI. Hybridization was performed in 1 X NaCl/sodium citrate solution (1 X NaCl/sodium citrate = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 X Denhardt's solution (1 X Denhardt's solution = bovine serum albumin, polyvinylpyrrolidine, and Ficoll all at 0.2 mg/ml), 50 mM sodium phosphate buffer (pH 7.0), 0.5% sodium dodecyl sulfate (SDS) at 60 °C. Filters were washed in several changes of 2 X NaCl/sodium citrate, 0.1 % SDS at room temperature followed by a wash in the same solution at 50 °C. Three positive clones, rNT18, rNT19, and rNT23 were identified and plaque purified. rNT18 contained the full length rat NT/N gene and was used for further analysis (Kislauskis et al. 1988).

**Plasmid Constructions.** All plasmids were propagated in the *E. coli* bacterial strain, HB101, and purified by standard protocols (Sambrook et al. 1989). For riboprobe synthesis, the following DNA fragments were subcloned into pGEM4 vector DNA (Promega). pGEM-E11-NT was produced by ligating a 489 nt Dra I-Eco RI fragment of rNT18 (Kislauskis et al. 1988), containing 5' flanking sequences, exon 1 and part of intron 1 of the rat NT/N gene, into Sma I-Eco RI digested pGEM 4. pGEM-rNT4 was produced by ligating a 336 nt Bgl II-Eco RV fragment of rNT18 (Kislauskis et al. 1988), containing exon 4 of the rat NT/N gene, into Sma I-Bam HI digested pGEM 4. A 562 nt Eco RI fragment derived from a subclone of the λT14 human α-tubulin gene (Lemischka and Sharp 1982) was ligated into Eco RI digested pGEM4 to generate a probe spanning part of intron 3 (165 nt) and exon 4 (383 nt) of the rat α-tubulin gene. A 944 nt Eco RI-Pvu II fragment of the human *fra-1* F89 clone (Matsui et al. 1990) and a
1008 nt Eco RI fragment of the human fra-2 F29 clone (Matsui et al. 1990) were subcloned into Eco RI-Sma I and Eco RI digested pGEM 4, respectively.

For the production of preproNT/N fusion proteins, the appropriate sequences of a NT/N cDNA clone (rbNT,(Alexander et al. 1989b) were subcloned, in-frame, into the pATH 2 bacterial expression vector (Kleid et al. 1981). pATH-rbNT was constructed by ligating the 1130 nt Pvu II - Bam HI fragment of the rat NT/N cDNA clone (rbNT), containing nearly the entire coding region (lacking the first 7 amino acids) along with 200 nucleotides of 3' untranslated region, into the Sma I and Eco RI sites of pATH 2. To obtain RNA coding for the full length preproNT/N, the 1120 nt Pvu II-Eco RI fragment of rbNT was ligated to the Hind III - Pvu II fragment of the canine NT/N cDNA and subcloned into the Hind III - Eco RI site of pGEM4 to produce pGEM-crhNT.

Production of antiserum to preproNT/N. Fusion protein production and purification was as described (Kleid et al. 1981). Briefly, HB101 cells, transformed with pATH-rbNT, were grown at 37 °C overnight in M9 CA media (6 g Na2HPO4, 3 g KH2PO4, 0.5 g NaCl, 1 g NH4Cl, 5 g casamino acids, 0.25 g MgSO4, 15 mg CaCl2, 2 g glucosuc, and 10 mg Thiamine B1 per liter of dH2O, pH 7.5) containing ampicillin (0.1 mg/ml) and l-tryptophan (10 μg/ml in 0.1 M KOH). A 1:100 dilution was grown in 100 ml M9 CA (lacking l-tryptophan) to mid-log, induced with indoleacrylic acid (10 μg/ml in absolute ethanol) and grown for an additional 6 hours at 37 °C with vigorous shaking. The cells were left overnight at 4 °C. Bacterial cells were pelleted (1,000 X g, 10 minutes), resuspended in 20 ml lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 3 mg/ml lysozyme, pH 7.4) and incubated on ice for 2 hours. An additional 30 minute
incubation was done after adding 1.4 ml of 5 M NaCl and 1.5 ml of 10% NP-40. This solution was sonicated to reduce viscosity and centrifuged at 8,000 X g for 20 minutes. The pellet was washed twice with 20 ml 1 M NaCl, 10 mM Tris-HCl, pH 7.4, once with 10 mM Tris-HCl, pH 7.4, resuspended in 150 µl 2x SDS/gel loading buffer (1x SDS/gel loading buffer = 10% glycerol, 5% β-mercaptoethanol (β-ME), 3% SDS, 62.5 mM Tris-HCl, 0.01% bromphenol blue, pH 6.8) and loaded on a 10% SDS/polyacrylamide gel (Laemmli 1970). The fusion protein was visualized by a modified procedure of Nelles and Bamberg (Nelles and Bamberg 1976). Briefly, the gel was washed 3 X in ice cold dH₂O, soaked in ice cold 0.25 M KCl, 5 mM β-ME for 5 minutes, and rinsed in ice cold 5 mM β-ME until precipitated bands were detected. The appropriate band was excised from the gel and used directly for immunizations.

Immunizations were performed using standard procedures (Harlow and Lane 1988). A gel slice containing approximately 300 µg of TrpE-NT/N was homogenized with Freund's complete adjuvant and equal amounts were injected subcutaneously into the backs of two New Zealand white rabbits. Two weeks later each rabbit was boosted with approximately 50 µg of TrpE-NT/N in Freund's incomplete adjuvant. Starting twelve days later blood was collected at three day intervals, and the serum was processed and titered. When the serum was determined to contain a high titer of antibodies to TrpE-NT/N, remaining blood was collected, the serum was stored at -20 °C and the rabbits were sacrificed. The IgG fraction was affinity purified on protein A beads using established procedures (Harlow and Lane 1988).
**Immunoprecipitation.** For titering the antiserum raised against TrpE-NT/N, an immunoprecipitation assay was developed. Sense-strand crhNT RNA was transcribed from pGEM-crhNT with T7 RNA polymerase following linearization with Nhe I. crhNT RNA was translated in rabbit reticulocyte lysate (Promega) containing $^{35}$S-methionine (800 Ci/mMole) as described (Titus 1991). 1 µl of lysate was mixed with 200 µl PBS-SDS (1 X PBS, 0.1% SDS; 1 X PBS = 8 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$, 0.24 g KH$_2$PO$_4$) along with various amounts of either pre-spun antiserum (10,000 X g, 10 minutes) or isolated IgG fraction and incubated on ice for 1 hour. The antibody-antigen complex was isolated by affinity absorption on formalin fixed Staph A as described (Kessler 1975). The antiserum-translation mix was incubated with 20 µl of washed Staph A on ice for 30 minutes. This mixture was layered on top of a sucrose cushion (1M sucrose, 3.65 X PBS, 0.5% NP-40, 1% deoxycholate, 1 mM EDTA, 0.1% SDS, 5 mM methionine) and spun at 10,000 X g, for 10 minutes. The pellet was washed 2 X with PBS-SDS and resuspended in 1 X SDS/gel loading buffer. The immunoprecipitated products were resolved by 15% SDS/PAGE (Laemmli 1970) and subjected to standard fluorographic techniques (Hames and Rickwood 1981). Staph A was washed and resuspended (10% w/v) in PBS-NDBS (1 X PBS, 0.5% NP-40, 1% deoxycholate, 0.1% SDS, 1mg/ml bovine serum albumin (BSA), 5 mM methionine).

ProNT/N was immunoprecipitated and analyzed from metabolically labeled PC12 cells (see below) by essentially the same method, except that cell extracts (equal counts brought up to 200 µl with PBS-SDS) were incubated with the appropriate amount of antiserum or IgG fraction. The wash and cushion buffers also contained 5 mM leucine with or in place of methionine.
To make radiolabeled molecular weight markers, pGEM-crhNT was linearized with the following restriction enzymes; Nhe I, Eco RV, Hha I, Bgl II, and Dde I. After transcription with T7 RNA polymerase, these RNAs were translated in rabbit reticulocyte lysate and immunoprecipitated as described above to produce $^{35}$S-labeled peptides of the following predicted molecular weights, 19670 (full length), 14030, 7542, 3850, and 2548 daltons, respectively.

In vivo metabolic labeling of PC12 cells. For continuous labeling, PC12 cells (~1 X 10⁶) were pelleted (500 X g) at 37 °C and washed once with 5 ml of DMEM lacking either leucine or methionine (Sigma) and dialyzed sera (10% horse and 5% fetal bovine), resuspended in 0.8 ml of the same media and placed in a 6 well culture plate. Sera was dialyzed in 1 X Earle's balanced saline (1 X EBS = 2.65 g CaCl₂, 2 g MgSO₄, 4 g KCl, 68 g NaCl, 1.4 g NaH₂PO₄ and 10 g glucose per liter dH₂O). These cells were incubated for 15 minutes in a CO₂ incubator at 37 °C. An additional 200 µl of the medium described above, containing $^3$H-leucine or $^{35}$S-methionine was added to the cells and the cells incubated for various times. At the end of the labeling time, cells were transferred to a 1.5 ml centrifuge tube, washed with ice cold 1 X PBS and placed on ice. The cells were pelleted (500 X g) at 4 °C for 5 minutes, resuspended in NP-40/DOC lysis buffer (1% NP-40, 0.5% deoxycholate in 1 X PBS) and incubated on ice for 30 minutes. The extracts were clarified by two successive centrifugations, 1500 X g followed by 10,000 X g, both for 5 minutes at 4 °C. Labeling efficiency was determined by TCA precipitation (see below) and equal counts were immunoprecipitated. Pulse-chase experiments were done essentially the same except that after a 30
minutes labeling period, cells were washed and incubated in serum-supplemented DMEM containing 10 X unlabeled methionine (10 mM).

**Nuclear run-on transcription assay.** Nuclei were isolated essentially as described (Ausubel et al. 1989; Greenberg et al. 1985). PC12 cells (approximately 5 X 10^7/time point) were harvested and washed twice with ice cold 1 X PBS. Cells were lysed in 4 ml NP-40 lysis buffer (0.5% Nonidet P40, 10 mM NaCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, pH 7.4) by gently vortexing (Penman 1969). Nuclei were pelleted by centrifugation (500 X g) for 5 minutes and the supernatant was transferred to a separate tube (cytoplasmic fraction). The nuclear pellet was washed in 4 ml NP-40 lysis buffer and repelleted as above. Nuclei were resuspended in 180 µl ice cold 2 X transcription buffer and immediately labeled (1 X transcription buffer = 35% glycerol, 10 mM Tris-HCl, 5 mM MgCl₂, 80 mM KCl, 0.2 mM EDTA, pH 7.5) (Celano et al. 1989)

*In vitro* labeling of nascent NT/N transcripts was performed as described (Celano et al. 1989). Essentially, nuclei in 1 X transcription buffer containing 0.5 mM dithiothreitol (DTT), 4 mM each of ATP, GTP, CTP and 20 µl (200 µCi) of α-[^32]P-UTP (3000 Ci/mmol) were incubated at 30 °C for 15 minutes with vigorous shaking. To this mixture, 10 µl of RNase-free DNase (10 mg/ml) and 10 µl of CaCl₂ (20 mM) were added and the mixture incubated at 30 °C for an additional 5 minutes. The reaction was ended by adding 2 µl of proteinase K (10 mg/ml), 25 µl 10 X SET (1 X SET = 0.5% SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) and 5 µl yeast RNA followed by incubation at 37 °C for 30 minutes. RNA was isolated by solubilizing nuclei in 550 µl guanidinium solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium sarcosyl, 100 mM...
β-ME, pH 7.0), 90 μl sodium acetate (pH 5.0), 900 μl phenol, and 180 μl chloroform on ice for 15 minutes. After centrifugation (10,000 X g) for 5 minutes, the aqueous phase was transferred to a microfuge tube and RNA was precipitated with 1 volume of isopropanol at -70 °C for 30 minutes. The RNA was recovered by centrifugation (10,000 X g) for 15 minutes, resuspended in 360 μl guanidinium solution, 40 μl sodium acetate (pH 5.0) and reprecipitated with 400 μl isopropanol as before. The RNA pellet was washed with 70 % ethanol, dried and resuspended in 100 μl diethylpyrocarbonate (DEPC)-treated dH2O. Equal counts (approximately 3 X 10^7 CPM) were hybridized to plasmid DNA bound to Zetabind nylon membranes using the protocol described for RNA blot analysis.

The following plasmid DNAs were used to detect specific run-on transcripts: Full length rat NT/N (rbNT), mouse c-fos (pGEMfos3), and c-jun (JAC1.1) cDNAs containing 700, 2140 and 2600 nt of coding sequences, respectively. A 366 nt Eco RI-Sph I fragment of mouse α-tubulin (MAT α1, Villasante et al. 1986)) and pGEM4 vector DNA were used as a positive and negative controls, respectively. Plasmid DNA at 5 μg/slot was denatured and nicked by boiling in 50 μl 0.1 N NaOH for 5 minutes. After denaturation, 360 μl of ice cold 6 X SSC (1 X SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0) was added and 400 μl of this mixture was loaded on Zetabind nylon membranes using a slot-blot apparatus (Schleicher and Schuell, Keene, N.H.). Filters were air-dried, baked under vacuum for 2 hours at 80°C and prewashed as described for RNA blot analysis.
RNA Isolation. RNA was prepared by a modification of the LiCl/urea procedure (Rhodes 1975). To isolate total RNA, cells were harvested, washed with 5 ml ice-cold 1 X PBS, resuspended in 1 ml of 1 X PBS, and lysed by the addition of an equal volume of 8 M urea, 8 M LiCl. The solution was sonicated to reduce viscosity (Branson J-17A sonicator, microprobe, 5 setting), incubated overnight at 4 °C, and the RNA was recovered by centrifugation at 10,000 X g for 30 minutes. RNA was resuspended in TES buffer (10 mM Tris-HCl, 5 mM EDTA, 0.1% SDS, pH 7.4), extracted with phenol and chloroform, ethanol precipitated, and dissolved in DEPC-treated H2O. For the preparation of nuclear and cytoplasmic RNA, nuclei were isolated as described for the nuclear run-on transcription assay. The nuclear pellet was washed with 2 ml double detergent buffer (1% NP-40, 0.5% deoxycholate, 10 mM NaCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH 7.4), the wash supernatant was pooled with the cytoplasmic fraction, and the final nuclear pellet was resuspended in 2 ml double detergent buffer. RNA was extracted from each fraction by addition of an equal volume of 8 M urea, 8 M LiCl and processed as described above. Synthetic sense strand E11-NT RNA (see below) was added just prior to addition of the LiCl/urea solution as an internal control for RNA recovery (1 ng/10^7 nuclei for nuclear RNA, 4 ng/10^7 cells for cytoplasmic and 5 ng/10^7 cells for total RNA). PolyA+ RNA was isolated on oligo(dT) affinity columns as described (Sambrook et al. 1989).

RNase Protection Assay. Riboprobe synthesis and RNase protection assays were performed as described (Melton et al. 1984; Zinn et al. 1983). ^32P-labeled antisense probe RNA was transcribed using T7 RNA polymerase and α-^32P-CTP following linearization of the pGEM-E11-NT or pGEM-rNT4 with Eco RI. Sense
RNA standards were transcribed using SP6 RNA polymerase following linearization of the pEII-NT template with Rsa I. RNA samples (10-25 µg nuclear RNA, 5-10 µg cytoplasmic or total RNA) were hybridized overnight with \(^{32}\text{P}\)-labeled probe (100,000 cpm) in hybridization buffer (80% formamide, 40 mM PIPES, pH 6.7, 0.4 M NaCl, 1 mM EDTA) at 45 °C. Samples were digested with nuclease P1 (20 µg/ml) and ribonuclease T1(1µg/ml) as described (Ausubel et al. 1989; Brewer and Ross 1988), extracted with phenol and chloroform, ethanol precipitated after the addition of carrier yeast RNA (10 µg), and analyzed on 8M urea/6% polyacrylamide sequencing gels. RNase protection products were quantitated either using a βetascope 603 Blot Analyzer (βetagen, Waltham, MA) or by scanning autoradiograms using a Hoeffer GS300 densitometer and GS-365 PS Data System. Signals resulting from the internal standard RNA were used to correct for small variations in sample recovery.

\(^{32}\text{P}\)-labeled antisense RNA (623 nucleotides) corresponding to the α-tubulin gene was transcribed with SP6 RNA polymerase after linearization of the template with Hind III. Hybridization to RNAs containing intron 3 results in a 562 nucleotide protected fragment while hybridization to spliced RNAs results in a 383 nucleotide product. To produce \(^{32}\text{P}\)-labeled RNA markers, a fragment of the rat gene, rNT 1.8 subcloned into pGEM 4 (Kislauskis et al. 1988), was linearized with the following restriction enzymes; Dra I, Hinf I, Ava II, Pvu II, Hae III, Rsa I, and Hind III. Following transcription with SP6 RNA polymerase, RNA fragments of the following molecular weights were obtained; 496, 305, 242, 196, 158, 113, and 65 nt, respectively.
RNA Blot Analysis. Total RNA (20 μg) was size-fractionated on 1% agarose gels containing 0.6 M formaldehyde and blotted onto Zetabind nylon membranes using standard procedures (Sambrook et al. 1989). Filters were incubated in 0.1 X SSC, 1% SDS at 65 °C for 1 hour, prehybridized in 50% formamide, 3 X SSC, 50 mM sodium phosphate, pH 7.0, 1% SDS, 5 X Denhardt's, 10 mM EDTA, and 1 mg/ml yeast RNA for 4 hours at 45 °C and hybridized overnight at 45 °C with 32P-labeled riboprobes in prehybridization buffer containing 1 X Denhardt's. Filters were washed with several changes of 1 X SSC, 0.1% SDS at room temperature followed by one or more washes with 0.1 X SSC, 0.1% SDS, and 0.1% sodium pyrophosphate at 60-65 °C. AP-1 cDNAs subcloned into RNA expression vectors were used to synthesize 32P-labeled antisense RNA probes as described (Ausubel et al. 1989). T7 RNA polymerase was used to transcribe c-fos (pGEMfos3), fra-1, and c-jun (JAC.1) after linearization of templates with either Bst EII (c-fos), Stu I (fra-1) or Nae I (c-jun). SP6 RNA polymerase was used to transcribe fos B (pGEM-AC113), fra-2, and jun-B (465.20) after linearization with Sma I, Bam HI, and Nar I, respectively. A 32P-labeled DNA probe for jun-D was prepared by random primed synthesis of a Sph I cDNA fragment of pXHJ-12.4 (Ryder et al. 1989).

Analysis of protein synthesis inhibition efficiency. PC12 cells were treated with either anisomycin or cycloheximide for various times and protein synthesis, RNA synthesis and cell viability was analyzed. Protein synthesis was determined by measuring the incorporation of 35S-methionine into acid insoluble material. 1 X 10⁶ cells were labeled with 35S-methionine (50 μCi) in serum-supplemented DMEM at 37 °C in a CO2 incubator. At the appropriate time
points, cells were harvested, washed in 1 X PBS, solubilized in 1 ml SUM buffer (1% SDS, 0.5 M urea, 1% β-ME) and boiled for 5 minutes. 20 μl of this solution were precipitated with 10% trichloroacetic acid (TCA) on ice for 30 minutes, passed through glass fiber filters and washed with ice-cold 5% TCA. The filters were air-dried, added to scintillation fluid, and counted on a LKB Rackbeta scintillation counter (Pharmacia LKB Biotechnolgies, Piscataway, N.J.). RNA synthesis was determined similarly, except cells were pulse-labeled with 20 μCi \(^3\)H-uridine. In both cases, acid-insoluble counts were compared to total cellular counts to adjust for the effects of protein synthesis inhibitors on methionine and uridine uptake (McKnight 1978). Cell viability was determined by dye exclusion. Viable cells were counted on a hemocytometer after treatment with 0.04% trypan blue in 1 X PBS.

**Protein Kinase C determinations.** PKC activity in PC12 cells was determined as described (Palfrey and Waseem 1985). Approximately 1 X 10^7 induced PC12 cells were harvested, washed in 10 ml ice cold 1 X PBS and resuspended in 1 ml ice cold extraction buffer (50 mM Tris-HCl, 50 mM β-ME, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EGTA, pH 7.5). This suspension was sonicated for 2 minutes and centrifuged (800 X g) for 10 minutes at 4 °C. The supernatant was decanted to a new tube and centrifuged (120,000 X g) for 30 minutes at 4 °C to pellet the membrane fraction. The supernatant was saved (cytosolic fraction) while the membrane pellet was resuspended in 1 ml ice cold extraction buffer with 10 μl Triton X-100 and incubated on ice 30 minutes. Both fractions were stored at -70 °C.
In vitro phosphorylation of histone H1 was used to assay for PKC activity in the cytosolic and membrane fractions of PC12 cells. Equal amounts of protein (5 μg) from each fraction (determined by Bradford assay) were mixed with 800 μl of 100 mM Tris-HCl, 5 mM MgSO4, 0.2 mg/ml histone H1, 50 μM γ-32P-ATP (500 mCi/mmol), pH 7.4. These reactions were incubated for 5 minutes at 30 °C and then placed on ice to stop the reaction. For PKC specific phosphorylation, 500 μM CaCl and 50 μg/ml phosphotidylserine were included, whereas 500 μM EGTA was included as a non-specific phosphorylation control. 20μl of the above samples were added to an equal volume of 2 X SDS/gel loading buffer, boiled for 5 minutes and loaded on 15% SDS/PAGE (Laemmli 1970). Phosphorylated histones were detected by autoradiography and quantitated by scanning the autoradiograms using a Hoeffer GS300 densitometer and GS-365 PS Data System.
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