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MOLECULAR AND TRANSGENIC STUDIES ON TYROSINE HYDROXYLASE GENE REGULATION: ANATOMICAL, DEVELOPMENTAL AND FUNCTIONAL ANALYSES.

by

NAN MIN

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Date — Chair of Examining Committee: Dr. R.J. Bodnar

5-1-97 — Kay Deaux

Date — Executive Officer in Psychology: Dr. K. Deaux

Dr. T.H. Joh

Dr. H.J. Son

Dr. T.E. Frumkes

Dr. V. Luine

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

MOLECULAR AND TRANSGENIC STUDIES ON TYROSINE HYDROXYLASE GENE REGULATION: ANATOMICAL, DEVELOPMENTAL AND FUNCTIONAL ANALYSES.

by

Nan Min

Adviser: Professor Richard Bodnar

Catecholaminergic neurotransmission regulates many physiological function in the central and peripheral nervous system. Catecholamines (CA) are important in the modulation of blood pressure, responses to stress, motor function, memory, learning, mood, appetite, and the mediation of psychotropic drug action. From its precursor tyrosine, CA biosynthesis occurs via a series of enzymatic reactions the first and rate-limiting step catalyzed by tyrosine hydroxylase (TH). Drugs that have major effects on CA neurotransmission are being used in the treatment of psychiatric and neurological conditions, such as schizophrenia , major affective disorders and Parkinson's disease. Pharmacological approaches may lack receptor specificity and cross-react across different neurotransmitter systems. Thus, I used molecular approaches to study CA neurotransmission function by investigating *in vivo* regulation of the TH gene. TH undergoes precise temporal, spatial and functional regulation at the transcriptional level.

To identify genetic regulatory elements of the rat TH gene that contribute to the regulation of its expression in CA cells, three fusion genes containing 0.15 kb, and 2.4 kb and 9.0 kb of the 5' flanking sequences of the rat TH gene linked to the *E. coli* lacZ (β–galactosidase) reporter gene were microinjected into the pronuclei of fertilized embryos to generate transgenic mice. Analyses of the transgene expression in the CNS revealed that the 9.0 kb promoter, but not the smaller sequences, drove the reporter expression to

catecholamine cells in the CNS of transgenic mice. In addition, the 9 kb TH promoter also directs CA cell lineage-specific expression of lacZ during embryogenesis. Thus, it appears to contain the cis-acting DNA elements necessary to interact with the environmental cues that specify differentiation. Finally, these TH 5' sequences are also sufficient to mediate cell-specific trans-synaptic regulation of reporter gene expression in two paradigms: downregulation of expression in the glomerular layer of the olfactory bulb and upregulation of expression in the locus ceruleus and VTA after reserpine administration. Thus, the 9.0 kb of rat TH 5' flanking sequences contain the important DNA elements to mediate appropriate spatio-temporal and trans-synaptic expression of a reporter gene to catecholamine cell in the CNS of transgenic mice.

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New York, May 1st 1997

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I. INTRODUCTION

1.1 Foreword

Central and peripheral catecholaminergic neurotransmission regulates many physiological functions. In the 1920s, catecholamines were first suggested as neurotransmitters by the demonstration of the release of catecholamine-like compounds from nerves (Loewi, 1921). Two decades later, von Euler identified the neurotrasmitter in sympathetic nerves as norepinephrine. The elucidation of functional roles for catecholamines was advanced by several developments. Fluorescent spectroscopy was developed as a sensitive and specific method for measuring catecholamines in the brain and other tissues. Reserpine, a drug introduced for the treatment of hypertension and schizophrenia, was found to deplete monoamines from the brain. Axelrod demonstrated that sympathetic nerves can be labeled with radioactive norepinephrine. In the 1960s, Falck and Hillarp devised methods for visualizing catecholamines in neurons by a histofluorescent technique.

From its precursor tyrosine, catecholamines biosynthesis occurs via a series of enzymatic reactions to produce dopamine, norepinephrine and epinephrine. The first and rate-limiting step is catalyzed by tyrosine hydroxylase (TH). Catecholamine levels remain steady even under marked alteration of neuronal activity by a precise control of catecholamine biosynthesis. Catecholamines are metabolized in the body by enzymatic activity and reuptake. The process of uptake and storage of catecholamine explain the actions of many drugs. Cocaine and antidepressant drugs blockthe reuptake of ^{3}H-norepinephrine. Amphetamine is also found to block reuptake as well as release catecholamines.

Investigating the mechanism of action of drugs effective in the treatment of psychiatric conditions, specially schizophrenia and major affective disorders, has provided important information about abnormalities associated with behavioral pathology. These drugs have major effects on catecholamine metabolism, thus implicating abnormal catecholaminergic neurotransmission in the etiology of these disorders. This hypothesis stemmed from the observation that tricyclic drugs and monoamine oxidase inhibitors prevent the inactivation of norepinephrine and also aleviate depression and that reserpine, a drug which depletes catecholamines, can induce depression. Most recently, attention has focused on the sensitivity of catecholamine receptors in affective disorders and the changes induced by antidepressants drugs which appear to normalize disturbed CA receptor sensitivity as mood improves. The recognition that antipsychotic drugs can block dopamine receptors stimulated productive research toward treating this complex disease.

Understanding the basic mechanisms in catecholamine neurotransmission led to the development of an effective therapy for Parkinson's disease, which is characterized by selective degeneration of dopaminergic nigro-striatal neurons. In 1959, Carlson found that the administration of reserpine to rats markedly reduced the dopamine content in the caudate nucleus and caused motor tremors. L-dopa adminstration, a dopamine precursor that crosses the blood-brain barrier, aleviated the tremors. Hornykiewicz examined post-mortem Parkinson's patient brains and determined that hardly any dopamine was present in their caudate nucleus. These observations led Cotzias to conduct experiments showing that prolongued administration of high doses of L-dopa relieves the symptoms of Parkinson's disease.

Despite the progress in catecholamine research brought by many scientific advances, much remain to be addressed. These powerful new technologies set the stage for investigators to ask more sophisticated and penetrating questions regarding the role of catecholamine in normal and pathological states. The use of pharmacological approaches to delineate catecholamine function and rectify pathological states has the limitation on relying in agonists and antagonist that lack receptor specificity and cross-react across different neurotransmitter systems. Emerging molecular technologies can circumvent some of the specificity problems. Thus, I used molecular approaches to study catecholamine neurotransmission function. The central theme of this dissertation is to investigate *in vivo* regulation of the Tyrosine Hydroxylase gene. Tyrosine Hydroxylase (TH), the first and rate limiting enzyme in the biosynthesis of the catecholamines, undergoes precise temporal, spatial and functional regulation at the transcriptional level. As a first step to understand the processes involved in the regulation of the TH gene, transgenic approaches were used to identify important sequences for the expression of the TH gene. The data demonstrated that 9 kilobase-pairs (kb) of 5' flanking sequences of the rat TH gene drive appropriate cell specific, developmental stage-, and trans-synaptic expression of a reporter gene. Thus, these DNA sequences contain some of the necessary DNA cis-elements to mediate TH expression. Unraveling the events which regulate TH, and thus catecholamine levels, at the molecular level may shed light into basic mechanisms underlying catecholamine modulated behavior.

1.2 Catecholamines

A fundamental property of neuronal cells is the ability to communicate with other cells via chemically mediated neurotransmission. In 1901, Langley identified an active substance "sympathin" released from sympathetic nerves which mimicked the effect of nerve stimulation. It was later identified by von Euler (1948) as the compound norepinephrine which, along with dopamine and epinephrine, is a catecholamine neurotransmitter. The catecholamines - characterized by a chemical structure containing a catechol (a benzene ring with two adjacent oxygen groups) and an amine side chain - act as neurotransmitters and/or neurohormones in the central nervous system (CNS) and peripheral tissues.

Catecholamines :

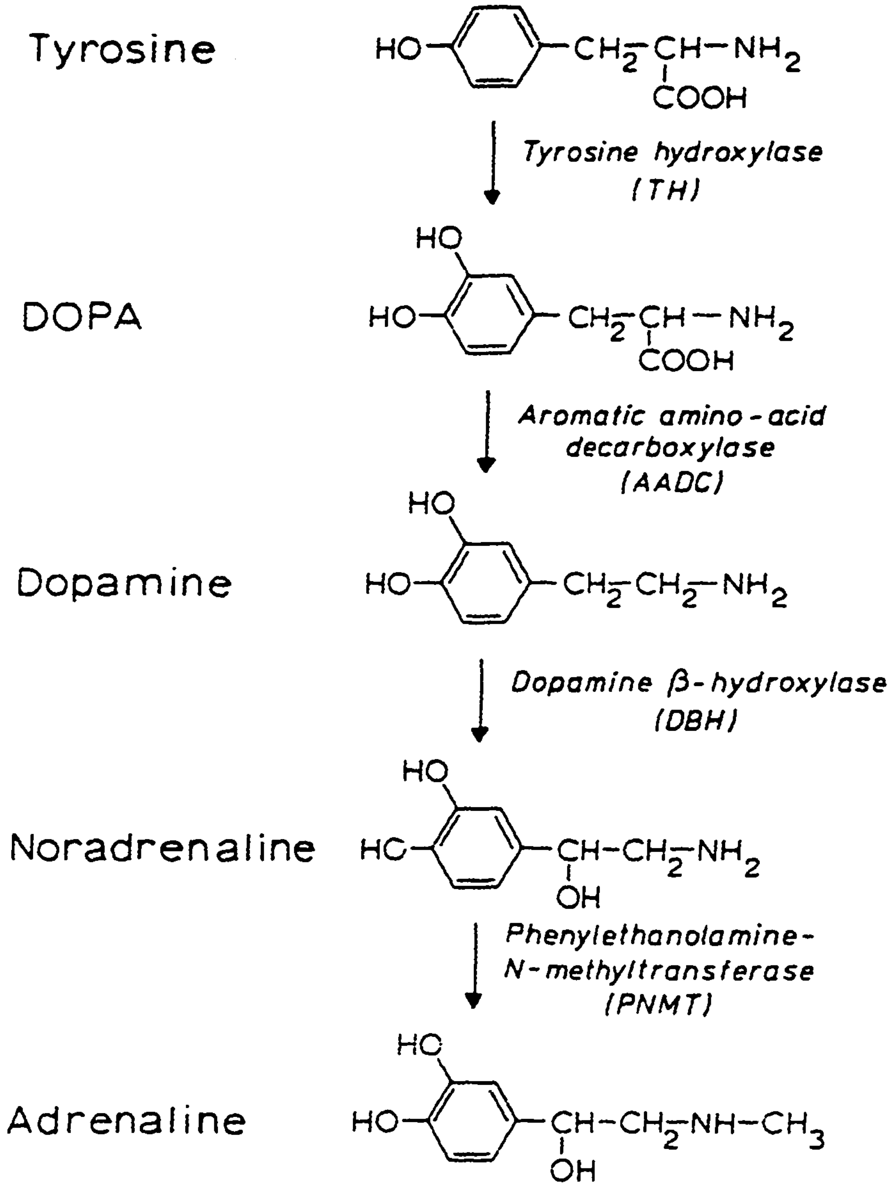

Fig 1.1:
Biosynthetic pathway of the catecholamines
Dopamine, Noradrenaline and adrenaline

1.2.1 Biosynthesis

The biosynthesis of catecholamines, as first postulated by Blaschko in 1939, involves a sequential enzymatic pathway from their common precursor L- Tyrosine, an essential dietary amino acid. The catecholamine biosynthetic pathway is illustrated in Figure 1.1. In 1964, Nagatsu and colleagues (Nagatsu, Levitt, & Udenfriend, 1964) demonstrated that TH catalyzes the first and rate-limiting step in the biosynthesis of the catecholamines. Except for Amino Acid decarboxylase (AADC; EC 4.1.1.28) which is expressed ubiquitously (Rahman, Nagatsu, & Kato, 1981), the expression of catecholamine biosynthetizing enzymes is cell- specific. TH is expressed in all cells that synthesize catecholamines. In the presence of oxygen and biopterin, TH catalyzes the addition of a hydroxyl group to L- Tyrosine, modifying it into L-Dopa. Next, AADC catalyzes the removal of the carboxyl group from L-Dopa to form dopamine. This is the final step in the biosynthetic pathway for dopamine-containing cells. In cells that synthesize epinephrine or norepinephrine, dopamine-β- hydroxylase (DBH) catalyzes an additional step which converts dopamine into norepinephrine. Limited only to epinephrine-producing cells, phenylethanolamine N-methyltransferase (PNMT) catalyzes the transfer of a methyl group from S-adenosylmethionine to the nitrogen of norepinephrine, converting it to epinephrine.

1.2.2 Anatomical Localization

Our understanding of the function of catecholamine-containing neurons has been advanced by the neuroanatomical visualization of these neurons and their axonal projections. Numerous investigators have used the Falck and Hillarp technique (Falck, Hillarp, Thieme, & Torp, 1962), which takes advantage of the fact that catecholamines form intensely fluorescent products in the presence of formaldehyde, to map the distribution of catecholaminergic cell bodies and pathways in the brain. Monoamines were established as neurotransmitters by histofluorochemical localization of the catecholaminergic neurons (Dahlstrom, & Fuxe, 1964). With the advent of tract-tracing and immunocytochemical methods utilizing, respectively, radiolabeled tyrosine topically injected into the cell body or terminal fields and antibodies directed either to TH , or to catecholamines itself, the mapping process has become much more specific. Furthermore, the use of specific antibodies raised against the purified biosynthesizing enzymes has permited a more precise localization of those enzymes using immunocytochemistry (ICC).

The combined data obtained using the histological techniques mentioned above revealed TH expression, thus catecholamine phenotype, to be limited to discrete sets of cells in the CNS and PNS. In the CNS, major expression sites include dopaminergic cells in the midbrain (substantia nigra and ventral tegmental area), diencephalon (hypothalamic nuclei), olfactory bulb (juxtaglomerular neurons), and the retina (amacrine cells), noradrenergic neurons in the pons (locus coeruleus), and the adrenergic and noradrenergic neurons in the medulla (C1/A1 and C2/A2). In the periphery, TH expression is found in the adrenal medullary chromaffin cells as well as sympathetic ganglia. It is therefore very likely that the TH gene requires multiple regulatory elements to allow for the variety of anatomically and functionally distinct cells that display the catecholamine phenotype.

Further insight in regards to the anatomical distribution, as its relates to function, was revealed by the use of a catecholaminergic-specific neurotoxin 6-hydroxydopamine (6-OHDA) . Using desipramine to protect noradrenergic cells from 6-OHDA, dopaminergic neurons can de selectively destroyed. Thus, the pathways of noradrenergic neurons and dopaminergic neurons can be established by a process of elimination.

(a) The Noradrenergic system (NA)

The NA systems can be subdivided into two groups: the locus coeruleus system and the lateral tegmental system, composed of NA neurons located in the dorsal motor vagus, nucleus tractus solitarius, and adjacent tegmentum and lateral tegmentum (Moore and Bloom, 1979). Major focus will be placed into delineating the locus coeruleus system to set up for the studies undertaken. Anatomical evidence obtained from fluorescence and immunohistochemistry in conjunction to physiological data obtained by iontophoresis characterize the NA neuron system as having a remarkably extensive projection system, with high degree of collateral arborization. The sites of termination spread to the spinal chord, brainstem, cerebellum, hypothalamus, thalamus, basal telencephalom, and entire isocortex.

The locus coeruleus, a compact cell group located in the rostral pons, contains half the NA neurons in the brain, about 1,500 on each side of the brain in the rat and 12,000 in human. Based on evidence obtained from histochemical techniques (Olson & Fuxe, 1971; Ungerstedt,1971) immunofluorescent techniques (Swanson & Hartman, 1975) and autoradiographic tracing (Segal et al, 1974) , the fibers from the locus coeruleus form the five major noradrenergic tracts, and it has both ascending and descending projections.. The three ascending tracts are the central tegmental tract (dorsal bundle), a central gray dorsal longitudinal fasciculus tract and a ventral tegmental-medial forebrain bundle tract. These ascending tract are mostly ipsilateral and follow vascular and fascicular routes to innervate all cortices, thalamic and hypothalamic nuclei, and the olfactory bulb. Another major fascicle ascends in the superior cerebellar peduncle to innervate the cerebellar cortex. The fifth major tract has descending projections that go primarily to the ventral horn spinal chord, and the brain stem itself (sensory nuclei).

(b) The Dopamine System (DA)

Located in the mesencephalon and diencephalon, the DA cell systems contrast with NE by appearing to be "local" systems with highly specified, topographically organized projections (Moore & Bloom, 1978). For the purpose of the studies undertaken, special focus will be placed on the mesencephalic DA system.

For ease of classification, the DA system can be viewed in terms of the efferent DA fibers. The interneurons systems with ultra-short projections, consist of the interplexiform amacrine-like neurons which connect inner and outer plexiform layers of the retina , and the periglomerular DA cells in the olfactory bulb, which link together mitral cell dendrites in separate glomeruli. The intermediate-lenght projection systçms include: the (a) tuberohypophysial DA cells, which projects from arcuate and periventricular nuclei into the intermediate lobe of the pituitary and the median eminence; (b) the incertohypothalamic neurons, which link the dorsal and posterior hypothalamus with the dorsal anterior hypothalamus and lateral septal nuclei; and (c) the medullary periventricular group, which include those dopamine cells around the dorsal motor nucleus of the vagus nerve, the nucleus tractus solitarius, and the cells scattered in the tegmental periaqueductal grey. The long lenght projection systems are the long projections linking the ventral tegmental and substantia nigra DA cells with three principal sets of targets: (a) nigro-striatal, the neostriatum (mainly the caudate and putamen), (b) mesocortical, the limbic cortex (medial prefrontal, cingulate, and entorhinal areas), and (c) mesolimbic, other limbic structures (the regions of the septum, olfactory tubercle, nucleus accumbens septi, amygdaloid complex, and piriform cortex.

1.2.3 Importance in Physiological Processes

Despite the restricted localization of catecholamine neurons to specific neural nuclei, which represent less than 1% of the total neuronal population, catecholaminergic innervation is widespread and plays a variety of roles in the function of the Central Nervous System (CNS) (Cooper, Bloom, & Roth, 1991; Moore, 1982). For instance, the epinephrine neurons of the medulla contribute to the regulation of blood pressure and cerebral blood flow (Reis, et al., 1984), locus coeruleus norepinephrine neurons in the pons are involved with the control of behavioral awareness and attentional processes, dopamine neurons in the hypothalamus are critical for regulation of pituitary hormonal function (Cooper, et al., 1991), and midbrain dopamine neurons contribute to the coordination of motor behavior. The importance of monoaminergic neurotransmission for normal brain functioning is further shown by the alteration of its activity or regulation in several neurological and psychiatric disorders including schizophrenia (Iversen , 1978; Carlsson, 1987), bipolar disorder (Egeland, et al., 1987), depression, and Parkinson's disease (Hornykiewicz, 1973).

The dopaminergic mesocorticolimbic function is involved in several aspects of behavior, in particular as it relates to reinforcing or associative properties, including: feeding (D'angio, & Scatton, 1989; Hoebel, Hernandez, Schwartz, Mark, & Hunter, 1989), reward (Wise, & Rompre, 1989), sensory motor gating (Swerdlow, Braff, Masten, & Geyer, 1990). Stress alters dopamine release in the n. accumbens and cortex (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989), and also in the amygdala (Ray, Henke, & Sullivan, 1987).

Olds & Milner (1954) suggested that rewarding stimulation activated brain circuitry relevant to natural incentives such as food and sexual contact. Several addictive substances- stimulants, opiates, nicotine, phencyclidine, and cannabis- synergize with rewarding medial forebrain bundle stimulation, and elevate - as it does stimulation itself (

Bauco et al,1994; Blaha & Philips,1990; Gratton et al., 1988) - dopamine concentrations in the nucleus accumbens and other dopamine terminal fields.

The deficit in the dopaminergic neurotransmission in the nigrostriatal pathway caused by the selective neuronal loss in the dopamine-containing cells in the substantia nigra is the relevant feature in Parkinson's disease. Such loss is correlated to pathophysiological manifestations such as rigidity, hypokinesia, and tremor. Amphetamine usually increases locomotor activity and stereotypy (Carlsson, 1970)

Based on physiological data, locus coeruleus neurons are selectively activated in vigilance tasks; thus it suggest that the noradrenergic system is involved in sustained arousal, attention (Aston-Jones, Rajkowski, Kubiak, & Alexinsky, 1994).

1.3 Tyrosine Hydroxylase

TH is a mixed-function oxidase that requires molecular oxygen and tyrosine as its substrates and biopterin as its cofactor. It catalyzes the addition of a hydroxyl group to the meta position of tyrosine, thus forming 3,4-hydroxy-L-phenylalanine (L-Dopa). TH has a K_m value for tyrosine in the micromolar range. TH is primarely a soluble enzyme, localysed in the cytosol of catecholamine-containing neuronal processes. Interactions with membrane constituents such as phosphatidylserine, or polyanions, such as heparin sulfate, have been shown to alter its kinetic characteristics.

1.3.1 Biochemical Properties

Despite their diversity in distribution and function, the catecholamines - dopamine, norepinephrine and epinephrine - are linked by a common biosynthetic pathway. The first step is the conversion of L-tyrosine to L-Dopa. Although the chemical reaction was proposed by Blaschko (Blaschko, 1957), the enzyme responsible for this reaction had long eluded indentification . In part, it was due to the high rate of auto-oxidation of tyrosine in boiled tissue homogenates. In fact, it was suggested that the hydroxylation of tyrosine *in vivo* occurred non-enzymatically (Fellman, & Devlin, 1958). However, Nagatsu and colleagues could not be persuaded that such an important step in catecholamine biosynthesis could occur non-enzymatically, and therefore unregulated. To demonstrate the existence of this elusive enzyme predicted as stereospecific, Undenfriend and Nagatsu developed a highly sensitive isotopic assay for the enzyme activity using the isomer D-(14C) tyrosine as the control. After determination that the enzyme was both membrane bound and soluble, they successfully isolated TH from bovine adrenal tissue homogenates by ammonium sulfate fractionation (Nagatsu, et al., 1964). Since this initial characterization, TH has been purified to homogeneity from several sources including rat striatum (Joh, Park, & Reis, 1978), and rat pheochromocytoma cells , PC12, (Markey, Kondo, Shenkman, & Goldstein, 1980).

TH requires tetrahydrobiopterin and molecular oxygen as obligatory cofactor and substrate, respectively, for the tyrosine hydroxylation reaction (Nagatsu, et al., 1964). The requirement of a tetrahydroxydropterin as a cofactor of the hydroxylating process was first discovered by Seymour Kaufman studying rat liver phenylalanine hydroxylase (PAH)(Kaufman, 1959).

The activity of TH, isolated from bovine adrenal or caudate nucleus, is stimulated by the addition of iron.

1.3.2 Structure

(a) TH protein

The molecular weight of the purified monomeric TH subunit, isolated from rat or bovine tissue, has been reported to be in the range of 59 - 61 kD (kilodalton) (Joh, et al., 1978; Markey, et al., 1980; Okuno, & Fujisawa, 1982; Richtand, Inagami, Misono, & Kuczenski, 1985; Vulliet, Langan, & Weiner, 1980). Analysis of the native enzyme isolated from adrenal gland and brain by gradient gels under non-denaturing conditions revealed a band in the range of 210 -220 kD (Markey, et al., 1980), or at 260 kD (Okuno, et al., 1982). Furthermore, cross-linking patterns following denaturing gel electrophoresis (SDS-PAGE) suggested an association of subunits in a tetramer conformation (Okuno, et al., 1982; Richtand, et al., 1985). Since the monomeric form is active (Joh, & Reis, 1975) and a single mRNA directs the production of the active enzyme (Horellou, Guibert, Leviel, & Mallet, 1986), it was suggested that TH might be a tetramer of identical subunits.

Proteolysis of the TH enzyme by trypsin generates a 34kD fragment which has removed a 17 kD N-terminal and a 5kD C-terminal fragments (Vigney, & Henry, 1981). Thus, the TH monomer is comprised of two functional regions: the 34kD central catalytic region and a 17 kD N-terminal regulatory region which regulates enzyme activity through phosphorylation and directs substrate specificity (Abate, Smith, & Joh, 1988).

(b) TH gene

The isolation of TH cDNA and genomic clones allowed for analysis of the molecular structure of the TH gene and shed light into its regulation at the translational level.

i. cDNA

Taking advantage of TH abundance in pheochromocytoma cells (PC12), partial cDNA clones of rat TH were isolated by screening expression libraries with TH antibody (Lamoroux, et al., 1982; Lewis, Tank, Weiner, & Chikaraishi, 1983). Subsequently, a full lenght rat TH cDNA was isolated, encoding 498 aminoacids (Grima, Lamouroux, Blanot, Biguet, & Mallet, 1985). The predicted protein was 55,905 kd which was lower than the molecular weight of the enzyme determined by SDS-PAGE (59-61 kd).

Characterization and cross species structural comparisons betweeen cDNAs clones indicate that 92% of rat and human aminoacid sequence are identical (Brown, Coker, & K.L., 1987; Grima, et al., 1985; Grima, et al., 1987; Kobayashi, et al., 1988; O'Malley, et al., 1987); quail and mammalian TH are 75-77% identical (Fauquet, Grima, Lamoroux, & Mallet, 1988); while Drosophila TH has only a 48-49% homology with vertebrates (Neckmeyer, & Quinn, 1989). The central and two N-terminus regions of the TH are highly conserved suggesting that these regions are critical for the integrity of enzyme activity: for instance, catalysis, enzyme regulation, or proper folding.

ii. Genomic DNA

The genomic structure of the TH gene was characterized, as clones from several species were isolated: rat (Brown, et al., 1987), bovine (D'Mello, Turzai, Gioio, & Kaplan, 1989), and human (O'Malley, et al., 1987 ; Stoll, & Goldman, 1991). There is a high degree of conservation of genomic structure among all species studied so far. The TH gene spans approximately 8 kb, and it is present in a single copy containing 13 primary exons (51 to 421 bp in lenght) and 12 introns.

iii. Comparison with related hydroxylases:
Phenylalanine (PAH) and Tryptophan (TPH) hydroxylases

Members of the biopteridin-dependent aromatic amino acid hydroxylase family (TH, TPH and PAH) share many physical and biochemical properties. Comparison of the TH sequence with PAH (Dahl, & Mercer, 1986; Ledley, Dilella, Kwok, & Woo, 1985) and TPH (Grennet, Ledley, Reed, & Woo, 1987) reveals a high degree of conservation in their primary structures. In addition, since the amino acid sequences of the middle portion (amino acids 187-324) of TH gene showed the highest homology to other aromatic amino acid hydroxylases (approximately 80%), these central domains might represent the catalytic site of the aromatic amino acid hydroxylases. The non-homologous domains are regions which may direct substrate binding and mediate enzyme regulation (Dahl, et al., 1986; Grennet, et al., 1987; Ledley, et al., 1985).

The conservation extends to the genomic structure. In the human gene, although the TH gene (13kb) is smaller than that the PAH [90kb, (DiLella, Kwok, Ledley, Marvit, & Woo, 1986)] and TPH [21kb, (Stoll, & Goldman, 1991)] genes, the overall organization, particularly the exon sizes and structure are very similar. The exon/intron boundaries were conserved with other aromatic amino acid hydroxylase (TPH and PAH) genes, suggesting their close evolutionary relationship (Brown, et al., 1987; Grennet, et al., 1987).

The TH gene has been mapped to human chromosome 11 (Craig, Buckle, Lamouroux, Mallet, & Craig, 1986), and , by homology, to chromosome 9 in mouse (Brilliant, Niemann, & Eicher, 1987); the PAH gene is located on human chromosome 12 (Lidsky, et al., 1984), and TPH has been mapped to 11p (Ledley, et al., 1987). Genetic evidence speculates that chromosomes 11 and 12 are ancestral homologues created by a process of tetraploidy. Thus, gene duplication and rearrangement are implicated in the evolution of these pterin-monooxygenase genes .

iv. Multiplicity of human cDNAs of TH

In human, TH molecules are encoded by at least three mRNAs, and most likely four, which differ in the 5' termini and involve alternative splicing (Grima, et al., 1987).

These various mRNAs have distinct anatomical distributions. The functional consequences of this variation represent another means of regulating catecholamine levels in normal and physiological states. Interestingly, the human TH gene is located in position 11p15, very close to Harvey-ras 1 and insulin genes (Craig, et al., 1986), genes that has been shown, in an Amish pedigree, to be in close linkage with an autosomal dominant form of bipolar disorder (Egeland, et al., 1987).

1.3.3 TH Regulation

As the initial and rate-limiting enzyme for the biosynthesis of catecholamines, TH activity determines the availability of catecholamines in neuronal and non-neuronal tissues. Because of its physiological importance, TH regulation has been studied intensively. Diverse and complex mechanisms control TH activity and modulate catecholamine synthesis in response to physiological demands. First, TH is modulated by end-product inhibition (Spector, Gordon, Sjoerdma, & Udenfriend, 1967). Thus, free intraneuronal catecholamine inhibits the activity of TH by competing at the site that binds the pterin cofactor; conversely, neuronal activity results in the release of catecholamines, a decrease in their cytoplasmic levels, and disinhibition of the enzyme. Further control of TH activity is achieved via two interdependent mechanisms (Joh, & Reis, 1978; O'Malley, et al., 1987). The first mechanism, activation, is a very rapid process that involves the modification of already existing molecules by a reversible, conformational alteration, which in this case is caused by phosphorylation of the enzyme molecule at its regulatory sites. The second mechanism, induction, is usually accompanied by an increased number of enzyme molecules through a *de novo* synthesis of novel enzymes. For this type of up-regulation process to take place, at least several hours should pass between the stimulus and up-regulation to allow for the transcription and translation of the genetic information.

a. Activation

Depolarization of catecholaminergic terminals causes an activation of TH. The kinetic characteristics of the enzyme changes so that it has a higher affinity for the pterin cofactor, and the enzyme becomes less sensitive to end-product inhibition. The increase in catalytic activity of TH is achieved though phosphorylation of the enzyme by protein kinases (Joh, et al., 1978; Yamaguchi, & Fujisawa, 1979). TH is the substrate of various protein kinases: cyclicAMP-dependent protein kinase (PKA, (Morgenroth, Hegstrand, Roth, & Greengard, 1975), cyclicGMP (cGMP)-dependent protein kinase (Roskoski, Vulliet, & Glass, 1987), Ca^{+2} calmodulin (CaM)-dependent protein kinase (El Mestikawy, Glowinski, & Hamon, 1983), and Ca^{+2} phospholipid-dependent kinase (PKC, (Albert, et al., 1984)). TH phosphorylation is always associated with a marked increase in the intrinsic enzyme activity, although the various protein kinases catalyze the incorporation of phosphate at different sites in the enzyme molecule (Campbell, Hardie, & Vulliet, 1986; Tachikawa, et al., 1987). Phosphorylation occurs at serine residues present in the N-terminus of TH monomer, in the rat: ser-8, ser-19, ser-40, and ser 153 (Campbell, et al., 1986). All four kinases appear to phosphorylate ser-40 *in vitro*; in addition, CaM-kinase phosphorylates TH on ser-19, and PKA can phosphorylate TH on ser-153. The kinase that phosphorylates TH in ser-8 has not yet been identified.

b. Induction

TH steady state level is not fixed, but rather, it flunctuates according to physiological requirements. Many pharmacological and physiological manipulations have been used to induce TH in catecholamine cells both *in vivo* and *in vitro*. The initial observations demonstrated an increase in TH activity when there was a chronic increase in the discharge of sympathetic neurons after reserpine administration, chemical deafferentation or stress (Kvetnansky, 1973; Muller, Thoenen, & Axelrod, 1969). Because the effect of increased TH activity was dependent upon the integrity of pre-ganglionic innervation, this phenomenon is referred to as trans-synaptic induction (Thoenen, Mueller, & Axelrod, 1969). Later on, it was demonstrated that the increase of TH activity is due to an increase in levels of the enzyme protein (Joh, Gagman, & Reis, 1973). As cDNA clones became available, it was ascertained that the mechanism underlying the accumulation of TH protein was due to an increase in the the mRNA levels. It has been determined that a variety of stimuli enhance TH gene transcription including: cAMP, nerve growth factor (NGF), epidermal growth factor (EGF), glucocorticoids, cold stress, and reserpine (Black, Chikaraishi, & Lewis, 1985; Faucon Biguet, Buda, Lamoroux, Samolyk, & Mallet, 1986; Gizang-Ginsberg, & Ziff, 1990; Lewis, & Chikaraishi, 1987; Lewis, et al., 1983; Stachowiak, Sebbane, Stricker, Zigmond, & Kaplan, 1985; Tank, Lewis, Chikaraishi, & Weiner, 1985; Wessel, & T.H., 1992).

1.4 Objectives of this work

TH governs the availability of catecholamines, and its expression undergoes exquisite spatial, temporal and functional regulation. Hence, complex genomic processes contribute to the phylogenetically conserved TH-expressing cell distribution, the dynamic developmental program and the physiologically induced alteration of functional levels. Control of eukaryotic gene expression is modulated by diverse mechanisms. In distinct systems and conditions, several genetic mechanisms may control tissue-specificity, for instance: DNA loss, amplification and rearrangements, transcription control elements and their cognate transcription factors, post-transcriptional regulation, alterations in chromatin structure and the role of nuclear oncogenes (Latchman, 1995). Distinct combinations of the

DNA-binding (trans-acting) factors that stimulate or inhibit the rate of transcription have been suggested to be one of the mechanisms that generate the diversity and specificity of mammalian gene expression. In this context, studies on the identification and characterization of the cis-acting regulatory elements of the gene are key to understanding the mechanisms underlying the expression of that gene. In this work, I will test to which degree the spatial, temporal, and physiological regulation of the TH gene is mediated by interactions among the cis- and trans-acting genetic regulatory elements. In other words, I will test if the cis-acting elements localized in the 5' flanking region of the gene are responsible for the characteristics of the catecholamine phenotype.

Two approaches have been used for mapping the cis-acting regulatory elements in a gene: an *in vitro* transfection system using cultured cell lines and an *in vivo* assay system using transgenic animals. Currently, several groups have attempted to delineate cis-acting elements important for the regulation of TH expression using *in vitro* cell culture. Using deletion mutational analyses, this laboratory has identified DNA cis-elements in the 5' TH flanking region that may be involved in the control of its transcription, for instance, a cAMP responsive element (CRE) located at -45 to -39 bp upstream of the cap site which is essential for basal and cAMP-mediated transcription of TH in two different TH-expressing cell lines (Kim, Lee, Carrol, & Joh, 1993). Additional putative elements include an AP-1 site acted upon by Fos-Jun heterodimers, as well as AP-2, SP-1, FSE and POU/Oct (Cambi, Fung, & Chikaraishi, 1989; Carroll, Kim, Kim, Goodman, & Joh, 1991). In addition, an AP1 motif and E box dyad (-205 to -182) were shown to be responsible for cell-specific expression of rat TH gene in PC8b cells (Yoon, & Chikaraishi, 1992). Such results could not be replicated *in vivo*, emphasizing the notion that the cell culture system is not adequate to delineate tissue- and developmental stage-specific elements essential for the TH gene expression. In addition, appropriate neural lines are not yet available and even when they are, isolated cells cannot reproduce the complexity of the nervous system.

A more rigorous and effective approach to delineate DNA elements, despite being costly and time-consuming, is the production and analysis of trangenic mice. Kaneda et al.(1991) produced transgenic animals carrying human TH gene (hTH) including 2.5 kb of 5' flanking region, the entire exons-introns and 0.5kb of 3' flanking region. These sequences were able to direct appropriate catecholaminergic tissue-specific expression, however, follow up studies of these transgenic animals revealed extensive ectopic expression in various non-catecholaminergic brain regions (Nagatsu, et al., 1991). The same investigators attempted to identify the minimum 5' flanking DNA sequences which direct tissue-specific CAT expression; only a larger 5.0 kb of hTH could drive reporter expression to some catecholaminergic tissues, yet at levels consistently lower than the endogenous TH gene, and abundant ectopic expression was detected in brain and somatic tissues (Sasaoka, et al., 1992). Banerjee et al. (1992) has reported that 4.8 kb of 5' flanking sequences of rat TH is sufficient to direct expression of TH gene to catecholaminergic neurons in transgenic animals. However, since the claim was based upon CAT enzyme assay using brain dissections, the investigators were unable to demonstrate cellular localization of the expression because neither CAT reporter immunocytochemistry or *in-situ* hybridization was performed. Furthermore, CAT reporter gene was not fully expressed at levels equivalent to the endogenous TH in the brain and periphery. Thus, it remains to be determined whether 4.8 kb of 5' flanking region of rat TH gene is sufficient for tissue-specific expression of the reporter gene at the cellular level.

The aims of this dissertation are:

Aim 1: To determine the 5' flanking sequence of the rat TH which is capable of confering tissue-specific expression by assaying the *in vivo* activity of progressively deleted 5' flanking sequences of TH fused to the reporter gene. Three fusion genes containing 0.15 kb, and 2.4kb and 9.0 kb of the 5' upstream region linked to the bacterial lacZ reporter gene, were microinjected into the pronuclei of the fertilized one-cell embryo to generate transgenic mice.

Aim 2: To determine whether the 9.0 kb of the TH promoter sequence can drive appropriate developmental-specific expression of the lacZ transgene which recapitulates catecholaminergic cell lineage development during embryogenesis.

Aim 3: To determine whether the 9.0 kb of TH promoter sequence also contains the DNA elements necessary for neurally mediated induction of TH transcription. I will test the inducibility of a lacZ reporter in two trans-synaptic paradigms. First, unilateral naris closure, which results in odor-deprivation, produces a profound downregulation of TH expression at both the protein and mRNA level in dopaminergic cells located in the glomerular layer of olfactory bulb (Baker, 1990; Baker, Morel, D.M., & J.A., 1993). Second, the administration of reserpine results in a compensatory upregulation of TH expression in the locus coeruleus and adrenal gland (Faucon Biguet, et al., 1986; Joh, et al., 1973; Muller, et al., 1969; Wessel, et al., 1992). Thus, if the DNA cis-acting elements mediating these functional changes of TH expression *in vivo* reside within the 9 kb of TH promoter region, alterations in lacZ reporter expression should parallel changes in endogenous TH.

Chapter Two:

5' Upstream DNA Sequence Of The Rat Tyrosine Hydroxylase Gene Directs High-Level And Tissue-Specific Expression To Catecholaminergic Neurons In The Central Nervous System Of Transgenic Mice.

by

Nan Min, Tong H. Joh, Kwang S. Kim, Chu Peng and Jin H. Son

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2.1 Foreword and summary

Tyrosine hydroxylase (TH), the first and rate limiting enzyme in the biosynthesis of catecholamine neurotransmitters, is expressed within central and peripheral catecholaminergic cells. To delineate DNA sequences necessary for tissue-specific expression of the rat TH gene, transgenic mice were produced containing 0.15 kb, 2.4 kb, and 9.0 kb of 5' flanking sequence fused to the *E.coli* lacZ (ß-galactosidase) reporter gene. The reporter gene expression in the transgenic animals was monitored by both X-gal histochemical staining and ß-galactosidase immunohistochemistry and compared to TH mRNA and protein expression. Transgenic mice bearing 9.0 kb, but not the smaller constructs with either 2.4 kb or 0.15 kb of 5' flanking sequence, fused to lacZ were able to direct high level expression of ß-galactosidase at levels equivalent to the endogenous TH in central catecholaminergic cells, and to a lesser degree to adrenal gland. Previously, 4.8 kb of 5' flanking region was reported to contain some tissue-specific element(s) determined by chloramphenicol acetyltransferase (CAT) assay using regional brain dissections and was not able to demonstrate cellular localization of the CAT expression (Banerjee, et al., 1992). Using histological procedures which allow for spatial resolution, this study demonstrated that the crucial catecholaminergic neuron-specific DNA element(s) resides between -9.0 kb and -2.4 kb of the 5' flanking region of the rat TH gene; this assertion is substantiated by the high-level of tissue-specific expression of lacZ in catecholaminergic cells.

2.2 INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.14.16.2) catalyses the first and rate-limiting step in the biosynthesis of the catecholamines: dopamine, norepinephrine, and epinephrine (Nagatsu, et al., 1964). Despite the involvement of catecholamines in diverse central nervous system (CNS) functions, TH-expressing neurons are contained within distinct brain nuclei (Hökfelt, Martensson, Björklund, Kleinau, & Goldstein, 1984; Moore, & Bloom, 1978). Major expression sites include dopaminergic cells in the midbrain (substantia nigra and ventral tegmental area), diencephalon (hypothalamic nuclei), olfactory bulb (juxtaglomerular neurons), and the retina (amacrine cells), noradrenergic neurons in the pons (locus coeruleus), and the adrenergic and noradrenergic neurons in the medulla (C1/A1 and C2/A2). In the periphery, TH expression is found in the adrenal medullary chromaffin cells and sympathetic ganglia. Thus, it is very likely that the TH gene requires multiple regulatory elements to allow for the variety of anatomically and functionally distinct cells that display the catecholamine phenotype.

Currently, several groups have attempted to delineate cis-acting elements important for the regulation of TH expression using *in vitro* cell culture (Cambi, et al., 1989; Fung, Yoon, & Chikaraishi, 1990; Gandelman, Coker III, Moffat, & O'Malley, 1990). We demonstrated that a cAMP response element (CRE), located at -45 to -38 upstream to the CAP site, mediates both basal and inducible rat TH gene transcription in two different TH-expressing cell lines (Kim, et al., 1993). In addition, an AP1 motif and E box dyad (-205 to -182) were shown to be responsible for cell-specific expression of rat TH gene in PC8b cells (Yoon, et al., 1992). Such results could not be replicated *in vivo*, emphasizing the notion that the cell culture system is not adequate to delineate tissue- and developmental stage-specific elements essential for the TH gene expression, as appropriate neural lines are not yet available and even then, isolated cells cannot reproduce the complexity of the nervous system.

In recent studies using transgenic mice, Kaneda et al. (1991) produced transgenic animals carrying human TH gene (hTH) including 2.5 kb of 5' flanking region, the entire exons-introns and 0.5kb of 3' flanking region. These sequences were able to direct appropriate catecholaminergic tissue-specific expression, however, follow up studies of these transgenic animals revealed extensive ectopic expression in various noncatecholaminergic brain region (Nagatsu, et al., 1991). The same investigators attempted to identify the minimum 5' flanking DNA sequences which direct tissue-specific CAT expression; only a larger 5.0 kb of hTH could drive reporter expression to some catecholaminergic tissues, yet at levels consistently lower than the endogenous TH gene, and abundant ectopic expression was detected in brain and somatic tissues (Sasaoka, et al., 1992). Banerjee et al. (1992) has reported that 4.8 kb of 5' flanking sequences of rat TH is sufficient to direct expression of TH gene to catecholaminergic neurons in transgenic animals. However, since the claim was based upon CAT enzyme assay using brain dissections, the investigators were unable to demonstrate cellular localization of the expression because neither CAT reporter immunocytochemistry or *in-situ* hybridization was performed. Furthermore, CAT reporter gene was not fully expressed at levels equivalent to the endogenous TH in the brain and periphery. Thus, it remains to be determined whether 4.8 kb of 5' flanking region of rat TH gene is sufficient for tissue-specific expression of the reporter gene at the cellular level.

This study reports the production of transgenic mice bearing a series of 9.0, 2.4, and 0.15 kb of 5' flanking region of rat TH fused to the E.coli lacZ (ß-galactosidase) gene. Our results demonstrated, first, that 9.0 kb of rat TH could drive high-level and tissue-specific expression of lacZ with sufficient cellular resolution. Second, the data indicate that the crucial catecholaminergic neuron-specific DNA elements reside between -9 kb and -2.4 kb of 5' flanking region of rat TH gene.

2.3 MATERIALS AND METHODS

2.3.1 DNA constructs: pTH0.15lacz, pTH2.4lacZ, pTH9.0lacZ

A positive clone about 18 kb long (λTH-1) was isolated from an EMBL-3 rat genomic library by using a rat TH cDNA probe, as described previously (Carroll, et al.,

1991) (see Figure 2.1). The λTH-1 was double digested with SalI and XhoI, which released the insert from the phage arms in a 10 kb and a 8 kb fragments that were subsequently subcloned into pUC19. The pTHEI plasmid contained 0.8 kb of rat TH 5' flanking sequences and the whole rat TH gene (13 exons and 12 introns). The other plasmid, pTHup, consisted of 8.1 kb of upstream rat TH sequences 5' from the XhoI site at -773.

The construction of pTH0.15 and pTH2.4 plasmids was previously described (Kim, et al., 1993). To generate the pTH9.0 construct, a 7.9 kb SalI/ KpnI fragment from pTHup was ligated to the SalI/ KpnI digested pTH2.4 plasmid. The KpnI was an internal restriction site located at -1.0 kb from the TH cap site. These three constructs contain 0.15, 2.4,and 9.0 kb of rat TH 5' sequences, the transcription initiation site, and 27 bps of 5' untranslated region. The TH-lacZ fusion constructs were obtained by ligating the three TH upstream constructs to a blunt-ended Pst I/ Bam HI fragment from the plasmid pßActPSDKlacZpA (obtained from Dr. J. Rossant, Mount Sinai, Canada).

2.3.2 Production and identification of transgenic animals

Transgenic animals were generated by pro-nuclear DNA microinjection as described (Hogan, Costantini, & Lacy, 1986; Huh, Park, Cho, Joh, & Son, 1994). The fusion genes pTH0.15lacZ, pTH2.4lacZ and pTH9.0lacZ were linearized, isolated from their plasmid backbones by size fractionation in a 0.8% agarose gel electrophoresis, and purified by cesium chloride gradient centrifugation. The purified DNA was microinjected into the pronuclei of (CBA/J x C57Bl/6J) F1 mouse zygotes. Eggs that were successfully microinjected were transferred into the oviducts of pseudo-pregnant foster mothers. The generated mice were analyzed by genomic Southern blot from tail DNA biopsies, using the entire transgene as a probe. The subsequent progeny were identified either by tail DNA dot-blot or polymerase chain reaction (PCR).

Figure 2.1. Representation of genomic clone of rat TH gene, plasmids pTHup and pTHEI and three pTHlacZ fusion constructs. The 18 kb λTH-1 contains 9.0 kb of 5' flanking sequence and the entire rat TH gene (13 exons depicted as filled boxes). There are two Sal I restriction sites on EMBL-3 phage arms (hatched boxes). Restriction sites represented: S = Sal I, Bg = Bgl II, K = Kpn I, Xh = XhoI, Sm = Sma I. Detailed constructions are described in Materials and Methods. The deletional series of pTH-lacZ constructs contain 0.15 kb, 2.4 kb, or 9.0 kb of rat TH 5' flanking sequences, the transcription initiation site, and 27 bp of 5' untranslated region. These sequences are fused to lacZ reporter gene.

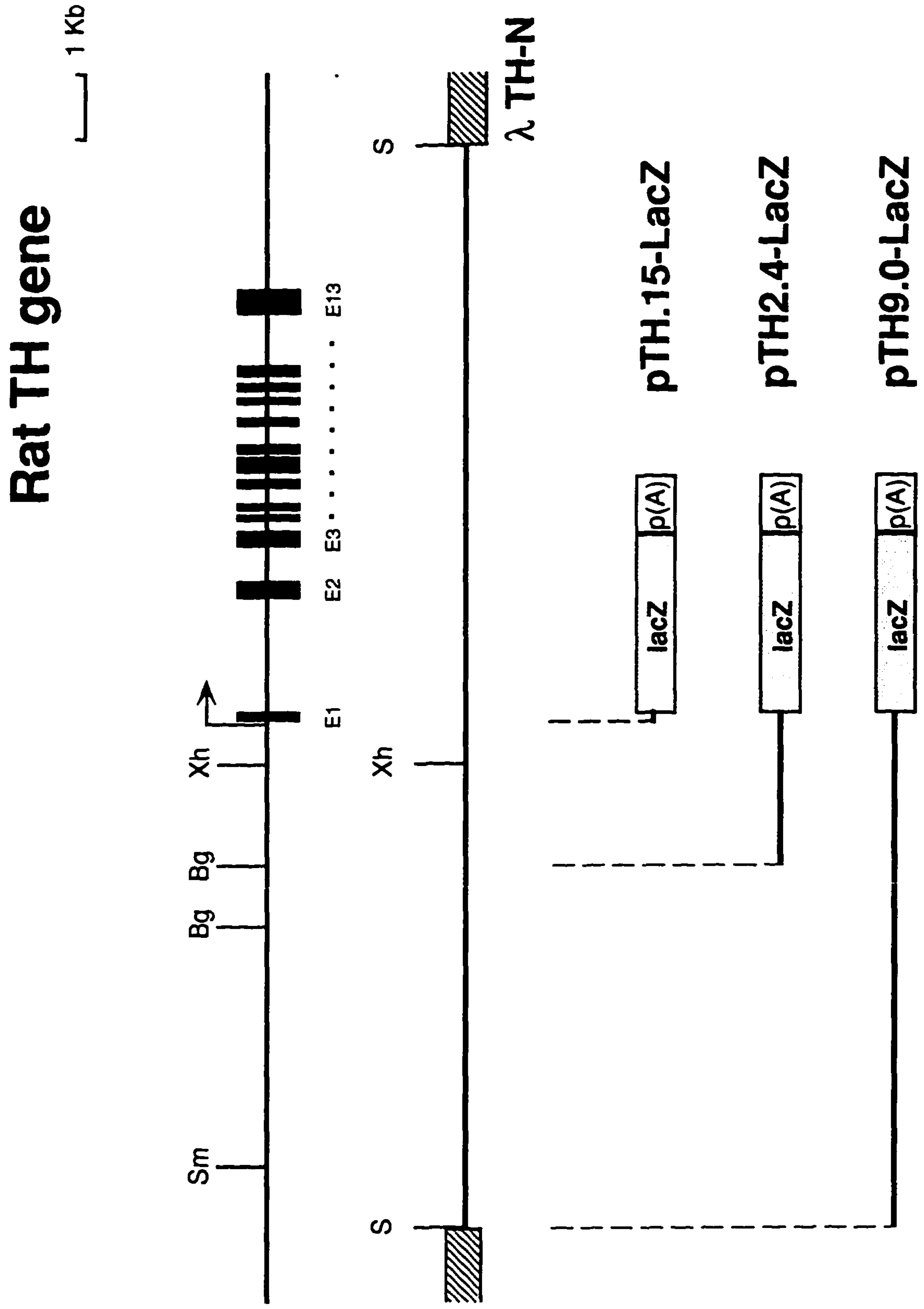
Rat TH gene
1 Kb
Sm
Bg
Bg
Xh
E1
E2
E3
E13
S
Xh
S
λ TH-N
lacZ
p(A)
pTH.15-LacZ
lacZ
p(A)
pTH2.4-LacZ
lacZ
p(A)
pTH9.0-LacZ

fig 2.1

2.3.3 Tissue preparation and X-gal histochemistry

To detect ß-galactosidase activity, X-gal histochemistry was performed as previously described (Huh, et al., 1994). Briefly, transgenic mice and controls were anesthetized with 2.5% Avertin (0.015 ml/g) perfused transcardially with a saline solution containing 0.5% $NaNO_2$ and 10 u/ml Heparin followed by the fixative solution (0.5% paraformaldehyde + 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)). Brains were removed, blocked and post-fixed in the same solution for 30-45 minutes, rinsed in phosphate buffer, then cryoprotected in a 30% sucrose solution. Either coronal or sagittal sections (100 µm) for the X-gal staining were obtained on a sliding microtome. To visualize the expression of the lacZ reporter gene, tissue sections were incubated overnight with a solution containing 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 0.15 M NaCl, 1 mM MgC12, 0.01% sodium deoxycholate, 0.02% NP-40, 0.2mg/ml X-gal in 10 mM phosphate buffer (pH 7.4). When appropriate incubation took place, expressing lines showed the characteristic insoluble blue color indicating the activity of the ß-galactosidase enzyme upon the X-gal substrate. After a brief wash with phosphate buffer, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped with Permount (Sigma).

2.3.4 Immunocytochemistry and *in situ* hybridization

The immunocytochemistry procedure was described in detail previously (Huh, et al., 1994), except that adjacent 40 µm sections were incubated overnight with either a rabbit anti-TH primary antibody (Prepared in our laboratory, at 1: 25,000 dilution) or a rabbit anti-ß-galactosidase antibody (Organon Teknika Co.,NC, at 1: 7,500 dilution). After washing, sections underwent an hour incubation with biotinylated anti-rabbit secondary antibodies (Vector Labs). The antigenic signal was amplified with an Avidin-biotin complex kit (ABC elite, Vector LABS), and visualized by incubation with 0.05% 3,3'-diaminobenzidine-tetrachloride (DAB) and H_2O_2 treatment.

TH *in situ* hybridization was performed on free floating sections as previously described (Stone, Grillo, Margolis, Joh, & Baker, 1991). Briefly, sections were prehybridized for 2 hours in a solution containing 50% formamide, 10% dextran sulphate, 1X Denhardt's, 2 x SSC, 50 mM DTT, and 1 mg/ml salmon sperm DNA, then hybridized overnight in the same solution with the addition of 1 x 10^7 cpm/ml of [^{35}S]labeled rat TH entire cDNA probe. Following stringent washing (to 0.1 x SSC, 48°C), sections were mounted onto slides and exposed 1 to 3 days to Kodak XAR-5 film to produce autoradiograms. Subsequently, slides were dipped in Kodak NBT-2 photoemulsion, exposed for 1 to 3 weeks (at 4°C), developed in Kodak D-19 solution, counterstained with cresyl violet, dehydrated in graded alcohols to xylene and coverslipped with Permount.

2.4 RESULTS

2.4.1 Production of transgenic mice bearing the pTH0.15lacZ, pTH2.4lacZ, and pTH9.0 lacZ construct

Based upon our *in vitro* data (Kim, et al., 1993), three different lacZ fusion constructs containing 0.15 kb, 2.4 kb, and 9.0 kb of rat TH 5' flanking region were analyzed to determine the relevant cis-acting elements which would confer strict tissue-specific expression of the TH gene. Table 2.1 summarizes the transgenic animals produced. The pTH9.0lacZ construct yielded nine transgenic founder lines. Additionally, six and seven founder lines were obtained from the pTH0.15lacZ construct and pTH2.4lacZ construct, respectively. Southern analysis characterized these founder lines with regard to transgene copy number and DNA rearrangements. The transgenes were found to be inserted at a single chromosomal locus without rearrangement and to be stably transmitted in a Mendelian fashion.

Initially, expression of the transgene was determined by X-gal histochemistry using sagittal 100 μm sections of brain from transgenic mice. The shortest construct, containing 150 bp of rat TH upstream, was unable to direct lacZ expression to any tissue analyzed. The pTH2.4lacZ construct directed lacZ reporter expression in one out of seven lines. Weak expression was seen in substantia nigra, locus coeruleus, and olfactory bulb of TH2.4-line 5 animals (see Table 2.1), and no expression was observed in adrenal gland. In addition, the low level of penetrance (10%) among the TH2.4-line 5 transgenic animal suggests that 2.4 kb of 5' flanking region does not contain sufficient information to direct expression of the lacZ gene to the appropriate cells. However, the pTH9.0lacZ construct could direct the tissue-specific lacZ expression in six out of nine transgenic lines including TH9.0-5, TH9.0-9, TH9.0-10, TH9.0-16, TH9.0-18, TH9.0-19, as shown in Table 2.1.

TABLE 2.1

Characterization of transgenic mouse lines containing the fusion gene constructs:

pTH0.15lacZ, pTH2.4lacZ, and pTH9.0lacZ

Transgene Construct	Founder lines	Sex	Transgene copy # (diploid)	ß-galactosidase CNS	AG
TH9.0lacZ	TH9.0-1	F	20	-	-
	TH9.0-5	M	4	+	+
	TH9.0-10	F	32	+	-
	TH9.0-15[a]	M	N.D.	N.D.	-
	TH9.0-16	F	2	+	-
	TH9.0-17	M	2	-	-
	TH9.0-18	M	8	+	-
	TH9.0-19	M	4	+[b]	-
TH2.4lacZ	TH2.4-2	M	4	-	-
	TH2.4-5	F	100	+[b]	-
	TH2.4-11	F	60	-	-
	TH2.4-11	M	80	-	-
	TH2.4-12	M	80	-	-
	TH2.4-21	M	40	-	-
TH.15lacZ	TH0.15-4	M	20	-	-
	TH0.15-5	F	40	-	-
	TH0.15-6	F	30	-	-
	TH0.15-7	M	2	-	-
	TH0.15-18	M	20	-	-
	TH0.15-21	M	10	-	-

ß-galactosidase expression was analyzed by X-gal histochemistry as described in Materials and Methods.

N.D. = not determined; + = present; - = not detected

[a] founder and progeny died before analysis

[b] incomplete penetrance of transgene expression

TABLE 2.2

Expression sites of pTH9.0lacZ transgene in the CNS and Adrenal gland of transgenic and non-transgenic mice

Expression sites	Transgenic mice		Non-transgenic mice	
	ß-galactosidase		ß-gal	TH
	TH9.0-5	TH9.0-16		
Traditional Catecholamine sites				
Hypothalamus (A11-A14)				
Periventricular N.	++	++	-	++
Paraventricular N.	++	++	-	++
Zona Incerta	++	++	-	++
Arcuate N.	+	+	-	++
Olfactory bulb	+++	++	-	+++
Substantia Nigra (A9)	+++	+++	-	+++
VTA (A10)	+++	+++	-	+++
Locus coeruleus (A4/A6)	+++	+++	-	+++
A5	+	+	-	+
A1/C1	+	+	-	+
A2/C2	+	+	-	+
Area Postrema	+	+	-	++
Adrenal Gland	+	-	-	+++
Nontraditional catecholamine sites[a]				
Accessory Olfactory Bulb	+	+	-	+
Anterior Olf. N.	+	+	-	+
Taenia Tecta/Septum/Fimbria	+	+	-	-
Amygdala	+	+	-	+
Interpeduncular Nucleus	+	+	-	-

The signs represent abundance of TH or ß-galactosidase immunoreactivity in the area: +++ = high, ++ = moderate, + = low, - = none.

[a] expression sites found in all expressing transgenic lines

2.4.2 Tissue-specific expression of lacZ reporter gene in catecholaminergic cells.

Catecholaminergic tissues were correctly targeted in all pTH9.0lacZ expressing lines. Tissue-specific expression of the transgene was further assessed in two representative transgenic lines, TH9.0-5 and TH9.0-16. As summarized in Table 2.2, the lacZ expression was observed in the dopaminergic substantia nigra, ventral tegmental area, hypothalamus (A11-A14) and olfactory bulb, in the noradrenergic locus coeruleus, A1, A2 and A5, in the adrenergic C1 and C2 neurons, in a few non-traditional catecholaminergic cells (see also Discussion) in the CNS and in the adrenal gland.

A sample of catecholaminergic tissues targeted can be seen in Figure 2.2; the composite represents the range of diverse TH-expressing phenotypes in CNS and periphery. The blue color denoting X-gal staining by ß-galactosidase activity was observed within the dopaminergic neurons of the substantia nigra and ventral tegmental area (VTA) (Figure 2.2A), the noradrenergic neurons in the locus coeruleus (Figure 2.2E), and the adrenergic cells of the C2 and C1 area in the metencephalon (Figure 2.2H). Also, in the periphery, adrenal medullary chromaffin cells displayed the characteristic blue stain, in spite of incomplete penetrance of the transgene expression (Figure 2.2D). X-gal staining was also observed in the remaining TH-expressing catecholaminergic tissues: hypothalamic dopaminergic nuclei (Figure 2.3), neurons in the raphe dorsalis, noradrenergic areas A1, A2, A5, A7, and juxtaglomerular cells in the olfactory bulb (Figure 2.4). The specificity of X-gal staining was further confirmed by immunocytochemical colocalization study in the next section.

Figure 2.2. Composite representing the diverse range of TH-expressing cell types in the CNS and periphery. Coronal section through the mesencephalon showing dopaminergic neurons of substantia nigra and ventral tegmental area stained for X-gal (A), TH (B), or ß-galactosidase immunocytochemistry (C). The cells expressing the lacZ reporter have an identical distribution and level of expression to endogenous TH expression. In (D), adrenal medullary chromaffin cells display X-gal staining. The similar expression of transgene and endogenous TH, both in level and in cell distribution, is demonstrated by the characteristic shape of the locus coeruleus which can be seen in this coronal section at the level of metencephalon; LC noradrenergic neurons displays X-gal staining (E), TH (F), or ß-galactosidase immunohistochemistry (G), Bar = 180 μm. Finally, in (H) arrows point to X-gal stained adrenergic cells of C2 (dorsal) and C1 (ventral) tegmental groups in the medulla. The bar equals 400 μm in A-C, 300 μm in D, 180 μm E-G and 540 μm in H.

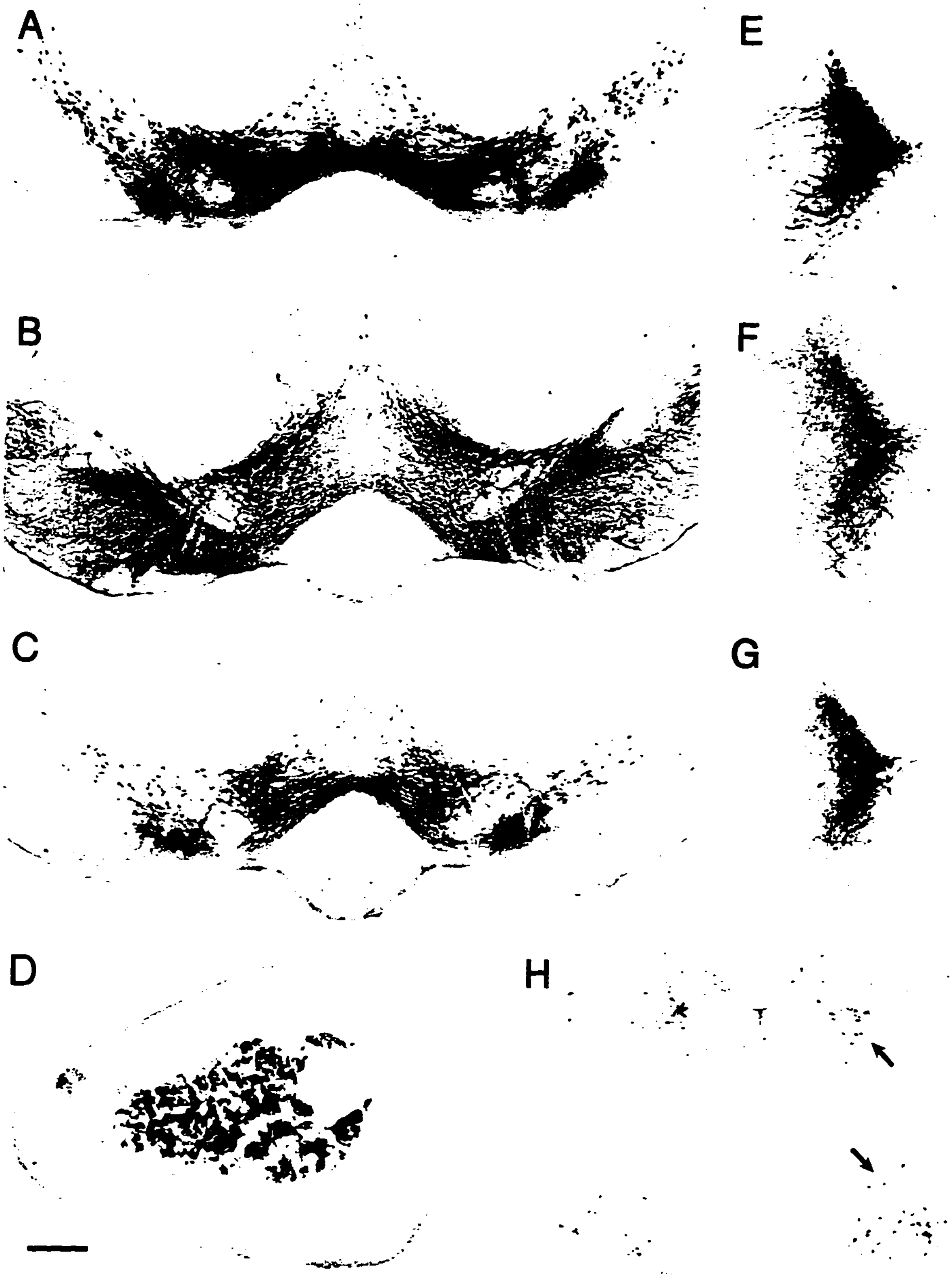

fig 2.2

2.4.3 Colocalization of the TH and ß-galactosidase immunoreactivity to catecholaminergic neurons.

An immunohistochemical study was carried out to demonstrate the colocalization of the TH and lacZ reporter gene expression. X-gal staining and ß-galactosidase immunostaining were coincident in all tissues observed. Comparable numbers of TH and ß-galactosidase immunoreactive cells were observed in their normal distribution within the substantia nigra and VTA (Figure 2.2B and 2.2C) and locus coeruleus (Figure 2.2F and 2.2G). This observation demonstrates that the endogenous TH and the transgene lacZ were expressed at comparable levels.

In the diencephalon, TH-expressing cells consist of dopaminergic neurons classically recognized into four different cell groups: A11, A12, A13, and A14 (Björklund, & Nobin, 1973). Systematic mapping of TH-immunoreactive cells in mouse brain revealed new groups and more complex distribution than originally described (Ruggiero, Baker, Joh, & Reis, 1984). Dopaminergic neurons are present in the periventricular and paraventricular nucleus confirmed by immunoreactivity to TH (Figure 2.3C) and ß-galactosidase (Figure 2.3A). Likewise, TH (Figure 2.3D) and ß-galactosidase (Figure 2.3B) immunoreactive neurons can be seen in the arcuate nucleus and the hypothalamic zona incerta.

Figure 2.3. Consecutive coronal brain sections through dopaminergic hypothalamic nuclei demonstrate lacZ expression which corresponds to endogenous TH expression. Dopaminergic cells in the paraventricular and periventricular nuclei are visualized by TH (C) or ß-galactosidase immunocytochemistry (A). Likewise, TH immunoreactive neurons (D) and ß-galactosidase immunoreactive neurons (B) are seen in the arcuate nucleus and zona incerta. The bar equals 140 µm in A and C and 70 µm in B and D.

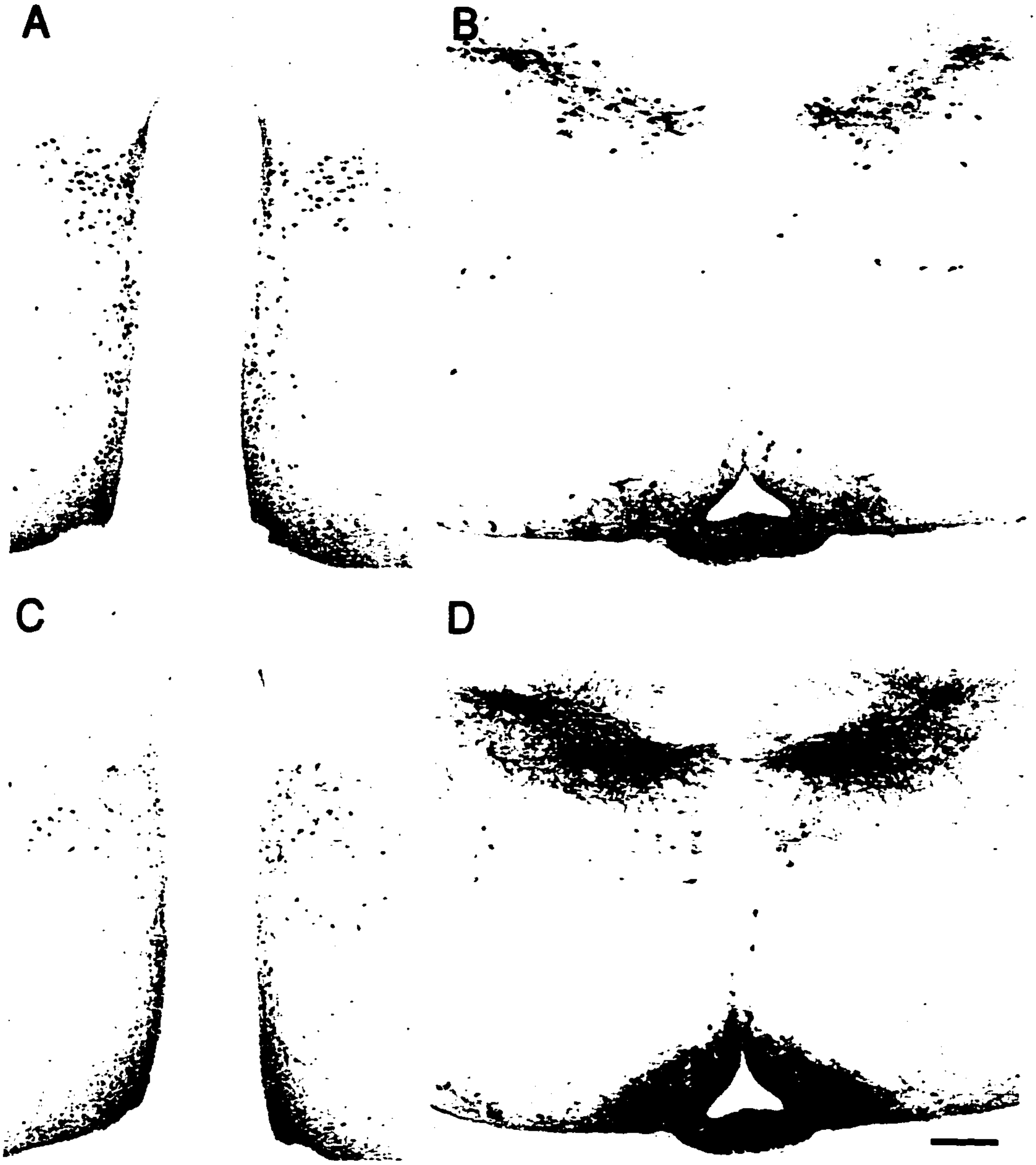

fig 2.3

2.4.4 Presence of reporter lacZ expression in nontraditional catecholamine sites

X-gal staining or ß-galactosidase immunoreactivity was observed in some nontraditional, i.e. not previously reported in the literature, areas (see Table 2.2). Some of this ectopic expression could be accounted for by a positional effect, where the sequences surrounding the location of insertion of the transgene exert an effect upon the expression of the reporter gene. It is characterized by being present only in an individual line, for instance, neurons in the inferior colliculus (line TH-10), neurons in the habenula (line TH-18), and ependymal cells (TH-9). In contrast, there were expression sites which were consistent across all expressing lines, thus inherent within the TH transgene sequences. The areas that exhibited such expression were the accessory olfactory bulb (Figure 2.4), the anterior olfactory nucleus, the interpeduncular nucleus, and two areas that encompassed cells from several nuclei not being restricted to nuclear boundaries: the first area included the lateral and medial septum, the taenia tecta and cells within the fimbria, the second area involved the amygdala, which were previously reported by Mezey (Mezey, 1989).

Figure 2.4. In (A) Cresyl violet counterstaining of a sagittal section though the olfactory bulb identifies the glomerular layer (GL) of main olfactory bulb and the accessory olfactory bulb (AOB). (B) Immunoreactive TH cells are restricted to the glomerular layer, however TH mRNA can be also be detected in the AOB (C), as illustrated by this autoradiogram depicting TH *in situ* hybridization. (D) ß-galactosidase immunohistochemistry is expressed not only in the traditional TH-expressing GL, but also in the AOB. For further discussion refer to text, Bar = 600 µm.

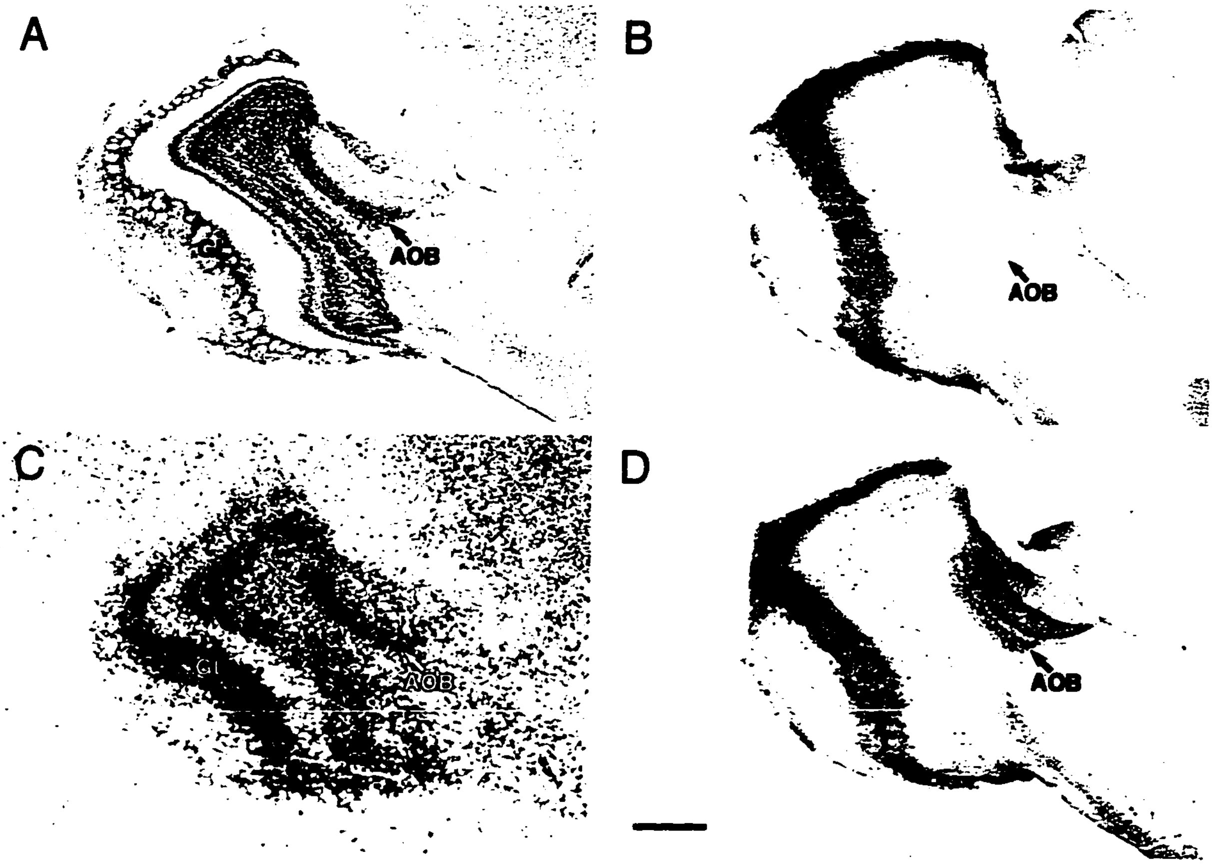

fig 2.4

2.5 DISCUSSION

This study demonstrated that 9.0 kb of 5' flanking sequence of rat TH gene contains the DNA elements necessary for directing high-level expression of the lacZ reporter gene to all catecholaminergic cells within the CNS. The high-level expression of the ß-galactosidase reporter parallels the endogenous TH levels. Our result is supported by other transgenic mice models using the 5' flanking region of the human (Sasaoka, et al., 1992) or rat (Banerjee, et al., 1992) TH gene. Although the human TH may be different from the rat TH gene, 5 kb of 5' flanking sequences of the human TH directed much lower levels of reporter gene expression than endogenous TH with numerous ectopic expression sites, which suggests 5 kb of flanking sequences is not sufficient to direct high level and tissue-specific expression in transgenic mice. Thus, the tissue-specific regulation of human TH gene seems to require not only 5' flanking sequence, but also exon-intron structure and 3' flanking sequence (Kaneda, et al., 1991). For rat TH, it is not clear the tissue specificity of reporter gene expression directed by the 4.8 kb of 5' flanking region because no cellular localization studies of CAT reporter gene expression were done. Instead CAT assays using regional brain dissections were used to detect the reporter gene expression, which does not allow for spatial resolution at the cellular level. In addition, the CAT reporter gene was not fully expressed at levels equivalent to the endogenous TH in the brain and periphery. Taken together, these data suggest that the DNA sequences between -9.0 kb and -4.8 kb of 5' flanking region are essential for high level, catecholaminergic neuron-specific expression in the CNS. Intriguingly, there was transgene expression in areas in which TH is not traditionally described, but this atypical expression was consistent across all lines and its level less subject to variability than the targeted catecholaminergic expression. For instance, our data showed ß-galactosidase expression in the accessory olfactory bulb. In mice but not rats, there is an apparent TH mRNA expression in the accessory olfactory bulb by *in-situ* hybridization but no TH protein is detected by immunocytochemistry (Figure 2.4; and unpublished observations, but see Figures in Stone et al., 1991). One plausible explanation is that post-transcriptional mechanisms may control the translational level of TH mRNA in this tissue and this post-transcriptional regulation was not extended to the translation of the lacZ reporter mRNA in the transgenic mice. Similar observations were made in the colchicine-treated hamster (Asmus, & Newman, 1993). Abundant TH mRNA was found in the medial amygdaloid nucleus and the reticular nucleus of the thalamus, but a small portion of the TH mRNA may be translated into TH protein.

Additionally, some of these non-traditional sites consisted of areas in which transient expression of TH was detected, for instance, the anterior olfactory nucleus (Nagatsu, et al., 1990), amygdala (Mezey, 1989; Verney, Gaspar, Febvret, & Berger, 1988), and cortex (Satoh, & Suzuki, 1990). It is suggested that 9.0 kb of rat TH contain the sequences which direct expression to these areas during a specific developmental stage, but a negative element may be lacking which normally switches off TH expression in the adult. Furthermore, the expression in the septum, amygdala, accessory olfactory bulb, and anterior olfactory nucleus reported in this study was also observed in a transgenic animal bearing 5 kb of upstream sequences of hTH (Sasaoka, et al., 1992). Moreover, TH-producing neurons have been found in various brain regions of many mammalian species outside the traditional catecholaminergic neurons (Gaspar, et al., 1987; Gouras, Rance, W.S., & Koliatsos, 1992; Kitahama, et al., 1990; Kitahama, et al., 1989; Kohler, Everitt, Pearson, & M., 1983; Kordower, Sladek, Fiandaca, Bing, & Gash, 1988). Thus, these novel expression sites herein reported may represent authentic TH expressing sites, but the steady state level of TH enzyme may be beyond current detection procedures.

The analysis of the lacZ expression in the adrenal medullary chromaffin cells yielded mixed results. Cells were correctly targeted in the adrenal medulla, but not at full penetrance, and the expression was seen only in one transgenic line (TH9.0-line 5). Consistent with previous work, even though the rat TH sequences tested in this work drive full expression in the CNS, these sequences may lack cis-acting elements to drive complete expression in peripheral cells. In fact, transgenic mice bearing 4.8 kb of rat TH fused to CAT reporter gene drove the expression of CAT in the adrenal gland at levels 20-fold lower than in the brain (Banerjee, et al., 1992). Likewise, when human TH was overexpressed in transgenic mice, there was a 50-fold increase of TH transcript levels in the brain as compared to a 5-fold increase in the adrenal gland (Kaneda, et al., 1991). Thus, it is possible that distinct regulatory mechanisms involving different DNA elements exist for differential TH gene expression in CNS and periphery.

In conclusion, the DNA sequence between -9.0 kb and -2.4 kb of the rat TH gene contain crucial DNA element(s) directing tissue-specific and high level expression to catecholaminergic cells in the CNS. Precise localization of the cis-acting element(s) in this region using transgenic mice will provide a valuable tool for the identification of trans-acting factors regulating TH gene expression in central catecholaminergic neurons.

Chapter Three:

Early Ontogeny Of Catecholaminergic Cell Lineage In Brain And Peripheral Neurons Monitored By Tyrosine Hydroxylase-lacZ Transgene

by

Jin H. Son, Nan Min, and Tong H. Joh

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3.1 Foreword and Summary

In the second study, I examined the early ontogeny of TH expression in transgenic mouse embryos by following the expression of a lacZ reporter, driven by the tissue-specific promoter of the rat TH gene. As the first and rate limiting enzyme in the biosynthetic pathway for catecholamine (CA) neurotransmitters, tyrosine hydroxylase (TH) is a specific phenotypic marker for CA cells in the central and peripheral nervous systems of adult animals. During embryogenesis, TH expression appears permanently within cells destined to be CA-secreting during adult life, and transiently in several cell types that will not express TH in adulthood. The lacZ reporter product, ß-Galactosidase (ß-Gal), visualized by X-gal staining, first became apparent in primordia of sensory ganglia serving the glossopharyngeal (IX) and vagal (X) cranial nerves at embryonic day (E)9.0. Between E9.5 and E10.5, ß-Gal expression extended to the remaining cranial sensory ganglia serving the trigeminal (V) and facial (VII) nerves, dorsal root ganglia, ventrolateral neural tube and sympathetic ganglion primordia. During that same period, the first ß-Gal expression in the embryonic brain also appeared within distinct regions, such as the ventral prosencephalon, the ventral and dorsolateral mesencephalon and the rostral and caudal rhombencephalon. The level of ß-Gal expression in all these tissues decreased at E13.5, but a distinct adult pattern of ß-Gal expression started to emerge in the substantia nigra and ventral tegmental area in the central nervous system and the adrenal medulla in the periphery. Our findings indicate that the proximal 9.0 kb of the 5' promoter region of the rat TH gene encodes sufficient information to direct development of the appropriate catecholaminergic lineage cells in the central and most peripheral nervous systems during embryogenesis.

3.2 INTRODUCTION

Neural differentiation begins with the induction of the neural ectoderm. Peripheral nervous system (PNS) lineages originate in the neural crest as the neural ectoderm elongates and folds to form the neural tube. Cells destined to form the central nervous system (CNS) arise from precursor cells near the inner surface of the neural tube (Jacobson, 1991). Thus, distinct cell lineages, destined respectively to comprise the PNS and CNS, originate early in embryonic development. Thereafter, many distinct neuronal cell lineages are established via mechanisms that remain largely unknown. Among the mechanisms that remain to be elucidated, determination of specific neurotransmitter phenotypes is an important aspect of neuronal and neuroendocrine cell development.

Differentiation into specific neurotransmitter phenotypes requires neurons to express specific genes for the biosynthesis, storage, release and synaptic inactivation of the neurotransmitter. An important phenotypic hallmark of all cells that synthesize and secrete catecholamine (CA) is expression of tyrosine hydroxylase (TH), which catalyzes the first and rate limiting step in the biosynthesis of the CA neurotransmitters dopamine, norepinephrine and epinephrine (Nagatsu, et al., 1964). In the adult brain, the expression of TH is restricted to a small number of distinct neuronal groups, including the dopaminergic neurons of substantia nigra (SN) and ventral tegmental area (VTA), tuberoinfundibular neurons of the hypothalamus, periglomerular cells of the olfactory bulb, and certain retinal amacrine cells, as well as the noradrenergic neurons of the locus coeruleus and subceruleus, and several smaller groups of noradrenergic and adrenergic neurons in the lower brainstem (Hökfelt, et al., 1984).

In prenatal rat brain, TH-positive cells are first detected immunohistochemically at embryonic day (E)12.5 (Specht, Pickel, Joh, & Reis, 1981a). At this stage, several populations of catecholaminergic cells, committed to become particular types of neurons, appear in a specific spatiotemporal pattern. TH-labeled neurons clustered in the ventral mesencephalon develop into the dopaminergic SN and VTA. Other TH-positive neurons, located in the rostral and caudal rhombencephalon, become the noradrenergic locus coeruleus in pons and the adrenergic/noradrenergic nuclei in the medulla, respectively. A small number of TH-positive neurons, which will become dopaminergic neurons in the hypothalamus (A11-A14), are scattered in the ventral prosencephalon. Between E12.5 and 14.5, postmitotic TH-positive neurons exhibit immature rounded shapes with a few smooth processes, while they migrate toward their adult destinations. After the completion of migration, they undergo cytodifferentiation to exhibit angular profiles with numerous immature dendrites. By E14.5, TH-labeled neurons in the rat brain are distributed in distinct anatomical locations similar to the adult pattern (Björklund, et al., 1973; Seiger, & Olson, 1973), except in the olfactory bulb, where dopaminergic cells start to appear at approximately E18 in rat embryos after the transient emergence of some intensely TH positive cells in the ventrolateral aspects of telencephalon at E15 (Baker, & Farbman, 1993; Nagatsu, et al., 1989). In the adult PNS, TH expression occurs sympathetic ganglia, sensory ganglia, a subpopulation of dorsal root ganglia neurons, some parasympathetic neurons, and neuroendocrine adrenal chromaffin cells (Katz, & Erb, 1990; Mercer, Hoyle, Kapur, Brinster, & Palmiter, 1991; Patterson, 1990; Price, & Mudge, 1983; Teitelman, H., Joh, & D.J., 1979). In contrast, the embryonic PNS contains many cells that express TH transiently. Examples of such cells include transiently TH-expressing cells in cranial sensory and dorsal root ganglia (Jonakait, Markey, Goldstein, & Black, 1984) as well as in ventrolateral portions of the neural tube (Teitelman, Gershon, Rothman, Joh, & Reis, 1981). In addition, reports of transient expression of TH in nephrogenic and gut mesenchyme (Cochard, Goldstein, & Black, 1978; Teitelman, et al., 1979) and embryonic pancreas (Teitelman, Joh, & Reis, 1981) demonstrate that expression of TH in early development is not confined to tissues arising from neural ectoderm.

Previous studies from many laboratories including ours have used TH-immunohistochemistry to monitor catecholaminergic differentiation during embryonic development (Baker, et al., 1993; Cochard, et al., 1978; Jonakait, et al., 1984; Specht, et al., 1981a; Specht, Pickel, Joh, & Reis, 1981b; Teitelman, et al., 1981). However, this approach may fail to detect the full spectrum of TH-positive cells due to the sensitivity of TH immunochemistry in early embryos. In addition, limited antibody penetration can restrict the degree to which spatiotemporal changes of TH expression in large tissue mounts can be visualized. Analysis of lacZ reporter gene expression in transgenic mice provides a new approach to neurodevelopment that circumvents some of the problems associated with immunocytochemistry. In addition, this approach provides important information about the function of the TH promoter driving lacZ expression. Previous work from this laboratory has shown that the proximal 9 kb of the rat TH gene directs high level, tissue-specific expression of the lacZ reporter in the CNS of adult transgenic mice (Min, Joh, Kim, C., & Son, 1994). The present study investigated the expression of this construct during embryonic development. Our results demonstrate that the proximal 9 kb of the 5' promoter of the rat TH gene directs CA cell lineage-specific expression of lacZ during embryogenesis.

3.3 MATERIALS AND METHODS

3.3.1 Transgenic Animals and Embryos.

Two representative transgenic lines, TH9.0-#5 and TH9.0-#16, were generated by pro-nuclear microinjection of the fusion gene containing 9.0 kb of 5' flanking region linked to the E.coli lacZ (ß-galactosidase) reporter gene as described (Min, et al., 1994). Pregnant B6CBAF1/J females (Jackson Laboratory, ME) were obtained by mating with adult male hemizygous transgenic mice. Since midnight was designated as embryonic day 0 (E0), the gestational age was designated as E0.5 when vaginal plugs were detected during morning inspection of females. Pregnant females were sacrificed on E8.5, E9.0, E9.5, E10.5, E11.5, E12.5, E13.5 and E15.5. The stage of each mouse embryo was confirmed by examining typical features of gestational stages (Theiler, 1989). At least three litters from TH9.0-#5 and TH9.0-#16 lines were investigated at each gestational age.

3.3.2 X-gal Staining of Embryos and Tissue Sections

To detect the developmental expression of ß-galactosidase activity, embryos from E8.5 to E11.5 were immersion-fixed for 45 minutes in 0.5% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Embryos from E12.5 to E15.5 were immersion-fixed for 15 minutes, an incision was made in the abdominal wall and the embryo was halved in the sagittal plane to facilitate the penetration of the enzyme substrate, X-gal and postfixed for 60 minutes X-gal staining was performed as described previously (Huh, et al., 1994). Embryos were incubated overnight with a solution containing 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 0.15 M NaCl, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-

40, 0.2mg/ml X-gal in 10 mM phosphate buffer (pH 7.4). After a brief wash with phosphate buffer, embryos were cryoprotected in a 30% sucrose solution overnight and transverse sections, 20-30 μm thick, were obtained on a cryostat at -22^{o}C and counterstained with neutral red. The transverse sections were examined and photographed with a Nikon Microphot FXA microscope and Diaphot-TMD inverted microscope. 100 μm sagittal sections of some E13.5 embryos were obtained after X-gal staining. Some embryos were cleared by incubating for 2 hrs in 3% H_2O_2 and photographed with a dissection microscope (Nikon SMZ-10).

3.4 RESULTS

3.4.1 Initial TH Expression Occurred in Transgenic Embryos at E9.0-E9.5

The developmental expression of the TH-lacZ construct was investigated starting at E8.5. As the anterior neuropore formed and closed at E9, the first confined expression of the ß-Galactosidase (ß-Gal) driven by the TH transgene promoter appeared simultaneously in primordia of sensory ganglia serving glossopharyngeal (IX) and vagal (X) nerves (Fig. 3.1A). About half a day later, at E9.5, primordia of the trigeminal (V) and geniculate (VII) ganglia demonstrated ß-Gal expression (Fig. 3.1B). Moreover, as the posterior neuropore began to constrict at E9.5, ß-Gal expression extended into the dorsolateral neuroepithelial lining of mesencephalon and spinal neural tube.

After complete closure of neural tube, at E10.5, the ß-Gal expression became apparent in the ventral prosencephalon, the ventral and dorsolateral mesencephalon, the caudal and rostral lateral rhombencephalon and the spinal neural tube. At this stage, the highest levels of ß-Gal expression were confined to cells of neural crest origin, such as the cranial sensory ganglia (V), (VII), (IX) and (X), primordia of the sympathetic ganglia near the dorsal aorta and dorsal root ganglia shown in Fig. 3.1C (For a coronal section of the neural tube, please see Fig. 3.3A). The expression continued over the next 1.5 days in most of these neuronal cell populations in a bilaterally symmetric pattern. By E13.5, the intensity of ß-Gal expression had decreased dramatically in a restricted pattern similar to the adult pattern (Fig. 3.1D and 3.1E). For the X-gal staining of the E12 and E13.5 embryos an incision was made in the embryo abdominal wall or the embryo was halved in the sagittal plane to facilitate the penetration of X-gal. In general, the ß-Gal expression pattern observed in both transgenic lines, TH9.0-#5

Fig. 3.1 Spatiotemporal expression pattern of the TH-lacZ transgene in whole-mount X-gal stained transgenic embryos at E9.0 to E13.5.

(A) Lateral view at E9.0 demonstrates the first expression of ß-Galactosidase activity in primordia of sensory ganglia serving glossopharyngeal (IX) and vagal (X) nerves; (B) Lateral view of E9.5 embryo with X-gal staining in the cranial sensory ganglia (V), (VII), (IX) and (X), dorsolateral neuroepithelial lining of mesencephalon and spinal neural tube; (C) Lateral view of E10.5 embryo with the highest levels of X-gal staining confined to the ventral prosencephalon, the ventral and dorsolateral mesencephalon, the caudal and rostral lateral rhombencephalon, spinal neural tube and the cranial sensory ganglia (V), (VII), (IX) and (X) and dorsal root ganglia; E12 (D) and E13.5 (E) embryos with the decreased ß-Gal expression in a restricted pattern. E12 embryo demonstrates the transient ß-Gal expression in the developing gut seen in the abdominal area. Halved E13.5 embryo shows transient X-gal staining in primordia of sinus hair follicles found in characteristic locations (arrow marked). drg, dorsal root ganglia; mc, mesencephalon; o, otic vesicle; rc, rhombencephalon; sc, spinal cord; primordia of sensory ganglia serving trigeminal (V), geniculate (VII), glossopharyngeal (IX) and vagal (X) sensory ganglia. Bar= 0.3 mm in A-C, 1.0 mm in D and 1.2 mm in E.

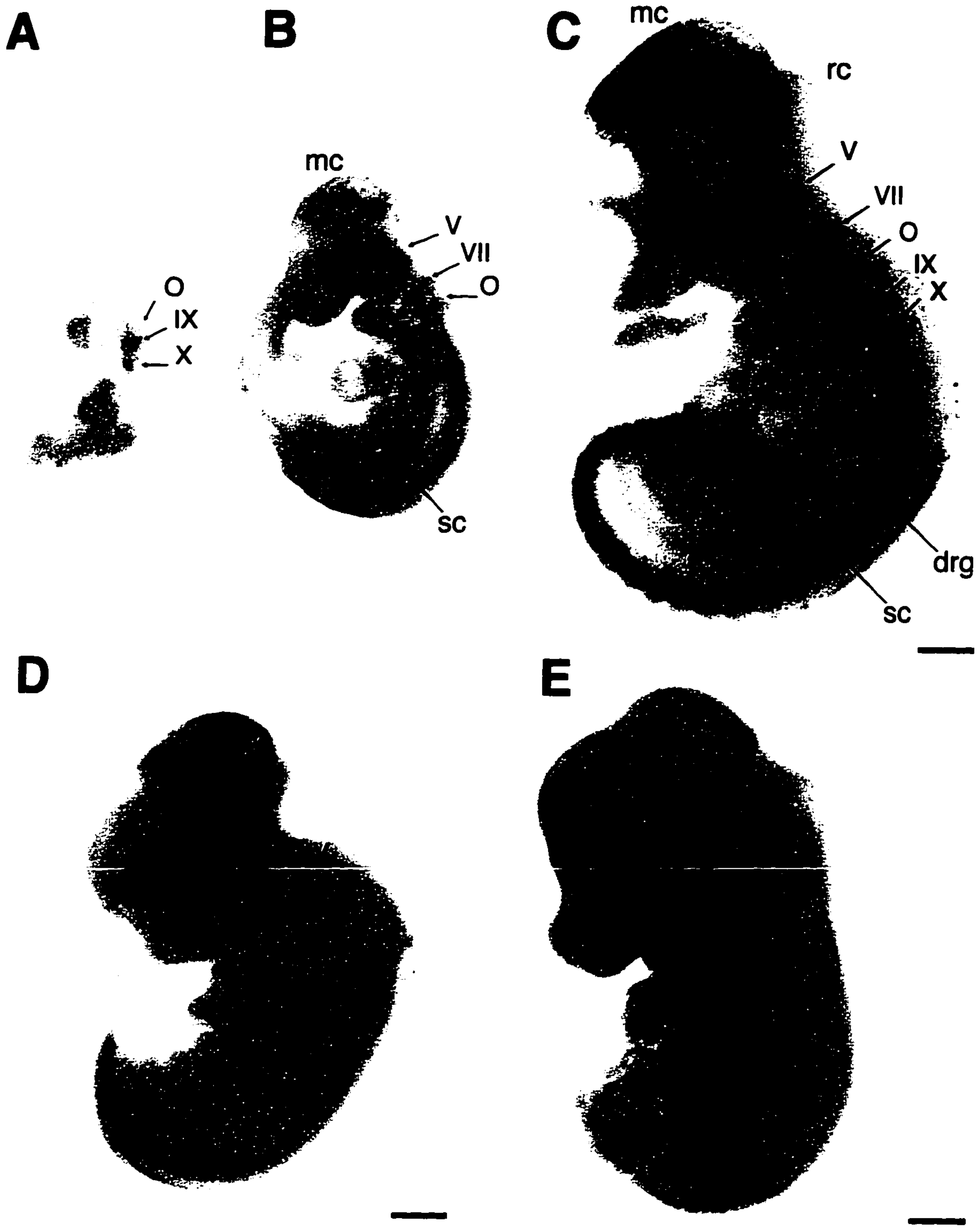

fig 3.1

and TH9.0-#16, demonstrate good correspondence with the previously described distribution of the TH-positive catecholaminergic tissues during embryonic development.

3.4.2 Specific and Transient Expression of TH in Midgestational Transgenic Embryos between E 10.5-E 13.5

The transgene expression reached its most widespread and intense levels between E10.5 and E12 (Fig. 3.1C and 3.1D). In the brain of E10.5 transgenic embryo postmitotic, but still immature ß-Gal positive neurons were present bilaterally in the dorsolateral mesencephalon (Fig. 3.2A). The distribution of these neurons was limited to the layer near the outer surface of the columnar neuroepithelium (Fig. 3.2B). ß-Gal expression in the dorsolateral mesencephalon started at E9.5, peaked at E10.5 and persisted at much lower levels through E12. By E13.5 the expression had completely disappeared, except for weak transient expression in the very caudal portion of tectum (Fig. 3.1E), and in neurons located within nuclei arranged in the adult pattern of CA-containing nuclei.

Additional distinct groups of ß-Gal positive cells were apparent in the ventral prosencephalon (Fig. 3.2C and 3.2D), the ventral mesencephalon (Fig. 3.2E and 3.2F) and the rostrolateral and caudal rhombencephalon (Fig. 3.2G and 3.2H) at E10.5. These ß-Gal positive neurons were localized near the outer surface of the neural tube, where postmitotic neurons migrate from the germinal neuroepithelial layer. At higher magnification, the ß-Gal positive, mostly postmitotic neurons displayed large rounded shapes and had very few processes (Fig. 3.2B and 3.2H).

At E12 the X-gal staining was more localized and less intense than in earlier embryos. For example, mesencephalic ß-Gal-positive cells were clustered on both sides of the midline along the mesencephalic flexure near the SN/VTA region. X-gal staining extended caudally close to the junction of the rhombencephalon (Fig. 3.1D). The rostral rhombencephalic ß-Gal-positive cells became more compact without any change in position at the junction of mesencephalon and rhombencephalon. Only a few stained cells were left in the ventral rhombencephalon rostral to the cervical flexure. As the

Fig. 3.2 X-gal staining pattern in sagittal sections of embryonic brain (E10.5) of the transgenic mice.

(A) Dorsal view demonstrating ß-Gal positive neurons present bilaterally in the dorsolateral mesencephalon; (B) Sagittal section through the dorsolateral mesencephalon with the limited distribution of the ß-Gal positive neurons to the layer near the outer surface of the columnar neuroepithelium; (C) ß-Gal positive cells (short arrow) located in the ventral prosencephalon, and (D) at higher magnification the ß-Gal positive immature neurons with very few precesses in rounded shape; (E) ß-Gal positive cells (short arrow) in the ventral mesencephalon and (F) higher magnification view of the ß-Gal positive cells in the ventral mesencephalon; (G) ß-Gal positive cells (short arrow) in the rostrolateral and caudal rhombencephalon and (H) higher magnification view of the ß-Gal positive cells in the rostrolateral rhombencephalon. iv, fourth ventricle; m, mesencephalic vesicle; mf, mesencephalic flexure; or, optic recess; ne, neuroepithelium; t, telencephalic vesicle.

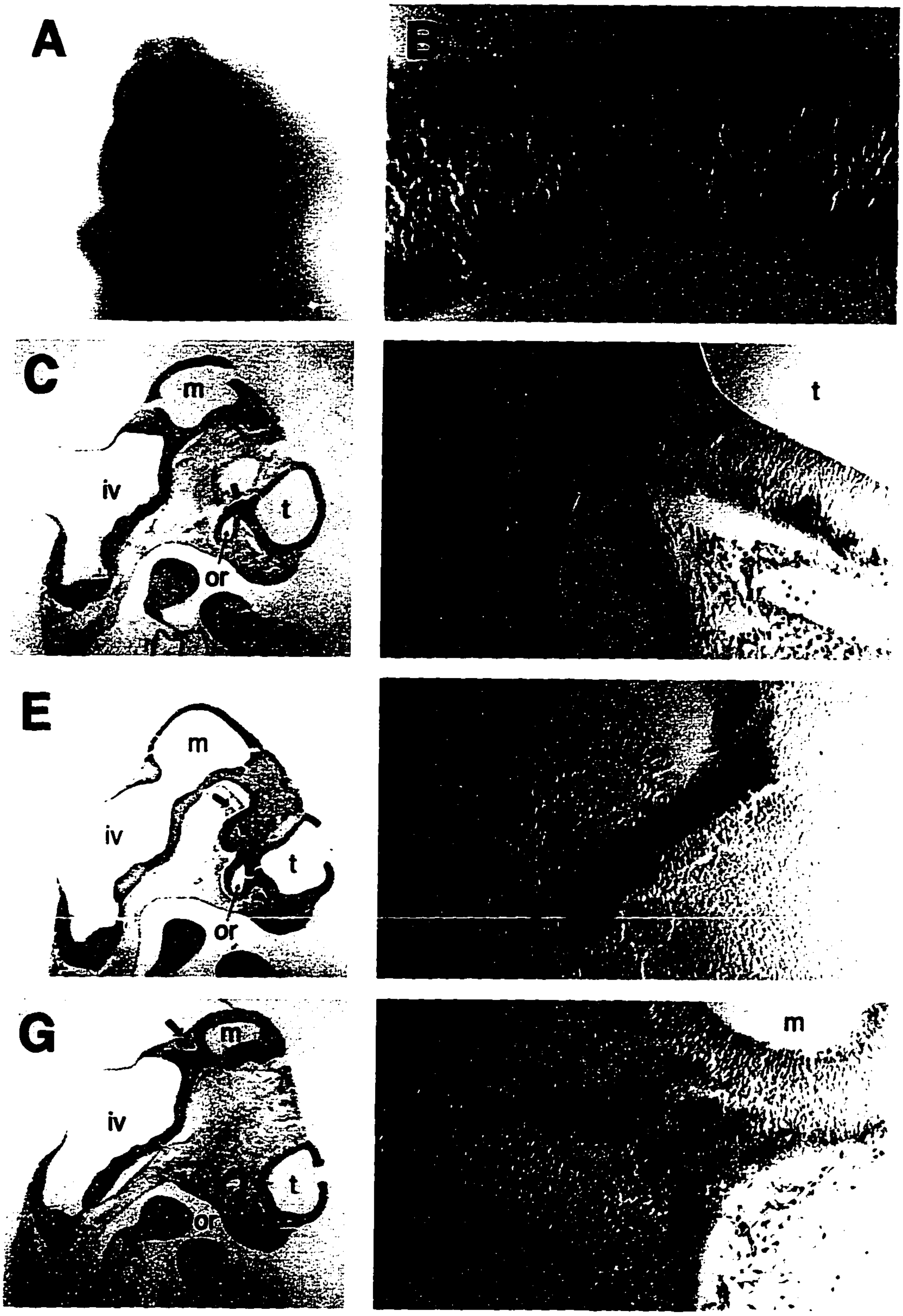

fig 3.2

embryonic brain further developed, an intensely labeled layer of cells subsequently appeared in the ventrolateral telencephalon, where the transient TH-immunoreactivity was observed in E15 rat embryos (Baker, et al., 1993). The lacZ staining then rapidly disappeared leaving only residual scattered staining by E13.5 (Fig. 3.1E). In E13.5 brains the mesencephalic dopaminergic neurons reside in distinct SN/VTA nuclei (A9-10) with dense lacZ staining also apparent over the mesencephalic flexure in the basal portion of the rostral mesencephalon. The noradrenergic nuclear complex (A4-7) in the brainstem showed weak ß-Gal expression, which became somewhat stronger by E15.5 (data not shown).

In the PNS, the dorsal root ganglia were X-gal stained ventrolaterally at E10.5, which sent processes into the developing spinal cord (Figs. 3.3A and 3.3B). At the same time the neural tube ß-Gal positive cells appeared between E9.5 and E10.5 (Fig. 3.3A). This expression corresponded precisely to the appearance of TH immunoreactivity in rat embryonic neural tube where motor neurons may develop. Rudimentary ß-Gal positive paravertebral sympathetic ganglia were formed symmetrically lateral to the dorsal aorta at E10.5 (Fig. 3.3A). The X-gal staining was increased by E12, paralleling the development of TH immunoreactivity in rat embryos of equivalent stages (Cochard, et al., 1978). In the trigeminal (V) ganglia, the ß-Gal expression was first observed at E9.5 and reached the highest level by E10.5. ß-Gal expression in these ganglia condensed bilaterally and ventrolaterally before forming the three distinctive ophthalmic, maxillary and mandibular branches (Fig. 3.3C). As the trigeminal processes reached the peripheral and central targets at E12, the ß-Gal expression was decreased dramatically in both left and right trigeminal ganglia. ß-Gal expression in adrenal chromaffin cells and in the nephrogenic mesenchyme emerged at E13.5 (Fig. 3.3D). ß-Gal expression in the adrenal chromaffin cells persisted into adulthood, whereas the expression in nephrogenic mesenchyme disappeared by E15.5.

The neural layer of retina, a few cells in the frontonasal processes (Fig. 3.1C), primordia of sinus hair follicles characteristically found in the jaw region (Fig. 3.1E) also demonstrated transient ß-Gal expression. Especially, the X-gal staining observed in the embryonic retina appeared first as the lens vesicle invaginated at E9.5 when the ophthalmic neural crest cells gathered in the surrounding mesenchyme. The staining diminished as the lens vesicle closed after detaching from the ectoderm (Fig. 3.3E). At the same time, the pigmented epithelial layer accumulated a number of cells with pigment granules derived from the ophthalmic neural crest cells. As retina fully developed, the X-gal stained dopaminergic neurons are clearly visible in the amacrine cell layer of the retina (Fig. 3.3F) where TH expression was demonstrated in rat retina (Park, et al., 1986). However, it remains unclear whether these ß-Gal positive amacrine cells arose from the earlier ß-Gal positive cells in the embryonic neural layer.

Fig. 3.3 Expression of ß-Gal activity in the developing peripheral nervous system and tissues.

(A) ß-Gal positive cells appear in coronal section of the dorsal root ganglia, ventrolateral portions of the neural tube and paravertebral sympathetic ganglia at E10.5; (B) In sagittal section through the dorsal root ganglia, the X-gal stained cells are present ventrolaterally, which send processes into the developing spinal cord (E10.5); (C) A sagittal section through the trigeminal ganglion demonstrating three distinctive ophthalmic, maxillary and mandibular nerve branches (E10.5); (D) In the sagittal section of E13.5 embryo ß-Gal expression in the adrenal chromaffin cells persists into adulthood and the transient ß-Gal expression appears in the nephrogenic mesenchyme; (E) The transient X-gal staining in the neural layer of embryonic retina at E10.5 and; (F) the X-gal stained dopaminergic neurons present in the amacrine cell layer of the adult retina. a, amacrine cell; ag, adrenal gland; da, dorsal aorta; drg, dorsal root ganglion; k, kidney; ln, lens vesicle; lv, left lobe of liver; nc, notochord; nt, neural tube; nu, neural layer of optic cup; ol, outer layer of optic cup; op, optic cup; sc, spinal cord; sg, paravertebral sympathetic ganglion; V, trigeminal ganglion; vt, vertebra.

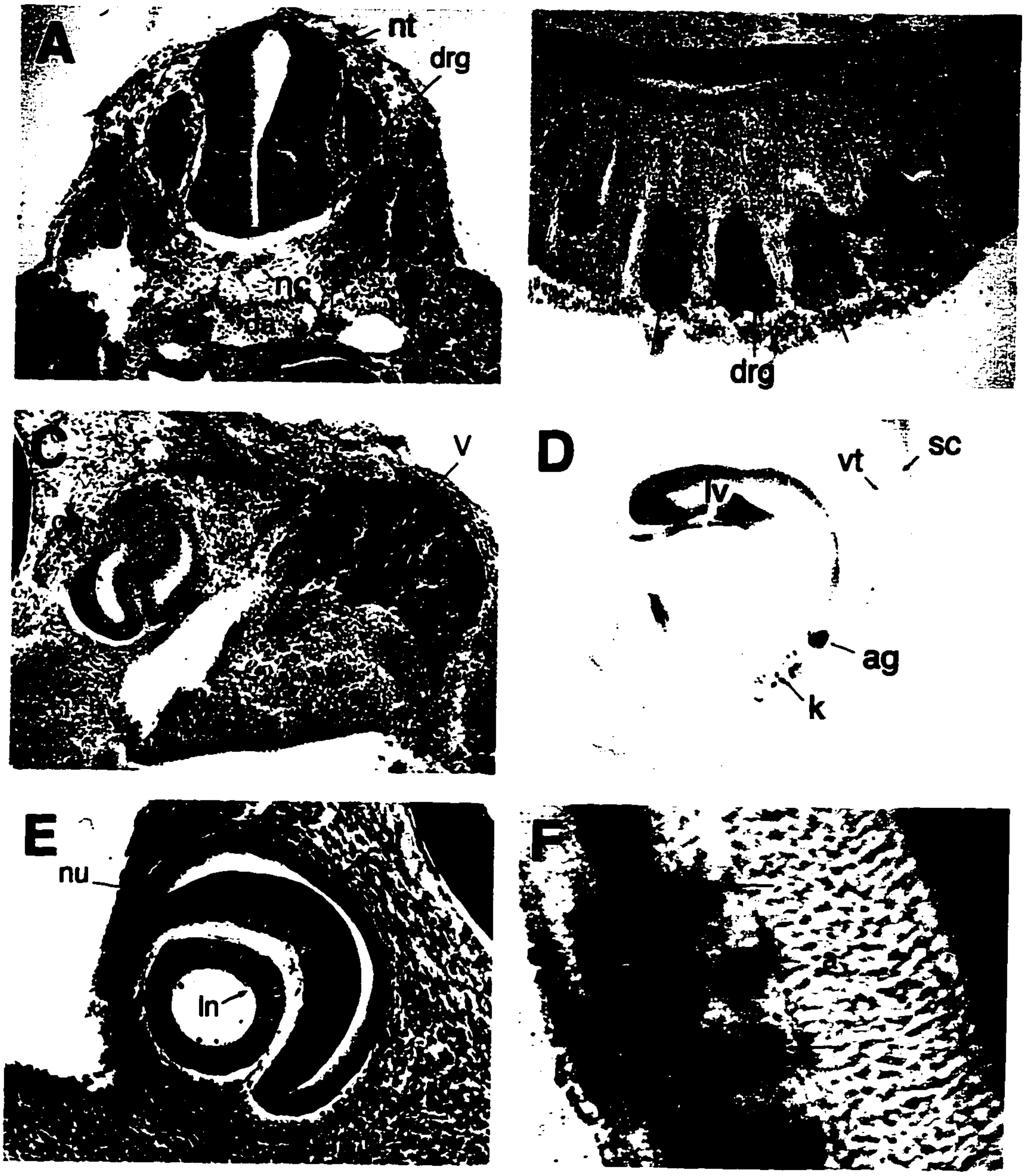

fig 3.3

3.5 DISCUSSION

We determined the temporal and spatial expression of the catecholaminergic phenotype using the specific marker transgene, TH-lacZ, during embryonic neural development. In a previous study (Min, et al., 1994) transgenic mice bearing 9.0 kb of 5' flanking sequence fused to lacZ reporter gene showed high levels of CA neuron-specific ß-Gal expression in the adult brain and adrenal medulla. In the present work, transgenic embryos showed CA cell lineage-specific expression of ß-Gal in both the CNS and PNS. These findings should allow us to identify specific DNA element(s) within the 5' promoter region of the TH gene responsible for CA lineage-specific expression. Understanding the function of this promoter region should help elucidate how various environmental cues influence CA neuronal differentiation.

3.5.1 TH Expression in Brains of Transgenic Embryos

The initial appearance of the dopaminergic neurons in the SN/VTA area was demonstrated in the basal portion of the mesencephalon of the fetal mouse at E13 by histofluorescence (Golden, 1973) and immunohistochemistry (Berger, et al., 1982). Thereafter, dopaminergic cells gradually extended to broad areas of the mesencephalon by E19, but the neurons are smaller and round to oval shape than in the adult brain. In the noradrenergic locus coeruleus of the pons the TH-positive cells first appeared bilaterally rostral to the pontine flexure of fetal mouse brain at E14 (Dreyfus, Markey, Goldstein, & Black, 1983). These observations in developing dopaminergic and noradrenergic neurons correspond precisely to the patterns of TH-lacZ transgene expression in the present study. ß-Gal positive cells, presumably postmitotic dopaminergic cells, were initially present in the rostral portion of mesencephalon at E10.5, which is equivalent to E12 to E12.5 rat embryos (Kaufman, 1992). These cells then migrate into the SN/VTA (A9-10) over the mesencephalic flexure by E13.5. Similarly, ß-Gal positive cells, probably representing noradrenergic precursor cells, appeared in the rostral rhombencephalon at E10.5 and localized in the developing locus coeruleus region at E13.5. These patterns again correspond exactly to previous TH immunohistochemical observations in both mouse and rat embryos (Berger, et al., 1982; Dreyfus, et al., 1983; Specht, et al., 1981a).

All catecholaminergic neurons share the common rate-limiting marker enzyme, TH. Divergence of the noradrenergic/adrenergic lineages are marked by expression of dopamine-ß-hydroxylase (DBH), which catalyzes the conversion of dopamine to norepinephrine. Experiments using human DBH-lacZ transgenes demonstrated X-gal staining in most catecholaminergic cell lineages except the dopaminergic lineage in the fetal mouse brain (Kapur, Hoyle, Mercer, Brinster, & Palmiter, 1991). Comparison of the distribution of ß-Gal positive cells from that study to that of ß-Gal positive cells in the present study suggests a partial overlap in TH-lacZ and DBH-lacZ expression in dorsolateral mesencephalon. This observation raises the interesting possibility that a common precursor pool in the dorsolateral mesencephalon gives rise to all the catecholaminergic neurons, which then migrate to appropriate sites. Thus, careful comparison of patterns of lacZ expression in DBH-lacZ transgenic mice to those of TH-lacZ mice may allow us to address the question regarding the ontogeny of all catecholaminergic lineages in the developing fetal brain. In addition, further in vivo and in vitro characterization of the phenotype of these ß-Gal positive cells can provide useful models to address the spatiotemporal regulation of the ontogeny of the dopaminergic, noradrenergic and adrenergic neurons.

3.5.2 TH Expression in Sensory Ganglia and Sympathoadrenal Cell Lineage

Two major subclasses of neural crest derivatives, sensory and sympathoadrenal cells migrate out from the edge of the neural ectoderm into the periphery and differentiate into sensory neurons, sympathetic neurons and adrenal chromaffin cells (Anderson, 1989). The cranial sensory ganglia are derived from the neural crest and/or placode (Le Douarin, 1986). The crest-derived ganglia are found in the proximal trigeminal (V), jugular-superior (proximal IX-X), proximal VII and dorsal root ganglia, while the placodal neurons are found in the distal portion of trigeminal (V), geniculate (VII), petrosal (IX), nodose (X) and acousticovastibular (VIII) ganglia (D'Amico-Martel, & Noden, 1983). Embryonic expression of TH is observed in most of these sensory ganglia, such as trigeminal, geniculate, jugular-superior, petrosal, nodose and dorsal root ganglia in the rat embryos between E10.5 and E15.5 (Jonakait, et al., 1984; Katz, et al., 1990), despite the fact that TH expression is extremely restricted in the adult sensory ganglia. In the TH-lacZ transgenic mouse embryos, ß-Gal expression was first apparent in primordia of the glossopharyngeal (IX) and vagal (X) ganglia at embryonic day (E) 9.0. Between E9.0 and E10.5, ß-Gal expression was expanded to the trigeminal (V), geniculate (VII) ganglia and dorsal root ganglia. Most of these expression sites either disappeared or were confined to subpopulation of neurons by E13.5. Mouse developmental stages are approximately 1.5 to 2 days earlier than rat stages during early development (Theiler, 1989). Allowing for this temporal adjustment, the transient expression of the transgene in the sensory ganglia appears to mimic perfectly the spatiotemporal expression of TH gene expression in rats as determined by TH immunohistochemistry.

Adrenal medullary chromaffin cells and sympathetic neurons are derived from committed sympathoadrenal progenitor cells. The differentiation of the progenitor cells depends upon both local environmental signals and acquisition of responsiveness to these signals. For example, the progenitor cells differentiate into chromaffin cells after migration into the adrenal gland containing a high content of glucocorticoid hormone (Doupe, Patterson, & Landis, 1985; Teitelman, et al., 1979). On the other hand, differentiation to sympathetic neurons seems to depend on the sequential availability of different neurotrophins with concomitant expression of their own receptors (Ernsberger, Sendtner, & Rohrer, 1989; Verdi, & Anderson, 1984). In the transgenic embryos ß-Gal expression appeared in sympathetic ganglion primordia at E10.5 and in the adrenal medulla at E13.5. From the previous TH expression studies performed in rat embryos, the initial expression of TH appears in sympathetic ganglion primordia at E12.5 rat embryos (36-37 somite stage) equivalent to E10.5 mouse embryos (Cochard, et al., 1978; Teitelman, et al., 1979) and in the adrenal anlage of E15 rat embryos equivalent to E13.5 mouse embryos (Teitelman, et al., 1979). The 9 kb of 5' upstream region of the rat TH therefore appears to contain the cis-acting DNA elements necessary to interact with the environmental cues that specify differentiation of neural crest progenitor cells into sympathetic neurons and the adrenal chromaffin cells.

Accumulating evidence suggests that some developing neurons and peripheral cells transiently express a CA phenotype prior to final differentiation (Baetge, Pintar, & Gershon, 1990; Jonakait, et al., 1984; Jonakait, Markey, Goldstein, Dreyfus, & Black, 1985; Landis, 1992). In our transgenic mice the transient TH expression was observed in both brain and peripheral tissues including dorsolateral mesencephalon, ventrolateral telencephalon, developing cranial sensory ganglia, ventrolateral neural tube, some enteric ganglia, primordia of sinus hair follicles, nephrogenic mesenchyme of developing kidney and neural layer of embryonic retina. However, the functional implications for this transient TH expression is not clear. One possibility is that transient TH expression may occur as a normal change of neurotransmitter property during development. For example, during postnatal innervation of the sweat glands, their noradrenergic sympathetic innervation changes into cholinergic sympathetic neurons (Landis, 1992). Alternatively, the transient TH expression may play some significant role in cell differentiation or neural development. In the leaner mouse mutant the disruption of transient developmental TH expression caused Purkinje and granule cells loss resulting in severe ataxia (Hess, & Wilson, 1991). Moreover, a number of observations have suggested that neurotransmitters including dopamine and norepinephrine, may play a significant regulatory role during the differentiation and development of neurons (Lauder, 1993; Leslie, 1993; Rowe, Messenger, & Warner, 1993; Zhou, Quaife, & Palmiter, 1995). However it should be carefully determined whether all transient TH expressing cells produce a trace of CA. Alternatively, the early catecholaminergic cells may undergo programmed cell death, migrate away from the transiently expression sites for further differentiation, or be present as an evolutionary remnant.

The transgenic lines described in this study will provide useful animal models for further investigation of the ontogeny of catecholaminergic neurons and catecholaminergic cell lineage-specific expression in both CNS and PNS. Manipulation of the TH promoter region should be useful for analyzing the interaction of various environmental cues with molecular mechanisms controlling neural differentiation during early development. The information presented here should provide a basis for identifying specific gene products affecting catecholaminergic differentiation.

Chapter Four:

A transgenic mouse model to study trans-synaptic regulation of tyrosine hydroxylase gene.

by

Nan Min, Tong H. Joh, Eric S. Corp, Harriet Baker, Joseph F. Cubells, and Jin H. Son

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4.1 Foreword and summary

The previous two studies demonstrated that 9kb of the rat tyrosine hydroxylase (TH) 5' flanking sequence directed appropriate spatio-temporal expression of a lacZ reporter gene to catecholaminergic cells in the CNS of transgenic mice. In the present study, specificity of transgene expression was further extended to demonstration of cell-specific functional regulation of lacZ expression using manipulations known to alter endogenous TH expression. Thus, if the DNA cis-acting elements mediating these functional changes of TH expression *in vivo* reside within the 9.0 kb of TH promoter region, alterations in lacZ reporter expression should parallel changes in endogenous TH. Naris closure induced an activity-dependent decrease of TH expression in the glomerular layer of the olfactory bulb that was paralleled by downregulation of the reporter expression. Acute reserpine administration (5mg/kg, s.c.) upregulates endogenous TH. Densitometry and image analysis were utilized to quantify lacZ expression. At 48 hours post-injection, there was a significant increase in OD values, thus X-gal staining, in the locus coeruleus and ventral tegmental area but not in the substantia nigra or olfactory bulb of reserpine treated transgenic animals. These data showed that the 9kb sequence also mediates cell-specific transynaptic regulation of reporter gene expression. Thus, analysis of this transgenic animal offers an useful model system to study *in vivo* regulation of TH gene expression.

4.2 INTRODUCTION

Synaptic transmission can regulate neuronal plasticity by modulating neurotransmitter biosynthesis via alteration in gene expression. Tyrosine hydroxylase (TH, EC 1.14.16.2), the first and rate-limiting enzyme in the catecholamine biosynthetic pathway (Nagatsu, et al., 1964), plays a pivotal role in the maintenance of homeostasis in response to environmental changes. TH expression undergoes exquisite spatial, temporal and functional regulation; hence, complex genomic processes contribute to the phylogenetically conserved TH-expressing cell distribution, the dynamic developmental program and the physiologically induced alteration of functional levels. Transcriptional (induction) and post-translational (activation) mechanisms control TH expression. There are a variety of stimuli that enhance TH gene transcription including: cAMP, nerve growth factor (NGF), epidermal growth factor (EGF), glucocorticoids, cold stress, and reserpine (Black, et al., 1985; Faucon Biguet, et al., 1986; Gizang-Ginsberg, et al., 1990; Lewis, et al., 1987; Lewis, et al., 1983; Stachowiak, et al., 1985; Tank, et al., 1985; Wessel, et al., 1992).

Using *in vitro* approaches, this laboratory has identified DNA cis-elements in the 5' TH flanking region that may be involved in TH transcriptional alteration by trans-neuronal stimuli, for instance, a cAMP responsive element (CRE) located at -45 to -39 bp upstream of the cap site which is essential for basal and cAMP-mediated transcription of TH (Kim, et al., 1993). Additional putative elements include an AP-1 site acted upon by Fos-Jun heterodimers, as well as AP-2, SP-1, FSE and POU/Oct (Cambi, et al., 1989; Carroll, et al., 1991). These DNA elements are included within 9.0 kb of 5' regulatory sequences of rat TH which are required to direct high-level and tissue specific expression of a lacZ (ß-galactosidase, ß-gal) reporter gene in transgenic mice (Min, et al., 1994). Smaller stretches of the rat and human TH upstream regions produced less specific, less reliable expression, associated with extensive ectopic expression (Banerjee, et al., 1992; Kaneda, et al., 1991; Sasaoka, et al., 1992).

To determine whether the 9.0 kb of TH promoter sequence also contains the DNA elements necessary for neurally mediated induction of TH transcription, we tested the inducibility of a lacZ reporter in two trans-synaptic paradigms. First, unilateral naris closure, which results in odor-deprivation, produces a profound downregulation of TH expression at both the protein and mRNA level in dopaminergic cells located in the glomerular layer of olfactory bulb (Baker, 1990; Baker, et al., 1993). Second, the administration of reserpine results in a compensatory upregulation of TH expression in the locus coeruleus and adrenal gland (Faucon Biguet, et al., 1986; Joh, et al., 1973; Muller, et al., 1969; Wessel, et al., 1992). Thus, if the DNA cis-acting elements mediating these functional changes of TH expression *in vivo* reside within the 9kb of TH promoter region, alterations in lacZ reporter expression should parallel changes in endogenous TH.

4.3 MATERIALS AND METHODS

4.3.1 Production and identification of transgenic animals

Mice from the transgenic line TH9.0LacZ-5 have been described previously (Min, et al., 1994). These mice display lacZ expression in a catecholamine cell-specific manner (Fig. 4.1).

4.3.2 Naris Closure

Adult mice were anesthetized with pentobarbitol (30 mg/kg) and their left naris cauterized with a bipolar coagulator as described previously (Baker, et al., 1993). Animals were analyzed between one and two months after naris closure.

4.3.3 Reserpine treatment

Adult mice were given a single injection of either reserpine (5 mg/kg, dissolved in glacial acetic acid) or vehicle subcutaneously at the interscapular area. Animals were sacrificed at 24 or 48 hours post-injection. The areas examined by X-gal histochemistry were the locus coeruleus (LC), substantia nigra (SN), ventral tegmental area (VTA), olfactory bulb (OB) and adrenal gland (AD).

Figure 4.1. Tissue-specific expression driven by 9.0 kb of rat TH regulatory sequences fused to the E.coli lacZ gene demonstrated by X-gal histochemistry in a sagittal section taken from a transgenic mouse from line TH9.0lacZ-5 (Min, et al., 1994). Abbreviations: ac, anterior commisure; Acc, accumbens; AOB, Accessory Olfactory Bulb; AON, Anterion Olfactory Nucleus; Cx, Cortex; Cb, Cerebellum; Hip, Hippocampus; IC, Inferior Colliculus; LC, Locus Coeruleus; ME, Medial Eminence; MOB,Main Olfactory Bulb; Str,Striatum; SN, Substantia Nigra; VTA, Ventral Tegmental Area; ZI,Zona Incerta.

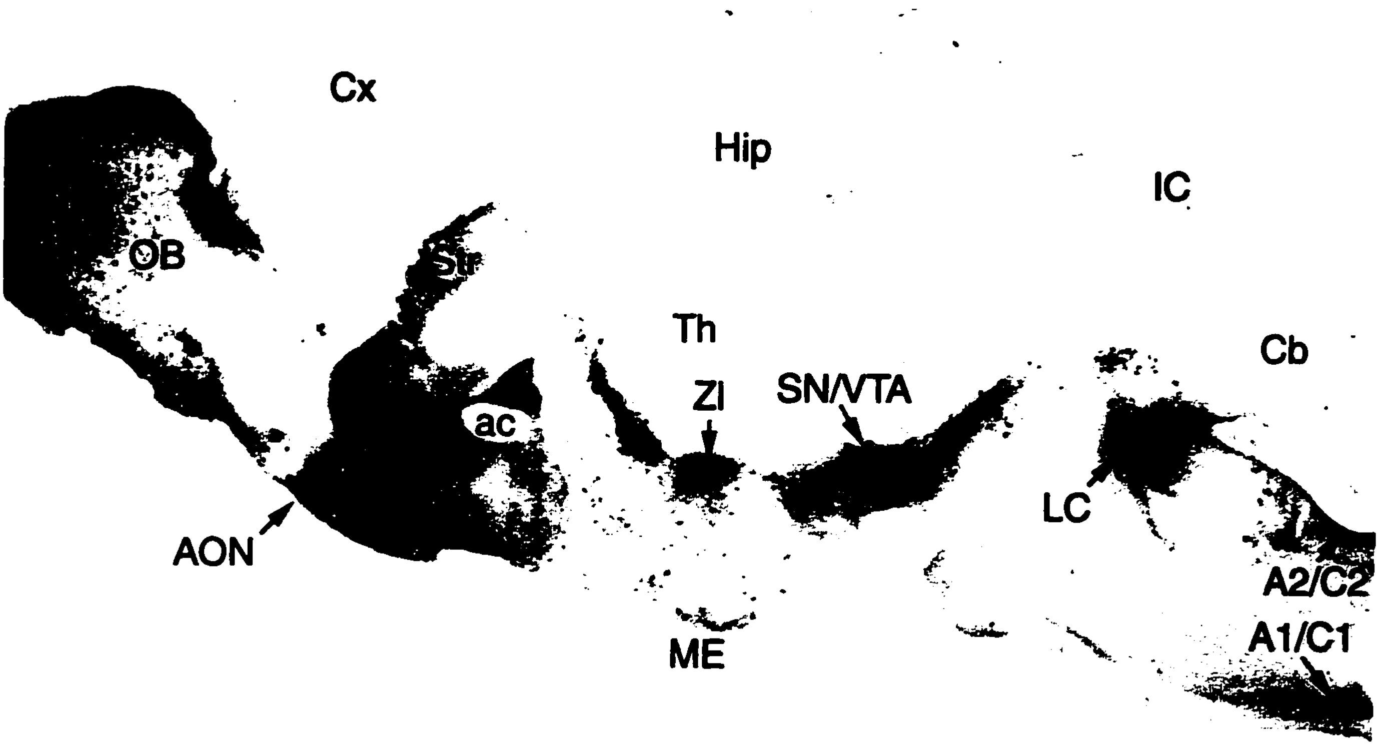

fig 4.1

4.3.4 Immunocytochemistry, *in situ* hybridization and X-gal staining

Histological procedures have been described in detail previously (Huh, et al., 1994; Min, et al., 1994). Briefly,

A. Immunocytochemistry (ICC) was performed in adjacent 40 μm sections using either a rabbit anti-bovine trypsinated TH antibody (prepared in our laboratory, at 1: 25,000 dilution) or a rabbit anti-ß-gal antibody (Organon Teknika Co., NC, at 1: 7,500 dilution).
B. TH *in situ* hybridization was performed on free floating sections using a 1.6kb rat TH cDNA labelled with ^{35}S dCTP by the random primer method (Stone, et al., 1991).
C. To visualize the expression of the lacZ reporter gene, sections were incubated with a solution containing 3.1mM potassium ferricyanide, 3.1mM potassium ferrocyanide, 0.15 M NaCl, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 0.2mg/ml X-gal in 10 mM phosphate buffer (pH 7.4). The activity of the bacterial ß-gal enzyme upon the X-gal substrate yields an insoluble blue dye. For quantification purposes, X-gal reactions in consecutive serial sections were incubated for different times (e.g. 15min, 1hr, 4hrs, and 16 hrs).

4.3.5. Quantitative analysis of ß-gal expression.

Computer-assisted digital analysis and densitometry were performed on slide mounted brain sections. Optical density (OD) measurements were obtained from corresponding areas from control and experimental groups using MCID software (M4 main image analysis program, version 1.2), (for alternative method see (Borsook, Falkowski, Rosen, Comb, & S.E., 1994)). OD is a measure of transmitted light; thus, an increase in saturation of the blue dye directly corresponds to an increase of OD (arbitrary units) at constant source of illumination. Anatomically matched brain regions sampled in this study included: medial and lateral glomerular layer of OB; SN and VTA taken at 4 levels: mesencephalon (A) at the level of the medial and lateral mammillary nucleus, (B) at the level of the interpeduncular fossa, (C) at the level of the interpeduncular nucleus, (D) at the level of the pontine VTA and Retro Rubral Fields (RRF-A8); LC at 3 levels (A) A6 pontine sections anterior to the facial nerve, (B) medial A4, (C) anterior A4; and several sections through-out the AD.

4.3.6 Statistical Analysis

Data derived from OD measures taken from right brain side were analyzed using separate three-way repeated measures ANOVAs (2x2x4; treatment group x sacrifice time x X-gal Reaction time) for each brain area. For descriptive purposes and graphic display, the rate of end-product formation (blue dye) was estimated by performing a non-linear regression of OD values using an one-phase exponential association model (Inplot). Results were plotted in a graph with OD as a function of the log of X-gal reaction time, and linear regression lines were computer generated.

4.4 RESULTS

4.4.1 Down-regulation of lacZ expression in juxtaglomerular cells of olfactory bulb following naris closure

As previously reported (Baker, et al., 1993), expression of TH mRNA (Fig. 4.2A and 4.2B) and TH immunoreactivity (Fig. 4.2 D,G,H) in the glomerular layer of the odor-deprived olfactory bulb (OB) is reduced in comparison to the intact side. TH mRNA down-regulation occurred in the deprived OBs, from both transgenic and non-transgenic animals (Fig. 4.2A,B). The presence of the transgene did not appear to affect endogenous TH regulation.

Both X-gal staining (Fig. 4.2 C,E,F) and ß-gal ICC (data not shown) demonstrated that naris closure produced changes in ß-galactosidase expression. In the odor-deprived bulb, down-regulation of TH expression was characterized by a decrease of TH immunoreactive cells and fibers (Fig. 4.2 G,H). In contrast, *lacZ* reporter gene down-regulation in the transgenic mice was characterized by a markedly reduced fiber content, whereas cell number remained about the same, albeit with less ß-gal immunoreactivity and less X-gal staining per cell (Fig. 4.2 E,F).

In the odor-deprived olfactory bulb of the transgenic mice, lacZ expression in granule and mitral cells and granule cells in the accessory olfactory bulb (non-traditional TH expression sites) did not exhibit the same degree of down-regulation (Fig. 4.2 C). The reporter expression was somewhat reduced in these non-traditional sites but not as markedly as the profound reduction in the expression in the glomerular layer. Thus, these areas in which expression occurs at the TH mRNA but not protein level in mice might be under distinct transcriptional control.

Figure 4.2. Naris closure induced downregulation of TH expression in the glomerular layer of the OB compared to the intact side: at the protein level in (B) TH ICC, (E,F) higher magnification; and the mRNA in (H) TH in-situ hybridization in non-transgenic and (G) transgenic animals. LacZ expression directed by the TH promoter mimics endogenous gene alterations visualized by X-gal staining in (A) or higher magnification (C,D).

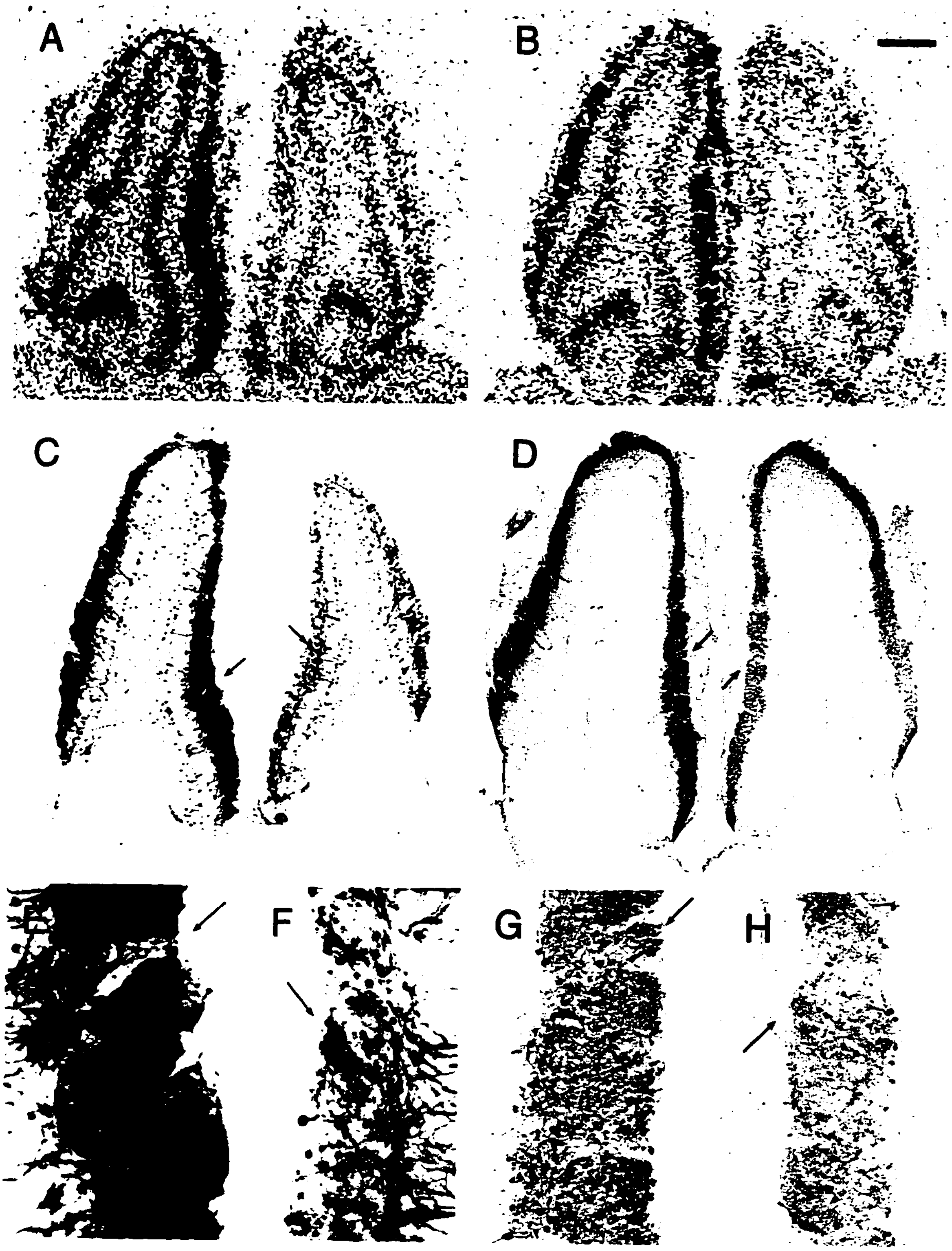

fig 4.2

4.4.2 Up-regulation of lacZ expression in locus coeruleus by reserpine treatment.

Noradrenergic cells in the LC of control and reserpine treated animals stained for X-gal for 4 hours are shown in Fig. 4.3. At 24 hours post-injection, reporter gene expression monitored by X-gal staining was equivalent in the LC cells of mice receiving either reserpine (24hrRes, Fig. 4.3 B) or vehicle (24hrVeh, Fig. 4.3A) injections. However, at 48 hours post-injection, reserpine induced a marked upregulation of transgene expression (48hrRes, Fig. 4.3D) characterized by enhanced X-gal staining compared to vehicle treated animals (48hrVeh, Fig. 4.3C). Qualitatively, in vehicle treated animals, overall staining was lighter and punctate areas of higher staining were distinguishable, whereas in reserpinized animals, staining acquired a continuous deep hue and leakage occurred into the surrounding parenchyma. This finding is in agreement with Borsook et al.,1994, when monitoring promoter activity using the X-gal assay, basal expression of the transgene appears as small blue puncta whereas induced expression appears as progressive cellular filling. Except that, as the X-gal reaction proceeds cellular filling by the blue dye invariably takes place and induction by experimental manipulations is characterized by an increase of rate of cellular filling and subsequent leakage.

To compare relative levels of X-gal staining quantitatively, we used densitometry and image analysis. A time-course analysis used digitized LC images (Fig. 4.4) to compare the X-gal staining in sections from 48hrVeh (Fig. 4.4A,C,E,G) and 48hrRes animals (Fig. 4.4B,D,F,H). The staining in 48hrRES animals appeared less than 15 minutes after the initiation of the X-gal reaction (Fig. 4.4B), while in the control animals expression was not detectable at this early point. In the LC, the effect of reserpine on lacZ expression was evident at all X-gal reaction times.

Graphic analysis of OD data (Fig. 4.5) indicated differential TH promoter induction by reserpine treatment in specific brain regions. There was a significant enhancement of reporter expression by reserpine treatment in LC [at level B, $F(1,8) = 6.05$, $p<.05$] and

VTA [at level C, F(1,8)= 11.22, p<.05] but not in SN and OB. Visual inspection indicated similar differences of the other sampled regions of LC and VTA but statistic analysis was not performed. In the LC, a small increase in OD induced by reserpine was already detectable at 24 hours post-treatment. The magnitude of this induction increased significantly over 48 hours, [in the LC the interaction (Sacrifice time x drug treatment), F(1,8) = 4.94, p=.0569]. There was a significant increase in OD over the time course (15min, 1h, 4hrs, 16hrs) of the X-gal reaction [in LC, F (3,24) = 115.26, p<.001]. These findings indicated that as the X-gal reaction proceeded, increasing amounts of blue dye accumulated and the reaction time must be acounted for when making intergroup comparisons across experiments. The graphic representation of log(reaction time) plotted against OD suggest that the X-gal reaction proceeds linearly. Qualitatively, differences in staining could be detected at any arbitrary time point; however the graphic analysis suggest that optimal comparisons should be made within the linear range of the regression line.

Consistent with our previous report (Min, et al., 1994), despite correct CNS expression, the reporter expression in the adult adrenal gland was neither fully penetrant nor trans-synaptically regulated (data not shown).

Figure 4.3. High power (40X) photomicrograph of LC cells stained by X-gal histochemistry for 4 hours from vehicle (A,C) and reserpine (B,D) treated animals sacrificed 24 hours (A,B) or 48 hours (C,D) post-injection. Light cellular staining and punctate areas of higher staining can be distinguished in the vehicle treated animals. In the reserpinized animals the staining acquires a deeper hue, and greater accumulation of the dye results in cellular leakage. Bar =

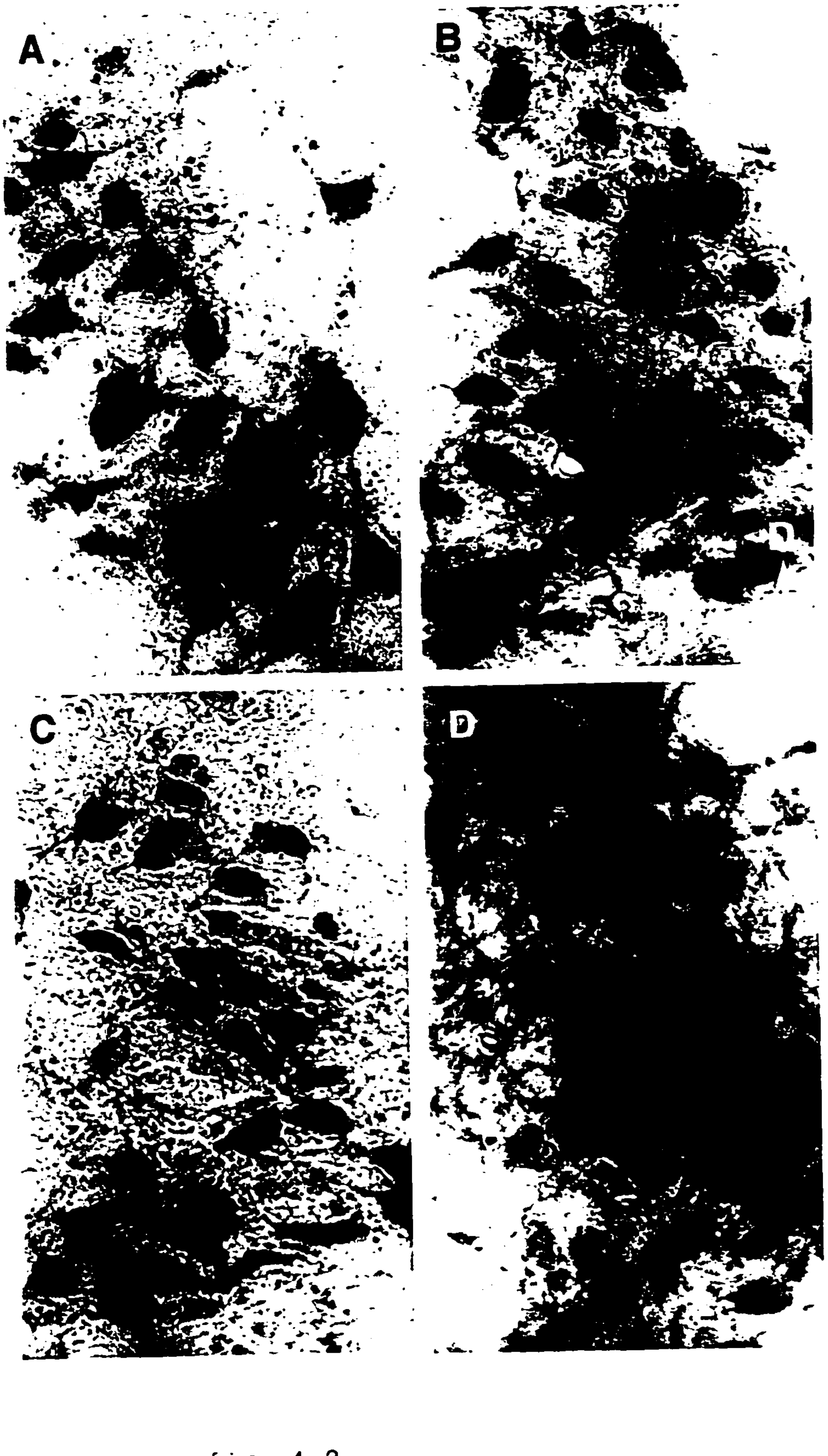

fig 4.3

Figure 4.4. Time course analysis comparing digitized images of LC from vehicle (A,C,E,G) and reserpine (B,D,F,H) animals sacrificed 48 hours post-injection at different X-gal reaction incubation times: 15 min (A,B), 1 hr (C,D), 4 hrs (E,F), and 16 hrs (G,H). The increase in X-gal staining in 48hrsRES animals can be detected at all time points. Bar =

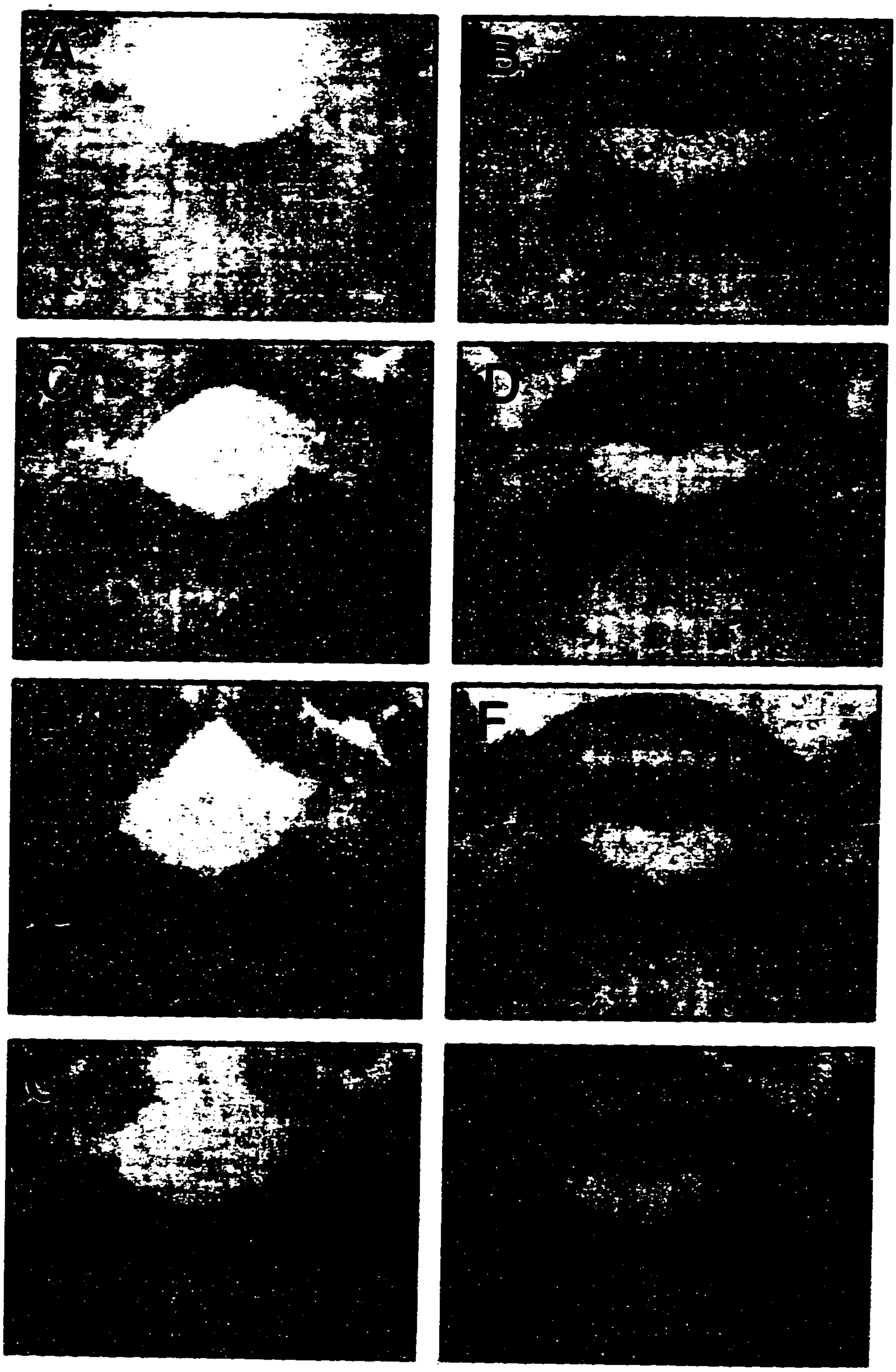

fig 4.4

Figure 4.5. Graphic representation of OD versus Log of Reaction time [Rx] in different brain areas demonstrates that reserpine induction of reporter gene expression in the 48hrsRes animals occurs in the LC (level B, medial A4) and to a lesser degree to VTA (level C, interpeduncular nucleus) but not OB or SN: (A) Locus Coeruleus, (B) Ventral Tegmental Area, (C) Olfactory bulb, and (D) Substantia Nigra.

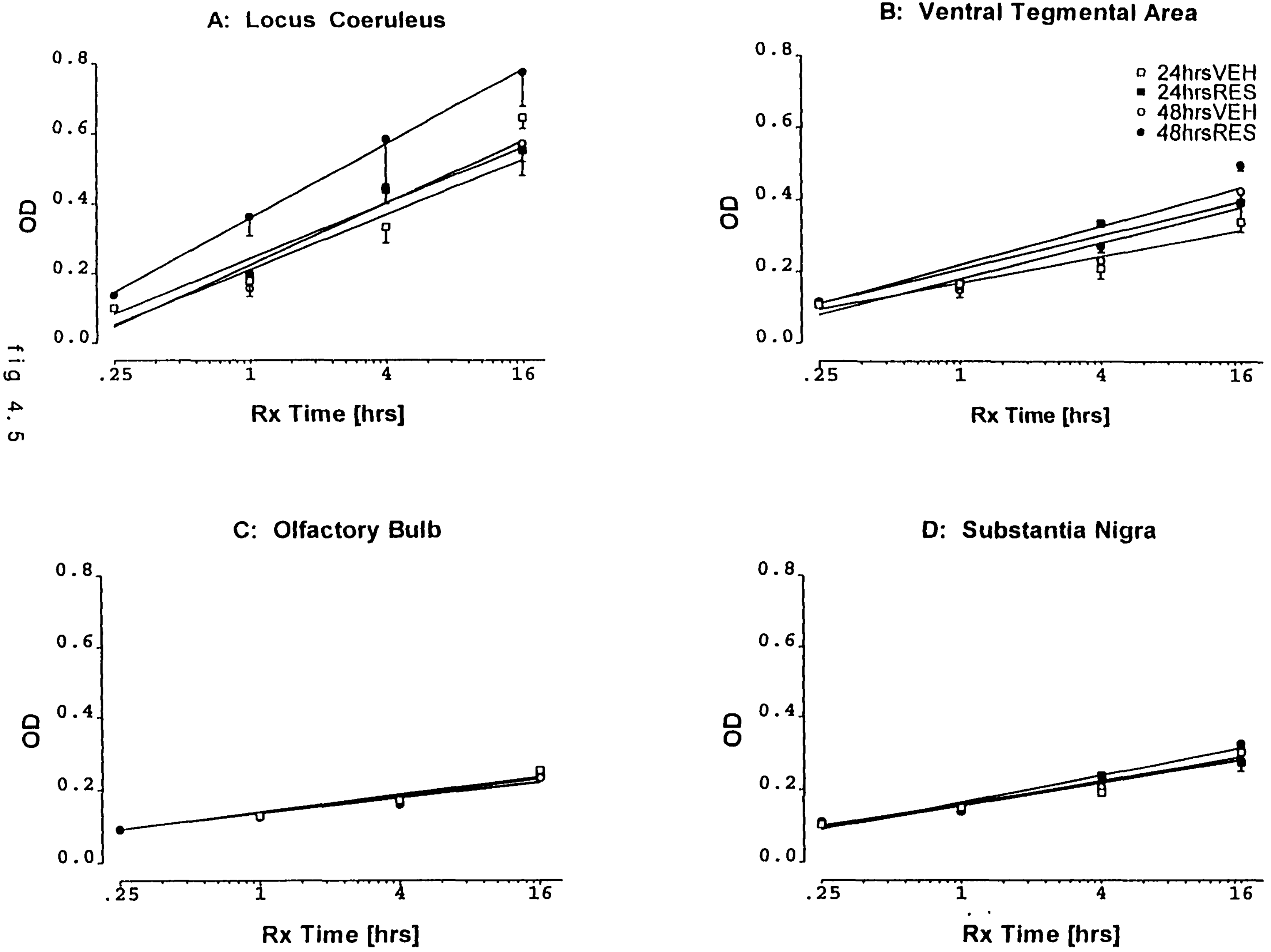
A: Locus Coeruleus
B: Ventral Tegmental Area
C: Olfactory Bulb
D: Substantia Nigra
OD
0.0
0.2
0.4
0.6
0.8
Rx Time [hrs]
.25
1
4
16
□ 24hrsVEH
■ 24hrsRES
○ 48hrsVEH
● 48hrsRES

fig 4.5

4.5 DISCUSSION

The present study demonstrates that the lacZ reporter expression, directed by 9.0 kb of rat TH promoter, can be regulated by trans-synaptic mechanisms in the CNS, *in vivo*. Thus, in addition to directing specific spatio-temporal expression to CNS catecholaminergic cells (Min, et al., 1994; Son, Min, & Joh, 1996), the 9.0 kb of 5' flanking sequences of the TH gene also mediates functional regulation of transgene expression in the LC and OB. Specificity of inducible expression directed by the TH promoter was demonstrated in two manipulations which regulated TH in a distinct manner. Activation of a transgene by empirical manipulations has been previously documented. For instance, in a Fos-lacZ transgenic mouse, the fusion protein could be induced by photic or seizure stimulation (Smeyne, et al., 1992). Induction was reported to occur either by increase in expression or by "recruitment" of additional cells which previously did not express the gene at detectable levels, or by both. Reports of down-regulation of a transgene are more scarce.

4.5.1 Transgene downregulation by naris closure indicates that TH expression in the glomerular layer is activity-dependent.

In this study, the decrease in transgene expression following naris closure resulted in diminished staining of ß-gal in cell bodies and marked reduction in fiber staining rather than a decrease in the number of ß-gal positive cells. These observations corroborates previous findings (Baker, 1990) that the effect of naris closure represents a functional downregulation, rather than cell death. Thus, the alterations of expression of the transgene in the olfactory periglomerular cells supports the notion that endogenous TH expression is activity dependent. Much debate underlies the nature of TH downregulation after deafferentation. Evidence that the phenomenon consists of a phenotypic modification, and is not the result of cell death, is supported by several lines of evidence. In a sub-population of juxtaglomerular which express both GABA and dopamine, GAD, the GABA biosynthetic enzyme, expression is not affected even though TH is down-regulated (Stone, et al., 1991); additionally, in reversible lesions, TH expression is restored when innervation can reach the OB again (Baker, Kawano, Margolis, & Joh, 1983). In another TH-transgenic model (Banerjee, et al., 1992) it was reported that deafferentation of the OB diminished CAT reporter expression driven by a 4.8 kb rat TH promoter, as measured biochemically in dissected OB tissue. However, these observations, while consistent with ours, could not determine transgene expression at the cellular level.

The present study not only localizes innervation-dependent TH transgene expression to the periglomerular cell, but also raises novel issues regarding the cellular expression of the lacZ driven by the 9.0 kb TH promoter. Regulation of the transgene was specific to the second-order neurons in the glomerular layer of OB. In mouse but not rats OB, TH mRNA expression which is not apparently translated onto TH protein occurs in sites such as accessory olfactory bulb and some mitral and granule cells in other layers (Min, et al., 1994). Rat-derived TH promoter directed expression in these non-traditional sites, thus exhibiting a mouse-specific pattern of expression. It is possible that such expression, if authentic, represents an evolutionary remnant. These observations suggest an epigenetic action of mouse transcription factor(s) upon putative phylogenetically conserved element(s) in the rat promoter. The expression in mice of mRNA which is not translated results in the same phenotype in rats despite a different mechanism, i.e. in both species no expression of TH protein occurs in these non-traditional sites. Interestingly, these non-traditional transgene expressing cells did not exhibit the profound activity-dependent down-regulation.

4.5.2 Both proximal and distal elements might be required for cell-specific induction by reserpine.

Reporter expression in the LC was induced by reserpine, and thus mimicked endogenous TH expression (Faucon Biguet, et al., 1986; Wessel, et al., 1992). The anatomic specificity of TH induction by reserpine was also exhibited by the reporter gene. Thus, we observed a large upregulation of reporter in nor-adrenergic LC cells and a small statistically significant upregulation in dopaminergic VTA, but not SN or OB, cells. Sub-populations of mesencephalic dopaminergic neurons possess different pharmacological (Chiodo, 1988) and anatomic properties. SN and VTA neurons have been shown to be physiologically different with respect to response to reserpine. A small increase in TH mRNA was found in the VTA three days after reserpine administration (Passineti, Morgan, Johnson, Millar, & Finch, 1990). In a Fos and TH double ICC study, differential effects of reserpine were demonstrated. That is, fos-like immunoreactive cells were co-localized with TH immunoreactive cells in most nor-adrenergic cell groups but not in dopaminergic cell groups except in the VTA where twenty percent of the cells were double labelled (Fritchy, Frondoza, & Grzanna, 1991).

Quantitative analysis of relative changes of X-gal staining support our qualitative observations with the added advantage that the data can be analyzed statistically. ICC results are not easily quantifiable (Benno, Tucker, Joh, & Reis, 1982), and intensity of chromogenic signal may not correlate with amount of protein (antigenic levels) since the signal is amplified several fold to produce a visible product. In contrast, changes in X-gal staining, appropriately controlled, can be attributable to alterations in number of protein molecules. The TH9.0lacZ transgenic mouse is thus a useful model for studying TH gene regulation.

In vitro, TH promoter analysis determined that only short proximal promoter sequences are sufficient to drive both basal and inducible expression in a catecholamine cell-specific manner (Yoon, et al., 1992); in contrast, much larger sequences are required for *in vivo* tissue-specific expression using transgenic analysis (Banerjee, et al., 1992; Min, et al., 1994). Even though DNA elements capable of altering TH transcriptional levels, such as CRE or AP-1, are located within few hundred-bp from the CAP site, it is important to stress that the reporter inducibility followed precise cell-specific regulation; for instance, reserpine inducible expression occurred in the noradrenergic cells of LC and VTA but not in the dopaminergic cells of either SN or OB. Taken together, *in vitro* findings would suggest that factors which alter TH transcriptional activity act upon elements located within the proximal TH promoter region, and *in vivo* analysis indicates that there are more distal elements which confer tissue-specific expression. Both proximal and distal elements must act in concert to confer full basal and inducible TH expression to CNS.

The current data do not preclude the existence of additional enhancer(s) or repressor(s) sites outside the 9.0 kb promoter. For instance, the lack of appropriate expression to peripheral cells by the 9.0 kb TH promoter strongly suggests that additional positive elements might be present elsewhere, for instance in intronic or 3' flanking sequences. In fact, elements located in 3' flanking sequences of the rat TH gene were reported to drive tissue specific expression in the PNS. Alternatively, the 9.0 kb sequence may contain negative element(s) which repress peripheral expression of TH in the adult sympathetic ganglia and adrenal medulla.

In conclusion, the 9.0 kb of rat TH promoter, which directs appropriate spatial-temporal expression in the CNS, also contains sequences sufficient to mediate cell-specific trans-synaptic regulation of reporter gene expression. This result also strengthens the specificity of expression driven by the 9.0 kb sequences. Furthermore, this transgenic model containing 9.0 kb of rat TH offers an useful and sensitive tool for studying both the cell specificity and functional regulation of TH expression. Further characterization of this promoter should delineate specific DNA elements involved in trans-synaptic regulation, and ultimately will help identify important trans-acting factors.

V. DISCUSSION

5.1. Summary and Interpretation of Results

The objective of this thesis work was to study the regulation of the TH gene by defining and characterizing a TH promoter (5' flanking sequences) which contained putative cis-acting DNA regulatory elements responsible for catecholamine tissue-specific, developmental and functional expression. To achieve this goal, three consecutive sets of experiments were carried out: (1) determination of the sequences important for tissue-specific expression by testing three DNA constructs, containing deleted amounts of the TH promoter fused to the *E.Coli* lacZ reporter gene, in transgenic mice, (2) monitoring of the transgene expression in transgenic mice during embryogenesis to determine whether the TH promoter could direct correct developmental stage-specific expression, and (3) characterization of functional regulation of transgene expression in two experimental paradigms.

In the first study, three recombinant DNA constructs, containing progressively deleted 5' upstream sequences of TH fused to the lacZ reporter gene pTH9.0lacZ, pTH2.4lacZ and pTH.15lacZ, were assayed for potential to drive catecholamine expression using transgenic mice. The analysis of transgene expression suggested that the shorter contructs, .15 and 2.4 kb of the TH 5' flanking region, did not contain sufficient information to direct expression of the lacZ gene to the appropriate cells. In contrast, pTH9.0lacZ, containing 9.0kb of 5' upstream region from the transcription initiation site was able to direct expression of the reporter to appropriate neurons in the CNS of transgenic mice with little ectopic expression. These data suggest that important cis-acting element(s) for tissue-specific expression of the rat TH gene resides within 9.0 kb of the 5' upstream region. Some of the non-traditional neurons were shown to express the transgene. This could take place if the TH promoter gene did not contain certain elements(s) that normally suppress the endogenous TH gene expression in those non-catecholamine neurons. Additionally, some transgenic-line specific ectopic expression was accountable for a position effect (integration site effect) that affected the expression of the transgene depending on its integration site on the mouse chromosome. Some spatiotemporal misregulations might also have occurred due to the lack of a negatively-acting element which modulates transient expression during a ceirtain stage of development that would normally be turned off at a later stage.

In the second study, the early ontogeny of TH expression was studied, by following the expression of the lacZ reporter driven by the 9.0 kb TH promoter, in transgenic mouse embryos. The lacZ reporter expression was first detected at embryonic day (E)9.0 in the primordia of sensory ganglia serving the glossopharyngeal (IX) and vagal (X) cranial nerves. Between E9.5 and E10.5, ß-Gal expression extended to the remaining cranial sensory ganglia serving the trigeminal (V) and facial (VII) nerves, dorsal root ganglia, ventrolateral neural tube and sympathetic ganglion primordia; in the embryonic brain the transgene also appeared within distinct regions, such as the ventral prosencephalon, the ventral and dorsolateral mesencephalon and the rostral and caudal rhombencephalon. The level of ß-Gal expression in all these tissues decreased at E13.5 with the emergence of a distinct adult pattern of ß-Gal expression in the substantia nigra and ventral tegmental area in the CNS and the adrenal medulla in the periphery. Our findings indicate that the proximal 9.0 kb of the 5' promoter region of the rat TH gene encodes sufficient information to direct development of the appropriate catecholaminergic lineage cells in the central and most peripheral nervous systems during embryogenesis.

In the third study, the specificity of the expression driven by the 9.0 kb promoter was further extended by demonstration of modifiable TH expression following two well characterized paradigms known to alter endogenous TH expression. Unilateral naris closure produces an activity-dependent decrease of TH expression in the glomerular layer of the ipsilateral olfactory bulb. Mimicking endogenous TH expression, LacZ expression was down-regulated. Acute reserpine administration upregulates endogenous TH. Using densitometry and image analysis to quantify lacZ expression, it was determined that at 48 hrs post-injection, there was a significant increase in OD values, thus X-gal staining, in the locus coeruleus and VTA but not in the substantia nigra or olfactory bulb of reserpine treated animals. These data showed that 9.0 kb sequence also mediates cell-specific trans-synaptic regulation of reporter gene expression.

Taken together, the previous studies have shown that 9.0 kb of 5' flanking sequences of the rat TH gene contain important DNA elements capable of regulating temporal, spatial and functional expression of the TH gene in transgenic mice.

5.2. Future Directions

5.2.1. Delineation of regulatory cis-elements within the 9.0 kb rat TH gene.

The 9.0 kb of the 5' flanking region of the rat TH gene contain several important regulatory DNA elements which direct basal and inducible expression, as well as potential cis-acting elements capable of temporal and spatial regulation. Sequence analysis of the TH promoter within the proximal 4.8 kb revealed several potential regulatory elements (Crews, & Chikaraishi, 1995). There are a large number of putative DNA binding sites which include TRE's (for AP-1 and AP-2), E-boxes (for HLH proteins), A-T rich octamer sites (for homeodomain proteins), CAAT boxes and sites for SP-1. Additionally, there are numerous potential binding sites for steroid receptor family hormones such as glucocorticoid and NGFI-B, for the NF-KB and for PU.1. Further deletional and site-mutagenesis analyses will be necessary to outline the functional contribution of these putative elements to TH transcription.

Finally, this extended sequence analysis also delineates a site, residing from -2342 to -2322, which is 57% identical (92% within a 12 bp core) to the consensus NRSE (neural restrictive silencer element) found in genes encoding SCG10 (Mori, Schoenherr, Vandenbergh, & Anderson, 1992), type II sodium channel (Kraner, Chong, Tsay, & Mandel, 1992) and synapsin (Li, Suzuki, Mori, & Greengard, 1993). The NRSE is a negative regulatory element which may repress gene expression in non-neuronal cells and found to be important for the cell-type specific expression of neuronal genes. The Dopamine β- hydroxylase (DBH) gene, that encodes for another catecholamine biosynthetic enzyme, contains a homologous sequence motif which resides in a 5' flanking upstream domain that has a cell type-specific silencer function (Ishiguro, Kim, & Joh, 1995). It will be of significant importance to address whether such a neuronal silencer exists in the regulatory region of the TH gene, primarily neuronal but also found in the adrenomedullary cells and transiently during development in non-neuronal cells, and the interactive role a such silencer would play to orquestrate the dynamic developmental changes amidst distinct TH-expressing cells.

The existence of DNA elements that may direct spatial expression to specific sites, for instance: a subtantia-nigra-specific element or a adrenal-specific element, has been speculated by the existence of several neuronal promoters that target to a subset of the appropriate expression sites. This laboratory has demonstrated that cis-acting elements responsible for pineal specific expression of the TPH gene *in vivo* may reside within 1.1 and 6 kb of the TPH 5' flanking sequences by demonstration of strong and specific transgene expression in the pineal gland which is dissociated from the correct raphe-specific expression (Huh, et al., 1994). Additional evidence for the existence of multiple and distinct elements that direct tissue-specific expression is evidentiated in a PNMT transgenic mice which directs correct expression to the retina and adrenal glands, but fails to direct expression to the adrenergic cells in the brain stem (Baetge, Behringer, Messing, Brinster, & Palmiter, 1988).

Extensive future work is necessary to delineate whether putative positive and negative regulatory elements are contained within the TH promoter. Furthermore, potential DNA elements suggested by sequence analysis still require further study to demonstrate that they are indeed functional. The characterization of cis-acting elements within the 9.0 kb sequences will greatly contribute to understanding the modulation of TH transcription.

5.2.2. Creation of immortalized catecholamine cell lines

Immortalized cell lines are important tools in studying molecular aspects of biological phenomenon. However, neuronal cell lines are difficult to develop because of the postmitotic and terminally differentiated nature of the neuron, and the scarcity of naturally occurring neuronal tumors to develop useful neuronal cell lines (Fung, et al., 1990).

Recently, a number of neuronal and nonneuronal immortalized cell lines were developed by targeted tumorigenesis to a specific cell type, achieved by expressing the simian virus (SV40) early region encoding the large T and small t antigens under the control of a specific tissue-specific promoter in transgenic mice (Hammang, et al., 1990; Hanahan, 1985; Mellon, et al., 1990; Rosenfeld, Crenshaw, & Lira, 1988). Additionally, a serotonergic cell line was established in this laboratory from pineal tumors derived from transgenic mice carrying a DNA construct consisting of 6.1 kb of the 5' flanking region of the mouse TPH gene fused to the SV40 T-antigen (Son, et al., 1996).

The existence of neuronal catecholamine cells would facilitate the study of TH regulation. Recently, two cell lines that have catecholamine characteristics have been developed: Path cells, derived from the an adrenal tumor, and Cath cells, derived from a CNS locus coeruleus-like tumor (Suri, & Chikaraishi,). In order to establish catecholamine cell lines, in particular dopamine specific, the 9.0 kb TH promoter fused to a temperature sensitive SV40 T-antigen has been microinjected into fertilized eggs to produce transgenic mice. The targeted tumorigenesis will result in the creation of catecholamine cell lines which have the advantage that their mitotic state (either proliferation or differentiation) can be manipulated by changing the cultures temperature. Conditional immortalization is an alternative way to develop catecholamine cell lines that can be established at specific developmental stages (Whitehead, VanEeden, Noble, Ataliotis, & Jat, 1993). Preliminary results from cell lines derived from the substantia nigra, locus coeruleus, and olfactory bulb suggest that immortalized cell are differentiated because TH expression can be detected. When finally established and characterized, these catecholamine-specific neuronal cell lines will be intrumental for studying the molecular mechanisms governing TH gene expression.

5.2.3. TH9.0lacz animal as a model system for the study of *in vivo* regulation of the TH gene

Transgenic animals produced in this study that exhibited a catecholamine specific expression of the lacZ reporter gene are a useful model system to investigate the mechanism for the modifiable TH induction. In the research for this dissertation, I demonstrated that the b-galactosidase activity (or intensity of X-gal staining) from the transgenic animals mimics the alterations in TH levels which occurs after experimental manipulations. Because the expression of the lacZ reporter gene is under the transcriptional control of the 9.0 kb of the TH 5' upstream region, the modulation of reporter expression can be used to monitor induction of TH (changes in mRNA levels). It is important to emphasize that the trans-synaptic TH induction occurred in a cell-specific manner. For instance, upregulation of transgene expression after reserpine treatment occurred in the locus coeruleus and VTA cells but not in olfactory or substantia nigra. The alteration in rate of expression was not a generalized induction effect, it was rather specific to the appropriate cells. Thus, both proximal elements which may alter rate of transcription and distal elements which confer cell-type specific expression were regulated in concert in order to exhibit the precise cell-specific trans-synaptic modulation of TH expression.

Another transgenic animal that contained 4.8 kb of the TH 5' flanking region linked to CAT (Banerjee, et al., 1992) was used to investigate changes in transgene expression in response to exposure to cold and immobilization stress (Augonis, Chikaraishi, & Tank, 1995). The results suggest that these stressors caused an induction of TH-CAT transgene in the adrenal medula, associated with an increase of endogenous TH protein expression. The TH induction was transient after a single immobilization and sustained after repeated immobilization or cold exposure for 2-3 days . In a cautionary note, the induction was detected in the adrenal gland, in spite of lack of full expression of the CAT transgene that was ten-fold lower than the expected TH endogenous levels in the adrenal (Banerjee, et al., 1992). In the TH9.0lacZ transgenic mice, a subset of adreno-medullary chromafin cells were correctedly targeted in one transgenic founder line, but their expression was variable in different individuals. However it might be unwarranted to draw conclusions over an expression that is not adequately regulated.

Currently, Dr. Nestler's group in Yale is using the TH9.0lacZ animal model to monitor alterations in TH levels in the mesolimbic projection from the VTA to the n. Accumbens after administration of addictive drugs. Preliminary observations indicate that this animal model will aid in screening drugs which have the potential for abuse in humans and also in screening possible drug treatments that may antagonize addictive effects and be used in the therapy of drug-dependent patients.

Thus, due to the ease and sensitiviy of the reporter expression assay, the transgenic animal produced in this work offers a useful way to study TH induction.

5.2.4 Characterization of trans-acting factors involved in TH gene regulation.

Interactions between tissue-specific cis- and trans-acting elements within the 9.0 kb of the 5' flanking region of the rat gene may play a role in modulating the expression of the transgene to catecholamine cells. Determination and characterization of transcription factors, that bind to the DNA elements present in the promoter region of the rat TH and alters its transcription rates, remains elusive.

This laboratory has determined the importance of a cyclic AMP response element (CRE) for the basal and inducible transcription of the TH gene (Kim, et al., 1993). Gel mobility shift assays using nuclear extracts from different cells were used to examine the role of CRE binding proteins (Tinti, et al., 1995). Based on differential mobility patterns, it was suggested that the peripheral (extracts from adrenal gland and PC12 cells) and central (extracts from olfactory bulb, substantia nigra and locus coeruleus) nervous systems express different CRE-binding proteins. Functional TH assays, by either reserpine administration to animals or forskolin treatment to cultures, produced an intense CRE-specific band that was not detectable in controls at same exposure times, indicating that CRE binding proteins play a role in trans-synaptic and cAMP-dependent increases in TH gene transcription.

A great deal of work lays ahead in identifying the transcription factors that regulates TH transcription, both in physiological and pathological cases. The creation of the immortalized cell lines will aid in the identification of such nuclear binding proteins, that influence TH transcription by binding in the cis- elements contained within the TH promoter.

5.2.5 Gene therapy

Parkinson's disease is a neurodegenerative disorder, the hallmark pathophysiological feature consisting of the death of nigro-striatal neurons. Recent data suggest that a variety of substances, in particular neurotrophic factors such as glial derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF), may support survival and regeneration of these neurons. Ultimately, these neurotrophic factors may be used in the therapy of neurodegenerative diseases. However, because these substances do not cross the blood-brain-barrier, the use of these substances is problematic. It has been suggested that a virus-mediated delivery system might be used to circumvent this problem. The Herpex simplex virus (HSV) has been used as a vector because it has amenable features that include a wide host range, an ability to infect postmitotic cells such as neurons, a capacity to be maintained indefinitely in a latent stage, and a high capacity for foreign gene insertion. HSV-1, a defective herpes simplex virus, has been developed to introduce genes into neurons (Geller, & Breakefield, 1988). Cells can be infected and expression of the desired proteins correctly targeted to the catecholamine cells, when using a designed retroviral vector such as HSV-1, Adenovirus (AV) or Adeno- associated Virus (AAV), carrying the 9.0 TH promoter fused to the gene encoding for the desired neurotrophic factor or any given peptide. Thus, using the tissue-specific 9.0 kb TH promoter within an appropriate vector system should be an effective therapeutic tool for the delivery of substances into the CNS.

5.3 Final Remarks

The main contribution of this dissertation is the identification and characterization of regulatory sequences of the rat TH promoter. These data provide the basis for studies that would seek to determine the functional contribution of these DNA sequences to complex mechanisms that govern cell-specific expression of neuronal genes. Furthermore, as in the case of the reporter gene, it is proposed that the 9.0 kb sequences can be used to drive the espression of a number of genes to catecholamine cells, and this will be useful both for investigative and clinical purposes. Finally, the transgenic mice produced with the TH9.0lacZ construct offer a useful tool for the study of *in vivo* TH regulation.

VI. BIBLIOGRAPHY

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