REGULATION OF THE HUMAN NEURONAL NITRIC OXIDE SYNTHASE GENE VIA ALTERNATE PROMOTERS

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ABSTRACT

Nitric oxide is a gaseous, free radical molecule that functions in the nervous system as an atypical neurotransmitter, second messenger, vasodilator, and potent neurotoxin, depending on cell type and level of expression. Physiologically, nitric oxide is thought to mediate long-term potentiation, the cellular correlate of learning and memory formation, as well as synaptic plasticity and remodeling. Under pathologic conditions such as cerebral ischemia, NOS1 overproduces nitric oxide, which is a key mediator of excitotoxic cell death and neurodegeneration. Our laboratory discovered that multiple, individually functioning promoters (5’1 and 5’2) regulate human NOS1 gene expression. The present work describes the discovery and cloning of a novel human NOS1 promoter, 5’3, and the demonstration of its unique, developmentally regulated expression pattern in the central nervous system. Promoter-specific alternative splicing contributes to NOS1 mRNA diversity in both the 5’ untranslated and coding regions of the gene. We generated several lines of transgenic mice that express reporter genes under the control of separate human NOS1 promoter complexes (PR(5’1+5’2) and PR(5’3+5’4)), and studied how these alternate promoters contribute to NOS1 expression in various physiologic and pathophysiologic states in vivo. To this end, transgene expression is documented throughout normal development, which demonstrates overlapping but distinct patterns of promoter use that contribute to the overall recapitulation of
endogenous NOS1 gene expression. In a murine model of striatal neurotoxicity, systemic methamphetamine administration results in promoter-specific transcriptional activation in the transgenic mice. NOS1 enzymatic activity is stimulated in motor neuron cell bodies after peripheral transection of the facial nerve; however, the transgenic NOS1 promoter complexes are insufficient to recapitulate NOS1 activation in this model. These findings help clarify how multiple promoters and mRNA diversity contribute to the complex regulation of the human NOS1 gene, and further our general understanding of transcriptional regulation of gene expression via alternate promoters.
Dedicated to my family

Danielle Lynn
Tyler Gregory
Matthew Josiah
Amanda Noelle
I am grateful to have worked under the guidance and friendship of my adviser, Tony Young, who led my graduate journey with a fine blend of high expectation, gentle urging, and the freedom to explore science in all of its pitfalls and triumphs. I truly appreciate the patience afforded me during the arduous challenge of the dual pursuit of graduate and professional degrees.

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Finally, I praise God, through whom all things are possible and meaningful. For His abundant gifts of life, talent, opportunity, joy, and for the sacrifice of His Son, I am eternally grateful.
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Nitric oxide

It’s an air pollutant, a cigarette smoke toxin, and a waste product of microbial metabolism. Yet nitric oxide (NO·), a potentially toxic free radical, has emerged as a unique and important biological messenger molecule involved in wide ranging physiological and pathophysiological processes. Synthesized by a small family of nitric oxide synthase (NOS) enzymes, NO· plays key roles in immune defense mechanisms, blood pressure regulation, and nervous system development and function. In the central and peripheral nervous systems, NO· is a neurotransmitter, a signaling molecule during neuronal development and plasticity, and a mediator of excitotoxic neurodegeneration in injury and disease states. NO· stands out as a unique messenger molecule based on its physical properties. Unlike traditional neurotransmitters, which are stored in synaptic vesicles, this small, highly reactive molecule freely diffuses across cell membranes and thus cannot be stored within the cell. Whereas classic neurotransmitters are released by exocytosis into the synaptic cleft, act on membrane-associated receptors, and are eliminated by reuptake or enzymatic degradation, NO·’s activity in the nervous system is regulated by entirely different means. NO· is synthesized on demand, rather than stored, diffuses in all directions across cell membranes, acts directly on target molecules, and is
quickly oxidized to inactive end products. For these reasons, and because too much NO\cdot can induce neurodegeneration, it becomes evident that tightly controlled mechanisms must exist to dynamically regulate the production of NO\cdot in neurons. Many studies\textsuperscript{1-4} demonstrate that regulation at the level of gene expression plays a major role in controlling the cellular levels of nitric oxide. This work seeks to gain a better understanding of the complex transcriptional regulation of the neuronal nitric oxide synthase gene.

**Nitric oxide synthases**

While these studies focus on regulation of the neuronal isoform of NOS, a brief overview of the NOS isomers will provide some context for the gene of interest. There are three major nitric oxide synthases encoded by three separate genes: neuronal (nNOS, NOS1), inducible or immunological (iNOS, NOS2), and endothelial (eNOS, NOS3). Named according to the tissues in which they were first described, all have been demonstrated in multiple tissue and cell types. Endothelial NOS is primarily expressed in vascular endothelium, where it plays a major role as a vasodilator and regulator of vascular tone\textsuperscript{5}, and has also been observed in CNS neurons. The inducible NOS (iNOS) is expressed in a wide variety of cells types, including (but not limited to) macrophages, neutrophils, hepatocytes, chondrocytes, smooth muscle cells, pancreatic islet cells, CNS astrocytes, fibroblasts, epithelial cells, and many cancer cell lines. NO\cdot derived from iNOS has important functions in antimicrobial action, neutrophil and lymphocyte regulation, and allograft rejection\textsuperscript{6}. Neuronal NOS, originally found in rat cerebellum, is expressed in neurons throughout the central and peripheral nervous systems, the enteric
nervous system, and in a handful of non-neuronal cell types such as skeletal muscle, cardiac muscle, penile corpus cavernosum, testes, bladder, and kidney.

All NOS isozymes catalyze an identical reaction, \( L\text{-arginine} + O_2 \rightarrow \text{citrulline} + \text{NO}^- \), and share a heme group and the cofactor requirements of NADPH, calcium/calmodulin (Ca\(^{2+}/\text{CaM}\)), tetrahydrobiopterin (BH\(_4\)), flavin mononucleotide (FMN), and flavin-adenine dinucleotide (FAD). All are active as homodimers. While all share a similar COOH-terminal reductase domain, an NH\(_2\)-terminal myristoylation site is present only on eNOS, and nNOS contains an extended NH\(_2\)-terminus that harbors protein-protein interaction (PDZ) domains. Both eNOS and nNOS are present in resting cells (therefore formerly coined “constitutive”), and following an increased intracellular \([\text{Ca}\(^{2+}\)]\), Ca\(^{2+}/\text{CaM}\) binding transiently activates the enzyme leading to the production of a small amount of NO\(^-\). These “constitutively” expressed enzymes are, in fact, induced under certain traumatic or pathological conditions by way of new RNA and protein synthesis\(^7\). This contrasts with iNOS, which is not typically present in resting cells; certain cytokines, microbial products, or lipopolysaccharide induce its expression. iNOS is further distinguished by its covalently bound calmodulin, lack of stimulation by Ca\(^{2+}\), and its prolonged (up to days) and robust NO\(^-\) production upon activation\(^8\).

**NOS1 physiology**

In the peripheral autonomic nervous system, NOS1-derived NO\(^-\) is a neuronal messenger molecule. In the gastrointestinal tract, for example, depolarization of the myenteric plexus neurons causes relaxation of smooth muscle associated with peristalsis. NOS inhibitors block this process, and NO\(^-\) has been identified as the non-adrenergic
non-cholinergic (NANC) neurotransmitter of the gastrointestinal system\textsuperscript{7-9}. NO$^-$ is also the NANC neurotransmitter that regulates penile erection. NOS1 is highly expressed in nerves and tissues of the penis, including the pelvic plexus, cavernosal nerve and plexus, the cavernosal arteries, and the periphery of the corpora cavernosa. Inhibitors of NOS block electrically stimulated penile erections in rats and block relaxation of isolated strips of corpus cavernosum\textsuperscript{10,11}. Cerebral blood flow may also be regulated by neuron-derived NO$^-$, as autonomic nerves that supply the adventitial layer of cerebral vessels express NOS1 immunoreactivity\textsuperscript{12}.

There is evidence that NO$^-$ produced in neurons regulates neurotransmitter release by activating cGMP-dependent protein phosphorylation cascades. Synaptosomes isolated from striatum and cerebral cortex release neurotransmitter in response to NMDA-receptor activation, and NOS inhibitors block the process. PC-12 (rat pheochromocytoma) cells treated with nerve growth factor (NGF) become competent to release neurotransmitters coincidentally with their ability to express neuronal NOS. NOS inhibitors block both acetylcholine and dopamine release from differentiated PC-12 cells, and the blockade is reversed with the addition of excess L-arginine\textsuperscript{13,14}. NO$^-$ stimulates the release of neurotransmitters by activation of guanylyl cyclase and elevation of intracellular cGMP, which activates cGMP-dependent protein kinases to augment the phosphorylation of synaptic vesicle proteins associated with neurotransmitter release\textsuperscript{15}.

Neuronal NOS is also thought to play a role as a retrograde messenger in long-term potentiation (LTP), a form of synaptic modulation that is a cellular correlate of learning and memory formation. NOS inhibitors attenuate LTP in hippocampal slices, and the effect is reversed by the addition of L-arginine or hemoglobin (which binds
extracellular NO·). In cultured hippocampal neurons, NO· application causes increased frequency of miniature excitatory postsynaptic potentials, and may elicit LTP\textsuperscript{16,17}. While NO·’s role in the molecular events of LTP has been controversial, these findings correlate well with functional tests of learning and memory. 7-nitroindazole (7-NI), a NOS1-specific inhibitor, impairs learning in a passive-avoidance task in day-old chicks\textsuperscript{18}. Rats are also impaired in spatial learning (water maze and radial arm maze) when injected with 7-NI\textsuperscript{19}.

**NOS1 in excitotoxicity**

Excitotoxins comprise a group of neurotoxic substances, including the excitatory amino acid neurotransmitter, glutamate, and its analogs such as kainic acid, quisqualic acid, and N-methyl-D-aspartate (NMDA). “Excitotoxicity” is the cascade of cellular events, triggered by excitation of glutamate receptors on CNS neurons, which lead to a characteristic delayed onset, calcium dependent cell death. Over stimulation of the NMDA subtype of glutamate receptor (NMDA-R), associated with a cation-permeable channel, allows a large influx of calcium into the cell. The increased intracellular Ca\textsuperscript{2+} leads to the activation of a number of cellular processes that may be involved in cell death, including the activation of protein kinases, proteases, and phospholipases and the activation of neuronal NOS\textsuperscript{8,15}. A wealth of studies has demonstrated that NOS1-derived NO· is a key mediator of NMDA-R dependent excitotoxic cell death. Arginine analogs, which inhibit NOS1, prevent neurotoxicity elicited by NMDA and related excitatory amino acids in rat primary cortical cultures. The effect is competitively reversed by L-arginine. NMDA toxicity is completely attenuated by growth of the cultures in arginine
free medium. The NO· donor, sodium nitroprusside (SNP), produces dose dependent cell death that parallels cGMP formation (NO· activates guanylyl cyclase). Furthermore, treatment with hemoglobin, which complexes NO·, prevents the neurotoxic effects of both NMDA and SNP. Additionally, susceptibility to NMDA neurotoxicity develops over time in primary brain cultures coincident with the expression of NOS1, and both NMDA and SNP toxicity develop with a similar dose-effect and time course. Finally, NO·’s role in NMDA-R mediated excitotoxicity is confirmed in cortical cultures from NOS1-deficient mice. NOS1-/- primary cultures, but not wild-type controls, are markedly resistant to NMDA-mediated neurotoxicity and are not protected by NOS inhibitors. Together these data firmly establish that NOS1 and NO· mediate excitotoxicity.

While NO· directly reacts with many cellular targets, including heme and iron-sulfur containing proteins, and nitrosylates and ribosylates proteins, the majority of evidence indicates that NO·’s toxic effects occur through an interaction with the superoxide (O2·−) anion to form peroxynitrite (ONOO−). ONOO− is a highly reactive molecule that nitrosylates tyrosine residues of proteins, initiates lipid peroxidation, directly damages DNA, and decomposes into the highly reactive NO2· and OH· free radicals. Indeed, cortical cultures from transgenic mice overexpressing superoxide dismutase (SOD1) (which diminishes O2·−) are resistant to NMDA mediated excitotoxicity.

Excitotoxicity is well established as a mechanism for cellular injury in ischemic brain injury, such as occurs in cerebrovascular infarction and reperfusion damage (e.g. “stroke”). Following an occlusive event, release of glutamate into the extracellular space
stimulates NMDA receptors, activating NOS, which rapidly (3-24 minutes) and markedly increases NO\(^{-}\) levels (from 10 nM to 2 µM) in the brain\(^{27}\). Brain nitrite (a stable NO\(^{-}\) metabolite) and cGMP levels also rise within 30 minutes of ischemia\(^{28}\). Notably, O\(_2\)\(^{-}\) and ONOO\(^{-}\) are also increased in ischemia\(^{29}\). Animal models of cerebral ischemia have been exploited to determine the roles of these reactive oxygen species in excitotoxic cell death. Inhibiting the production of NO\(^{-}\) or the accumulation of O\(_2\)\(^{-}\) would be expected to diminish brain injury following ischemia. Consistent with this notion is the observation that animals treated with SOD before focal ischemia and in transgenic mice overexpressing SOD, the infarct volume following focal ischemia is markedly attenuated\(^{30}\). Additionally, neuronal NOS selective inhibitors reduce infarct volume following middle cerebral artery (MCA) occlusion in mice, rats, and cats\(^{23}\). NOS1\(^{-/-}\) mice have similarly decreased infarct volumes following MCA\(^{31}\) or carotid artery\(^{32}\) occlusion.

The NOS1 gene

The human neuronal NOS gene is located on chromosome 12 at position 12q24.2-24.3\(^{33,34}\). A large and structurally complex gene, human NOS1 is comprised of 29 exons and 28 introns, spanning more than 240 kb as a single copy gene in the haploid human genome\(^{35}\). The primary translation initiation and termination sites are located in exons 2 and 29, respectively, encoding an open reading frame that is 4302 bp and 1434 amino acids in length. The two other human NOS genes, eNOS and iNOS, share considerable homology with nNOS with respect to genomic organization, size of exons, and location of intronic splice junctions, suggesting that the three isoforms are derived by gene
duplication events\textsuperscript{35-37}. The only other known closely related human gene is cytochrome P-450 reductase, though the homology is limited to the C-terminal reductase domain of the NOS isoforms, especially in the exons responsible for binding NADPH\textsuperscript{1}.

Homologous nNOS cDNAs have been described in rat\textsuperscript{38}, mouse\textsuperscript{39}, rabbit (GenBank Accession #U91584), and drosophila\textsuperscript{40}, though the human gene is the most extensively characterized. The coding sequences of the three mammalian homologs share 86-88\% nucleotide sequence identity with human NOS1, while drosophila NOS has approximately 60\% identity.

**NOS1 mRNA diversity**

As previously discussed, NOS1 is a widely expressed but tightly controlled gene which plays many roles, such as in neuronal development, differentiation, signaling, plasticity, and toxicity. One can imagine the regulatory complexity required for a single gene to be appropriately expressed in so many diverse cellular environments. It appears that much of this regulation occurs through the production of a very large number of alternate mRNA transcripts. Indeed, the degree of mRNA diversity observed in nNOS is unprecedented in any mammalian gene characterized to date\textsuperscript{41}. While some of these alternative transcripts result in structurally different NOS1 proteins (and potentially different biologic functions), most of the mRNA variants differ only in untranslated regions and therefore do not affect protein structure. These numerous transcripts are generated by multiple mechanisms, including allelic variation, alternative promoter usage, alternative splicing, and the use of alternative polyadenylation signals. A brief
discussion of these mechanisms follows, while a detailed examination of alternate promoter usage and splicing are the subject of a subsequent chapter in this work.

Multiple alleles of the human NOS1 gene exist, based on the number of microsatellite CA repeats found within the 3’-UTR of exon 29. These alleles occur in normal individuals. While several neurodegenerative diseases are caused by (or cause) expansion of unstable microsatellites, it remains to be determined whether or not this mechanism contributes to changes in NOS1 mRNA transcript generation, stability, processing, or localization.

At the time of this writing, at least ten alternative first exons have been identified in the 5’ genomic regions of the human NOS1 gene. These alternate exons, in general, splice to a common exon 2 that contains the translation initiation codon (AUG). Thus, the alternate first exons encode multiple transcripts with diverse 5’-UTRs that do not affect protein structure, but rather produce a single full length NOS1 protein. Genomic cloning reveals that these exons are spread over a region greater than 105 kb, and it is likely that each exon is transcribed from its own unique promoter. This notion is supported by evidence that each of the variants has tissue-specific expression patterns. Additionally, our laboratory has demonstrated for several of the exons that their 5’-flanking regions are sufficient to drive the expression of a reporter gene in vitro (and unpublished data). The cloning and characterization of some of these alternate promoters/exons is the subject of the following chapter. In addition to the alternative promoters that reside upstream of exon 2, two testis-specific promoters and exons (T1 and T1b) have been cloned from the genomic region corresponding to intron 3 of the full length NOS1. Transcripts initiating from these two promoters generally include a novel
second exon (Tex 2), and are spliced to exon 4 of the full length NOS1. As translation begins in exon 5 in these testicular mRNAs, a truncated protein (TnNOS) is produced which lacks NH₂-terminal domains but retains catalytic NOS activity\textsuperscript{45}.

Alternative splicing is another mechanism that generates multiple NOS1 transcripts that differ in size, 5'UTR, and translational start sites. One well characterized example of NOS1 alternative splicing occurs during skeletal muscle development. In developing skeletal muscle, NO\textsuperscript{−} facilitates fusion of cultured myoblasts\textsuperscript{46} and mediates retrograde synaptic signaling in myocyte neuronal co-cultures\textsuperscript{47}. In mature skeletal muscle, calcium influx associated with muscle depolarization is linked to NO\textsuperscript{−} formation, which in turn stimulates guanylyl cyclase, a cascade that modulates contractile force\textsuperscript{48,49}. An alternative splicing event occurs in skeletal and cardiac muscle that inserts a 102 bp sequence between exons 16 and 17, which are incorporated into the translated polypeptide\textsuperscript{50}. The resultant NOS1 isoform, nNOSµ, retains catalytic activity and calcium/calmodulin-dependent regulation similar to that of brain NOS1. The 34 amino acid insert contains possible protein phosphorylation sites, and may function as a substrate for calcium/calmodulin-dependent protein kinase\textsuperscript{51}.

Another example of alternative splicing that generates alternate NOS1 proteins was described during the characterization of NOS1 mutant mice, which were created by the targeted deletion of exon 2\textsuperscript{52}. Residual brain NOS catalytic activity in these mice is attributed to the alternatively spliced nNOS isoforms nNOSβ and nNOSγ, which lack exon 2. Two NOS1 transcripts with alternate 5’ ends were identified in these “knockout” mice, each splicing to a common acceptor site in exon 3. Neither alternate 5’ exon contains an in-frame ATG. The nNOSβ transcript initiates translation at a cryptic CTG
codon within the 5’ exon, encoding six unique amino acids that continue in frame with those encoded in exon 3 to form the 136 kD nNOSβ. Alternately, translation beginning from the first in-frame ATG, which falls in exon 5, encodes the 125 kD nNOSγ protein. nNOSβ also occurs in wild type mice, and accounts for approximately 5% of total NOS1 mRNA. Additionally, alternative splicing of exon 2 occurs in a region-specific manner in the brain, found predominantly in the pediculopontine tegmental nucleus, and in striatal and cortical interneurons\(^5\). nNOSβ is not detected in the cerebellum of wild type mice, a structure that specifically lacks residual NOS activity in the exon 2\(^{ΔΔ}\) mice\(^5\). The biologic significance of these truncated proteins may lie in sub-cellular localization.

Exon 2 encodes PDZ domains that enable NOS1 to be anchored to a complex of synaptic membrane-bound proteins, while the truncated NOS1 variants that lack the PDZ domains are present unbound in the cytoplasm\(^5\). Several other examples of alternative splicing important in NOS1 regulation have been described, some of which will be discussed in the following chapter.

Yet another source of NOS1 transcript diversity is the use of multiple polyadenylation signals. The entire 3’UTR and flanking 3’ genomic region has been cloned and characterized for the human gene\(^\text{35}\). Exon 29 resides within the 3’UTR, and contains four putative polyadenylation sites spanning approximately 200 nt. The alternative use of these sites generates NOS1 mRNAs with distinct 3’UTRs, and potentially different function. While there is currently no data indicating a biologic consequence of generating these different transcripts, the very long 3’UTR and its conservation across species may imply biologically important 3’UTR \textit{cis}-elements and function. Notably, despite the presence of a canonical polyadenylation signal
[AATAAA], the predominant site for poly(A) addition appears to be at one of the three nonconsensus sequences [ATTAAA], which is generally considered a rare and inefficient signal\textsuperscript{54}. The detection of transcripts using primarily the noncanonical sequence may reflect either its preferred use in the context of NOS1 regulation, or may represent an artifact of enhanced stability of shortened transcripts cleaved at this upstream polyadenylation signal.

**Regulation of gene expression by alternative promoters**

Promoters have been defined as modulatory DNA structures containing a complex array of cis-acting regulatory elements required for accurate and efficient initiation of transcription and for controlling expression of a gene\textsuperscript{55}. Alternative promoter use has been observed for many genes, and is now recognized as a major mechanism of transcriptional regulation in eukaryotes. In essence, alternative promoter use constitutes a versatile mechanism to create diversity and flexibility in the regulation of gene expression, especially important in gene expression that varies by tissue type, developmental state, or in response to endogenous and exogenous signaling. This mechanism, for example, either allows a single gene to respond in different cell types to the same extracellular signals, or enables different responses within one cell to different signals. In addition to developmental, tissue-, cell type-, and state-specific regulation, alternative promoters affect mRNA stability, translational efficiency, and the structure of the amino terminus of the encoded protein. Each of these mechanisms may be used in isolation or in combination, resulting in the potential for widely diverse mRNA structure and function derived from a single gene.
Alternative promoters enable the control of ubiquitous vs. tissue-specific gene expression. One such example is the human porphobilinogen deaminase gene (PBGD), which harbors both a housekeeping and a tissue-specific promoter. The upstream housekeeping promoter is ubiquitously expressed, and contains structural features typical of housekeeping genes, while the downstream promoter is active only in erythroid cells and displays structural similarity to the β-globin gene promoters\textsuperscript{56}. In this case, each promoter contains a translational start site, such that one PBGD isoform is present in all cells, and the other only in erythroid cells.

Multiple promoters are known to regulate the level of expression of genes as well, by either producing transcripts with different responsiveness, or with altered stability or translation efficiency. The glucokinase gene contains alternative tissue-specific promoters that are differentially affected by hormonal stimuli. The upstream promoter leading to exon 1β is active mainly in pancreatic β islet cells, while the downstream promoter leading to exon 1L is active exclusively in hepatocytes. Both promoters harbor translation initiation sites, so that tissue specific protein isoforms are encoded. Hormones apparently do not regulate the β cell promoter, while the hepatocyte promoter is both dramatically activated and inactivated by insulin and glucagon, respectively\textsuperscript{57}. The HOX-5.1 gene is an example of a gene with two promoters that give rise to mRNA isoforms that differ in their stability\textsuperscript{58}. Alterations in the normally highly unstable oncogene \textit{myc} mRNA may be attributable to alternate transcripts, and it is known that one \textit{myc} mRNA isoform is more efficiently translated than another\textsuperscript{59}. In the iron regulatory element (IRE), some of the long GC-rich leader exons produced by alternative promoters cannot be translated efficiently\textsuperscript{60}.
Several genes use alternative promoters during development, such as the insulin-like growth factor family of genes. Both IGF-I and IGF-II are regulated by multiple promoters active in varied tissues and are subject to developmental and tissue-specific regulation. During fetal development, promoters P2, P3, and P4 of the IGF-II gene are active. In the liver, they are shut off after birth, at which time the P1 promoter becomes activated\(^6\). The IGF-I gene’s promoter P1 is expressed in most fetal and postnatal tissues, while P2 is primarily expressed after birth and is restricted to the liver. P2, and not P1, is responsive to pituitary growth hormone which effects its action by stimulating IGF-I in the liver\(^6\). The IGF gene family thus exemplifies how alternative promoters enable genes to respond to diverse cellular, developmental, growth, and metabolic conditions.

A final example that demonstrates the functional relevance of multiple promoter use is in the case of promoter shifting in oncogenic transformation of the \(myc\) gene, which contains two closely linked, but independently regulated promoters. P2 is the predominant, downstream promoter and accounts for up to 90% of \(myc\) RNA in normal tissue, while the upstream promoter P1 normally generates less than 25% of the transcripts\(^5\). Chromosomal translocation of the MYC gene locus on chromosome 8 to one of the loci carrying the immunoglobulin heavy- and light-chain genes is characteristic of Burkitt’s lymphoma. In these tumor cells, there is a shift in promoter use such that 50-90% of \(myc\) mRNA is derived from the P1 promoter\(^5,6\). Apparently, paused or premature termination of RNA polymerase II at the \(myc\) P2 promoter impedes transcription from the P1 promoter on the normal \(myc\) gene. The translocated \(myc\) gene in Burkitt’s lymphoma has lost the ability to retain RNA polymerase II at the P2
promoter, probably from interaction with the regulatory elements of the adjacent immunoglobulin locus\textsuperscript{64,65}. Alternative promoter use is now recognized as a common, important transcriptional mechanism for regulating developmental, tissue, and state-dependent gene expression.
CHAPTER 2
Diversity of Human NOS1 Transcripts

Introduction

NOS1 expression in the brain is widespread but clearly limited to specific neural systems and cell types. In the cerebellum, for example, NOS expression occurs in patches of granule cell neurons that receive excitatory mossy fiber afferent connections, and in molecular layer interneurons, but not in purkinje neurons. In the striatum, NOS1 expression is limited to medium aspiny neurons, and is excluded from medium spiny and large cholinergic neuronal populations. Robust NOS1 expression is observed in nearly all olfactory bulb neurons, contrasting with the cerebral cortex in which expression occurs in only about 1% of cortical neurons. Additionally, NOS1 is dynamically regulated during development and synaptogenesis, in response to nerve injury, and in neurodegenerative conditions such as stroke and Huntington’s disease.

The expression of NOS1 in specific neuronal systems, yet across a broad spectrum of tissues and in response to both intrinsic and exogenous stimuli, obviates the need for tightly controlled, dynamically regulated NO· production. Many studies indicate that regulation at the level of gene expression plays a major role in tissue and state-specific generation of nitric oxide. A key question arises: How does a
single gene accommodate such a wide variety physiologic and pathophysiologic signals? While the specific molecular mechanisms are poorly understood, at least part of this regulation is accounted for by the generation of a large number of alternate NOS1 transcripts in a tissue and state-dependent fashion\textsuperscript{42,73,77}. As previously discussed, NOS1 mRNA diversity results from allelic variation, alternative promoter use, alternative splicing, and the use of alternate polyadenylation signals. Two of these mechanisms, multiple promoter use and alternative splicing, are further explored in this chapter.

Young and colleagues\textsuperscript{44} in our laboratory discovered that human NOS1 cDNAs cloned from cerebellum had two distinct 5’ exons spliced to a common second exon. Genomic cloning and sequence analysis revealed that the unique exons, designated 5’1 and 5’2, are located within 300 bp of each other but are separated from exon 2 by an intron that is at least 20 kb in length. The genomic regions upstream of 5’1 and 5’2 each have a distinct promoter activity, and are able to drive the expression of a reporter gene in \textit{in vitro} transfection studies. The promoter regions are structurally different from one another. Exon 5’1 is embedded in a CpG island and its associated promoter lacks an identifiable TATA box, resulting in transcription initiation at multiple sites. In contrast, the 5’2 promoter lies outside of the G+C-rich domain, and primer extension shows that transcription initiates at just two sites that flank an initiator element. Clearly these represent two different classes of promoter, and it is likely that they are differentially regulated \textit{in vivo}. The 5’1+5’2 promoter complex, which includes the 2.3 kb genomic region immediately upstream and including exon 5’2 and part of 5’1, strongly drives the expression of the luciferase reporter gene in HeLa cells. This suggests that this region lacks the critical cis-acting element(s) responsible for CNS-restricted expression.
However, a longer genomic fragment (4.3 kb), fused to the β-gal reporter gene, is expressed exclusively in the central nervous system of Drosophila embryos in transient assays\(^7\). While these studies demonstrate that the 5’1 and 5’2 promoter complex is sufficient for neuronal expression, it remains to be determined how each promoter region, in isolation, contributes to the complex pattern of NOS1 gene expression \textit{in vivo}. The structural elements, which differ between the 5’1 and 5’2 promoters, suggest that they are differentially regulated and expressed. A precedent for tissue-specific alternate promoter use exists in the testis-specific NOS1 transcripts described by Wang \textit{et. al.}\(^4\). In this case, the T1 and T1b alternate first exons reside in the third intron of the full-length NOS1 gene. Transcripts initiating there may include a novel second exon (Tex2), are spliced to exon 4 (numbered according to full length NOS1), and initiate translation in exon 5. These testis-specific transcripts therefore give rise to an NH\(_2\)-terminal truncated TnNOS protein, which lacks protein-protein interaction and localization domains, yet retains NOS catalytic activity. Whether or not tissue or state-dependent alternative promoter use occurs for the 5’1 and 5’2 exons, and any other promoters that may reside upstream of exon 2, remains to be experimentally demonstrated.

Alternative splicing is another mechanism employed by the cell to generate multiple NOS1 transcripts, with many variations on the theme. These variations include single exon insertions or deletions, multiple exon skipping, and alternative usage of splicing sites. These different splicing events affect either the 5’UTR only; the translation initiation site; the coding region with in-frame insertions or deletions; or the coding region resulting in reading frame-shifts. One such example is the alternate splicing event, described in this chapter, which inserts an 89 bp exon between a first exon.
and the common second exon. This appears to be a tightly regulated process, as only transcripts including specific first exons are able to incorporate the novel exon. Other examples of alternative splicing that alter only the 5’UTR include a 22 bp mouse exon inserted similarly to the human 89 bp novel exon, and the aforementioned Tex 2 cassette insertion between Tex 1 and exon 4 in the TnNOS 5’UTR. Such molecular events are rare, and are of unknown biological significance. Splicing events that alter the translation start site are also observed. When exon 2 was deleted to create nNOS knockout mice, minor transcripts were discovered that lack exon 2 but encode truncated proteins that retain NOS catalytic activity. These alternate mRNAs were subsequently found to exist in wild type mice and humans. Additionally, the transcripts that lack exon 2 demonstrate exon 1 and tissue specificity, indicating the involvement of regulatory mechanisms. The biologic significance of these truncated proteins may lie in cellular localization, as exon 2 encodes PDZ domains that enable NOS1 to be anchored to a complex of synaptic membrane-bound proteins, and the truncated NOS1 variants are present in the cytoplasm. Alternative splicing events within the coding region that retain the reading frame have also been described. A 315 nucleotide deletion corresponding to exons 9 and 10 occurs in various tissues of human and mouse, and even drosophila. These exons encode a highly conserved 105 amino acid domain adjacent to the calmodulin-binding domain. The resulting protein may be involved in synaptogenesis in the rodent brain. An in-frame cassette insertion of the 102 nt exon occurs between exons 16 and 17, which also shows tissue specificity in the rat and human. Finally, alternative splicing within the NOS1 gene can result in frame-shifts within the coding region. The 56 nt Tex 2 insertion between exons 3 and 4 of the full
length NOS1 transcript is observed in several human tissues\textsuperscript{45}. The resultant frame shift introduces a stop codon within exon 6, and therefore encodes a COOH-terminal truncated protein. The cassette deletion of exon 10 alone (as opposed to 9/10) is also detected in various human tissues, and causes translation to stop within exon 11\textsuperscript{35}. It is not known whether these truncated proteins are translated\textit{ in vivo}. The predicted proteins would likely lack NOS enzymatic activity, though they could potentially interrupt dimerization of the full-length protein. In this chapter, alternative splicing events that alter the 5’ end of NOS1 mRNA are described. These appear to be differentially regulated events that are state- and first-exon dependent.

**Materials and Methods**

\textit{Human tissues}: Frozen human brain and skeletal muscle tissue samples were obtained through the Cooperative Human Tissue Network at The Ohio State University Hospital (funded in part by the National Cancer Institute), and from the Harvard Brain Tissue Resource Center (supported in part by PHS grant number MH/NS 31862). Because tissues were collected up to twelve hours post-mortem, RNA quality and quantity varied between samples due to degradation before freezing. Only those samples with intact total RNA, as determined by ribosomal RNA band integrity on formaldehyde gel electrophoresis, were used in further experiments. Frozen fetal brain samples were obtained from spontaneously aborted fetuses from the Central Laboratory for Human Embryology, University of Washington (supported in part by the National Institute of Child Health and Human Development of the National Institutes of Health).
**RT-PCR:** Total RNA was isolated from tissue samples using standard guanidinium isothiocyanate based techniques (\(^8^1\) and using TRIZOL Reagent and protocol (GibcoBRL)). 2 µg total RNA was used for first strand cDNA synthesis with 100 units SuperScript II (GibcoBRL) reverse transcriptase according to the manufacturer’s protocol. The products were diluted 10X and used for PCR with the following conditions: 1/20\(^{th}\) of cDNA template, 1X PCR Buffer (GibcoBRL), 0.2 mM (each) dNTP mix, 2.5 µM (each) primer, 1-4 mM MgCl\(_2\), 0-10% DMSO, 2.5 u Taq DNA polymerase (Gibco BRL), final volume 50 µl H\(_2\)O. Generally the following cycles were used: 95° C, 5 min; then 5 cycles of 30 seconds at 95°C and 1 minute at 72°C; then 35 cycles of 30 seconds at 95° for denaturation, 30 seconds at 59° for annealing, and 45 seconds at 72°C for extension. For specific primer pairs, the following conditions were employed: Ex3 Fwd/Ex6 Rev (2mM MgCl\(_2\), 10% DMSO); 5’1 Fwd/Ex2 Rev2 (1mM MgCl\(_2\), 7% DMSO); 5’2 Fwd/Ex2 Rev2 (2mM MgCl\(_2\), 7% DMSO); 5’3 Fwd/Ex2 Rev2 (2mM MgCl\(_2\), 0% DMSO); 5’4 Fwd/Ex2 Rev2 (3 mM MgCl\(_2\), 2% DMSO); GAPDH Fwd/GAPDH Rev (2 mM MgCl\(_2\), 0% DMSO).

**5’ rapid amplification of cDNA ends:** 5’RACE was based on the protocol of Frohman\(^8^2\). First strand cDNA from various tissues was reverse transcribed (SuperScript II, BRL) from an exon 2-specific antisense primer (GSP-RT). T4 polynucleotide kinase (GibcoBRL) in the presence of dATP was used to append a poly(A) tail to the cDNA. A poly(T) “anchor” primer (Q\(_T\)) was hybridized to the poly(A) tail of the cDNA, and PCR amplification was carried out using a sense primer (Q\(_0\), which hybridizes to Q\(_T\)) and a nested exon 2 antisense primer (GSP-1). These products were subjected to a second
round of amplification, using nested sense (Q1) and antisense (GSP-2) primers. The thermal cycling conditions were altered from those in Frohman82; they were as follows: 0.2 mM each dNTP, 10% DMSO, 0.5 µM primers, 2 mM MgCl₂. Products were run on a 1% agarose gel, Southern blotted, and probed with an exon 2-specific oligonucleotide (Ex2 Rev1). Primer sequences are listed in the appendix.

DNA cloning, sequencing, primer extension: PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen) according to the manufacturer’s protocol. DNA sequencing was carried out using either the standard dideoxy thermal cycle sequencing (dsDNA Cycle Sequencing System, Gibco BRL) or samples were submitted to the Ohio State University DNA Sequencing Lab for automated sequencing on Prism ABI machines. Primer extension experiments were carried out according to established protocol81, using either the PE 5’3 Rev or PE 5’4 Rev primers (appendix).

Results

5’1 and 5’2 transcripts are co-expressed in human brain

NOS1 mRNA transcripts initiating with the 5’1 and 5’2 first exons were isolated from human cerebellum44, but their distribution throughout the rest of the brain has not been reported. RT-PCR (reverse transcription-polymerase chain reaction) experiments were performed to determine which brain tissues express these alternate NOS1 transcripts, and whether or not the alternate promoters are expressed in a tissue-specific manner in human brain. Human brain samples obtained include: Brain stem and pons; temporal lobe and hippocampus; cerebellum; occipital lobe cortex; temporal lobe; and
frontal lobe. The tissues showed no evidence of abnormal pathologic changes. RNA from human cerebellum, which was previously shown to express the 5’1 and 5’2 transcripts\textsuperscript{44}, was used as a positive control.

PCR primers were designed to detect NOS1 transcripts initiating with either the 5’1 or 5’2 first exon. Upstream primers (5’1 Fwd and 5’2 Fwd) are specific to either of the alternate untranslated 5’ exons, and both are paired with a reverse primer (Ex2 Rev1) that is complimentary to the 5’ end of exon 2 upstream of the translation start site. The expected 5’1 and 5’2 amplicons are 149 and 98 bp, respectively. Primers that amplify a 433 bp NOS1 coding region (Ex3 Fwd and Ex6 Rev) (fig. 2.1, top) serve as a positive control to detect the presence of full-length NOS1 transcripts initiating with any known or unknown 5’exons.

The results are shown in figure 2.1. The 5’1, 5’2, and coding region transcripts are all detected in every brain tissue tested, with the exception of occipital lobe, in which the 5’2 transcript is apparently lacking. However, in a separate experiment using the same batch of occipital lobe total RNA (but freshly reverse-transcribed cDNA), the 5’2 transcript was detected (data not shown). These experiments were repeated several times with similar results. The band intensity of the 5’1 and 5’2 amplicons varies between samples, possibly indicating varied expression levels of the transcripts in these tissues. Because the PCR experiment was not designed to be quantitative, however, no specific conclusions regarding differential expression levels can be made. In addition to the expected bands in the gel, a faint band of approximately 240 bp is detected by the 5’1 Fwd + Ex2 Rev1 primer pair in several brain regions (brain stem and pons; temporal lobe
Figure 2.1. 5’1 and 5’2 NOS1 transcripts are co-expressed throughout the adult brain.
Top: Schematic diagram of NOS1 mRNA with alternate first exons. Arrows indicate positions of primers used for RT-PCR experiments. The coding region is shaded. Bottom: RT-PCR products visualized on ethidium bromide stained gel. “C” = 433 bp coding region amplified with primers from exons 3 and 6. “1” and “2” = 149 and 98 bp untranslated regions amplified with primers from exons 5’1 and 5’2, respectively, paired with the common exon 2 antisense primer. BS = brainstem; TL = temporal lobe; HC = hippocampus. “cerebellum (+)” indicates RNA template from tissue previously demonstrated to contain all three transcripts. “(−)” = no RNA template; M = molecular weight marker.
and hippocampus; cerebellum; occipital lobe; and frontal lobe). This band represents an alternatively spliced transcript containing a novel 89 bp exon, which is discussed below.

**Discovery of two additional human NOS1 alternate first exons, 5'3 and 5'4**

NOS1 mRNA is expressed in human skeletal muscle, but transcripts beginning with either the 5’1 or 5’2 exon are present at only minimal levels in this tissue. The 5’1 and 5’2 amplicons are not visible by ethidium bromide stained agarose gel, but are detected after Southern blotting and hybridization to a radioactive probe. Because the 5’1 and 5’2 transcripts do not appear to account for the abundant skeletal muscle NOS1 mRNA, we hypothesized that one or more alternate NOS1 transcripts might exist in skeletal muscle that, like those previously described, differed only at their 5’ ends. Furthermore, we sought to determine whether other transcripts encode NOS1 within the human brain.

5’-RACE (*rapid amplification of cDNA ends*) was used to detect and clone any novel NOS1 transcripts from human cerebellum, hippocampus, pooled fetal brain (Clontech), normal skeletal muscle, and two skeletal muscle tumors (sarcoma and liposarcoma). These skeletal muscle tumor samples were received from the Cooperative Human Tissue Network before normal tissue became available. For the 5’-RACE experiment, the primer (GSP-RT, i.e. “gene specific primer-reverse transcription”) for 1st strand synthesis via reverse transcription is targeted to exon 2, just upstream of the AUG translation start site. The nested gene specific primers (GSP-1, GSP-2) lie upstream, within the 5’UTR of exon 2 (fig. 2.2a). Thus, by experimental design, only those transcripts that contain exon 2 will be detected.
**Figure 2.2. 5′RACE cloning and sequence of human NOS1 exon 5′3.**

A) Schematic of primers used for 5′RACE cloning. GSP-RT used for 1st strand cDNA synthesis, GSP-1 and GSP-2 used for nested amplification of 5′ ends of NOS1 transcripts. The line with asterisk (——*) denotes the exon 2a-specific oligonucleotide (Ex2 Rev1) used for the Southern blot shown in (B). B) Ethidium bromide gel (top) and Southern blot (bottom) of the 5′RACE products. (-) control is NOS1 coding region (exon 3-6) RT-PCR product. (+) controls are 5′1- and 5′2-Ex2 RT-PCR products, known to contain exon 2 DNA. “No template” lanes are 5′RACE negative controls. Cerebellum A and B are identical except that different amounts of GSP-RT primer (20 and 50 ng, respectively) were used in the 1st strand cDNA synthesis step. 5′RACE products of both the 1st and 2nd rounds of amplification were run on the gel (left and right for each pair, respectively). C) Sequence of cloned ~850 bp 5′RACE product indicated in (B). Novel 5′3 exon is underlined, and is spliced to the common exon 2 splice acceptor site.
A single NOS1-specific novel transcript was detected by 5’RACE, amplified from the skeletal muscle/liposarcoma RNA sample. To confirm that the liposarcoma 5’RACE product was authentic, all 5’RACE amplicons were Southern blotted and probed with a NOS1 exon 2-specific oligonucleotide directed at the 5’ end of the exon (Ex2 Rev1) (fig. 2.2b). The only signal detected corresponds to the liposarcoma 5’RACE product, which was subsequently band isolated and cloned into the pSK⁺ (Stratagene) plasmid vector. The DNA sequence of the clone was determined (fig. 2.2c), which revealed 88 nucleotides of a novel human 5’ exon, now designated 5’3. The 5’3 exon splices to exon 2 at the same splice acceptor site used by the 5’1 and 5’2 transcripts.

A sequence homology search (NCBI BLAST) of Genbank reveals that the 88 nt of 5’3 shares 73% nucleotide identity with the rat NOS1 alternatively spliced exon 1b, including one stretch of 92% sequence identity over 27 bp. Lee et. al. report that transcripts initiating with exon 1b are limited to fetal tissue in the rat. Another NOS1 alternate first exon, called 1c, is revealed within 200 nt upstream of exon 1b on the rat genomic clone. Exon 1c mRNA expression was reported only in kidney, and no human homologue has previously been described. However, the sequence of a human genomic clone (λNOS17) containing exon 5’3 (cloned by Shawn Pierson in our laboratory) reveals a 1c-like region just upstream of 5’3 (Fig 2.3), suggesting that yet another human NOS1 alternate first exon exists. A sense primer directed at this region was designed to determine if human NOS1 transcripts exist that initiate with this sequence. Indeed, RT-PCR confirmed expression of this exon, named 5’4, in adult brain, skeletal muscle, sarcoma, and liposarcoma (see below and Fig 2.6). Sequencing of 5’4-containing
Figure 2.3. Comparison of human and rat genomic clones containing homologous alternate 5' exons.
A) Genomic organization of human and rat homologous exons. B) Sequence similarity between homologous exons. Known human transcriptional start sites are indicated by arrows. Splice sites are indicated by arrowheads (black = obligate, grey = conditional).
RT-PCR products confirm that exon 5’4 is spliced to exon 2 at the same splice acceptor site used by the other major NOS1 transcripts.

Multiple transcriptional initiation sites exist for the 5’1 and 5’2 exons. We sought to determine whether, like 5’1 and 5’2, transcription of 5’3 and/or 5’4 starts at multiple loci, and if so, to define those sites. Primer extension experiments failed to reveal the start site(s) of 5’3, as the reverse transcriptase enzyme prematurely stops 3 nt upstream of the primer (PE 5’3 Rev). This occurred in several experiments, presumably reflecting an artifact due to secondary RNA structure, rather than a true transcriptional start site. However, two unique 5’RACE products were cloned from both temporal lobe and liposarcoma RNA, whose sequences terminate at either of two adenosine residues that are putative transcriptional initiation sites, 51 and 86 nt upstream of the PE 5’3 Rev primer. Thus, exon 5’3 is either 84 or 114 bp long, depending on which site is used (fig. 2.4). Both of these putative transcriptional start sites fall within a consensus initiator (Inr) element. The Inr consensus consists of A at +1 and T at +3 (5’-ANT-3’), surrounded by two to three pyrimidine residues within the +4, +5, and –1 through –5 positions. The Inr sequences in exon 5’3 are as follows: 5’-ACCCCaGTgC-3’ and 5’-TGTTAGTGc-3’ for the upstream and downstream start sites, respectively (+1 A indicated in bold). RT-PCR experiments using a sense primer (Intron 5’4/5’3), which hybridizes immediately upstream of the first start site, fail to amplify any product (data not shown), suggesting that transcription of the 5’3 mRNA does not initiate upstream of this primer.

While no 5’RACE clones were generated that contained exon 5’4, primer extension from a 5’4-specific oligonucleotide (PE 5’4 Rev) does indicate a single
Figure 2.4. Human genomic sequence containing NOS1 alternate first exons 5’4 and 5’3. Exon 5’4 is underlined. Exon 5’3 is double underlined. Primers used for PCR and Primer Extension experiments are indicated by arrows. Nucleotides in bold indicate transcriptional start sites, as determined by Primer Extension (PE), 5’RACE cloning (RACE), or a putative site based on sequence. The consensus Initiator sequence is 5’-YYYYYANTYY-3’ (where A is +1 and 2 or 3 pyrimidines(Y) are found within -1 to -5, and +4 to +5).
transcriptional start site. In this case, the +1 C residue lies 35 nt upstream of the primer, so that exon 5’4 is 149 nt in length. This putative start site does not fall within an initiator element, nor is there an identifiable TATA box in the upstream region. A consensus Inr element (5’-GCCTCACTCA-3’) does exist 4 bp downstream from the indicated +1 C, though primer extension fails to detect a signal corresponding to initiation from this site. RT-PCR experiments with sense primers (i.e. 5’4a Fwd) upstream of the +1 C, paired with several reverse primers from exons 2 and 4 (i.e. Ex2 Rev1, Ex4 Rev1), do not detect any transcripts (data not shown), supporting the location of the start site indicated by primer extension.

5’3 and 5’4 mRNAs are developmentally regulated

As previously described, transcripts initiating with exons 5’1 and 5’2 are co-expressed throughout the adult brain. The rat exon 1b, homologous to human exon 5’3, is expressed only in fetal tissue according to Lee et. al. 73. Additionally, exon 1c, the 5’4 homologue, was described only in rat kidney. To determine whether expression of any of the known human NOS1 transcripts are also spatially and/or developmentally regulated, RT-PCR experiments were conducted using both fetal and adult brain, normal skeletal muscle, and skeletal muscle-derived liposarcoma and sarcoma samples. Fetal whole brain samples were obtained from spontaneously aborted fetuses of age 85, 101, and 115 days old. Pooled fetal brain total RNA (Clontech) was also obtained, which includes RNA from brains of 16 spontaneously aborted fetuses ranging in age from 16 to 32 weeks gestation. Adult brain tissues include cerebellum, temporal lobe, dentate gyrus of the hippocampus, and striatum, all structures known to express NOS1.
RT-PCR experiments were carried out using primers designed to amplify each alternate NOS1 transcript. Specifically, sense primers 5’1 Fwd, 5’2 Fwd, 5’3 Fwd, and 5’4 Fwd were each paired with the common antisense Ex2 Rev2 primer, and PCR conditions were optimized for each pair (see materials and methods). The expected amplicons for the 5’1, 5’2, 5’3, and 5’4 transcripts are 385, 334, 349, and 282 bp, respectively. Ex3 Fwd and Ex6 Rev primers were used to amplify a 433 bp NOS1 coding region fragment, and a 358 bp GAPDH positive control was amplified as well. The results for 5’1, 5’2, and 5’3 are shown in figure 2.5. The coding region, common to NOS1 transcripts initiating with any of the alternate first exons, is detected both adult and fetal brain, as well as in skeletal muscle and the two solid tumors. While no specific quantitative conclusions can be drawn, the data suggest that the NOS1 transcripts are relatively abundant throughout the adult brain and in the tumor samples, and relatively less abundant in skeletal muscle and the two earlier time points in fetal development. As was previously demonstrated, 5’1 and 5’2 transcripts are co-expressed throughout the adult brain, and here both are shown to be present throughout fetal development as well. 5’1 and 5’2 transcripts are only weakly detected in liposarcoma and sarcoma, respectively, and are not detected in skeletal muscle.

Exon 5’3 is expressed in the adult brain as well, though striatal expression is relatively weak. Liposarcoma and sarcoma demonstrate the highest levels of 5’3 expression, perhaps explaining the success of 5’RACE cloning from the tumor sample. Weak skeletal muscle expression of 5’3 is also evident, which is visible on the ethidium bromide-stained gel when slightly overexposed (not shown). Clearly, however, transcripts starting with exon 5’3 are not detected in any of the fetal brain samples.
Figure 2.5. RT-PCR of alternate human NOS1 transcripts.
PCR primers and amplicon sizes described in text. FB = fetal brain. MW = pCAT/HinF1 molecular weight marker.
These experiments were repeated several times with identical results. To confirm that fetal brain does not express 5’3, further RT-PCR experiments were conducted, in which the 5’3 Fwd primer was paired with antisense primers from exons 2, 4, or 6 (Ex2 Rev1, Ex4 Rev, and Ex6 Rev). No 5’3-containing transcripts were detected (data not shown). These data are important to rule out the possibility that alternatively spliced fetal 5’3 transcripts exist that lack exon 2, which would have been missed in the initial experiment. Additionally, the exon 5’3-exon 6 RT-PCR products were Southern blotted and probed with a radioactively labeled exon 3-specific oligonucleotide (Ex3 Rev); still, no signal was detected in the fetal brain samples.

RT-PCR to amplify 5’4-containing transcripts has proven difficult to optimize, with multiple non-specific products dominating the ethidium bromide-stained gel. Upon Southern blotting of these products, however, a single NOS1-specific product is revealed by hybridization with an exon 2-specific oligonucleotide probe (Ex2 Rev1) (Fig. 2.6). Like 5’3, 5’4 transcripts are detected in adult cerebellum, temporal lobe, dentate gyrus of the hippocampus, and very weakly in skeletal muscle. However, relative expression levels in these tissues do not mirror 5’3, as the 5’4 signal is strongest in dentate gyrus, weak in temporal lobe, and absent from striatum. Fetal brain samples also completely lack 5’4 transcripts, indicating a developmentally regulated expression pattern similar to 5’3. Like 5’3, the 5’4 mRNAs are also abundantly expressed in the tumor samples. Also evident in sarcoma and liposarcoma are larger, alternatively spliced transcripts that contain exon 5’4; these are discussed below.
Figure 2.6. Southern blot of 5’4 RT-PCR products compared to ethidium bromide stained 5’3 RT-PCR products. A nested exon 2-specific oligonucleotide probe (Ex2 Rev1) was used for hybridization. Detail in text. FB = fetal brain.
Alternative splicing of NOS1 mRNAs is 5’ exon-specific

The NOS1 gene was knocked out in mice by targeted deletion of the second exon, which includes the translation initiation site. NOS1 mRNA and protein levels are greatly reduced in these mice, but residual NOS catalytic activity in the brain revealed the presence of a splice variant, nNOSβ, which lacks exon 2 and its encoded PDZ domain. We conducted PCR experiments to determine whether similar alternatively spliced human NOS1 transcripts exist in the brain. First, as diagrammed in figure 2.7a, sense primers from each of the alternate first exons (5’1, 5’2, or 5’3) were paired with a common antisense primer from exon 6 (Ex6 Rev), and RT-PCR was carried out using template RNA from fetal (pooled and 85, 101, and 115 day), adult brain (cerebellum and dentate gyrus), sarcoma and liposarcoma. In each case, this generated an abundant “full length” amplicon (i.e. containing exon 5’N and exons 2 through 6) of the expected size (~1.67 kb), as well as several shorter, less abundant products. These shorter amplicons varied in number and intensity between samples, but at least one was visible in each combination of primer pair and tissue type. To determine whether any of these shorter transcripts are NOS1-specific, the amplification products were Southern blotted and hybridized to an exon 3-specific oligonucleotide probe (Ex3 Fwd) (fig. 2.7b). This revealed that transcripts initiating with each of the alternate first exons (5’1, 5’2, and 5’3) exist both in the full-length form and as shorter alternative splice variants. Based on size alone, some of the alternatively spliced products (~530 bp) were thought to lack exon 2 (which is 1143 bp).

To determine the structure of these alternative splice variants, the first round RT-PCR products were run on a low melting point gel and excised together, being careful to
Figure 2.7. Alternative splicing generates diverse NOS1 transcripts.
A) First round RT-PCR diagram. Primers indicated by arrows. NOS1 coding region shaded. B) Southern blot of PCR products generated as in (A), probed with exon 3-specific oligonucleotide (Ex3 Fwd). Cb = cerebellum, DG = dentate gyrus, Lipo = liposarcoma, Sarc = sarcoma, FB = pooled fetal brain, 85/101/115 d = fetal brain of noted gestational age in days. C) Second round, nested PCR diagram, with schematic of cloning strategy. Details in text.
Figure 2.8. Alternative splicing of human NOS1 mRNAs.
A) “Full length” mRNAs generated from either 5’1, 5’2, or 5’3, with +1 ATG starter methionine indicated. B-D are 5’1 alternate transcripts, E is a 5’2 alternate transcript, F&G are 5’3 alternate transcripts. B) Splice variant with insertion of novel exon 2a and 506 nt deletion within exon 2, eliminating the +1 ATG. May initiate translation at +753 CTG (exon 3), encoding truncated nNOSβ. C) Splice variant lacking exon 2, known to encode nNOSβ. D) Splice variant with deletions in exon 5’1, exon 4, lacking exons 2 and 3, and with a 5 nt (AAGCG) insertion (I); may initiate transcription at +1694 ATG (exon 5), encoding nNOSγ. A-G: Putative translated regions are shaded, and the resultant NOS1 variant proteins (see text) are indicated on the right.

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exclude the full-length amplicons. These pooled short products then served as templates for a second round of nested PCR, in which the original 5’ sense primers (5’1, 5’2, or 5’3 Fwd) were paired with a nested exon 4-specific antisense primer (Ex4 Rev) (fig 2.7c). The products were then cloned and sequenced; the alternative splice variants are diagrammed in figure 2.8. Exon 5’1-containing clones reveal three alternative splice products in addition to the full-length transcript: The first contains exon 5’1 spliced directly to exon 3 (i.e. lacking exon 2 in its entirety); the second consists of exon 5’1 followed by a novel 89 bp exon (“2a”), and exon 2 that contains a 556 bp internal deletion (nucleotides #503-1059 of GenBank accession #U17299); the third begins in exon 5’1 but lacks the 3’ 60 nt, lacks exons 2 and 3 in their entirety, has an insertion of 5 nt (AAGCG) between exons 5’1 and 4, and lacks the 5’ 20 nt of exon 4. 5’2-containing clones revealed only one alternative splice product in addition to the full-length transcript, which lacks exon 2 in its entirety. Two 5’3-containing clones were found. The first, as expected, lacks exon 2, while the second lacks all but the 3’ 111 bp of exon 2.

The nested PCR experiment, by design, only detects transcripts that are shorter than the "full length" transcript. However, we hypothesized that transcripts would exist which contain the novel exon 2a in the context of deletion free NOS1 mRNAs, which would not have been detected in the nested PCR experiment. RT-PCR amplifications with either the 5'1 Fwd or 5'2 Fwd sense primer, paired with the Ex2 Rev1 antisense primer, each give rise to two bands visible by ethidium bromide staining: the expected "full length" band and a less abundant band approximately 90 bp larger. The presence of a unique EcoRI site within exon 2a facilitated a simple experiment to test the hypothesis
Figure 2.9. Alternatively spliced 5’1 and 5’2 NOS1 transcripts include exon 2a.
A) Diagram of the two proposed 5’1-containing PCR products shown in (B), amplified with the 5’1 Fwd and Ex2 Rev primers (arrows). The line with asterisk (——*) denotes the exon 2a-specific oligonucleotide used for the Southern blots shown in (B) and (C). B&C) PCR products generated with 5’1 Fwd (B) and either 5’1 Fwd, 5’2 Fwd, or 5’3 Fwd (C) and Ex2 Rev primers, without and with EcoR1 digestion. These were Southern blotted (right half of (B&C) and probed with an exon 2a-specific oligonucleotide.
that the larger PCR product is a NOS1 transcript containing the novel 89 bp exon 2a (diagrammed in fig. 2.9a). The 5’1 PCR products were digested with EcoR1, Southern blotted, and probed with an exon 2a-specific oligonucleotide (Ex2a Rev) (fig. 2.9b). EcoR1 cut the upper band, but not the lower band, and only the upper band (and its digestion product) are detected by Southern hybridization. Exon 2a was detected in 5’2 initiated transcripts, but was not detected in either 5’3 or 5’4 (not shown) initiated transcripts (fig. 2.9c). These data suggest that transcripts beginning with 5'1 and 5'2, but not 5'3 or 5'4, are able to incorporate the novel 89 bp exon 2a. At the time this exon was discovered, BLAST sequence homology search revealed no similar known sequences. However, Wang et. al. 42 recently described an alternatively spliced exon in human, called “AS,” that is identical in sequence to exon 2a. This exon is located downstream of the 5’1 and 5’2 exons, within the same genomic EcoR1 fragment.

RT-PCR using primers 5’4 Fwd and Ex2 Rev1, paired with Southern detection, revealed a 5’4 containing transcript present in sarcoma and liposarcoma that is approximately 350 bp larger than expected. This product did not hybridize with an exon 2a specific probe (not shown). Based on the size of the insertion, we hypothesized that in this case the mRNA contains exons 5’4 and 5’3, including the “intronic” region between them, spliced to exon 2. This is supported by Southern blot data, which shows that the large, but not the smaller (i.e. 5’4 spliced to exon 2) transcript, hybridizes to an exon 5’3-specific oligonucleotide probe (5’3b) (fig. 2.10). Transcripts containing both 5’4 and 5’3 are not detected in tissues other than sarcoma and liposarcoma, and similar RT-PCR and Southern blot experiments failed to detect any transcripts that contain both the 5’2 and 5’1 exons in any tissue.
Figure 2.10. NOS1 mRNAs initiating with 5’4 are processed aberrantly in cancers.

Diagram (top) of alternate splicing events; PCR primers (5’4 Fwd and Ex2 Rev1) are indicated by arrows. Oligonucleotide probes used for Southern blot (bottom) are indicated by (***) and (•••). S=sarcoma, L=liposarcoma.
Discussion

The primary purpose of this work was to elucidate how alternative promoters contribute to the complex transcriptional regulation of the human neuronal NOS gene. Initial experiments showed that the 5’1 and 5’2 promoters are essentially co-expressed throughout the adult brain. However, with the discovery of exons 5’3 and 5’4, alternate developmental regulation of human NOS1 transcripts was demonstrated for the first time. Additionally, alternative splicing was shown to contribute to NOS1 transcript diversity, and also appears to be first exon-specific.

As detected by PCR, NOS1 transcripts initiating with 5’1 or 5’2 are expressed throughout the adult brain, with no appreciable regional differences. Because the two promoters are structurally different, it may have been expected a priori that differential expression patterns would be evident. It is possible that the two transcripts are in fact co-expressed in vivo, which might occur if the two closely linked promoters share proximal cis-acting elements. In this scenario, the transcriptional machinery could bind to the general locus, facilitating its interaction with the promoters in a coordinated fashion. Finer levels of regulation specific to each promoter may then occur in this setting, such that cell specific or state specific expression differences are feasible. Another likely explanation is that the transcripts are co-expressed in different cell populations within the same tissue. In these experiments, template DNA was isolated from tissue blocks representing different brain regions, each of which certainly harbors many different populations of neurons and other cells. To address this issue, experiments are currently underway using in situ hybridization with alternate first exon-specific probes to determine differential transcript expression at a cellular level of resolution. A third
possible explanation is that the promoters are active in the same neurons, but serve different functions. For example, one may be basally expressed while the other is induced in response to specific stimuli. This concept is supported by the fact that 5’1 and 5’2 represent functionally distinct promoter classes. Exon 5’1 is embedded within a CpG island, lacks identifiable TATA and Inr elements, and is transcribed from a large number of initiation sites\(^44\), making this promoter similar to those of a large number of “housekeeping” genes\(^84\). In contrast, exon 5’2 is less G+C rich, harbors an initiator element\(^85\), and appears to initiate transcription at a limited number of sites\(^1,44\). In fact, these proposed explanations for the observed co-expression of 5’1 and 5’2 are not mutually exclusive, and all may be valid.

Tissue-specific and developmentally regulated NOS1 expression has been demonstrated in rodents\(^51,73,86\). However, exons 5’3 and 5’4 represent the first example of human NOS1 alternate transcripts that are developmentally regulated. While the specific timing of 5’3 and 5’4 induction remains to be determined, it is tempting to speculate that the promoters for these transcripts account for some of the observed roles of NOS1 in development. For example, spatial and temporal NOS1 protein and mRNA expression patterns occur during the development of the CNS\(^87-89\). These changes seem to correlate with differential susceptibility of the cells, at particular developmental stages, toward specific inductors, such as nerve growth factor\(^90\), and GABA and neurotrophins\(^91\). Developmentally regulated NO\(^-\) expression may be involved in refinement of the developing nervous system through the processes of apoptosis and differentiation. For example, NO\(^-\) triggers the switch to growth arrest during differentiation of PC12 cells into neuronal cells in response to NGF, an event accompanied by induction of NOS
activity\textsuperscript{92}. NO\textsuperscript{-} has a demonstrated role in the activity-dependent arborization of spinal motor neuron dendrites in developing mice\textsuperscript{93}. Furthermore, a developmental role for NOS1 in the human neocortex has been suggested by its spatial and temporal expression in the embryonic brain\textsuperscript{69}. As previously noted, the rat homologues of exon 5’3 and 5’4 (exons B1 and C1), were detected only in E18 embryo and adult kidney, respectively\textsuperscript{73}, by means of RNase protection assays. The data presented here for the human transcripts are not consistent with those reported for the rat homologues by Lee \textit{et. al}. It is possible that exons sharing sequence homology are regulated differently, even oppositely, between species. However, Brenman \textit{et al}\textsuperscript{51} showed mouse 5’3 mRNA, via Northern blots, expressed in adult brain and not in the embryo, consistent with the human data presented here. Parsimony would suggest, then, that the developmental regulation observed for the human gene is conserved in rodents as well.

Computer analysis using the Transcription Element Search System (http://www.cbil.upenn.edu/tess) of the sequence upstream of exon 5’3 and including exon 5’4 reveals several putative binding sites (Fig.2.11) for trans-acting factors which may contribute to this promoter activity. The region from –438 to +84 (with +1 defined as the A of the 3’ initiator element) lacks an identifiable TATA element. However, a possible binding site for the transcription initiation complex exists at –67, which has a G+C rich region with homologous sequences for binding sites of the initiation factor TFIID and the transcription factors SP-1 and MAZ. Two other consensus SP-1 sites are also found within this region. It should be noted that virtually every simple Inr studied has been shown to be active in the presence of upstream SP-1 sites\textsuperscript{84}, and this arrangement is sufficient to initiate transcription at specific nucleotides in the absence of
Figure 2.11. Putative cis-acting regulatory elements in the 5’3’ promoter region. The genomic sequence including the 5’3’ (double underline) and 5’4’ (single underline) alternative first exons. The upstream transcription start site for exon 5’3’, as determined by 5’ RACE, is indicated by “+1” and is in bold. The putative binding sites for transcriptional trans-acting regulatory elements are indicated by boxes, or underlined in the case of overlapping sites.
a TATA sequence. Two binding sites for AP-2 are present. AP-2 is a DNA-binding protein expressed in neurons and regulated by retinoic acid. Correspondingly, NOS1 mRNA is increased in a human neuroblastoma cell line following trans-retinoic acid-induced neuronal differentiation\textsuperscript{94}. In addition, position –48 contains a putative binding site for Oct-2.1, a member of the POU family of transcription factors that has been shown to specifically upregulate the NOS1 5’1, but not the 5’2 promoter\textsuperscript{95}. While these potential \textit{cis}-elements are attractive candidates for differential regulation of NOS1 transcription, specific protein-binding, mutational, and expression studies will be needed to demonstrate functional relevance. To initiate these studies, Shawn Pierson has cloned various 5’3-containing fragments and fused them to a reporter gene. Preliminary experiments demonstrate expression of 5’3 in HeLa and in neuroblastoma cell lines.

Alternative splicing appears to play an important role in the complex regulation of NOS1 gene expression. In addition to the splicing events that lead to the deletion of exon 2, which are well described in the literature, evidence is presented here of alternative splicing events that appear to be first exon and tissue dependent. Transcripts were observed that lack all or part of exon 2, including the major translation initiation site. As previously discussed, these are likely to produce truncated NOS1 proteins that lack the PDZ domain, important for protein binding and localization. The truncated region also includes the binding site for PIN (protein inhibitor of nNOS)\textsuperscript{96}, and its exclusion is likely to alter the enzymatic activity of NOS1. One major finding here is the novel alternatively spliced exon 2a, which is only included in transcripts initiating with 5’1 and 5’2. The sequence of exon 2a lacks an AUG start codon, and therefore is unlikely to alter the translational start site of the full length protein. Furthermore, short open reading frames
(ORF) that have been shown to disrupt the scanning process of the translational machinery\(^9\) are unlikely. Its exclusion from 5’3 and 5’4-containing transcripts suggests another level of tightly regulated NOS1 expression. The finding of an exon insertion within a 5’UTR that does not affect the open reading frame is thought to be a rare event, but has been described in a small number of mammalian genes, including connexin-32\(^9\), nuclear respiratory factor-1\(^9\), and GM3 synthase\(^1\). The functional relevance of the exon insertion in these genes has yet to be experimentally demonstrated. One may speculate that exon 2a harbors either secondary structural elements or *cis*-acting elements for RNA binding proteins that may alter the translational efficiency of the transcripts. These regulatory mechanisms are known to exist in other genes, such as fibronectin, interferon-\(\gamma\), and ferritin, and the iron responsive element (IRE) is a well characterized 5’UTR *cis*-element that mediates translational control in many transcripts (references \(^1\)). It is intriguing that 5’3 and 5’4 transcripts are not detected that contain the alternatively spliced exon 2a. This first exon specificity may result from a positional effect, in that exon 2a lies in close proximity to the exon 5’1 and 5’2 cluster but is greater than 70 kb downstream of the 5’3 and 5’4 cluster. Alternatively, the different first exons could contain *cis* elements that differentially interact with the spliceosome-DNA complex.

5’1 and 5’3 transcripts were found that apparently spliced in unconventional locations, resulting in partial exon deletions. It is unknown whether these mRNAs are translated, but they each eliminate the exon 2 AUG and theoretically could encode N-terminal truncated proteins similar to nNOS\(\beta\). Another plausible explanation is that they represent aberrant transcripts present in the nucleus that are inefficiently or not translated,
but are detected here by the exquisite sensitivity of RT-PCR combined with Southern hybridization. In this case, little functional relevance would be assigned to these low level transcripts. Another unconventional splice variation occurs when transcription initiates with exon 5′4, producing a “read through” mRNA that includes the intron and exon 5′3 before splicing to exon 2. For this to occur the 3′ splice donor site of exon 5′4 may be missed by the splicing machinery, with the next available donor site found downstream in exon 5′3. Interestingly, this splice variant was detected only in the two skeletal muscle-derived tumor samples. While the major product is produced by the standard splicing events (Fig 2.10), perhaps in the altered nuclear environment of the tumor cell the 3′ splice donor site is less efficiently bound by the spliceosome, or other cancer-specific factors alter RNA processing. Aberrant RNA processing has been described for a number of genes in cancer and disease, including BRCA2, NF1, tuberous sclerosis (TSC2), FGF receptor 2, and fragile histidine triad (FHIT) in thyroid tumors. In the case of the TSC2 RNA, a splice acceptor mutation disrupts a putative polypyrimidine structure in an intron, resulting in intron retention and use of a downstream cryptic splice acceptor in the adjacent exon. Although no point mutations have been identified in the exon 5′4 or 5′4 sequences in the tumor samples, it is tempting to speculate that a similar mechanism is responsible for the intron inclusion in these altered cell types. While altered RNA processing often results from splice site mutations, the FHIT mRNA is aberrantly processed despite having no splice site or other identified sequence changes. In this case, relaxation of mRNA splice control appears to be a feature of follicular cell-derived thyroid neoplasms. In the case of the FGF receptor 2 gene, where alternate exon inclusion determines receptor subtype, alternative splicing
events appear to be dictated by cis-elements within introns that interact with the polypyrromidine tract binding protein (PTB)\textsuperscript{105}. Further work on NOS1 alternative splicing and intron inclusion should focus on the presence of splice site mutations, polypyrromidine tracts, or other cis-elements capable of contributing to preferential use of splice sites in different cell states.
CHAPTER 3
Analysis of Transgene Expression in the Central Nervous System

Introduction

Studies of the mouse and rat NOS1 genes have provided much important information about the structure and function of neuronal nitric oxide synthase. While many aspects of NOS1 gene structure and regulation are homologous between rodents and humans, analysis of the alternate promoters and transcripts reveals some important differences, as described in the previous chapter. Because we are ultimately interested in the role of NOS1 gene regulation in human physiology and disease, much of our attention has been focused on the human gene. Studies of human genes are burdened by some inherent limitations, however. Tissue samples can be difficult to obtain, and vary in quality depending in collection and processing procedures. More importantly, practical and ethical factors prohibit properly controlled experimental design, toxic or injurious treatments, or developmental studies. These circumstances obviate the need for animal models in research. We are faced with a dilemma, then, when we desire to study uniquely human traits in ways that are not practically or ethically possible. This is the case with human nNOS, in which the structure (and likely the function) of the 5’ regulatory region of the gene differs from that of the rodent genes. One way to overcome
this dilemma is through the creation of transgenic mice, in which the human gene may be studied in ways not possible in its natural context.

As previously discussed, multiple promoters regulate human NOS1 gene expression, several of which show specificity to the central nervous system. Little is known about how each of the promoters contributes to the complex pattern of NOS1 expression with regards to basal expression, expression during normal development or plasticity, or in response to disease or injury in the CNS. We sought to determine the individual contribution of each of these promoters to directing NOS1 expression in the CNS by creating transgenic mice in which either the 5’1+5’2 or the 5’3+5’4 promoter region of NOS1 to drive the expression of the β-galactosidase reporter gene in vivo. Over twenty lines were analyzed to determine the pattern of expression of the transgenes in the developing embryo and in the adult CNS. The transgene expression patterns were compared to the expression of endogenous rodent and human NOS1, as reported in the literature and as determined in our laboratory. While there is some variation between lines harboring the same transgene, both the 5’1+5’2 and the 5’3+5’4 NOS1 promoter regions are able to direct β-gal expression in neurons in a manner consistent with the expression pattern of endogenous NOS1. The populations of neurons expressing either of the two transgenes overlap, but reveal subsets of cells that demonstrate promoter-specific transgene expression. It is expected that these lines of transgenic mice will facilitate future studies of the regulation of the human NOS1 gene in ways that are not possible by focusing on the endogenous gene.

In order to validate the transgenic model for NOS1 promoter function, it is necessary to determine how closely the expression patterns of the transgenes recapitulate
the endogenous expression of neuronal NOS. A human promoter driving expression in mouse complicates such a comparison, as endogenous expression in the two species differs in some aspects of development and in certain adult tissues. For example, one of the CNS structures in which NOS1 is most strongly expressed is the accessory olfactory bulb\textsuperscript{107}, a structure that does not exist in the human brain. Also, in the adult rodent cerebellum NOS1 is strongly expressed in granule cells and their processes, but not in purkinje cells\textsuperscript{68}. This contrasts with the human cerebellar expression pattern, in which granule cells show only very weak expression, while purkinje cells do express NOS1\textsuperscript{108}. Despite these differences, the known pattern of NOS1 expression is consistent between rodent and human in the majority of tissues and cell types. In a multitude of studies, the map of NOS1 gene expression in these species has been determined at the RNA level by Northern blot, RT-PCR, and in situ hybridization; and at the protein level by Western blot, NADPH-diaphorase histochemistry, and immunohistochemistry. In this chapter, a detailed analysis of CNS expression of the PR(5’1+5’2) and PR(5’3+5’4) transgenes is compared to the known endogenous NOS1 expression patterns in mouse\textsuperscript{109}, rat\textsuperscript{68,110,111}, non-human primate\textsuperscript{112}, and human\textsuperscript{113,114} brain\textsuperscript{115}.

**Materials and Methods**

*Screening for transgenic status:* Offspring of transgenic breeding pairs were screened for transgenic status. Ear or tail clips were digested in 1X PCR buffer with added detergents (0.45% NP40, 0.45% TWEEN 20) and 100 µg Proteinase K (total volume 20 µl) at 55°C overnight with periodic agitation, followed by denaturation of Proteinase K by boiling for 10 minutes. 5 µl of the digestion product was used for PCR amplification of a 250 bp lacZ target under the following
conditions: 1X PCR Buffer (GibcoBRL), 2.5 mM MgCl2, 0.2 mM (each) dNTP, 0.5 µM (each) lacZ Fwd and Rev primers (Appendix), 0.5 units Taq polymerase (GibcoBRL) in a total reaction volume of 50 µl. After 5 minutes denaturation at 95°C, 30 PCR cycles were carried out, 30 seconds at 93°C, 30 seconds at 59°C, and 45 seconds at 72°C. Products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

_Tissue fixation:_ Adult or one-week-old mice are anesthetized with Avertin (0.33 ml/g) administered intraperitoneally. Avertin is 2:1 (w/w) tribromoethanol:tert-amyl-alcohol, diluted 80 fold in isotonic saline (0.9% sodium chloride). The mice are perfused transcardially (i.e. through the left ventricle with a 26 gauge needle, and an incision in the right atrium for outflow) with 15 ml of ice cold 2% paraformaldehyde (buffered with 0.1 M PIPES (pH 6.9), 2 mM MgCl₂, and 2 mM EGTA) via peristaltic pump at approximately 3 ml/min. The brain (or other tissue of interest) is dissected out and immersed in the perfusion fixative for an additional hour at 4°C. The tissue is subsequently cryoprotected by immersion in 30% (w/v) sucrose (in 0.1 M phosphate buffered saline pH 7.3, with 1 mM MgCl₂) at 4°C until the tissue is equilibrated (i.e. when it sinks), usually 24 hours. Tissues are embedded in O.C.T., and 40 µm frozen sections are collected via cabinet cryostat. Slides are then dried overnight on the 37°C slide warmer, and stored at room temperature. Embryos are collected from timed pregnant females, who are sacrificed by CO₂ asphyxiation, and immediately rinsed in cold PBS and immersed in ice-cold 2% paraformaldehyde (buffered, as above) for 3 hours. They are subsequently cryoprotected in 20% sucrose solution, and sectioned as above. Prior to fixation, tail clips are collected to screen for transgenic status by PCR.
**Histochemical staining for transgenic β-galactosidase expression:** 25-40 µm tissue sections from perfusion fixed animals are stained with X-gal substrate: 1 mg/ml 4-Cl-5-Br-3-indolyl-β-galactosidase (X-gal); 0.1 M PBS; 5 mM potassium ferricyanide; 5 mM potassium ferrocyanide; 2 mM MgCl$_2$; 0.02% (w/v) NP-40; and 0.01% (w/v) sodium deoxycholate. Staining is by immersion at 37°C in the dark, for 2-24 hours, depending on transgene expression level. Sections are then rinsed in 0.1 M PBS before counterstaining with neutral red. After ethanol series dehydration and clearing (Histoclear), slides are coverslipped with Permount and visualized by light microscopy. For X-gal staining of whole mount tissues, the same procedure is followed as for sections, except that post-perfusion fixation is 30 minutes and the cryoprotection step is eliminated.

**Histochemical staining for NADPH-diaphorase:** 40 µm tissue sections from perfusion fixed animals are stained for NADPH-diaphorase activity by immersion in the staining solution: 50 mM Tris-Cl (pH 8.0); 0.6 mM nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH); 0.25 mM nitro blue tetrazolium (NBT); 0.1% Triton X-100. Sections are stained for 30-90 minutes at 37°C in the dark, and then rinsed in 50 mM Tris-Cl (pH 8.0) before ethanol dehydration and coverslipping.

**Immunohistochemistry:** Brains of transgenic mice were perfusion fixed as above, using 2% paraformaldehyde, and subsequently post-fixed for three hours in the same solution. 25µm sections were collected, and dried overnight. Following a 5-minute rinse
in 1X PBS, slides were placed in 0.3% H2O2/0.3% serum/PBS for 5 minutes, and rinsed again in PBS. They were then placed in blocking solution (5% Bovine Serum Albumin, 10% lamb serum, 0.2% Triton X-100) for 20 minutes, and rinsed in PBS. The slides were incubated with the primary antibody, α-NOS1 (rabbit polyclonal IgG, Santa Cruz Biotechnology, sc-648) at 1:200 dilution for three hours at 37°C in a humid chamber. After two PBS rinses, the slides were exposed to the secondary antibody, goat α-rabbit IgG-biotin conjugate (Santa Cruz) at 1:500 dilution, for 30 minutes at 37°C. The sections were then stained with the Vectastain Elite ABC (avidin-biotin complex) reagent and DAB + Ni substrate according to the manufacturer’s protocol (Vector Laboratories). For X-gal/NOS1-immunohistochemical double-labeling experiments, the slides were subjected to X-gal histochemical staining as above, except that they were not counterstained or dehydrated prior to immunohistochemistry.

*Description of transgenes:* The NOS1 promoter-reporter gene constructs used to create the transgenic mice were designed by Anthony Young and John Oberdick, and the molecular cloning was carried out by Linda Zhang. A schematic of the transgenic constructs is given in figure 3.1. The first transgene contains the 4.3 kb promoter region upstream of the human NOS1 5’1 and 5’2 exons, henceforth called PR(5’1+5’2). The 3’ end of this genomic fragment lies at the Sma1 site within exon 5’1, 83 nucleotides upstream from this exon’s 3’ splice site. The second transgene contains the 2.6 kb promoter region upstream of the human NOS1 5’3 and 5’4 exons, called PR(5’3+5’4). The 3’ end of this genomic fragment is the Stu1 site, 12 nt upstream of the 3’ splice site. Each of these NOS1 promoter fragments was then separately cloned upstream of the...
Figure 3.1. Schematic diagram of the L7/Lac-Z constructs that were used in the creation of transgenic mice. All were cloned into the pBlueScript SK+ vector in a similar fashion, and differ only in the promoter region immediately upstream of the L7 structural gene.
L7/lacZ reporter gene cassette. This cassette consists of the 3 kb *Escherichia coli* β-galactosidase gene, lacZ, cloned into the unique BamH1 site within exon 3 of the mouse L7/pcp-2 gene\(^{116}\). L7 is a natural, small gene that has been frequently used in transgenic analysis\(^{116}\). Reporter genes, such as lac Z, have been inserted into the L7 gene, and these constructs are uniquely expressed in Purkinje cells of the cerebellum. Dr. Oberdick has altered the four-exon L7 gene so that it lacks all potential translation initiation sequences (ATG), in all reading frames, ensuring that translation begins within the β-gal sequence. Thus, the L7 promoter has been effectively eliminated, and the L7/lacZ fusion gene is placed under transcriptional control of either the 4.3 kb PR(5′1+5′2) or 2.6 kb PR(5′3+5′4) human NOS1 promoters. The 5’ UTR sequences of NOS1 have been shown to diminish expression of closely linked reporters\(^{42}\). The L7 DNA thus serves as a spacer to potentially enhance lacZ expression, and provides a genetic environment favoring proper transcript splicing (J. Oberdick, personal communication). A promoterless control transgene was also created, which is identical to the other constructs except that no exogenous DNA was cloned upstream of the L7 gene. The promoter-reporter constructs were introduced into the mouse genome by standard microinjection techniques, performed by Jan Parker-Thornberg at the O.S.U. Transgenic Mouse Facility.

**Results**

**Classification of different lines, nomenclature**

Nine lines of mice harboring the PR(5′1+5′2)-L7/LacZ transgene were established, four of which have been extensively analyzed to determine the expression pattern of β-galactosidase in the brain. The other lines either did not express β-
galactosidase in the CNS, or bred insufficiently for rigorous analysis. The four lines that do demonstrate CNS transgene expression are lines 3, 18, 73, and 99, named according to the numbers assigned to each founder. These lines contain approximately 25-100 copies of the transgene, based on Southern blotting performed by Linda Zhang. Lines 3 and 18 were derived from a single founder (number 1, F₀¹), the offspring of which exhibited two distinct phenotypes. Several F₁ offspring (of F₀¹) were out-bred with nontransgenic mice to achieve phenotypic homogeneity. A line was considered homogenous when all transgenic pups were determined to share one of the phenotypes expressed by the F₁ mice.

Twelve founders containing the PR(5’3+5’4)-L7/LacZ transgene were established. For initial analysis, four founders were sacrificed, three of which (2, 5, and 12) demonstrated NOS1-like transgene expression. The remaining eight founders were bred, four of which have expression patterns that correlate significantly with endogenous NOS1 expression in the brain (lines 19, 81, 87, and 88). Transgenic offspring of founder 88 had two distinct phenotypes, only one of which was limited to extremely weak expression in the cerebral cortex. Thus, offspring of founder 88 were bred to phenotypic homogeneity (as described with founder 1, above), and the high-expressing line retains the founder’s number.

Expression during development

**PR(5’1+5’2)-L7/Lac-Z**

Samples of mice from three lines harboring the PR(5’1+5’2)-L7/LacZ transgene (18, 73, and 99) were analyzed at developmental times ranging from embryonic day 14 (E14) to postnatal day 7 (P7). Whole embryos were examined both in whole mount and
microscopically in 30 µm sections. At postnatal stages, brains were removed and examined both whole and in sections.

At the earliest developmental stage analyzed (E14), there is limited transgene expression in the central nervous system. At this stage, line 99 has the strongest and most widely distributed transgene expression among the PR(5+1+5’2) lines analyzed. Line 99 embryos show strong expression in a well-delineated area of the dorsal brain stem-spinal cord transition and in other discrete brain stem nuclei, including the trigeminal ganglion. Additionally, dense staining is visible in the external granule cell layer of the developing cerebellum (fig 3.2B), and the serotonergic nucleus of the raphe dorsalis. A broad cluster of cells in the dorsal thalamus stains strongly, as does the dorsal lateral lemniscus. Moderate expression is also evident in the fornix (as a grouping of positive cells), the lamina terminalis (a tight midline cluster), dentate nucleus of the cerebellum, and the cerebellar peduncles. A few scattered cells are observed in the horizontal nucleus of the diagonal band of Broca, globus pallidus (striatum), tectum, and tegmentum. In the spinal cord, expression is limited to a band of cells on the ventral border of the sacral region of the cord (fig 3.2C). These cells are better visualized in coronal sections of this portion of the spinal cord, where they clearly occupy a venterolateral position within the ventral horns (fig. 3.2D). In line 99 embryos, the only non-neural tissue that expresses the transgene is choroid plexus within the cerebral ventricles.

The CNS expression in line 73 E16 embryos is limited to strong staining in the anterior thalamus, and moderate staining of the mammillothalamic tract. These embryos have more extensive non-neural staining than line 99, with blue cells evident in ossification centers of developing bones, and in the smooth muscle layers of the
**Figure 3.2. Line 99 E14 transgene expression.**  A) Sagittal section of whole embryo. Box indicates detail shown in (B). Arrowheads indicate the sacral region shown in (C).  B) Detail of box shown in (A). DBS = dorsal brain stem; CB = cerebellum; TG = trigeminal ganglion.  C) Detail of area delineated by arrowheads in (A).  D) Coronal section through the sacral spinal column of a littermate, indicating transgene expression in the ventrolateral spinal cord.
gastrointestinal tract, very similar to the pattern seen in line 18 (fig. 3.3). Transgene expression is entirely non-neural in E14 and E15 line 18 embryos. That the brain and spinal cord lack β-galactosidase activity is apparent in the whole mount embryos (fig. 3.3B,C), and confirmed in microscopic examination of sagittal sections. Tissues that do express the transgene in line 18 include the ossification centers of developing bone, especially surrounding vertebrae, the base of the skull, ribs, and long bones (fig. 3.3F). The tracheal cartilage is also well delineated (fig. 3.3G). Additionally, skeletal muscle (especially near vertebrae) and gastrointestinal smooth muscle stain blue in these embryos (fig. 3.3E).

On the day of birth (P0), line 73 brains show more widespread transgene expression than at the earlier developmental stage, as observed in both whole mount and sagittal sections. Strong staining is still observed in the anterior thalamus, now joined in intensity by the mammilotegmental tract, medial septum, medial habenular nucleus, and external granule cell layer of the cerebellum. Other brain loci that have initiated a moderate level of expression by P0 include the dentate gyrus of the hippocampus, the middle cerebellar peduncle, the inferior colliculus, and pons. Weak staining is evident in scattered cells throughout the cervical spinal cord. Additionally, non-neural expression is noted in the choroid plexus within the left ventricle.

Whereas E14 embryos of line 18 mice lack any CNS transgene β-galactosidase activity, brains examined at P1 actively express the transgene in several structures. Blue cells are observed in posterior thalamic nuclei (i.e. the ventral posterior nucleus), which is in contrast to the anterior staining observed in line 73. Moderate expression occurs in the ventral tegmental nucleus, interpeduncular nucleus, deep cerebellar nuclei (i.e. dentate
Figure 3.3. E14 embryos stained with X-gal. A) wild type. B-H) line 18 PR(5'1+5'2). B) sagittal view. C) dorsal view. Boxes in (D) indicate detail shown in (E) and (F) of gastrointestinal smooth muscle and ossification centers, respectively. The box in (H) indicates detail of tracheal cartilage shown in (G).
nucleus), hippocampus, and amygdaloid area. Cerebellar staining is evident in the granule cell layer, which contrasts with line 73’s expression in the external granule layer. Line 18 brains at this stage also show scattered expression in the caudate putamen and cortex (especially deep layers), in pattern that resembles the adult expression of both the transgene and endogenous NOS1.

Seven-day-old (P7) line 18 brains were also analyzed for transgene expression. Whole mount staining (fig. 3.4) demonstrates strong β-gal activity in the cerebellum, including the parafloccular lobes. Prominent staining is also seen in the pons and distinct brain stem nuclei, with weak staining observed in the olfactory bulb, basal forebrain, amygdaloid area, superior colliculus, and dorsal cervical spinal cord. Neocortical staining is not appreciated on whole mount at this developmental stage, though positive cortical neurons are observed in thin sections of both P1 and adult brains.

**PR(5’3+5’4)-L7/LacZ**

E14 embryos of three lines (81, 87, 88) carrying the PR(5’3+5’4)-L7/LacZ transgene were examined for β-galactosidase activity, both in whole mount and in sagittal sections. The only evidence for neural expression apparent on whole mount is in line 88, in which a thin blue vertical stripe is seen on either side of the vertebral column. On gross examination, this pattern resembles that seen in line 18 (PR(5’1+5’2) transgene) embryos. However, unlike E14 line 18 embryos, the line 88 sagittal sections clearly demonstrate that this stripe results from strong transgene expression in the dorsal root ganglia (DRG) of the sensory spinal nerves (fig. 3.5A). Apart from the expression in the DRG of line 88, line 81 and line 88 embryos share a similar expression pattern on
Figure 3.4. X-gal staining of 7 day old brains from line 18 PR(5’1+5’2) mice. Superior (A) and inferior (B) views demonstrate cerebellar, olfactory bulb, brainstem and pons staining.
Figure 3.5. Embryonic expression of the PR(5'3+5'4) transgene in line 88 mice.
whole mount. In each case, staining is discretely localized to the hip and shoulder joints. β-gal activity is not visible within the brain of either line on whole mount. Whole mount E14 embryos of line 87 do not appear to express the transgene. Indeed, these embryos are indistinguishable from nontransgenic littermates even after 24 hours of immersion in the X-gal staining solution.

Limited transgene expression in line 87 is revealed, however, at the microscopic level. Weak staining is evident in a narrow band of cells on the midsagittal ventral edge of the spinal cord (not shown), in a pattern similar to line 99. These few cells are the sole visible expressers in line 87 E14 embryos. Sagittal sections of line 88 E14 embryos show more robust transgene expression within the CNS. In addition to the aforementioned dorsal root ganglia, strong β-gal activity is seen in the trigeminal (cranial nerve V) ganglion (fig. 3.5B), posterior pituitary (fig. 3.5C) and the glossopharyngeal (cranial nerve XII) ganglion. Moderate expression exists in the pons, pretectal and tectal areas, and the vestibular nucleus in continuity with the developing cerebellum. Positive cells are also scattered throughout the spinal cord. Outside of the nervous system, there is strong staining in the shoulder and hip synovium, but not at other bone and joint locations. The tongue is also weakly stained. Interestingly, in line 88 there is also strong staining in the adrenal gland (fig. 3.5D), a tissue in which endogenous NOS1 is strongly expressed in the embryo.\textsuperscript{53} No transgene expression is detected in the neural tissue of line 87 embryos.

**Expression in the adult CNS**

The adult pattern of PR(5’1+5’2)-driven β-galactosidase expression was extensively analyzed in four lines of mice (3, 18, 73, 99), at three to six months of age.
Phenotypic homogeneity was confirmed by comparing transgene expression patterns in at least three siblings from a litter, and checked in each subsequent generation. Two patterns of expression became apparent within line 99 after microscopic comparison. Mice exhibiting each pattern are currently being out-bred to nontransgenics to ensure a stable phenotype. The data herein referred to as line 99 are limited to only one of the phenotypes, which will be considered a single line. The other sub-line will be assigned another number, and its phenotype described in a future work. For all lines, the reported expression data represent collective information gathered from whole mount and 40 µm coronal and sagittal sections. The list of positively staining loci is extensive in several lines; therefore, the structures are ordered anatomically to simplify organization and allow more direct comparison between lines. For each line, telencephalic and diencephalic (forebrain) structures are listed first, followed by those in the mesencephalon (midbrain), and rhombencephalon (hindbrain: pons, medulla oblongata, and cerebellum).

Line 3 mice exhibit a characteristic pattern of β-gal activity, with strong, discrete staining in a relatively few (compared to other lines) number of CNS structures. The most prominently stained structures in the brain are the olfactory bulb, superior colliculus, and cerebellum, which are clearly visible on whole mount (fig. 3.6A). Within the olfactory bulb, the internal granule layer is darkly stained, while other cell populations show little or no β-gal activity. Positive cells are scattered throughout the frontal cerebral cortex, which are limited in distribution to the superficial cortical layers. Moderately strong expression is seen in the periventricular gray matter. Transgene expression in the superior colliculus is particularly strong, and is specifically limited to
Figure 3.6. Line 3 PR(5’1+5’2) transgene and wild type NOS1 expression. A) Whole mount of adult line 3 brain, sagittal view of midline structures. Note the prominently stained olfactory bulb (OB), pons, superior colliculus (SC), and posterior cerebellar lobules (Cb). B) Coronal section with prominent lac-Z staining in the superior colliculus (line 3, left), which closely resembles the NOS1 immunohistochemical signal (wild type, right, reproduced from Bredt et. al. (1991)).
cells of the superficial gray layer of this structure, a pattern strikingly similar to NOS1 (fig. 3.6B). It should be noted that an identical staining pattern in the superior colliculus is observed in line 17 mice (these mice were poor breeders, and their analysis has been limited and therefore not addressed herein). Abundant β-gal expression is apparent in the pons, a structure stained in every transgenic mouse harboring the PR(5’1+5’2)-L7/LacZ transgene. The lateral parabrachial nuclei are stained abundantly as well, and weakly staining cells are scattered throughout the brain stem. The staining within the cerebellum is intense, and the pattern of expression is characteristic of several of the transgenic lines. The granule cell layer is dark blue in its full thickness (fig. 3.13b), but the strong expression is limited to lobules VII-IX of the cerebellar cortex, giving a distinctive wedge-shaped expression pattern. The level of expression is several-fold lower in the granule cell layer of more anterior lobules. Notably, the parafloccular lobes are negative. Scattered stellate and basket neurons of the molecular layer exhibit β-gal activity throughout the entire cerebellum, though somewhat more frequently within the posterior lobules. Other tissues samples were collected and analyzed from line 3 mice. In the eye, some retinal neurons stain positively, while no expression is evident in spinal cord, skeletal muscle (vastus medialis), or kidney.

Line 18 mice exhibit the most robust and diverse β-galactosidase activity of all the transgenic lines analyzed. Nearly the entire brain is blue after staining, though areas that lack expression provide evidence that the transgene is not merely ubiquitously expressed in the CNS. Many telencephalic structures are positive. The olfactory bulb is darkly stained, and thin sections reveal that the transgene expression is strong within every layer of this structure (fig. 3.7A). Nearly all cells within the granule layer are
stained, as are approximately half of the cell bodies that form the olfactory glomeruli. In the cerebral cortex, transgene expressing neurons are distributed evenly through all six cortical layers, in all lobes of the neocortex (fig. 3.7C). Approximately 1-3% of all cortical cells are positive in line 18 transgenic mice, a number comparable to that estimated for NOS1\textsuperscript{117} (fig. 3.7D). Transgene expression within the caudate putamen also closely resembles the NOS1 pattern (fig. 3.7E,F). Moderately stained cells are found throughout the amygdaloid complex and the basal nucleus of the stria terminalis, while weakly stained neurons are evident in the nucleus accumbens (core and shell), ventral region of the lateral septal nucleus, and septofimbrial nucleus. Moderate $\beta$-gal activity is seen in scattered large cell bodies throughout all layers and regions of the hippocampal formation, including the dentate gyrus (fig. 3.7B). Several structures of the diencephalon display $\beta$-gal expression in line 18 mice. Strong expression occurs in a subset of hypothalamic nuclei, in both major subdivisions. In the medial zone, the posterior, dorsomedial, venteromedial, and hypothalamic paraventricular nuclei express the transgene. Positive lateral zone structures include the supraoptic, lateral, and supramammillary nuclei (which actually overlap both zones). In contrast to the hypothalamic nuclei, which are difficult to distinguish due to overlapping groups of neurons, the many nuclei of the thalamus are sharply delineated. Likewise, robust transgene expression in the thalamus follows sharply defined borders, and positive nuclei are generally anterior thalamic structures (fig. 3.7G). These include the parataenial, anterodorsal, anteromedial, anterior paraventricular, angular, and ventral medial nuclei. Closely apposed to the thalamus is the strongly stained venterolateral geniculate nucleus, in which both the magno- and parvocellular parts express the transgene. Midbrain
Figure 3.7. Line 18 X-gal staining compared to NOS1 expression. X-gal staining of Line 18 PR(5’1+5’2) sagittal sections of adult brain (A, B, C, E, G), and comparable NOS1 immunohistochemical images (D, F, H, reproduced from Bredt, et. al. (1991)). A) Olfactory bulb. B) Hippocampus. C&D) Cortex. E&F) Caudate Putamen. G) Anterior thalamus. H) Sagittal brain, with thalamus indicated (italic text and arrow added to original image).
structures that exhibit β-gal activity are the interpeduncular nuclei (especially the rostral subnucleus), raphe nuclei (medial and dorsal), and tectal parabigeminal nuclei; all display well-defined cell clusters of moderate staining intensity. Neuronal groups residing in the hindbrain are equally well represented. These include the paratrochlear nucleus and periaqueductal gray matter, as well as several tegmental nuclei. Like all PR(5’1+5’2) lines, strong staining in the pons is a characteristic feature. Transgene expression is also strong in the cerebellum, though the positive cell types differ dramatically from those seen in line 3 brains. In line 18 cerebellum, the vast majority of molecular layer interneurons (basket and stellate cells) are strongly stained, and approximately 30% of purkinje cells contain small inclusions of blue precipitate (fig. 3.8A). The granule cells apparently lack transgene expression in this line, but another source of β-galactosidase activity exists within the superficial aspect of the granular layer. These discrete blue patches, larger than granule cells, may represent glia, golgi neurons, or cerebellar glomeruli; the latter are large synaptic complexes where excitatory mossy fiber afferents synapese with dendrites of granule and golgi neurons. Also unlike line 3, the expression pattern in these cerebella extends with equal intensity into the parafloccular lobes (fig. 3.4). In these mice, coronal sections were made of the thoracic spinal cord, which revealed clear (but relatively weak) transgene expression in small neurons scattered throughout the gray matter of the dorsal columns and in ependymal cells surrounding the central canal. Also considered part of the central nervous system, the retina of line 18 mice exhibits β-gal activity in ganglion and amacrine neurons. Other components of the eye that stain blue are portions of the choroid layer, corneal epithelium, and stroma. Two
Figure 3.8. Variable transgene expression in cerebellum between line 18 and line 3. Lac-Z expression in cerebellum of line 18 (A) and line 3 (B) PR(5’1+5’2) adult transgenic mice. M = molecular layer; P = Purkinje layer; G = granule cell layer.
tissues that express the NOS1 gene, skeletal muscle and kidney, lack any evidence of PR(5’1+5’2)-L7/LacZ expression.

Brains of line 73 mice are more intensely stained than in any other line, while the expression pattern is more limited than most. Whereas the other brains are submerged in X-gal staining solution between six and 24 hours, line 73 whole brain and sections require less than 30 minutes to develop good color, and reach saturation in most positive structures within two hours. Transgene expression in the olfactory bulb proper is limited to periglomerular cells, approximately 20% of which stain dark blue. Strong staining occurs in the medial portion of the olfactory nucleus, but not in the accessory olfactory bulb. The accessory olfactory bulb, which is not present in humans, exhibits very strong NOS1 expression in the mouse and rat. Fibers of the olfactory tract also show β-gal activity. In the basal forebrain, both the medial and lateral septal nuclei are very strongly stained. The anterior region of the hippocampus displays only scattered positive cells, though fibers of the hippocampal-cortical tract are clearly stained. Other forebrain structures that are notably lacking any expression are the cortex and caudate putamen. Within the hypothalamus, subsets of cells within many nuclei, including the suprachiasmatic and preoptic nuclei, are stained. Also unique to line 73, all discernable nuclei of the thalamus exhibit transgene expression, at a high level (fig. 3.9). Another striking feature of the line 73 expression phenotype is the intense staining of the medial habenular nucleus and its associated fiber tracts. While expression in the medial habenular nucleus is characteristic of lines expressing the PR(5’3+5’4)-L7/LacZ transgene, no other lines mice carrying the 5’1+5’2 promoter construct exhibit expression in this structure. The medial habenular nucleus is opaquely stained, so that individual
Figure 3.9. PR(5'1+5'2) transgene expression in line 73 adult mice. A,B,C) lateral septum and anterior, middle, and posterior thalamic nuclei, respectively. LSD = lateral septal thalamic nucleus, dorsal part; fi = fimbria of hippocampus; PVA = paraventricular thalamic nucleus, anterior part; AT = anterior thalamic nuclei (ventral, dorsal, and medial); ReN = reuniens thalamic nucleus. D,E,F) Medial habenular nucleus (MHN) and fasiculus retroflexus (FR) shown in sequential sections. 40X magnification.
cell bodies are not distinguishable, and this staining intensity persists into the retroflexus fasciculus. The fiber tracts connecting the two structures are darkly stained, as are the fibers of the habenular commissure (fig. 3.9). In contrast to these highly expressing structures, the lateral habenular nucleus is devoid of staining. Transgene expression in the midbrain is limited to the bilateral interpeduncular nuclei, which are, like most positive structures, intensely stained. Within the hindbrain, the pons is relatively weakly stained, while the vestibular and cochlear nuclei show moderate transgene expression levels. Finally, the cerebellar cortex in line 73 is intensely stained in all areas, including the parafloccular lobules. The granule cell neurons constitute the highest expression level, and staining is also seen in their parallel fiber processes that extend to the molecular layer. Basket and stellate interneurons within the molecular layer are also positive, but the purkinje neurons are notably lacking expression. There are scant positive cells in the deep cerebellar nuclei and brainstem. Tissues outside of the central nervous system have not been evaluated in adult line 73 mice.

PR(5’1+5’2)-L7/LacZ expression in line 99 is widely distributed, but in general staining intensity is weak and the number of positive cells within identified structures considerably fewer when compared to other lines. There are a handful of exceptions, however, where as many as 50% of cells within a locus are deeply stained. The olfactory bulb is negative, while approximately 15% of anterior olfactory nucleus cells are blue. The lateral olfactory tract (area 2) is one of the intensely stained structures within the line 99 CNS, in which approximately half of cells express the transgene. In the cerebral cortex, a small number of cells (< 1%) have β-galactosidase activity. These are distributed primarily in the deeper layers of most cortical areas, including the frontal
association, sensory, and orbital regions. Scattered staining is observed within the amygdaloid complex; in both the anterior and paraamygdaloid areas, and specifically within the cortical amygdaloid nucleus. Rare positive cells are noted in both lateral and medial septal nuclei of the basal forebrain, with a cluster of staining at the diagonal band (nucleus of the vertical limb). The dentate gyrus of the hippocampus contains another of the densely staining areas, as approximately 30% of granule cells within this formation are positive. Also within the hippocampal formation is an intensely stained subiculum, presubiculum, and moderately stained lateral entorhinal cortex. The rest of the hippocampal formation contains only a few scattered cells that express the reporter gene. The caudate putamen exhibits scarce blue cells, which number approximately 50-fold less than in the NOS1-like line 18. Throughout the diencephalon, rare, scattered blue cells are found in various loci, including the claustrum, premammillary nuclei, and some thalamic structures. The parataenial thalamic nucleus is the exception, in which approximately 5% of cells demonstrate β-gal activity. Midbrain staining is evident in a lone structure; the substantia nigra pars compacta shows moderate expression, with about 20% of its cells positive. In the hindbrain, the pons exhibits particularly strong transgene expression. The lateral parabrachial nucleus and various tegmental nuclei have weak β-gal activity, and positive neurons are found throughout brain stem nuclei. Of the brain stem nuclei, the nucleus ambiguus stands out with an intense staining pattern. The nucleus ambiguus contains the motor nuclei of the glossopharyngeal and vagus cranial nerves (CN IX and X, respectively). The cerebellar cortex lacks β-galactosidase activity in all but a few rare cells, apparently randomly distributed cells. No transgene expression is detected in spinal cord, eye (including retina), skeletal muscle, or kidney in line 99 mice.
PR(5’3+5’4)-L7/LacZ

Considering the plethora of founder mice generated following microinjections of the PR(5’3+5’4)-L7/LacZ molecule, breeding and analyzing 12 lines of mice harboring the same transgene was not practical. Instead, four founders (numbers 2, 5, 11, and 12) were sacrificed and analyzed for their expression patterns without the possibility of perpetuating their lines for use in future studies. Nonetheless, the data generated from these individual mice contributes to our understanding of NOS1 promoter-specific expression patterns. The brain of founder #2 was divided in the midsagittal plane, and half stained in whole mount, the other half sectioned coronally for microscopic examination. The remaining three founders were similarly divided, but only examined in whole mount. The eight founders that were not sacrificed were bred, and several transgenic pups from each litter were assayed for β-galactosidase activity in the CNS. Half of the founders either produced insufficient numbers of offspring or their pups lacked adequate CNS transgene expression to warrant further study. Four lines (from founders 19, 81, 87, and 88) were established, enabling the detailed analysis of CNS transgene expression.

Founder #2 reveals expression of the transgene in the olfactory bulb. Light staining is apparent throughout the cortex on whole mount. In the hippocampal formation, only the entorhinal cortex and subiculum are stained. In the basal forebrain, intense staining in most cells is evident in the lateral septal nucleus (fig 3.10A), an area where expression is characteristic of mice carrying the 5’3+5’4-driven transgene. Structures of the diencephalon that have β-galactosidase activity include the weak but well-delineated staining in the venterolateral geniculate nucleus in the thalamic area (fig
Figure 3.10. Founder 2, PR(5’3+5’4) transgene expression. A) LSN = lateral septal nucleus, 25X. B) VGN = venterolateral geniculate nucleus, 50X. C) MHN = medial habenular nucleus, 100X. D) Posterior cerebellum, 200X. E) Anterior cerebellum, 200X.
3.10B), the zona incerta of the subthalamus, and very intense staining of the medial habenular nucleus (fig 3.10C), the latter being another characteristic feature of the lines with this transgene. The posterior area of the hypothalamus shows moderate transgene expression. In the midbrain, the dorsal raphe nucleus and the substantia nigra (pars reticulata) each demonstrate transgene expression of moderate intensity. Both the pons and cerebellum exhibit β-gal activity in the hindbrain. The pons is relatively weakly stained, while the cerebellar cortex displays the familiar wedge-shaped expression pattern. Within the posterior cerebellar lobules, the intensely staining granule cells are the prominent source of the blue precipitate (fig 3.10D). The level of expression in granule cells falls dramatically, however, in the anterior lobules, in which staining is limited to other cell types (or glomeruli) within the granule layer (fig 3.10E). The purkinje neurons and molecular layer are negative throughout. Some staining is evident within the brain stem and spinal cord on whole mount, but specific structures are not identified.

Founders #5 and #12 exhibit quite similar transgene expression patterns to each other. Both display moderate levels of β-galactosidase activity in the cerebral cortex, hypothalamus, and thalamus. Weak staining is seen in the pons, brain stem, and cervical spinal cord of both mice. Transgene expression is intense in the superior colliculus of founder #5, in contrast to the very weak staining of founder #12. Likewise, expression in the medial habenular nucleus is present in both brains, but is significantly stronger in founder #5. Although not as intense as in founder #2, both #5 and #12 exhibit granule cell expression in the posterior lobules of the cerebellar cortex. Additionally, some purkinje cells show transgene expression in the anterior lobes. Founder #11 contained
the fewest positive structures and the weakest overall staining intensity. In this brain, only the cortex and medial habenular nucleus are moderately stained. Very weak expression is also visualized in the hypothalamic and thalamic areas, pons, and spinal cord.

Brains of line 19 mice were examined both in whole mount and coronal sections. Overall, the PR(5’3+5’4)-L7/LacZ transgene is moderately expressed in these mice. Some brain tissues, while clearly containing positive cells, display only the weakest β-galactosidase activity, which is missed without careful examination of tissue sections at high power magnification. Within the olfactory bulb, for example, only the rare mitral neuron stains blue near the glomeruli. Similarly weak expression occurs in the lateral olfactory tract. Staining is more abundant in the accessory olfactory bulb, where approximately 5% of cells are positive. The cerebral cortex contains transgene-expressing cells in the motor and orbital areas, where 1-2% of cells in the middle cortical layers are blue. The only positive cells within the dentate gyrus of the hippocampus are in the polymorph layer. However, stronger staining is observed in the cingulum and entorhinal cortex, which are part of the limbic system within the hippocampal formation. In the amygdala, weak and infrequent blue cells lie only in the posterior area. The lateral septal nucleus and parasubiculum exhibit moderate transgene expression. In the diencephalon, the thalamus is moderately stained in the parataenial, anterodorsal, and anteroventral nuclei. Very weak staining is observed in the lateral hypothalamic area and in the medial habenular nucleus. Mesencephalic transgene expression is strong in the parabigeminal nuclei, and moderate in both the raphe nuclei and the superficial layer of the superior colliculus. In the hindbrain, the pons exhibits the most intense β-gal activity.
of any brain structure. Several other loci have moderate transgene expression levels. These are the lateral parabrachial nucleus, paratrochlear nucleus, vestibular nucleus, periaqueductal gray, and tegmental nuclei. Scattered positively staining cells are also evident in the medulla and spinal cord. Very little $\beta$-gal activity is seen in the granule cell layer of the cerebellum, where only “splinters” of blue are observed in the midline. Minimal staining is also observed in the Purkinje cell layer, but the limited expression makes specific cell-type identification impossible.

Transgene expression in line 81 brains resembles that seen in line 19 in several aspects. The overall staining intensity is relatively weak, though thalamic nuclei in particular are moderately stained. Most other positive structures display only scattered and weakly staining neurons. This is the case in the glomeruli of the olfactory bulb and the caudate putamen. The cerebral cortex expression pattern resembles that seen in line 19, as the middle layers of the orbital region contain positive cells. The entorhinal cortex is one of the more intensely stained structures in the brain, and represents the sole component of the hippocampal formation that expresses the transgene. Many thalamic nuclei stain equally strong, including the parataenial, anterodorsal, and anteroventral nuclei, which are also positive in line 19. Additionally, the line 81 thalamus reveals transgene expression in the paraventricular, reuniens, and geniculate nuclei of the thalamus. The characteristic medial habenular staining is present, but is very weak in this line. Moderate staining in the dorsal fornix is evident. Line 81 is one of the few lines of transgenic mice that do not exhibit transgene expression in the fibers of the pontine nuclei (i.e. the pons). Weak but detectable transgene expression is noted within the dentate and interpositus nuclei (deep cerebellar nuclei), and moderate staining in the vestibular
The brain stem and spinal cord show scattered cells, as in line 19. The granule cells of the cerebellum are not stained, but sporadic blue non-granule cells are observed in the granule cell layer. Like in line 19, there is ill-defined staining within the purkinje cell layer of the cerebral cortex.

Brains of line 87 mice demonstrate moderate β-gal activity overall, and share many features of the expression pattern with the other PR(5′3+5′4)-L7/LacZ transgene-containing mice. The olfactory bulb is more intensely stained than lines 19 and 81, resulting from the positive granule cell layer in this line. On whole mount the cortex is very lightly stained, and microscopic examination reveals only sporadic (<1%) blue cells throughout the motor cortex. One exception is a small cluster of darkly stained neurons, the barrel field of the somatosensory cortex (fig. 3.11A). In the caudate putamen, moderately stained but infrequent cells are noted. The basal forebrain exhibits moderate transgene expression within the lateral septal nucleus (ventral area) and septofimbrial nucleus. Like several of the other PR(5′3+5′4) lines, specific anterior thalamic nuclei are strongly stained. These include the parataenial, anterodorsal, and interanterior nuclei in line 87. Of these, the parataenial nucleus has the highest transgene expression level. The dorsal fornix is positively stained, as is the medial habenular nucleus, though only moderately so. In the midbrain, the deep mesencephalic nucleus displays sporadic positive cells, and the raphe nuclei are moderately stained. Also in the midbrain, the parabigeminal nuclei stand out with intense transgene expression (fig. 3.11B). Identified by equal staining intensity are the lateral parabrachial nuclei in the hindbrain. Strong expression is evident throughout the microcellular tegmental nuclei. Moderate staining is seen in several areas of the periaqueductal gray. The pons is strongly stained in line 87.
Figure 3.11. Expression of the PR(5’3+5’4) transgene in line 87 adult mice. Discrete β-galactosidase staining in the barrel field of the primary somatosensory cortex (A) and in the parabigeminal nucleus (PBG) and microcellular tegmental nucleus (MiTg) of the midbrain. VCx = visual cortex, S = subiculum.
mice. Occasional groupings of positive cells are noted in the brain stem and cervical spinal cord. The wedge shaped pattern of expression, as seen in founder 2, is once again noted in the whole mount cerebellum, particularly in the lateral portions of the inferoposterior lobules. Microscopic examination reveals that as high as 20% of granule cells are positive within these lobules, while the other areas have no detectable staining in granule cells. Sporadic, larger blue cells are seen in the granule cell layer throughout the entire cerebellar cortex.

Among the lines harboring the PR(5’3+5’4) transgene, line 88 stands out as having the highest expression levels throughout the CNS. On whole mount, nearly the entire brain is stained dark blue, though discrete structures are clearly identifiable (fig. 3.12A). As with the highly expressing line 18 brains, well-delineated negative structures confirm that the transgene is not simply expressed ubiquitously in the brain. Moderate staining is present in all cellular layers of the olfactory bulb, but not in the accessory olfactory bulb. The cortex reveals intense staining that rivals any other line, with approximately 80% of all cortical cells darkly stained across both the motor and sensory areas. This intense staining occurs through the full thickness of the cerebral cortex, with the exception of the most superficial layer of cells (fig. 3.12B). The caudate putamen is also intensely stained, with approximately 15% of neurons expressing the transgene (fig. 3.12C). This amounts to an approximately five-fold higher number of positive cells than are revealed by NOS1 immunohistochemistry. Positive cells are scattered throughout the hippocampus, so that this structure is one of the more weakly stained overall. Both the lateral and medial septal nuclei have a moderately high level of transgene expression (fig. 3.12D). Within the diencephalon, the bed nucleus of the anterior commissure is darkly
Figure 3.12. Line 88, PR(5’3+5’4) transgene expression. X-gal staining in adult mice. A) Whole mount, cut and viewed in mid-sagittal plane. B-E) Coronal sections. B) Cortex, 100X. C) Striatum, 100X. D) Medial habenular nucleus (MHN). Also shown is dentate gyrus of hippocampus (DG). E) Lateral septal nucleus (LS) and medial septal nucleus (MS). The cut right edge is the midline. GCC = genu of corpus callosum, LV = lateral ventricle.
stained, as are several thalamic nuclei. In the thalamus, the anterodorsal thalamic nucleus is particularly intensely stained. Moderate transgene expression occurs throughout the hypothalamic area. The familiar medial habenular nucleus is one of the most intensely stained structures in the brain of line 88 mice (fig. 3.12E). In the midbrain, the substantia nigra pars compacta expresses the transgene strongly, and moderate staining is apparent throughout the inferior and superior colliculi. In the hindbrain, the anteroventral and ventral cochlear nuclei also express the transgene at a high level, and areas of β-galactosidase activity are apparent throughout the reticular formation, extending through the brain stem and spinal cord. Surprisingly, the pons in line 88 mice lacks any evidence of transgene expression. The granule cells of the cerebellum are also negative. The dark staining apparent in the posterior lobules of the cerebellum is accounted for by transgene expression within purkinje cells (fig. 3.12F). The distribution of positive purkinje cells, however, is limited to the midline (vermis), and elsewhere positive neurons are evident in the molecular layer. In the weakly stained regions of the cerebellum, scattered large cells are seen in the granule and purkinje cell layers, which appear to be neither granule nor purkinje neurons.

Transgene expression occurs in NOS1 neurons

To document appropriate expression of the transgenes, it is important to determine whether or not they are being expressed in the same cells as the endogenous NOS1 gene. To this end, double label immunohistochemistry experiments were carried out on sections of line 88 transgenic mouse brains. The sections were first processed for β-galactosidase activity, and subsequently exposed to a highly specific polyclonal anti-
Figure 3.13. NOS1 and Lac-Z coexpression. Line 88 PR(5’3+5’4) striatal neuron coexpressing the L7/Lac-Z transgene (blue) and NOS1 (brown). 400X magnification.
NOS1 antibody to label NOS1-containing neurons (fig 3.13). Within the striatum, approximately 30% of transgene-positive cells were also NOS1 positive. Conversely, approximately 50% of NOS1-positive cells (medium aspiny neurons) also expressed the PR(5'1+5'2)-driven reporter. Within the spinal cord approximately 95% of β-gal positive cells are also immunopositive for NOS1, and approximately 75% of NOS1 neurons coexpress the transgene. These data clearly demonstrate that, at least in the spinal cord of line 18 mice, the PR(5'3+5'4) transgene is appropriately expressed in a majority subset of NOS1 neurons.

**Discussion**

Clearly, each of the human NOS1 promoter complexes is able to drive the expression of the lacZ gene in diverse tissues of the mouse central nervous system. Two main questions arise: 1) To what extent do the transgenic models faithfully recapitulate the expression pattern of the endogenous NOS1 gene? 2) What does transgene expression mapping reveal about human NOS1 promoter-specific differential expression, in terms of developmental and tissue-specific regulation?

**Correlation of transgene and endogenous NOS1 expression patterns**

Of fundamental importance in discerning the utility of transgenic models of NOS1 gene expression is determining how faithfully the transgene is expressed in relation to the native gene’s expression pattern. To make this determination, of course, requires an accurate description of normal physiologic NOS1 expression in the central nervous system. While no complete description exists in a single published work, several
reviews and many original works\textsuperscript{109-111,113,115,118,119} have addressed this issue, and from these a fairly detailed consensus expression map emerges. Various methods have been used to detect NOS1 expression in the brain, including detecting mRNA by northern blotting, RT-PCR, and \textit{in situ} hybridization; protein by western blotting and immunohistochemistry; and catalytic activity by enzymatic assay or NADPH diaphorase histochemistry.

Since the demonstration that neuronal NADPH diaphorase is a nitric oxide synthase\textsuperscript{120,121}, the simple histochemical reaction to detect NADPH diaphorase activity, in which tetrazolium salts are reduced to visible formazans, has become the most widely used method to detect NOS1 activity in the brain. Unfortunately, of those methods listed above, this indirect assay can also be the least specific method for detecting NOS1, as other sources of NADPH diaphorase activity exist in the brain\textsuperscript{122,123}. However, the correlation of NADPH diaphorase activity with NOS1 immunohistochemistry in the brain is extremely high following proper tissue preparation, namely strong formaldehyde fixation. Among the sources of NADPH diaphorase activity in the brain, it appears that only NOS1-dependent diaphorase activity remains active under these conditions\textsuperscript{115}. For the discussion that follows, NADPH diaphorase activity will be considered as equivalent to NOS1 expression, because all of the data reviewed were obtained by either NADPH diaphorase histochemistry following formaldehyde fixation or by NOS1 immunohistochemistry, or both. Indeed, where both methods are used, colocalization is found in the vast majority of cases\textsuperscript{115}. An exhaustive treatment of NOS1 expression would be redundant here. However, to effectively compare the transgene expression patterns with that of NOS1, certain key findings from the literature will be discussed,
along with some functional correlations. Unless otherwise noted, data is from the review by Blottner, et. al.\textsuperscript{115}, and from references therein.

*Olfactory bulb.* In the rat main olfactory bulb, NOS is localized in neurons of the granule and glomerular cell layers as well as in occasional neurons of the inner and outer plexiform layers, but not in mitral cells, the secondary olfactory neurons\textsuperscript{110}. In the accessory olfactory bulb, the granular layer, fascicular bundles and dense fiber plexuses are intensely stained, representing some of the highest levels of NOS1 expression in the rat brain\textsuperscript{117}. Similar NOS1 expression in both structures has been reported in mouse. Neuronal NOS is expressed in the anterior olfactory nucleus and olfactory tubercle. Transgene expression reported herein varies between lines in the olfactory bulb and associated structures. In line 18 strong β-gal activity is seen in all cell layers of the olfactory bulb, in line 3 strong staining occurs only within the internal granule layer, while in line 73 expression is limited to the periglomerular cells of the main olfactory bulb (fig. 3.14). The transgene expression pattern observed in line 18 closely resembles the endogenous NOS1 mRNA expression pattern as visualized by in situ hybridization (fig 3.14C). Three of the PR(5’3+5’4) lines (19, 81, and 87) have weak to moderate staining in the main olfactory bulb, while the others lack any expression in this structure. Of all the lines, only 73 and 99 display transgene expression in the anterior olfactory nucleus, and only line 19 exhibits expression in the accessory olfactory bulb. The lack of transgene expression in most lines in the accessory olfactory bulb is intriguing. While NOS1 is abundantly present in this structure in rodents, the structure does not exist in the human brain; thus, it is perhaps appropriate that the human promoter fragments generally do not direct expression in this rodent tissue. In the developing rat brain, NOS1 protein is
Figure 3.14. Olfactory bulb PR(5’1+5’2) expression differs between transgenic lines, and comparison with NOS1 expression. A) Line 18, with expression throughout the olfactory bulb. B) Line 73, with expression limited to glomerular layer. C) Wild type mouse, in situ hybridization with NOS1-specific oligonucleotide probes. GCL = granule cell layer, GL = glomerular layer, PL = plexiform layer.
first detected in the olfactory bulb at postnatal day 3 (P3)\textsuperscript{88}. In the developing transgenic mice, β-galactosidase activity is not detected in the olfactory bulb in any line examined from E14 through P1. Light olfactory bulb staining is observed, however, in seven-day-old line 18 brains, correlating well with the timing of NOS1 induction.

Cerebral cortex. NOS1-positive neurons are evenly distributed throughout the cortical layers in the adult rat (layers II, III, IV, and VI) and cat (all layers), across all functional regions. It is estimated that 1% of rat cortical neurons express NOS1\textsuperscript{117}. Many fusiform, triangular, and multipolar cells are positive, while cortical pyramidal neurons are generally negative. The pyramidal cells, which express soluble guanylyl cyclase, are thought to be the major targets for NO· in the cerebral cortex. Many NOS-immunoreactive neurons in the cortex express relatively high levels of NMDA receptor mRNA (NR1). Transgene expression in the cortex varies considerably from line to line, ranging from none (lines 3 & 73) to expression in all cortical areas (line 18). Among the transgenic lines examined, line 18 most closely approximates both the distribution and frequency of cortical NOS1 expression (fig. 3.7C,D). This holds true in the developing cortex as well. In most studies, NOS1 immunoreactivity in the developing rat cortex is first detected at E19 to P0\textsuperscript{70,88}, though two report expression as early as E14 in the pre-cortex\textsuperscript{124,125}. In either case, expression is initially extremely weak, with strong upregulation occurring perinatally, followed by a drop to adult levels of expression during the first postnatal week of life. The sharp rise to maximal expression is thought to correspond with the period of neuronal migration and synaptogenesis, in which a role for NO· has been implicated\textsuperscript{70,125}. Cortical β-galactosidase activity is not detected in any of the transgenic lines during embryonic development, but is first seen in the P1 cortex of
line 18 mice. The positive neurons at this stage are distributed mostly in the deep cortical layers, with a frequency and staining intensity that approximates the adult expression level in this line.

**Basal ganglia.** NOS1 has been demonstrated in populations of medium-sized aspiny interneurons (1-2% of all striatal neurons) in the mouse, rat, cat, and human striatum. The striatum contains the highest activity of guanylyl cyclase and cGMP phosphodiesterase in the brain\(^{126}\), and these enzymes colocalize with NOS1. NOS1 expression is also observed in the globus pallidus, which relays the basal ganglia output to thalamic motor nuclei\(^{114}\). The highest overall transgene expressers of each construct, lines 18 and 88, exhibit relatively high levels of β-gal activity in the striatum. Positive striatal cell counts in line 18 are essentially equivalent to NOS1 (fig. 3.7E,F), and line 88 has approximately 5-fold more. On the other extreme, several lines of each construct lack any striatal β-gal expression, and line 99 displays only occasional positive neurons. With respect to developmental expression patterns, NADPH diaphorase activity is first detected in the striatum at embryonic day 18\(^{88}\). NOS1 immunoreactivity, first detected at P0, peaks during the first postnatal week, and then tapers off to adult levels\(^{124}\). At 13 gestational weeks (GW), striatal NOS1 activity is the first detected in the developing human brain, and like in the rat brain expression peaks between the 20\(^{th}\) and 36\(^{th}\) gestational weeks before returning to adult levels\(^{108}\). Two of the transgenic lines, carrying the PR(5'1+5'2) promoter fragment, exhibit early expression within the basal ganglia. In line 99, scattered β-gal-positive cells are detected in the globus pallidus at E14, while a similar expression pattern is not observed until P1 in line 18 striatum. These initial induction time points do approximate that seen for NOS1, though the current data
are insufficient to determine whether or not transgene expression, under the control of the human promoters, undergoes a developmental burst before attaining adult levels.

**Hippocampus and limbic system.** NOS1-containing interneurons and diffuse fiber networks are preferentially located in CA1-CA3 fields of the hippocampal formation, with a concentration in CA1. These positive cells are scattered throughout the molecular layer and stratum oriens of the hippocampus. CA1 pyramidal neurons have not been shown to express NOS1. In the dentate gyrus, basket cells scattered throughout the granule cell layer stain for NOS1. Reports of NOS1 expression in the granule cells of the rat hippocampus conflict dramatically, claiming either intense\(^{117}\) or no\(^{110}\) staining. Likewise, the transgenic mice exhibit a range of hippocampal expression, ranging from no staining (lines 3, 2, 81, 87) to strong (line 99) granule cell staining. Line 18 demonstrates an expression pattern that is most similar to that reported in the majority of studies, in which positive neurons are scattered throughout the hippocampus, in the molecular layer, stratum oriens, and non-granule cells of the granular layer (fig. 3.7B). NADPH diaphorase activity is noted as early as E15 in the developing rat hippocampus, and in the amygdala by postnatal day three. Two lines of transgenic mice, 18 and 73, exhibit β-gal activity in the hippocampus during brain development, which is first detected just after birth (P1 and P0, respectively). Expression in the amygdala also occurs at postnatal day one in line 18.

NOS1 positive cells are also found within the adult entorhinal cortex\(^{112}\), part of the hippocampal formation. Half of the PR(5’1+5’2) lines and three of five of the PR(5’3+5’4) lines have expression in the entorhinal cortex, which serves as a relay point between the neocortex and the limbic system\(^{127}\). Within the basal forebrain, distinct
populations of magnocellular NOS neurons have been described in the septal area and the nucleus of the diagonal band of Broca, neurons which innervate the hippocampus. Most of these NOS1-containing neurons are cholinergic, and express the low-affinity nerve growth factor receptor p75 through which NOS1 is activated. Of the transgenic lines, lines 2 and 73 boast the highest overall expression in the septal nuclei, at levels considerably greater than that reported for NOS1. The other lines display β-gal expression patterns more in accord with the endogenous NOS distribution. Line 99 exhibits a cluster of positive cells in the nucleus of the diagonal band of Broca, closely resembling the NOS1 expression pattern in this structure.

In the amygdala, many NOS-positive nerve cells occur in the medial amygdaloid nucleus, and densely staining fibers are detected in amygdaloid nuclei that are in continuity with fibers of the piriform cortex. Dense neuronal populations in the bed nucleus of the stria terminalis are also stained for NOS. Lines 18 and 99 exhibit strong and moderate transgene expression, respectively, throughout the amygdala. Line 19 has sparse positive cells in this area, while all other transgenic lines lack expression in the amygdaloid nuclei. The highly expressing brains from each construct, lines 18 and 88, however, each display strong staining in the bed nucleus of the stria terminalis, which receives major afferent fibers from the amygdala via the stria terminalis.

*Diencephalon.* In the rat, cat, and human, neurons that express NOS1 are found primarily within the magnocellular neurosecretory system of the hypothalamus, such as in the supraoptic nucleus, paraventricular nucleus, and accessory magnocellular cell groups. Cells within these structures exhibit a wide variation in NOS staining intensity. In rat magnocellular nuclei, NAPDH diaphorase has been co-localized with
oxytocin but not vasopressin, suggesting that oxytocin neurons are the major source of NO· production in the hypothalamic-pituitary system. A subset of parvocellular neurons expresses NOS1, as do scattered cell bodies throughout the mammillary nuclear complex. The major efferent connections of the hypothalamus arise in the mammillary nuclei, innervating the anterior nucleus of the thalamus, the reticular formation of the midbrain tectum, and nuclei of autonomic nerves in the brain stem and spinal cord. This distribution of NOS expression suggests that NO· may be involved in the regulation of the posterior pituitary. In the hypothalamus, the endogenous NOS1 expression pattern is most closely matched by line 18 transgenic mice, in which the supraoptic and paraventricular nuclei, as well as the medial and ventral supramammillary nuclei, exhibit β-galactosidase activity. Line 73 exhibits relatively weak transgene expression throughout the hypothalamic area, including the suprachiasmatic nucleus, a structure that has recently been shown to express NOS1. In line 99, scattered positive cells are limited to the premammillary nuclei. The brains of founder 2 and of line 19 display weak staining in the hypothalamus, primarily in the posterior hypothal area.

In the rat thalamus, scattered populations of NOS-containing neurons are mainly distributed in the paraventricular nucleus, the anteroventral nucleus, the central medial nucleus, and the reuniens nucleus, while other thalamic nuclei contain sparsely stained cell bodies and fibers. In a single study NOS1-positive neurons were not identified in the human thalamus. In line 73 mice the entire thalamus is heavily stained (fig. 3.9). In contrast, other lines display more discrete thalamic transgene expression. For example, in line 18, expression occurs in the well-delineated anteroventral nucleus, closely mimicking the NOS1 expression observed in the rat (fig. 3.7G,H). These mice also
express the transgene in the parataenial and paraventricular nuclei, the latter of which displays prominent NOS1 immunostaining. Line 99 mice also display weak anterior thalamic and reuniens nucleus staining in scattered somata. Four of the five PR(5’3+5’4) lines (all but founder 2) exhibit strong anterior thalamic staining, including the parataenial, anterodorsal, and anteroventral nuclei. Of these, line 81 has the most widespread expression pattern, with the paraventricular and reuniens nuclei also stained. The anterior thalamic nuclei function as a relay center in the limbic system, receiving their principle afferents from the mammillary body of the hypothalamus, and sending output fibers to the cingulate gyrus\(^{127}\). Of particular interest is the thalamic parataenial nucleus, which is strongly stained in lines 99, 18, 19, 81, and 86. This thalamic nucleus is thought be an important thalamic link between limbic and striatal processing\(^ {132}\).

While only scant NOS1 activity is detected in the adult parataenial nucleus, strong expression occurs during development of the thalamus. Strong NOS1 immunoreactivity is maintained from E17 to P1, is weaker from P3 through P15, and is virtually absent in the parataenial nucleus of 30-day-old rats\(^ {133}\). It is possible that adult expression of the transgenes in this thalamic nucleus is properly localized, but inappropriately timed.

Thalamic transgenic β-galactosidase activity is apparent during development as well. In line 99 E14, line 73 E16, and line 18 P1 brains, strong staining occurs in the dorsal, anterior, and ventral thalamic nuclei, respectively.

NOS1 expression in the adult thalamus also occurs in the medial and lateral geniculate nuclei. NOS1-positive neurons are scarce in the dorsolateral geniculate nucleus, but densely populate the venterolateral geniculate nucleus in the rat\(^ {110}\). Line 18 transgenic mice closely match this expression pattern, with strong staining limited to the
ventrolateral geniculate nucleus. Lines 2 and 19 exhibit similar but less abundant transgene expression in this structure (line 2 shown in fig. 3.10). The lateral geniculate nucleus functions as a major relay nucleus in vision processing, receiving input fibers directly from retinal ganglion cells via the optic nerve and tract, and then sending efferents to the primary visual cortex. In the rat retina, NOS1 is present in a subpopulation of GABAergic amacrine cells of the inner nuclear and/or ganglionic layer, and in humans NADPH diaphorase activity has been found in cone photoreceptor cells. Staining for β-galactosidase activity was performed on sections of eye from lines 3, 18, and 99. Of these, line 3 and 18 exhibit transgene expression in retinal cells that appear to be amacrine and ganglion neurons.

NOS1 expression has been documented in the habenula, a midline structure that lies just superior to the thalamus. The lateral habenular nucleus contains a dense network of strongly stained fibers (but not cell bodies), while only sparse positive fibers have been reported in the medial habenular nucleus. A few NOS-containing fibers are also distributed through the habenular commissure and in the fasciculus retroflexus. These brain regions are involved in emotional control, sexual arousal, REM sleep, and seizures. Neurons of the habenular nuclei boast a novel type of purinergic synapse in the central nervous system. ATP is the fast-acting neurotransmitter, which is released from nerve terminals that originate in septal nuclei of the basal forebrain. The co-transmitter, if any, is thought to be glutamate. β-galactosidase activity in medial habenular nucleus of the transgenic mice is characteristic of the PR(5’3+5’4) lines, which do not exhibit lateral habenular expression. The level of expression varies, with line 88 and founder 2 brains (figs. 3.12E and 3.10C, respectively) displaying the most intense staining. While this
pattern contradicts that reported for NOS in the literature, \textit{in situ} hybridization data from this work clearly demonstrate NOS1 mRNA expression in the medial, but not the lateral, habenular nucleus of the rat (fig. 3.15). One possible explanation for this apparent disparity is that the NOS1 mRNAs are transcribed in medial habenular neuronal cell bodies, and the protein is transported along their projections into the commissure, lateral habenula, and fasciculus retroflexus. Alternatively, the NOS1 mRNA may not be translated \textit{in vivo} in this structure, whereas the transgenic lacZ transcript eludes such post-transcriptional regulation. Of the PR(5’1+5’2) transgene-containing mice, only line 73 exhibits habenular β-gal activity, which is extremely intense. In these mice, not only is the medial habenular nucleus darkly stained, but so too are the fibers of the habenular commissure and the fasciculus retroflexus (fig. 3.9). The sheath of the fasciculus retroflexus carries axons between the lateral habenular nucleus and monoaminergic targets in the midbrain. This portion of the tract is selectively degenerated in response to drugs of abuse with strong dopaminergic actions, such as cocaine and amphetamines. Indeed, with certain drugs, such as cocaine, these fibers are the only known targets for degeneration in the CNS. The core of the fasciculus retroflexus, which carries cholinergic fibers from the medial habenula to the interpeduncular nucleus (which is also stained in line 73 brains), is very specifically degenerated in response to high levels of systemic nicotine administration\textsuperscript{136}. Given that the PR(5’3+5’4) fragment drives transgene expression specifically in neurons of the medial habenular nucleus, it is tempting to speculate that promoter specific NOS regulation may play a role in the response to nicotine in these cholinergic fibers.
Figure 3.15. NOS1 mRNA expression in the hippocampus and medial habenular nucleus. *In situ* hybridization using pooled oligonucleotide probes targeting the NOS1 coding region. CA1 = CA1 region of hippocampus; MHN = medial habenular nucleus.
Midbrain. NOS1 is detected in many neuronal groups throughout the mesencephalon. Strong staining occurs in the superior colliculus and interpeduncular nucleus, while moderate expression is observed in the dorsal raphe nucleus and substantia nigra. In the rat superior colliculus, the most prominent NOS-containing neurons populate the superficial gray layer. The expression pattern is strikingly similar to the β-gal pattern observed in line 3 (fig. 3.6) and 17 mice (the latter line was not propagated, and so is not described here). The superficial gray layer is also specifically stained in founder 5 and line 19 mice. The superior colliculus is part of the visual system, receiving inputs from the optic nerve by way of the lateral geniculate nucleus, and also from the visual cortex. Glutamatergic neurons of the superficial gray layer are thought to be important in visiosensory and visiomotor function\(^{137}\). Efferent nerves form the spinotectal and spinobulbar tracts, which are probably responsible for the reflex movements of the eyes, head, and neck in response to visual stimuli\(^{127}\). The aforementioned interpeduncular nucleus receives fibers from the habenula. Strong transgene expression occurs in this cell group in lines 18 and 73, but is totally absent in PR(5’3+5’4) transgenic mice. The dorsal raphe nucleus consists mostly of serotonergic neurons that project diffusely throughout the CNS, but in particular to the frontal cortex and striatum. Lines 18, 2, 19, and 87 all share transgene expression in this structure. The substantia nigra, which is concerned with muscle tone, communicates with the cerebral cortex, spinal cord, hypothalamus, and basal ganglia. In Parkinson’s disease, NO\(^-\) mediated excitotoxicity is one candidate implicated in the neurodegeneration of dopaminergic neurons originating in the substantia nigra and terminating in the striatum (nigrostriatal neurons)\(^{15}\). The transgenic lines 88 and 99 exhibit moderately strong β-gal
staining within the substantia nigra pars compacta; some expression is also observed in the substantia nigra pars reticulata of founder 2.

**Hindbrain.** The most intense NADPH diaphorase staining of neurons in the rat brain occurs in the pedunculopontine and laterodorsal tegmental nuclei\textsuperscript{110}, yet these two brainstem loci lack significant β-galactosidase activity in all transgenic lines examined. Several of the transgenic lines, representing both constructs, do display expression throughout the tegmentum, though no common pattern is discernable between lines. NOS-containing cell bodies are detected in the vestibular nuclei of rat and human\textsuperscript{110,113}, and correspondingly, lines 73, 19, and 81 each exhibit transgene expression within the vestibular nuclear complex. The vestibular nuclei receive direct input from the balance organs of the inner ear (utricle, saccule, and semicircular canal) via the vestibular nerve (cranial nerve VIII), in addition to fibers from the cerebellum. Efferent axons target the cerebellum, spinal cord, and cranial nerves III, IV, and VI (which control the muscles of eye movement). These well-studied neuronal pathways function in maintaining balance by influencing muscle tone of the limbs and trunk, and in coordination of head and eye movement, so that visual fixation on an object can be maintained\textsuperscript{127}. Among the lower brainstem centers involved in central autonomic control, NOS neurons occur in the dorsal motor nucleus of the vagus, nucleus ambiguus, nucleus tractus solitarius, and the nucleus of the medulla oblongata\textsuperscript{110,111}. NOS-expressing nerve cells are also present in many reticular nuclei, such as the parabrachial nucleus. All but one of the transgenic lines have significant β-galactosidase activity scattered throughout these lower brainstem regions, though discrete staining in specific loci can only be identified in a few cases. For example, the nucleus of the medulla oblongata is clearly stained in line 73 brains, and
approximately 50% of cell bodies in the nucleus ambiguus are intensely stained in line 99 mice. Additionally, the lateral parabrachial nuclei express the PR(5'1+5'2) transgenes in lines 3 and 99. Within the pons, NOS-positive nerve fibers course throughout the pontine nuclei and transverse fiber network, and staining is prominent in the pontine reticular nuclei, but cells bodies in the pontine nuclei are unstained in the rat. In the human brain, NOS expression in the pontine nuclei peaks at 20-36 gestational weeks, and remains weakly positive in the mature brain\textsuperscript{108}. As previously described, transgene expression in these pontine fibers is characteristic of the PR(5'1+5'2) transgenic mice, and occurs in the majority of PR(5'3+5'4) mice as well. Afferents from the crus cerebri of the midbrain, via the corticopontine tract, innervate the pontine nuclei, which in turn project fibers to the cerebellum. These connections form the main neural pathway linking the cerebral cortex to the cerebellum.

\textit{Cerebellum.} Neuronal NOS cDNA was first isolated from rat cerebellum\textsuperscript{107}, which boasts some of the highest expression levels of NOS in the brain. Consequently, NOS1 expression in the cerebellum has been well characterized. Within the rodent cerebellar cortex, NOS1 mRNA is most strongly expressed in the abundant somata of granule neurons in the granule cell layer. Protein localization, however, whether by NADPH diaphorase histochemistry or by NOS1 immunohistochemistry, is much stronger in the molecular layer, primarily due to NOS1 being transported to the granule neurons’ parallel fibers which extend throughout this layer. NOS1 immunoreactivity is still observed in the granule cell layer, but is weak due in part to the scant cytoplasm of granule cells. Glomeruli within the granule cell layer also demonstrate NOS1 expression. These structures are large synaptic complexes formed by the terminals of excitatory
glutamergic mossy fiber and inhibitory Golgi cell afferents contacting dendrites of granule cells. Both NOS1 mRNA and protein are found in the basket and stellate interneurons of the molecular layer. The purkinje neurons are devoid of NOS1 expression under normal physiologic conditions. The NOS1 expression pattern observed in the human cerebellum, however, differs somewhat from that seen in the rodent. In a study of NOS1 expression during human brain development, NOS1 immunoreactivity was very weak in granule cells and their processes, and present in purkinje cells, from 20 gestational weeks through 11 years old (the oldest in the study)\textsuperscript{108}. Transgene expression patterns vary considerably in the cerebellum from line to line, resembling different aspects of rodent and human NOS1 expression in each case. In line 3, β-gal activity in the cerebellum closely resembles that seen in rodents, as granule cells (in some regions, see below) and basket and stellate neurons are strongly stained, while purkinje cells are negative (fig 3.8B). In the cerebellar cortex of line 18 mice, approximately 70% of molecular layer interneurons express the transgene, as do a majority of the purkinje cells (fig 3.8A). In the granule cell layer of these mice, occasional staining is observed in non-granule cell structures, which are likely glomeruli based on their size and distribution.

Line 73 mice exhibit the most intense cerebellar transgene expression among the different lines. The granule cell layer throughout the entire cerebellar cortex is intensely stained such that individual cell borders are often obliterated. The granule cell processes (parallel fibers) are also lightly stained in the molecular layer. While these processes contain abundant NOS1 protein, presumably the transgenic β-galactosidase protein is not subject to the same translocation machinery as the endogenous NOS1. This distribution of the β-gal activity could result from another transport system or by simple diffusion
based on the abundance of expression in the cell bodies of granule neurons. Neuronal somata in the molecular layer are strongly stained, especially in the deep portion, which may indicate a preference for expression in basket cells over stellate cells. Line 73 mice lack transgene expression in purkinje cells. β-galactosidase expression is virtually absent in the cerebellum of line 99 mice. Only extremely rare cells (i.e. 2-3 per 40 µm section across the entire cerebellum), distributed apparently randomly, display blue staining. While it is possible that these represent a rare, novel cell type, more likely these cells simply express the transgene aberrantly. Two PR(5'3+5'4) lines, 2 and 87, exhibit strong staining in the granule cells, which, like line 3, occurs only in the posterior cerebellar lobules (i.e. lobules 7-9), forming a wedge-shape pattern easily seen in whole mount (e.g. fig. 3.6A). Consistency between lines occurs in the sporadic non-granule cell staining within the granule cell layer in four of the PR(5'3+5'4) lines (all but line 19). Like in the granule layer of line 18, these positively staining structures are likely glomeruli. The transgene expression pattern in line 88 is notably different, in that granule cells are negative, but purkinje cells are strongly stained in the familiar posterior wedge pattern. The purkinje neurons are positive only within the vermis of the cerebellum, again unique among the transgenic lines. Basket and stellate neurons in the molecular layer are positively stained, but in a distribution complimentary to that seen in purkinje cells (i.e. in the cerebellar hemispheres, and not in the vermis). Line 88 is the only PR(5'3+5'4)-containing transgenic line to exhibit β-gal activity in the molecular layer.

With such varied cerebellar expression patterns evident among transgenic mice with both constructs, it is difficult to say how closely the expression patterns recapitulate that seen for endogenous NOS1. The analysis is confounded by the species-specific
differences in cerebellar NOS1 expression, and by differences in cellular compartmentalization of the NOS1 and transgenic β-galactosidase proteins. For example, in rodents NOS1 mRNA is highly expressed in granule cell bodies, but most of the protein is transported to their parallel fibers in the molecular layer. Since the β-gal protein does not share protein-protein interaction domains with NOS1, it is not expected to be transported in a similar fashion, if at all. Therefore, although NOS1 protein is scarce in granule cell somata, abundant β-gal activity in these cell bodies could be predicted, and therefore representative of the rodent NOS1 expression pattern. Human granule cells apparently express NOS1 at much lower levels than do rodents. As several of the transgenic lines lack granule cell staining, it is possible that in these cases cis-acting inhibitory elements of the human promoter override the permissive environment of the mouse transcriptional machinery. Of course, the lack of transgene expression could also simply reflect the normal variation observed between lines observed in many other structures, presumably due to transgene integration-site effects. Also of particular interest is the wedge-shaped expression pattern common to several transgenic lines representing both promoter complexes. It is known that mouse cerebellar NOS1 is expressed regionally in parasagittal bands\textsuperscript{138}, but there are no reports of expression that is limited to posterior lobules in adults of any species. However, in the developing rat brain, NOS1 mRNA expression is first detected in the cerebellum at postnatal day 1 (P1), becomes restricted to the superficial layers of the posterior folia at postnatal day 6 (P6), and attains the normal adult expression pattern throughout the entire cerebellum by P21. Furthermore, NOS1 mRNA expression occurs primarily in the molecular and purkinje layers at P6, before shifting exclusively to the granule cell and molecular layers by P21.
Thus, the possibility exists that in the transgenic mice exhibiting posterior lobule \( \beta \)-gal activity, the human promoters recapitulate a mid-developmental stage of cerebellar NOS1 expression. Perhaps the human promoter fragments lack sufficient regulatory elements to direct expression through the complete developmental program in the mouse.

**Overall expression patterns and promoter-specific transgene expression**

Despite clear line-to-line variation in PR(5’1+5’2)-L7/LacZ transgene expression patterns, some overall trends emerge. The fibers and cells of the pons exhibit transgene expression in all four lines examined, as do various brain stem nuclei. While the specific number of cells and \( \beta \)-galactosidase activity level varies somewhat between lines, the pons is the only discrete CNS structure that is positive across all lines. Several brain loci are positive in three of the four lines. These include the olfactory bulb, frontal association area of the cerebral cortex, hippocampus, lateral septal nucleus, thalamus, and granule cells of the cerebellum. Additionally, many CNS structures demonstrate expression activity in two of the four lines. This list includes: In the telencephalon—the olfactory bulb, anterior olfactory nucleus, olfactory tract, sensory and orbital cerebral cortex, dentate gyrus of the hippocampus, entorhinal limbic cortex, medial septal nucleus, and caudate putamen; in the diencephalon—the hypothalamus and mammillary nuclei; in the mesencephalon—the interpeduncular nuclei and superficial gray layer of the superior colliculus; and in the rhombencephalon—the lateral parabrachial nuclei, tegmental nuclei, and the molecular layer of the cerebellum.

In a similar fashion, trends in expression localization are observed among the lines of mice containing the PR(5’3+5’4)-L7/LacZ transgene. For the sake of this
summary, five “lines” of mice are considered: the four true lines (19, 81, 87, and 88) as well as the single brain analyzed from founder #2 (which was the only one examined microscopically). The cortex, cerebellum, medial habenular nucleus, and spinal cord stand out as brain structures in which the transgene is expressed in all five lines. Like all commonly expressing structures, the intensity of β-gal activity and specific cell counts varies from line to line, but the trend is clear in these loci. Several brain tissues exhibit transgene expression in four out of five lines. These include the olfactory bulb, lateral septal nucleus, anteroventral thalamic nucleus, the non-granule cells within the granule cell layer of the cerebellum, and brain stem nuclei. Furthermore, the following structures are positive in three of the five lines: motor cerebral cortex, entorhinal limbic cortex, caudate putamen, parataenial thalamic nucleus, raphe nuclei, pons, and granule cells of the cerebellum. This data is summarized for both transgene constructs in Table 3.1, which indicates all structures in which expression occurs in at least half of the transgenic lines for either promoter-reporter construct.

There is significant variation in the pattern of reporter gene expression between the different lines of mice created with a single transgene. The noted trends become important in distinguishing the actual contribution that each transgene’s cis-elements confer upon the observed pattern of expression. For example, it is likely that the locus of chromosomal integration of the transgene, which naturally differs for each line, is a major contributor to the variability in expression observed between lines. At the transgene integration site, factors such as chromatin structure and flanking transcriptional modulators (e.g. enhancers, promoters, silencers, etc.) are likely to affect transgene expression in ways not attributable to the transgene structure itself. Of course, the extent
Table 3.1. Comparison of transgene expression between individual lines of both PR(5’1+5’2) and PR(5’3+5’4) transgenic mice. CNS structures that demonstrate transgene expression in ≥ 75% of lines under the control of a promoter complex are indicated in bold.
to which these extrinsic factors affect the transcriptional regulation of the transgene represents an unknown variable that confounds transgene expression analysis. In some cases, transgene expression in a cell type that normally exhibits NOS1 expression may be reduced or silenced entirely by flanking cis-acting elements. In others, ectopic expression may result from transgene integration near an unrelated, strong promoter, for example. The evidence that expression within certain tissues or cell types is dependent upon the transgene’s intrinsic regulatory elements lies within the trends observed across lines of transgenic mice. When the reporter gene expression occurs in a specific tissue in all or nearly all lines of mice harboring a transgene, it is likely that such consistency is transgene-specific. This, coupled with evidence that transgene expression occurs in a tissue or cell type known to express the endogenous NOS1 gene, leads to the parsimonious conclusion that the observed expression pattern is not only dependent on the transgene’s cis-elements, but that it recapitulates NOS1 gene expression in that tissue. In brain loci that express the transgene in only one or a minority of lines, it is more difficult to ascribe transcriptional regulation to either integration variables or intrinsic transgene factors. Chromosomal integration site probably contributes significantly to both the variability of positive tissues between lines, and to the range of expression level within a single brain structure between lines. As will be discussed below, even in the cases where a positive structure is unique to a single transgenic line, PR(5’1+5’2)- and PR(5’3+5’4)-directed β-gal expression occurs exclusively in tissues that have been shown to express endogenous neuronal NOS. The fact that no ectopic transgene expression is observed in the adult CNS supports the notion that both promoter constructs direct transgene expression in NOS1-specific patterns. Extrinsic factors, such as
chromosomal integration site, likely confer a supplemental level of transcriptional control.

It also becomes clear, from examining the summary data in Table 3.1 and represented graphically in Fig. 3.16, that each promoter construct is preferentially expressed in an overlapping, yet significantly different set of CNS tissues. For example, β-gal expression in the lateral septum, thalamus, pons, and brain stem is common to both transgenes. However, only the PR(5’1+5’2) promoter complex drives expression in the anterior olfactory nuclei, frontal association cortex, mammillary nuclei, and interpeduncular nuclei. Although no identified structures are unique to the PR(5’3+5’4) construct, a strong association with this promoter complex is observed within the cerebral motor cortex, medial habenular nucleus, raphe nuclei, and spinal cord. Thus, it appears that the two promoter regions direct transgene expression in a tissue-specific manner. Some structures (e.g. islands of Calleja, bed nucleus of the stria terminalis, and the gracile nucleus) were shown to express NOS1 but neither of the promoter-reporter constructs, which is expected for recombinant molecules comprised of only a fraction of the large and complex NOS1 promoter regions. Under normal physiologic conditions, NOS1 is expressed in a wide array of cell and tissue types within the CNS. The tissue-specificity of each promoter complex that is observed in these transgenic models supports the hypothesis that the differential use of multiple promoters accounts for NOS1 expression in such diverse tissues within the central nervous system.

A summary model of regional permissive vs. nonpermissive NOS1 gene expression in the central nervous system is proposed. In this model, neurons of certain CNS regions or functional systems contain a cellular milieu that is sufficient, or
Figure 3.16. Schematic representation of overlapping subsets of central nervous system neurons in the PR(5’1+5’2) and PR(5’3+5’4) transgenic mice. The outermost rectangle represents the entire CNS. The large oval represents all NOS1 expressing neurons, such that CNS regions shown to express transgenic β-galactosidase are subsets of NOS1 expressing neurons. The subsets of brain structures expressing either transgene are overlapping but distinct. Representative CNS structures that characteristically fall within specific transgene expression groups are indicated in boxes. Note that transgene expression is not observed in tissues not known to express NOS1.
permissive, for NOS1 gene expression at the transcriptional level. Within this permissive environment, the endogenous gene is variably expressed depending on cell state and signaling. Likewise, these cells harbor sufficient transcriptional machinery, or lack certain inhibitors, such that the various transgenes are subject to transcriptional activation. Conversely, there appear to be regions and neuronal systems that do not express NOS1 under any condition, perhaps due to either the lack of crucial activating transcription factors or the constitutive expression of inhibitors of NOS1 transcription. As an example of the latter, neuron-specific gene expression is achieved for NOS1 (and other neural genes) in part by the presence of the cis-acting SNOG element (synaptosomal-associated protein of 25 kD, neuronal nitric oxide synthase, GAP-43)\textsuperscript{139}, which binds to a repressor constitutively expressed in non-neural cells. Thus, the NOS1 gene harbors a cis-element that is always subject to negative regulation, but overcomes this in the absence of the inhibitor. Perhaps a similar repressor exists in a subset of CNS neurons that renders them nonpermissive to NOS1 expression. Expression of the PR(5'1+5'2) and PR(5'3+5'4) transgenes is apparently restricted the same set of permissive neurons as is the endogenous gene. One can speculate that the transgenes contain sequences from the endogenous promoter that are sufficient for binding these negative regulatory factors, though these hypotheses are yet to be tested experimentally.

**Conclusion**

In the present study, two human NOS1 promoter regions were fused to a lacZ reporter gene, and introduced into transgenic mice. Despite considerable variation in transgene expression patterns between lines, clear trends emerge in the data. These
trends indicate that both the PR(5'1+5'2)-L7/LacZ and PR(5'3+5'4)-L7/LacZ transgenes are appropriately expressed in the central nervous system. The two transgenes’ expression patterns partially overlap, but also demonstrate tissue-specific differential promoter use. The transgenes are expressed exclusively in tissues known to express NOS1, each faithfully recapitulating a subset of the total NOS1 expression pattern in the central nervous system.

Data presented in the previous chapter demonstrates that transcripts derived from the 5’1, 5’2, 5’3, and 5’4 promoters are co-expressed in a number of human CNS tissues. The transgenic models both corroborate and expand these data in that the two promoter complexes exhibit expression in several of the same tissues, and also in subsets of CNS cell populations specific to one or the other transgene. Many of these tissue-specific expression patterns, which were not uncovered by RT-PCR experiments, are revealed by the nature of the transgenic model, which allows single cell resolution of transgene expression. Taken together, these data suggest that transgenes under the control of the human 5’1+5’2 and 5’3+5’4 promoter regions adequately represent NOS1 gene regulation, both in terms of appropriate CNS localization and promoter-specific expression. It has been hypothesized that multiple promoters account for developmental, tissue, and state-specific NOS1 expression in the central nervous system, but very little evidence has been presented in the literature to demonstrate this. The transgenic models will be useful for further studies aimed at elucidating the role of alternate NOS1 promoters in response to physiological and pathophysiological environments that dynamically regulate NOS1 expression. For example, transgene regulation in response to nerve injury and drug toxicity are explored in the following chapter. It is expected that
these transgenic mice, along with others currently being established, will be used in many
other experimental paradigms of promoter-specific NOS1 gene regulation. Additionally,
these promoter complexes may be used to drive the expression of virtually any structural
gene in a NOS1-restricted pattern in the CNS, either for NOS1 studies or otherwise.
CHAPTER 4
NOS1 Promoter-Specific Response to Injury

Introduction

NOS1, as previously discussed, is transcriptionally regulated in response to a wide variety of endogenous and exogenous stimuli. In an effort to better understand how differential promoter activation contributes to the regulation of NOS1 gene expression, the PR(5'1+5'2) and PR(5'3+5'4) transgenic mice were subjected to two injury paradigms known to induce NOS1 expression. Both peripheral nerve injury and neurotoxic drug exposure have been shown to activate NOS1 transcription, but no evidence of alternate promoter use in these injuries has been offered to date.

A solid body of evidence exists demonstrating that, following axonal injury, a dramatic induction of NOS1 occurs in many types of central and peripheral neurons that normally lack the enzyme (refs in\(^1\)). The potential role of NOS1 in neurodegeneration, neuroprotection, synaptic remodeling, and target muscle reinnervation in these injuries is controversial. On the one hand, NO is thought to play a role in motor neuron differentiation, as evidenced in the ventral horn of the spinal cord\(^1\), and also in neuromuscular synaptogenesis\(^1\). On the other hand, NO may be involved in the death of neuronal cell bodies disconnected from their distal targets\(^1\). To further fuel the controversy, it appears that the effects of NO vary between neuroprotective and
neurotoxic depending on the maturity of the involved neurons and the specific type of axonal injury. For example, NADPH-d was induced after sciatic axotomy in neonatal spinal motor neurons, many of which died after the injury, but not in adult spinal motor neurons, in which degenerative changes were not prominent after sciatic nerve transection\textsuperscript{143}. In contrast, NADPH-d activity is induced in both immature and mature spinal motor neurons by root avulsion, an experimental model that results in severe damage of the parent cell bodies in adulthood as well\textsuperscript{144}. The induction of NADPH-d and NOS has been well demonstrated in the motor neurons of several brainstem nuclei (hypoglossal, vagus, and facial) after various types of nerve injury to their associated cranial nerves\textsuperscript{140,145-147}, but the fate of these motor neurons is highly variable.

Injury of the facial nerve in rats has emerged as a model for studying the role of NOS1 in response to axonal injury. The facial motor neurons constitute a relatively homogenous cell population, and their reactions to axonal injury and regeneration have been extensively studied\textsuperscript{148,149 150}. Facial nerve compression causes increased expression of NADPH-d in the facial motor nucleus, and the time course of expression corresponds well with the recovery of facial muscle function\textsuperscript{146}. However, L-NAME administration following facial nerve compression did not change recovery time. Additionally, there is no obvious facial motor neuron death following axonal compression. In contrast, avulsion traumatically injures FMN cell bodies\textsuperscript{150}, and transection induces cell death of immature, but not mature facial motor neurons\textsuperscript{151}.

In order to gain an understanding of the regulation of the NOS1 promoters in response to nerve injury, we considered the transection of the facial nerve in adult mice as an appropriate model. As was previously shown in rats, a transection injury followed
by a survival time of one week lead to a maximal induction of NADPH-d activity in the ipsilateral facial motor nucleus\textsuperscript{151}. NOS1 mRNA induction, as demonstrated by \textit{in situ} hybridization, is also observed with a similar time course in the same injury model\textsuperscript{152}. We sought to determine which of the alternate promoters is responsible for NOS1 induction in this model, and therefore applied this transection model to our transgenic mice. Concurrently, we would determine whether or not NOS1 expression was similarly induced in the mouse facial motor nucleus as it is in the rat model, studying both the transgene and the endogenous gene. In these studies, the PR(5’1+5’2) and PR(5’3+5’4) transgenic mice proved insufficient to determine differential promoter activation in response to nerve injury. However, preliminary data do suggest that the endogenous mouse NOS1 gene is transcriptionally activated in the facial motor nucleus following facial nerve transection.

Methamphetamine (METH) is a psychostimulant drug of abuse, which has been shown to be neurotoxic to specific central neuronal populations. As a class of drugs, amphetamines are indirect sympathomimetics, exhibiting their effect by triggering the massive release of newly synthesized dopamine (DA) from presynaptic vesicles, with an increase in extracellular dopamine concentration in the nigrostriatal system. High doses of amphetamines cause long-lasting neurotoxicity associated with a marked decrease in dopamine level in the striatum\textsuperscript{153-155}. In mice, for example, systemic METH administration causes significant depletion of dopamine (DA), serotonin (5-HT), and their metabolites\textsuperscript{156}, primarily affecting striatal dopaminergic systems\textsuperscript{157}. In fact, a similar depletion in striatal dopamine nerve terminal markers was demonstrated in humans, in the post-mortem brains of chronic METH users\textsuperscript{158}. 

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Evidence has accumulated that glutamate and reactive oxygen species (ROS) are involved in METH toxicity in the CNS, and studies demonstrating a role for NOS1 followed. For example, 7-NI, a NOS1-specific inhibitor, significantly attenuates the toxic effects of METH both \textit{in vitro}\textsuperscript{159} and \textit{in vivo}\textsuperscript{160,161}. In addition, NOS1 -/- mice are protected against dopaminergic neurotoxicity\textsuperscript{162}. Direct evidence that methamphetamine administration causes induction of NOS1 in the striatum was shown by NADPH-d and NOS1 immunohistochemistry\textsuperscript{163}. To date, it is not known which of the NOS1 transcripts is (are) induced, or how alternate promoter activation contributes to NOS1 gene expression in this model of drug induced neurotoxicity. In the current studies, we demonstrate that the two NOS1 promoter complexes may each be activated in response to methamphetamine neurotoxicity, but in different ways.

\textbf{Materials and Methods}

\textit{Facial nerve axotomy.} Wild type and transgenic mice were deeply anesthetized with Avertin as previously described (Chapter 3). The adequacy of anesthesia was determined by testing for lack of both the corneal reflex and the pain withdrawal reflex (foot pad pinch). White petrolatum was applied to the eyes to prevent corneal drying. The mice were shaved behind the right ear, and prepped with betadyne solution in the usual sterile fashion. A 4-5 mm incision was made behind the ear, and blunt dissection was used to expose the facial nerve down to its exit from the stylomastoid foramen. The facial nerve was transected sharply as close as possible to the foramen. The incision was closed with staples and cleaned with betadyne. 5 cc of warmed normal saline was administered subcutaneously, and the animals were allowed to recover in a 37°C
incubator. Successful surgery was evaluated by full recovery and lack of whisker movement on the injured side. Sham operations were performed in identical fashion, except that the facial nerve was not transected. The animals were sacrificed after seven days, were perfusion fixed, and striatal tissue processed for β-galactosidase histochemistry as previously described (Chapter 3).

*In situ* hybridization: Fresh frozen 12-15 µm brain tissue sections were collected on a cryostat, and thaw mounted onto slides (Fisher SuperFrost Plus) for one minute on a slide warmer at 37°C. Slides were stored at -80°C with desiccant until use. 5 µM pooled NOS1-specific oligonucleotide probes (Appendix) were labeled with an α<sup>33</sup>P-poly(A) tail to high specific activity (> 2 X 10<sup>8</sup> cpm/µg) with terminal transferase (rTdT, GibcoBRL) according to the manufacturer’s protocol. Free nucleotides were removed with a NucTrap (Stratagene) column. The slides were thawed at room temperature in a sealed container for 15 minutes, fixed in 4% paraformaldehyde for 10 minutes at room temperature, and rinsed twice in 1X PBS. They were then treated in 0.25% acetic anhydride freshly brought up in 0.1 M triethanolamine (TEA) (pH 8.0) for 10 minutes at room temperature, and then dehydrated by standard ethanol series and allowed to air dry. Labeled probe, diluted in hybridization solution (Amresco, 50% formamide) at 1.5 X 10<sup>6</sup> cpm/100 µl/slide, was added to each slide, coverslipped with paraformaldehyde, and incubated overnight in a humid chamber at room temperature. All pre-hybridization and hybridization solutions were RNase-free. Post-hybridization washes were as follows (15 minutes each): twice in 2X SSC @ 37°C; twice in 2X SSC @ 45°C; twice in 1X SSC @
37°C; four times in 1X SSC @ 50°C. Slides were again dehydrated in an ethanol series, allowed to air dry, and apposed to single emulsion X-ray film (Kodak) for 48-72 hours at -80°C. Following macroautoradiography, slides were dipped in undiluted NTB-2 emulsion (Kodak) in the dark, dried vertically for one hour, and then stored at 4°C for 10-15 days with desiccant in a sealed box, at which time they were developed with Dektol (Kodak) developer and fixer according to the manufacturer’s protocol.

*Methamphetamine administration:* Age and sex matched adult mice were administered either 10 mg/kg methamphetamine (Sigma) or saline intraperitoneally. Mice were injected four times at two hour intervals, and then sacrificed between one hour and 7 days following the final drug or saline dose. The animals were perfusion fixed, and striatal tissue processed for β-galactosidase histochemistry as previously described (Chapter 3).

**Results**

**Transgenes not detected in facial motor nucleus after nerve injury**

Unilateral facial nerve axotomies were performed on wild type, PR(5'1+5'2), and PR(5'3+5'4) transgenic mice. Mice ranged in age from five to 13 weeks at the time of axotomy, and were sacrificed at either one or two weeks after surgery. These ages and survival times were chosen to correspond with the maximal level of NOS induction in the transected facial motor neurons as previously demonstrated\(^\text{147}\). When assayed for β-galactosidase activity, no transgene expression was evident in any of the transgenic lines (lines 3, 18, 73, 99, 19, 81, 87, 88) at any time point studied. NADPH-diaphorase...
staining of brain stem sections of PR(5'1+5'2) transgenic (fig. 4.1a) and wild type mice (not shown) reveal a robust induction of NADPH-diaphorase activity in the motor neurons within the FMN ipsilateral, but not contralateral, to the injury. This diaphorase data validates the nerve injury model and technique. Additionally, diaphorase activity is seen bilaterally in vascular structures, likely as a result of endothelial NOS activity in local vessels. This vascular staining is present throughout the brainstem, and is seen in non-injured mice as well.

NOS1 mRNA is also induced following axotomy, as demonstrated by *in situ* hybridization with pooled oligonucleotide probes (fig 4.1b) that hybridize to the NOS1 coding region. Note that the signal intensity was initially high enough to be seen by the naked eye on macroautoradiography, but is greatly diminished in this image as an artifact of the counterstaining procedure. This suggests that the NADPH-diaphorase activity observed after facial nerve transection does in fact represent NOS1 expression, rather than a nonspecific diaphorase activity. Further attempts were made to detect alternate NOS1 transcripts in the FMN following nerve injury. *In situ* hybridization using oligonucleotide probes designed to hybridize to each of the alternate first exons was attempted. However, inadequate specificity of the hybridization signal routinely confounded the interpretation of the results, which were ultimately inconclusive.

Ongoing experiments aim to enhance the signal intensity and specificity in the detection of alternate NOS1 transcripts in this injury model.
Figure 4.1. Facial motor nuclei following facial nerve transection. A) NADPH-diaphorase staining in the FMN ipsilateral to the axotomy in an adult wild type mouse. B) *in situ* hybridization with pooled NOS1 coding region probes, demonstrating NOS1 mRNA induction in the FMN ipsilateral to the injury (indicated by arrows).
Methamphetamine alters transgene expression in striatum

PR(5'1+5'2) and PR(5'3+5'4) transgenic mice treated with intraperitoneal methamphetamine injections were examined for striatal β-galactosidase activity. Initial experiments were carried out (line 99, PR(5'1+5'2)) to determine the time course for lac-Z expression in this model. Mice were sacrificed one, four, or 24 hours after the final methamphetamine (or saline) injection, and the number of blue cells per high power field were manually counted. These data are shown in figure 4.2. In this line of transgenic mice, there is no significant difference in β-galactosidase expression between saline and methamphetamine treated groups. The data are the most variable at the one-hour time point, and showed little difference between 24-hour and 7-day survival. 24-hour survival was deemed adequate to detect transgene expression, and because this survival time correlates well with known NOS1 induction following methamphetamine administration, this time point was used in subsequent experiments.

The quantity and relative intensity of lac-Z positive cells in striatal tissue sections of PR(5'1+5'2) and PR(5'3+5'4) transgenic mice, treated with either saline or methamphetamine (10 mg/kg x 4), are summarized in figure 4.3. The number of β-galactosidase positive cells in each striatal slice was standardized (to 0.1 µm² tissue area) to account for variations in tissue area measured by the image analysis software. In addition to the number of positive neurons, the relative intensity of gene expression was measured by calculating the integrated optical density of the blue X-gal precipitant in each striatal tissue section (as in). There is no difference in either the number of striatal neurons or the expression level of the reporter in line 3 mice, which carry the PR(5'1+5'2) transgene. Line 18, which also has the PR(5'1+5'2) transgene, shows no
Figure 4.2: Time course of β-galactosidase expression in striatum of Line 99 mice following systemic methamphetamine administration. Time points are 1 hour, 24 hours, and 7 days survival after the last injection. n = 4 for each treatment group. Results are presented as mean ± std. dev.
Figure 4.3: β-galactosidase expression in striatum of transgenic mice after systemic methamphetamine administration. A) Mean number of blue cells (lac-Z positive) per standard tissue area. B) Mean signal intensity, as determined by integrated optical density (I.O.D.). Data are presented as mean ± S.E.M.. * p < 0.01; ** p < 0.001; Two-tailed Student’s t test was used to determine significance of difference between control and METH for each transgenic line.
difference in the number of lac-Z positive neurons, but their relative intensity does 
increase significantly by approximately 15% (p < .01) after the administration of 
 systemic methamphetamine at toxic levels. In contrast, line 88, which harbors the 
 PR(5’3+5’4) transgene, shows a significant 262% increase (p < .001) in the number of 
 positive striatal neurons following methamphetamine administration, while the relative 
 intensity remains unchanged. Line 99 were also analyzed, and failed to show any 
significant difference in either number or intensity of striatal β-galactosidase positive 
neurons. These findings suggest that the PR(5’1+5’2) and PR(5’3+5’4) transgenes, at least 
in some lines, are differently regulated in response to systemic methamphetamine.

**Discussion**

The purpose of these experiments was to examine the activation of the 
 PR(5’1+5’2) and PR(5’3+5’4) promoter-reporter transgenes in response to two types of 
neuronal injury. In the facial nerve transection model of peripheral nerve injury, the 
transgenes fail to express in the facial motor nuclei of injured mice. However, the 
endogenous mouse NOS1 mRNA is activated in this model. In the model of striatal 
toxicity via methamphetamine administration, the transgenic mice display promoter-
specific differential transgene activation.

It is unclear why neither transgene is detected in the FMN following facial nerve 
transection. The *in situ* hybridization and NADPH-d data presented here demonstrate 
that the endogenous mouse NOS1 gene and protein are upregulated in response to facial 
nerve transection. Because the human 5’1-, 5’2-, and 5’3-containing NOS1 mRNAs are 
thought to be responsible for the majority of NOS1 expression in the central nervous
system (chapter 2 and Wang, et al.\textsuperscript{42}), it was anticipated that transgenes containing these promoters would also be transcriptionally activated in this model. One explanation is that the gene fragments used to construct the transgenes contain less than the full promoter that would be necessary for accurate recapitulation of NOS1 gene expression. Although the PR(5'1+5'2) and PR(5'3+5'4) transgenes contain enough information for basal expression in a fairly NOS1-specific pattern (chapter 3), they may lack crucial \textit{cis}-acting elements required for induction by inter- or intracellular signals. If these sequences exist in the native gene, they may reside either further upstream or downstream (such as in the > 20 kb intronic DNA separating the alternate first exons from the common exon 2) from the promoter fragments used in the current constructs. Another possibility is that, despite general homology between the rodent and human NOS1 genes, the human promoters fail to bind and/or respond to mouse-specific transactivating factors. Despite a high degree of sequence homology, the human and mouse 5’1 and 5’2 untranslated regions function differently, at least in terms of specific splicing events. For example, the sequence surrounding the exon 5’2 downstream splice site is conserved between human and mouse (Fig. 4.4), yet splicing at this locus is only observed in the human gene (see chapter 2), such that a small human-specific intron exists. In this case, the resultant human and rodent 5’1-containing NOS1 mRNAs must be different immediately downstream of exon 5’2, and this structural difference could account, in part, for species-specific differences in transcriptional or translational activation.

These explanations for a lack of transgene expression in the FMN of mice presume that one or more of the promoters contained in the transgenes do in fact account for NOS1 activation in facial nerve injury. It is feasible that another of the known (or
unknown) NOS1 alternate promoters responds to this type of injury. For example, a recent study indicates that transcription initiating within exon 2 may play a significant role in Ca$^{2+}$ dependent NOS1 gene induction in the murine CNS$^{43}$. In this study, the exon 2 promoter sequence was robustly activated in response to neuron depolarization, while the other CNS-cluster alternate first exons were either moderately induced (exon 1b, the 5’1 homologue) or not induced. Because of the location of the exon 2 promoter sequence, which falls immediately upstream of the major transcriptional start site, the alternate transcript initiating there would not have been detected in the 5’RACE cloning strategy described in chapter 2 of this work. Similarly, the PR(5'1+5'2) and PR(5'3+5'4) constructs lack exon 2 sequence, which may in fact be the major CNS inducible human NOS1 promoter. To this end, Deyu Zhang and Tony Young in our laboratory have identified and cloned the human NOS1 exon 2 promoter, and the creation of transgenic mice harboring an exon 2 promoter-L7/lacZ reporter transgene is currently in progress.
The existence of the exon 2 promoter provides an attractive and plausible mechanism for NOS1 transcriptional activation in response to nerve injury. The Ca\textsuperscript{2+}-responsive promoter harbors two Ca\textsuperscript{2+}/cAMP response elements (CRE), which bind to CREB and ATF-2 in vitro\textsuperscript{43}. Many processes that are thought to be regulated by NOS1, including neuronal injury and plasticity\textsuperscript{76}, are Ca\textsuperscript{2+}-dependent. Because NOS1 catalytic activity is also Ca\textsuperscript{2+}-dependent, it follows that Ca\textsuperscript{2+} influx into neurons could activate both NOS1 transcription and catalytic activity in a synergistic manner to maximize NO\textsuperscript{·} production. Transgenic mice harboring the PR(E2)-L7/lacZ transgene will be instrumental in determining the role of differential regulation of NOS1 expression via alternate transcripts in a variety of existing NOS1 induction models, including facial nerve transection and plasticity.

The role of the PR(5'1+5'2) and PR(5'3+5'4) transgenes in NOS1 activation in response to methamphetamine is also difficult to determine because of mixed findings in the current set of experiments. Of the three PR(5'1+5'2) transgenic lines, two failed to show reproducible lac-Z inductions. Line 18 mice demonstrate a small but significant up-regulation in transgene expression, as determined by relative signal intensity. In contrast, the PR(5'3+5'4) line 88 mice respond by dramatically increasing the number, but not the intensity, or lac-Z neurons. While many studies\textsuperscript{165-168,162,169} implicate the role of NO\textsuperscript{·} in amphetamine associated dopaminergic system toxicity, only one study to date directly measures neuronal NOS protein expression in methamphetamine-treated animals\textsuperscript{163}. In that study, NOS1 immunohistochemistry demonstrated an increase in the number of NOS1 positive striatal neurons, but not an increased staining intensity. In fact,
the two-fold recruitment of neurons reported in that study correlates well with the 2.5-fold recruitment observed in the line 88 transgenic mice.

The question that arises is how might methamphetamine treatment cause an increased number of striatal neurons to activate NOS1 transcription? Again, the answer is unclear because it is not known whether NO• plays a causative or a reactive role in this paradigm of neuronal toxicity. For example, NO• has been shown to cause a Ca\textsuperscript{2+}-dependent dopamine (DA) release from rat striatal slices\textsuperscript{165}, while another study contends that NO• suppresses DA release\textsuperscript{166}. What is clear is that amphetamine and its analogs (methamphetamine, MPTP, MDMA) cause a massive release of DA from presynaptic vesicles, with a resultant short term extracellular spike and a long term depletion of DA in the nigrostriatal system. Furthermore, it appears that NOS1/NO• inhibition protects striatal neurons from amphetamine toxicity\textsuperscript{162,167-169}. A number of studies have also shown that glutamate release increases striatal DA release during methamphetamine exposure\textsuperscript{170,171}. It seems that methamphetamine can cause extracellular glutamate release, causing NMDA receptor-mediated, Ca\textsuperscript{2+}-dependent NOS1 activation. The enhanced NO• levels could then damage dopaminergic neurons, resulting in dopamine depletion.

This model would in fact predict the recruitment of new striatal neurons to activate NOS1 transcription, as is demonstrated in line 88 mice, based on widespread extracellular glutamate release and activation of NMDA receptors in the striatum. The time course of NOS1 induction in different injury models is also of interest. Unlike the delayed time course of NOS1 activation seen in the facial nerve injury model, following induced cerebral ischemia NOS1 mRNA and immunoreactivity are acutely elevated at
one and four hours, respectively, decline within 48 hours, and return to baseline within seven days\textsuperscript{172}. Cerebral ischemia, like methamphetamine toxicity, elicits its toxic effects via glutamate-mediated excitotoxic mechanisms, including the activation of neuronal NOS. Correspondingly, the NOS1 immunoreactivity in methamphetamine-exposed mice is highest at one and 24 hours, and declines by one week\textsuperscript{163}, suggesting a similar activation mechanism in these two models of neuronal damage. Both the PR(5'1+5'2) and PR(5'3+5'4) promoter regions contain cis-elements that could potentially mediate this type of NOS1 induction. For example, the PR(5'1+5'2) complex has sites for the protein kinase C (PKC)-activated transcription factors NF-κB, AP2, and AP1, in addition to the protein kinase A (PKA)-activated transcription factor CREB. Likewise, the 5'3 promoter region harbors two AP2 sites, which may respond to PKC activation, as well as SP-1, MAZ, and Oct-2.1 sites (as discussed in chapter 2). In our laboratory Dr. Terrie Rife demonstrated that the 4.3 kb PR(5'1+5'2) promoter, in response to PKC activation via phorbol myristic acid (PMA), drives the expression of a reporter gene in PC-12 cells induced to differentiate with nerve growth factor, in a time course very similar to that described for ischemic and methamphetamine-induced striatal NOS1 expression. It is plausible that PKC-mediated activation of either the PR(5'1+5'2) or PR(5'3+5'4) complexes accounts for the observed NOS1 induction following methamphetamine exposure. Experiments are ongoing in our laboratory to elucidate the specific transcription factors that bind to the promoters and mediate their activation. It will also be interesting to test the PR(E2) transgenic mice in the methamphetamine injury model, considering the demonstrated activation of that promoter via CREB/CRE binding.
If the modest induction of the PR(5'1+5'2) transgene in line 18 accurately represents the endogenous gene activation subsequent to methamphetamine administration, it is not clear that this would be physiologically relevant. Furthermore, because the other two PR(5'1+5'2) lines tested failed to show lac-Z induction, one must question the significance of the line 18 results. However, as discussed previously, variable transgene expression between lines harboring the same transgene is to be expected, and in fact it has been demonstrated (chapter 3). First, it is feasible that a small rise in transcriptional activity provides templates for a further rise in translational activation, culminating in a significant elevation in NOS1 protein. Since NOS1 is a catalytic enzyme, even small increases in total protein could result in large increases in catalytic activity, producing large amounts of NO. As discussed previously, Ca$^{2+}$-mediated NOS1 activation may work simultaneously and synergistically at both transcriptional and translational levels. As such, even a 15% increase in NOS1 mRNA would contribute significantly to induction of NOS1 protein levels. It will be informative to determine whether the PR(5'1+5'2) complex is Ca$^{2+}$-responsive, and to compare this response to that of the PR(E2) transgene in similar assays.

This set of experiments, in which the PR(5'1+5'2)- and PR(5'3+5'4)-L7/lacZ transgenes were tested for differential regulation in response to two neuronal injury mechanisms, yielded mixed results. While the data do not clearly delineate the role of the various NOS1 promoters in response to injury or toxicity, they do support the validity of this approach for further studies. The transgenes are expressed in appropriate, NOS1-specific tissues; the assay for transgene expression is easily performed; transgene expression is consistent between tissue samples; and the results are quantifiable by
computer image analysis. Whether in studies of neuronal damage, plasticity, or development, these and future lines of NOS1 promoter-containing transgenes should prove invaluable in elucidating the molecular mechanisms responsible for the differential regulation of alternate promoters in the human neuronal nitric oxide synthase gene.


24. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for


APPENDIX

Oligonucleotide/Primer Sequences
(alternate names indicated in parentheses)

RT-PCR

5'1 Fwd:  CGG AGG ATT CAG AAC CCG GAG
5'2 Fwd:  GGT GAG GAG CTA CTT AGC G
5'3 Fwd (5'3b):  ATT AGT GCC GCT GGC CTC TC
5'4 Fwd (5'4b):  CCA CCT CTA AAT GAA AGA AAG
Ex2 Rev1: (NOS-A):  CGG ATC AGA TCT GAG GCA TC
Ex2 Rev2:  AAC TCG AGT CAG CGT CAC CCA CTC ATG G
Ex2a Rev:  ACT ATG GCT AGG TAG TTT GG
Ex3 Fwd (NOS-C):  CAA GTC ACA CAA ACC TCT G
Ex4 Rev:  TCT CCC AGT TCT TGA CC
Ex6 Rev (NOS-D):  GTT CAC CTC TTC CAG CCT TTC C
Intron 5'4/5'3 (Int 5’4/3):  CCT GGT GTC AAT TAA ACC C
GAPDH Fwd:  CCG GAT CCT GGG AAG CTT GTC ATC AAC GG
GAPDH Rev:  GGC TCG AGG CAG TGA TGG CAT GGA CTG

5'RACE

GSP-RT:  AAC TCG AGC TGT CTG AAG ACC TCA CAA TGC
GSP-1:  AAC TCG AGT CAG CGT CAC CCA CTC ATG G
GSP-2:  AAC TCG AGT CGG TGG CAT GAT TTC CT
5'RACE Q1:  CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT T
5'RACE Q0:  CCA GTG AGC AGA GTG ACG
5'RACE Q1:  GAG GAC TCG AGC TCA AGC

Primer extension

PE 5'3 Rev:  CAT CTC CGC AAG GCC TCA CCC TGG GCA TGG TGG
PE 5'4 Rev:  CTG GGG ACA GCC AGG ATC GAG TGC ACT GTG TCT GC
In situ hybridization

mNOS ISH A: TGA GCT ACT CCG GGC AAG AAC CGG CTG CAG AGT TTC TGT CGC CTC
mNOS ISH B: GGC CTT GGG CAT GCT GAG GTC CGT TAC TGG GAC CTG GGA CCC TGT
mNOS ISH E1a: TTC TTA ATG TCC CTG CTT TAT CCT ATC ACC GCT GAA ACG AAT TAG
5’2 Fwd T7: TTG GAT CCT AAT ACG ACT CAC TAT AGG GAG ATA GGA TAA AGG AGG GAC