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SECOND MESSENGER SYSTEM MODULATION OF AROMATIC L-AMINO ACID DECARBOXYLASE AND TYROSINE HYDROXYLASE IN NORMAL AND MPTP LESIONED MICE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

By

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* * * * *

The Ohio State University

1995

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# VITA

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<td>July 31, 1966</td>
<td>Born- Barberton, OH</td>
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## PUBLICATIONS


Hadjiconstantinou M., Young E.A. and Neff N.H. Nigrostriatal aromatic L-amino acid decarboxylase (AAAD) activity is increased transiently after treatment with a phorbol ester. **Amer. College Neuropharm. Abstr.** (1993) pp 219

Young E.A., Neff N.H. and Hadjiconstantinou M. Second messenger mediated effects on L-aromatic amino acid decarboxylase and tyrosine hydroxylase in normal and MPTP-lesioned mouse striatum. **Amer. Soc. Neurochem.** (1994) 26:


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<tr>
<td>AAAD</td>
<td>aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>8-Br-cGMP</td>
<td>8-bromo-cyclic-3',5'-guanosine monophosphate</td>
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<td>catechol-O-methyl-transferase</td>
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<td>CRE</td>
<td>cAMP-response element</td>
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<tr>
<td>CREB</td>
<td>cAMP-response element binding protein</td>
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<td>DA</td>
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<td>dihydroxyphenylacetic acid</td>
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<td>epinephrine</td>
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<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<td>H₂O</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>homovanillic acid</td>
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<td>icv</td>
<td>intracerebroventricular</td>
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<td>ip</td>
<td>intraperitoneal</td>
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<td>IP₃</td>
<td>inositol triphosphate</td>
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<td>internal standard</td>
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<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxy-L-phenylalanine</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-pyridinium</td>
</tr>
<tr>
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<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MTHP</td>
<td>DL-6-methyl-5,6,7,8-tetrahydropyridine</td>
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<tr>
<td>NE</td>
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<td>ODS</td>
<td>octadecylsilane</td>
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<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
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<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
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<td>phenylethanolamine N-methyl transferase</td>
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<td>prot</td>
<td>protein</td>
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<tr>
<td>SEM</td>
<td>standard error of the means</td>
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<td>TCA</td>
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<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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INTRODUCTION

A. Dopamine and its Role in Brain Function

The principal dopaminergic projection systems are the nigrostriatal, mesolimbic, mesocortical, tuberohypophysial and incertohypothalamic (Figure 1). There are also dopaminergic interneurons in the retina and in the olfactory bulb. Mesocortical and mesolimbic projections connect the ventral tegmental area with medial prefrontal, cingulate, piriform and entorhinal cortices, nucleus accumbens, amygdala, and regions of the septum and olfactory tubercle. These connections may be important for emotional and motivated behaviors and appear to be involved in certain forms of drug addiction and mental disorders including depression, schizophrenia-like states and obsessive-compulsive disorder (Bertolucci-D'Angio et al., 1990, and Fibiger and Phillips, 1988). The tuberohypophysial projections connect the arcuate and periventricular nuclei to the intermediate lobe of the pituitary and the median eminence. These neurons play an important role in regulating the release of pituitary hormones, especially prolactin (Weiner and Molinoff, 1989). The incertohypothalamic system links the dorsal posterior
Figure 1. Schematic diagram illustrating the distribution of the main central neuronal pathways containing dopamine. The stippled regions indicate the major nerve terminal areas and their cell groups of origin. The cell groups in this figure are named according to the nomenclature of Dahlström and Fuxe (1965). Figure from Chapter 10, Biochemical Basis of Neuropharmacology, Cooper, Bloom and Roth, eds. (1991).
hypothalamus with the anterior hypothalamus and the lateral septal nuclei. No definitive role has been attributed to this pathway, though it may be involved in integrative control of the autonomic nervous system (Albanese et al., 1986).

The major dopaminergic tract, the nigrostriatal projection, runs in the medial forebrain bundle from dopaminergic cell bodies in the substantia nigra pars compacta to the striatum (caudate/putamen). On the basis of pharmacological observations its existence was postulated around the turn of the century, however, it was not until the late 1960's with improved morphological techniques that there was solid anatomical demonstration of this pathway (Hattori, 1993). In the striatum, these somatotypically organized projections synapse on cholinergic interneurons and on gamma-butyric acid (GABA) projection neurons.

The function of the nigrostriatal projections seems to be to regulate the activity of circuits linking the cerebral cortex, basal ganglia and thalamus which are involved in motor control (Figure 2). These circuits control not only strictly sensorimotor aspects of movement programming, but also conditional aspects of planning movements, program selection and motor memory and retrieval (Graybiel, 1990).

From the striatum, there are two major output pathways which are differently influenced by the dopaminergic inputs (Côté and Crutcher, 1991). First the "direct" pathway, is the striatal projection to the internal segment
Figure 2. Major anatomical connections of the basal ganglia. A. The caudate nucleus and putamen receive almost all afferent input to the basal ganglia. B. The internuclear connections include topographically organized connections between all of the nuclei of the basal ganglia. C. The principal target of efferent connections from the basal ganglia is the thalamus. From Kandel, Schwartz and Jessel, Principles of Neural Science, 3rd edition (1991).
of the globus pallidus and the pars reticulata of the substantia nigra (the output nuclei of the basal ganglia), which then project to the thalamus. The "indirect" pathway is the circuit from the striatum to the external segment of the globus pallidus, which projects to the subthalamic nucleus. The subthalamic nucleus in turn projects back to both pallidal segments and the substantia nigra (Kandell et al., 1991). Dopamine (DA) has two effects: one stimulatory on the striatal GABAergic neurons of the "direct" pathway and the other inhibitory on the "indirect" pathway (Carlson, 1993). Both effects should thus be stimulatory on motor activity. Loss of dopaminergic activity in the striatum effectively results in inhibition of the "direct" pathway and increased inhibition by the "indirect" pathway (Young and Penney, 1989), with the net result of loss of mobility. This is precisely what is seen in Parkinson's disease (PD), where there is degeneration of the dopaminergic neurons of the substantia nigra. A discussion of PD will appear later.

B. Dopamine Synthesis and Metabolism

Catecholamines are a group of structurally related neurotransmitters including DA, norepinephrine (NE), and epinephrine (Epi), synthesized from the amino acid, L-tyrosine (Figure 3). Dietary tyrosine crosses the blood brain barrier via a non-selective amino acid transport. The type of catecholamine
Figure 3. Catecholamine biosynthetic pathway. Tyrosine hydroxylase is the first and presumably the rate-limiting enzyme in the pathway. From Joh, Hwang, and Abate. in Neurtransmitter Enzymes: Neuromethods Vol 5, 1986.
made and released by a particular neuron depends on the synthetic enzymes it contains.

The first two enzymes, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AAAD) are present in all catecholaminergic neurons. Dopamine-β-hydroxylase (DBH) is present in noradrenergic neurons, while phenylethanolamine N-methyl-transferase (PNMT) is present only in neurons synthesizing Epi. These enzymes are also found in the adrenal medullary chromaffin cells where Epi and NE are synthesized and then released into the circulation. All four enzymes have been purified, allowing for study of their substrate specificity, cofactors and kinetics. They have also recently been cloned and sequenced allowing more detailed examination of their regulation (Nagatsu, 1991).

The first enzyme in the cascade is TH (E.C. 1.14.16.2), which is found both in the cytosol and as a membrane-associated protein. It is a homotetramer, with each subunit having a molecular weight of approximately 60,000 kDa (Joh et al., 1986). The TH gene has been cloned and sequenced from several sources including rat (Grima et al., 1985) and human (Kaneda et al., 1987) pheochromocytoma cells, and bovine adrenal gland (D'Mello et al., 1988). The enzyme is a mixed function oxidase which converts L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and requires molecular oxygen, iron and tetrahydrobiopterin for this conversion (Nagatsu et al., 1964). Phenylalanine can also serve as a source of L-DOPA through
conversion to tyrosine and then to L-DOPA (Ishimitu et al., 1980). The conversion of tyrosine to L-DOPA by TH is thought to be the rate-limiting step for catecholamine synthesis (Levitt et al., 1965), and is subject to a variety of regulatory controls which will be discussed later. The affinity of TH for cofactor varies depending on the state of activation of the enzyme, but generally appears to be in the range of $10^{-4}$ to $10^{-5}$ M for the natural cofactor, (6R)-L-erythro-tetrahydrobiopterin (Oka et al, 1981).

The second step in the synthetic pathway is AAAD (E.C. 4.1.1.28). AAAD is found in catecholaminergic and serotonergic neurons, the adrenals and the pineal gland. It is also found in liver, kidney and various tissues where no neurotransmitters are produced (Rahman et al., 1981). This enzyme is primarily cytosolic and is thought to be a homodimer, with each subunit having a molecular weight of about 50,000 kDa (Ichinose et al., 1985). The cDNA of AAAD has been cloned and sequenced from several sources including bovine adrenal glands (Gudehithlu et al., 1992), guinea-pig kidney (Taketoshi et al., 1990), and human pheochromocytoma cells (Ichinose et al., 1989).

AAAD converts the L-DOPA to DA using pyridoxal phosphate as a cofactor (Christenson et al., 1972). This enzyme is fairly non-selective in that it also decarboxylates other aromatic L-amino acids including tyrosine, 5-hydroxytryptophan, phenylalanine, and tryptophan (Lancaster and Sourkes, 1972). Although AAAD is not considered to be the rate-limiting enzyme for
the synthesis of DA, it is rate-limiting for the synthesis of the trace amines (Dyck et al., 1983), and becomes the rate-limiting enzyme for DA synthesis in Parkinson's disease patients receiving L-DOPA therapy. The $K_m$ for pyridoxal phosphate is about 300 nM and the $K_m$ for L-DOPA is about 100 $\mu$M (Park et al., 1992). A more thorough discussion of AAAD follows later.

The two enzymes for the metabolism of catecholamines are catechol-O-methyl transferase (COMT), which transfers a methyl group from $S$-adenosylmethionine to the $m$-hydroxyl group of catecholamines, and monoamine oxidase (MAO), which converts the catecholamine to an aldehyde intermediate. The aldehyde intermediate formed by MAO is rapidly converted either to an acid or alcohol, by aldehyde dehydrogenase or aldehyde reductase, respectively.

MAO is located in the outer mitochondrial membrane and present in both neurons and non-neuronal cells (Abell, 1986). There are two types of MAO which differ in substrate specificity and localization (Urwyler and Von Wartburg, 1980). MAO A has a preference for NE and 5-HT, while the preferred substrate for MAO B is phenylethylamine. DA, tyramine and tryptamine are substrates for either MAO (Schoepp and Azzaro, 1981). COMT is primarily a cytosolic enzyme, although varying amounts are membrane associated depending on tissue type. From immunohistochemical studies, it appears to be located extraneuronally (Kopin, 1985).
In the striatum, 80 - 90% of DA is converted to dihydroxyphenylacetic acid (DOPAC) by MAO within the dopaminergic nerve terminals after reuptake (Fagervall and Ross, 1986). DOPAC can also be formed extraneuronally following DA release. DA is converted to homovanillic acid (HVA) at extraneuronal sites by sequential action of COMT and MAO (Cooper et al., 1991). It is thought that striatal DOPAC content reflects the recent history of DA biotransformation rather than the extent of DA release. In contrast, 3-methoxy-tyramine and HVA are formed extraneuronally and may better reflect the level of DA in the synaptic cleft (Altar et al., 1987).

C. Enzyme Regulation

In order for an organism (or an individual cell) to adapt and respond to changes in its environment, there must be some way of regulating relevant metabolic processes. As illustrated in Figure 4, an extracellular signal, hormone, neurotransmitter or drug binds at a receptor on the cell surface and causes the generation of a signal within the cell. This results in the generation of second, third or other messengers which eventually might trigger a cascade of events that will bring about alterations of the cellular milieu and responsiveness, including changes in enzyme activity. These changes can occur at the transcriptional, translational or post-translational levels.
Figure 4. Signals in the brain. Extracellular signals (first messengers) produce specific biological responses in target neurons via a series of intracellular signals (second, third, etc., messengers). Second messengers in the brain include cAMP, cGMP, Ca\(^{2+}\), and diacylglycerol. cAMP and cGMP produce most of their second-messenger actions through the activation of virtually one type of cAMP-dependent protein kinase and one type of cGMP-dependent protein kinase, respectively. The former enzyme exhibits a broad substrate specificity and the later a more restricted specificity. Ca\(^{2+}\) exerts many of its second-messenger actions through the activation of Ca\(^{2+}\)-dependent protein kinases, as well as through a variety of physiological effectors other than protein kinases. Ca\(^{2+}\) activates protein kinases in conjunction with calmodulin or diacylglycerol. There are at least 5 types of Ca\(^{2+}\)/calmodulin-dependent protein kinases in the brain: (1) phosphorylase kinase, which phosphorylates only phosphorylase (and possibly glycogen synthase); (2) myosin light-chain kinase, which phosphorylates only myosin light chain; (3) Ca\(^{2+}\)/calmodulin-dependent protein kinase I; (4) Ca\(^{2+}\)/calmodulin-dependent protein kinase II; and (5) Ca\(^{2+}\)/calmodulin-dependent protein kinase III. The substrate specificities of the latter three enzymes have not been established with certainty; (4) Ca\(^{2+}\)/calmodulin-dependent protein kinase II appears to have a broad substrate specificity, whereas Ca\(^{2+}\)/calmodulin-dependent protein kinases I and III appear to phosphorylate only a small number of substrates. Brain also contains a high levels of protein tyrosine kinase activity and numerous substrates for this activity; however, the exact number of such kinases and the nature of the cellular messengers that regulate them have not been established. In some cases, the phosphorylation of substrate proteins, or third messengers, appears to result directly in the biological response. In other cases, it seems to produce the biological responses indirectly through fourth, fifth, sixth, etc., messengers. From Nestler and Greengard (1983).
Figure 4.
The cellular responses to a stimulus can be either transient or long-lasting depending on the type and duration of the generated signal, and the intracellular pathways activated in response. Generally, regulation is categorized as either short- or long-term. Short-term regulation of enzyme activity occurs rapidly (within seconds to minutes) in response to momentary changes in neuronal activity or pharmacological manipulation and is accomplished without changes in total enzyme protein levels. Mechanisms such as phosphorylation, which can change the affinity of the enzyme for substrate or cofactors, and susceptibility to end-product inhibition (where product interferes with binding of substrate or cofactor and decreases enzymatic conversion to product) are involved. Long-term regulation involves the alteration of the turn-over of an enzyme, and provides a gradual and delayed method of control taking hours to days to develop. These changes are dependent on increased gene transcription and \textit{de novo} protein synthesis, or decreased degradation of pre-existing enzyme.

Phosphorylation and dephosphorylation of intracellular proteins constitutes a major route by which receptors (and their second messengers) transiently influence metabolic processes (Figure 5). The best studied intracellular signals are the second messengers: cAMP, cGMP, diacylglycerol (DAG), inositol triphosphate (IP$_3$), and intracellular calcium (Ca$^{2+}$). Second messenger activity can be mediated by cAMP- and cGMP-dependent protein kinases (PKAs and PKGs) as well as calcium-calmodulin (CaMKin II) and
Figure 5. Schematic diagram of the role played by protein phosphorylation in mediating some of the biological effects of a variety of regulatory agents. Many of these agents regulate protein phosphorylation through altering intracellular levels of a second messenger, cAMP, cGMP, or Ca^{2+}. Other agents appear to regulate protein phosphorylation through mechanisms that do not involve these second messengers. Most drugs regulate protein phosphorylation by affecting the ability of first messengers to alter second-messenger levels (curved arrows). A small number of drugs (e.g., phosphodiesterase inhibitors, Ca^{2+} channel blockers) regulate protein phosphorylation by directly altering second-messenger levels (straight arrows). From Nestler and Greengard (1989).
calcium-phosphatidylserine (PKC) dependent kinases. Protein kinases and phosphatases control a wide range of cellular events, such as cell division, signalling, differentiation and metabolism (Inagaki et al., 1994).

G-protein coupled receptors linked to adenylate cyclase can either increase or decrease its ability to generate cAMP (Kebabian, 1992), and hence, modulate PKAs. Forskolin, a diterpene, directly activates adenylate cyclase to increase cAMP levels (Laurenza et al., 1989) and indirectly activates PKA. G-protein coupled receptors linked to phospholipase C regulate the levels of DAG and IP$_3$, which in turn influence intracellular Ca$^{2+}$ levels and the activity of PKC (Nishizuka, 1984). Phorbol esters, including phorbol-12-myristate-13-acetate (PMA), directly activate PKC (Tanaka and Nishizuka, 1994). Forskolin and PMA are commonly used compounds for studying second messenger mediated effects.

Second messengers and protein kinases display a number of interesting properties which allow amplification, integration, convergence and modulation of multiple simultaneous internal and external signals. There can be additional layers of regulation through cross-talk between signalling pathways. Many signal transduction pathways are themselves altered by phosphorylation of component proteins (Girault, 1993). For instance, PKC has been shown to phosphorylate adenylate cyclase and increase cAMP generation (Yoshimasa et al., 1987), which could then increase PKA activity. PKA can effect PKC activity: it can phosphorylate certain IP$_3$ receptors and
may modulate the release of Ca\textsuperscript{2+} from the endoplasmic reticulum; and it has also been shown to up-regulate some Ca\textsuperscript{2+}-activated potassium channels and down-regulate others in the brain (Cohen, 1992), which would in turn influence PKC activity. These sorts of interactions allow for coordination of multiple extracellular signals at any given time, and also for integration over time.

Phosphorylation requires the transfer by protein kinases of phosphate molecules from ATP to a protein substrate, making it a phosphoprotein (Kötter, 1994). Protein phosphatases reverse this process. The net phosphorylation state (and activity) of these phosphoproteins can be altered by influencing the activity of the kinases or phosphatases that act upon them. A change in phosphorylation state of substrate proteins generally influences their activity over a period of seconds to minutes and is rapidly reversible.

There are two major protein kinase families, classified by which amino acid residues are phosphorylated on substrate proteins. The first, well studied group of kinases are classed as serine/threonine protein kinases, of which PKA, PKC and CaMKin II are members (Walaas and Greengard, 1991). The other family, referred to as tyrosine protein kinases, phosphorylates substrate proteins only on tyrosine residues (Cadena and Gill, 1992). Protein kinases act only at specific sites on a substrate protein by recognizing a motif, or portion of the amino acid sequence, surrounding the phosphorylation site (Kemp and Pearson, 1990). The phosphate acceptor site (serine or threonine residue) is
surrounded by determinant residues which are usually the charged amino acids, arginine and lysine. The position of these determinant residues determines the ability of a particular kinase to recognize the phosphate acceptor site.

There are four major classes of serine/threonine phosphatases (Cohen, 1989), as well as other phosphatases which only dephosphorylate tyrosine residues (Fischer et al., 1991). Three of the major serine/threonine phosphatases (protein phosphatases 1, 2A and 2C) have broad and overlapping substrate specificities and factors governing recognition of substrate are not yet understood (Cohen et al., 1989). Phosphatase activity may be modulated directly by binding of a second messenger or indirectly by second messengers (and protein kinases) acting on protein phosphatase inhibitory proteins (Nestler and Greengard, 1989). Okadaic acid has become a very useful tool for studying the involvement of phosphatases in biological responses since it is a very potent and selective inhibitor of type 1 and 2A protein phosphatases (Cohen, 1990).

Second messenger activated kinases not only produce covalent modification of preexisting proteins to alter their activity, but can also induce the synthesis of new proteins by altering gene expression (Kandell et al., 1991). These are more enduring changes which take place on the order of hours, days or even longer. A gene is composed of two parts: a coding region which encodes the amino acid sequence for a protein and a regulatory region located
upstream (5') of the coding region. The regulatory region consists of promoter and enhancer sequences which bind proteins that control the level at which the gene will be transcribed by RNA polymerase II (Hill and Treisman, 1995). An example of an enhancer element is the cAMP response element (CRE) and its transactivating protein, CREB. CREB proteins specifically recognize the CRE sequence and bind there (Gonzales et al., 1991).

Figure 6 shows a schematic of CRE/CREB activation. In this case, the β-adrenergic receptor coupled to adenylate cyclase, through the G-protein, Gs, increases cAMP and results in activation of PKA. When the cAMP pathway is sufficiently activated, the catalytic subunit of PKA translocates to the nucleus and phosphorylates CREB, so that it can bind to the CRE and facilitate binding of RNA polymerase to the gene's promoter and increase the frequency of transcriptional initiation (Hunter and Karin, 1992). Other promoter sequences such as the API sequence which are bound by homo- or hetero-dimers of FOS and JUN proteins in different combinations either enhance or repress transcription of a gene (Edwards, 1994). There may be several of these enhancer elements in the regulatory region of a gene allowing the gene product to be synthesized in response to several signals and make the gene subject to complex regulation when multiple signals are integrated.
Figure 6. CREB Pathway. A schematic illustration of the protein kinases and protein phosphatases that regulate activation of the CREB transcription factor. From Hunter (1995).
D. Regulation of Tyrosine Hydroxylase

TH activity has been found to be regulated by neuronal activity and neurotransmitters. This being the rate-limiting step in catecholamine synthesis (Levitt et al., 1965), much research has gone into understanding regulation at this step. TH is subject to end-product inhibition by DA and other catecholamines which compete for the tetrahydrobiopterin binding site on the enzyme (Ames et al., 1978). TH activity is also regulated by phosphorylation, which changes activity of individual enzyme molecules. The increased activity is the result of changes in the affinity of TH for tetrahydrobiopterin and this raises the possibility that changes in the concentration of the cofactor or in the affinity of TH for the cofactor would significantly affect the rate of tyrosine hydroxylation (Zigmond et al., 1989). Finally, there are regulatory controls at the level of the TH gene, which change levels of TH expression.

Acute activation of TH is produced by phosphorylation of the enzyme at one or more sites. Haycock et al. (1990) have demonstrated the presence of four phosphorylation sites at serines 8, 19, 31 and 40 on the enzyme. These sites are substrates for various kinases, including PKA, CaMKII and PKC.

Forskolin has been shown to stimulate TH activity in slices and synaptosomes from rat striatum and hypothalamus, and in slices from bovine retina (Katz et al., 1983). Under conditions which activate PKA in
striatal synaptosomes (addition of dibutyrl-cAMP, magnesium and ATP), there is incorporation of phosphate into TH, accompanied by a decrease in the $K_m$ for tetrahydrobiopterin and an increased $V_{max}$ (Goldstein et al., 1976). There are also concomitant increases in the $K_i$ for the inhibition of TH by catecholamines, reducing the extent of end-product inhibition that occurs. Extracts of soluble TH from rat striatum are also activated under conditions which activate PKC (addition of Ca$^{2+}$, magnesium and ATP) (Iuvone, 1984). Direct activation of PKC by the addition of phorbol esters increases TH $V_{max}$ while decreasing the $K_m$ for cofactor in striatal synaptosomal preparations (Onali and Olianas, 1986). Katz et al. (1983) noted that potassium-induced depolarization and forskolin exerted additive stimulatory effects on DA synthesis in striatal synaptosomes suggesting that the two worked independently. The depolarization-induced activation of TH appears to be Ca$^{2+}$-dependent as treatments which block Ca$^{2+}$-dependent processes block depolarization-induced increases in TH activity and the depolarization activated enzyme cannot be further activated by incubation with exogenously supplied PKC (El Mestikawy et al., 1985).

It appears that the increase in TH activity mediated by PKA is due to phosphorylation of serine 40 (Daubner et al., 1992). Funakoshi et al. (1991) used purified TH and activated kinase subunits to show that PKA phosphorylation of TH at serine 40 activates the enzyme. PKC and CaMKin II also increase phosphorylation at serine 40, however, they do so to a lesser
extent and this does not appear to be sufficient for TH activation. A study of tryptic peptides of activated TH showed that the peptide fragment phosphorylated in PMA-treated PC12 cells is different from that phosphorylated in forskolin-treated cells (Tachikawa et al., 1987). So, PKC activation of TH occurs through phosphorylation of a different serine residue on the enzyme than is phosphorylated by PKA. CaMKin II phosphorylates at serine 40 and additionally at serine 19. Phosphorylation of serine 19 by CaMKin II leads to activation of TH, although this appears to require the presence of an “activator” protein (Atkinson et al., 1987).

Protein phosphatase 2A has been shown to be the major TH phosphatase in the striatum (Haavik et al., 1989). Protein phosphatase 2C may play a minor role (10% or less of total TH phosphatase activity) and there does not appear to be any TH-specific phosphatases.

Regulation of TH activity is also accomplished through modulation of the amount of enzyme protein present within the cell. Changes in enzyme protein content are achieved at the level of DNA transcription to RNA, translation to new protein and degradation of existing protein. The TH gene has been cloned from several sources and sequenced, as have its upstream regulatory elements (Nagatsu, 1991). A great deal of the research on the regulation of TH gene expression has been done in PC12 cells, autonomic nervous system ganglia and adrenal glands. TH activity and mRNA are increased in these tissues by reserpine (Masserano and Weiner, 1983, and
Tank et al., 1985. TH mRNA also increases in the locus ceruleus and the ventral tegmental area, however, TH in the substantia nigra is not effected by reserpine (Pasinetti et al., 1990, Wessel and Joh, 1992). Antipsychotic treatments and electrical stimulation modulate TH activity in the substantia nigra through changes in TH mRNA levels and de novo protein synthesis (Masserano and Weiner, 1983). Various stressors (immobilization, cold, inescapable shock) modulate TH activity in the locus ceruleus and hypothalamus (Masserano and Weiner, 1983). Dexamethasone increases TH activity (Tischler et al., 1983), protein levels (Tank et al., 1986) as well as mRNA levels (Kim et al., 1993) in PC12 cells. In addition to increasing mRNA levels, glucocorticoids increase the stability of the TH mRNA (Fossom et al., 1992) and its translation (Baetge et al., 1981).

Forskolin increases TH mRNA several fold after prolonged exposure in the substantia nigra (Leviel et al., 1991). Nicotine (Hiremagalur et al., 1993) and cAMP analogues (Tank et al., 1986 and Lewis et al., 1987) also increase transcription of the TH gene in PC12 cells apparently by activation of PKA. This is consistent with the presence of a CRE in the upstream regulatory region of the TH gene (Nagatsu, 1991). Kim et al. (1994) demonstrated that PKA, via the CRE, plays a crucial role for both basal and cAMP-inducible transcription of the TH gene in PC12 cells. They also showed that PKA and PKC induce TH transcription by independent mechanisms. Activation of PKC in PC12 cells also appears to rapidly induce TH gene transcription as well
as play a role in modulation of post-transcriptional TH expression (Vyas et al., 1990). At -204 in the TH gene there is a sequence resembling the AP1 binding site to which the protein complex FOS/JUN has been shown to bind and to induce transcription following PKC activation.

E. Regulation of Aromatic L-Amino Acid Decarboxylase

AAAD is a relatively nonspecific enzyme found in both neuronal and non-neuronal tissues (Bowsher and Henry, 1986). In neuronal tissues, it is involved in the synthesis of catecholamines, serotonin, and the trace amines. AAAD is also found in high concentration in the liver, kidney, and stomach, although its function there is unclear, suggesting roles other than in catecholamine synthesis. In both rat and human, there is a single gene coding for AAAD in both neuronal and non-neuronal tissues, however, two mRNAs are produced: one is specific for tissue of neuronal origin and the other is specific for tissues of non-neuronal origin (Albert et al., 1987, Krieger et al., 1991, and Sumi-Ichinose et al., 1995).

AAAD is not considered to be the rate-limiting enzyme for catecholamine synthesis (Levitt et al., 1965). However, AAAD is the rate-limiting enzyme for trace amine synthesis (Dyck et al., 1983) and for DA synthesis in Parkinson's Disease (PD) patients being treated with L-DOPA. It has been generally assumed that AAAD is not modulated by neuronal
Recent work in the retina and striatum have provided evidence that this assumption deserves reconsideration.

Evidence shows that AAAD in the retina is regulated by both physiological stimuli and by pharmacological agents. Exposure of dark-adapted rats to light increases retinal AAAD activity to twice the levels observed in the dark (Hadjiconstantinou et al., 1988). This increase appears to be due to an increase in enzyme protein levels as evidenced by immunoprecipitation and by the finding that cycloheximide blocks the increase of AAAD activity. Administration of $\alpha_2$-adrenoceptor antagonists (Rossetti et al., 1989) or $D_1$ DA receptor antagonists (Rossetti et al., 1990) in the dark also result in a doubling of AAAD activity. Administration of $D_1$ agonists in the light brings AAAD activity down to dark levels. $\alpha_2$-Adrenoceptor agonists suppress both the light-induced rise in AAAD activity and the rise induced by the $\alpha_2$-antagonists.

Some observations in the retina suggest mechanisms other than changes in AAAD protein levels may be involved in the modulation of AAAD activity (Hadjiconstantinou et al., 1988). For example, when the lights are turned off, there is a biphasic decline in AAAD activity. AAAD activity drops rapidly at first (half-life of 3 minutes) and then more slowly (half-life of 73 minutes). The later decline may be accounted for by degradation of AAAD protein, but the early decline is too rapid and apparently rapidly reversible.
Whereas it takes roughly 3 hours of exposure to room light for AAAD to reach its maximal activity in fully dark-adapted animals exposed to light, only about 15 minutes to reach its full activity in the light is required if animals are returned from 30 minutes in the dark. Clearly, two or more mechanisms may be responsible for the rise and fall of retinal AAAD activity. The rapid, early occurring changes in activity could involve a regulatory mechanism which changes AAAD activity, followed by slower changes in AAAD protein levels.

Recently, it has been demonstrated that DA receptor antagonists increase striatal AAAD activity in both rats and mice (Zhu et al., 1992 and Hadjiconstantinou et al., 1993). Studies by Hadjiconstantinou et al. (1993) showed that acute administration of DA receptor antagonists results in an early, at about 15 min, and a late, at about 3 - 6 hours, increase of AAAD activity. The initial transient increase of striatal AAAD after DA receptor antagonist occurs too fast for synthesis of new molecules. The late increase is dependent on an increase in enzyme levels and can be prevented by administering protein synthesis inhibitors. Zhu et al. (1992) demonstrated that administration of either SCH-23390, a D₁ antagonist, or pimozide, a D₂ antagonist, significantly increased rat striatal AAAD activity (16 - 33% and 25 - 41%, respectively). The activity was increased within 30 minutes and the increases lasted 2 - 4 hours. Inhibition of protein synthesis by cycloheximide had no effects on the increases in AAAD activity when studied 30 minutes
later. The D₂ agonist, bromocryptine, reduced striatal AAAD activity by 23% (Zhu et al., 1993). These data also suggest the presence of regulatory mechanisms other than protein synthesis. The early increase is apparently due to an activation of the enzyme, possibly involving direct phosphorylation of AAAD.

So far, most evidence indicates changes in enzyme protein synthesis is a mechanism for control of AAAD activity. Subchronic administration of DA receptor antagonists, including haloperidol, resulted in an increase of enzyme activity, which was dependent on synthesis of new enzyme (Hadjiconstantinou et al., 1993). Chronic treatment with neuroleptics, haloperidol or loxapine, has been shown to elevate AAAD mRNA in the midbrain (Buckland et al., 1992). Chronic administration of the D₁ antagonist, SCH 23390 (Cho et al., 1995a) and acute administration of MK-801, an antagonist of the N-methyl-D-aspartate glutamate receptor (Hadjiconstantinou et al., 1995) have also been shown to increase midbrain AAAD mRNA levels. Two MAO inhibitors, pargyline and deprenyl, increase AAAD mRNA in the midbrain after administration (Cho et al, 1995b). It has also been shown that irreversible MAO B inhibitors (Li et al., 1992) and NSD-1015, an AAAD inhibitor (Li et al., 1993), increase AAAD gene expression in PC12 cells. The mechanism for the effects of these latter compounds are unclear.
There is additional circumstantial evidence that mechanisms other than control of enzyme levels may also be involved. Studies with native and recombinant bovine AAAD demonstrated the existence of two charge isoforms of AAAD (Park et al., 1992). These two forms are thought to result not from differences in primary structure, rather they may be the consequence of post-translational modification. The deduced amino acid sequences of bovine adrenal AAAD from our laboratory and from Kang and Joh (1990) show six potential phosphorylation sites. These same sites are also present in human AAAD amino acid sequence of Ichinose et al., (1989). However, knowledge of the primary structure is not sufficient to determine whether a "phosphorylatable" residue is indeed phosphorylated by a kinase in intact cells, and, potential phosphorylation sites predicted from the amino acid sequence must be confirmed by the appropriate biochemical experiments (Girault, 1993). PKA, PKC, CAM Kin II and proline-directed protein kinase are candidate kinases for AAAD phosphorylation (Table 1). Potential phosphatases would include the type 2A phosphatase which is the major phosphatase for TH in the striatum (Haavik et al., 1989). These kinases and phosphatases have roles in TH phosphorylation and dephosphorylation and are present in the dopaminergic neurons of the substantia nigra (Haycock and Haycock, 1991 and Haavik et al., 1989), further supporting their potential role in AAAD regulation.
Table 1. Protein Kinases, Phosphorylation Site Motifs, and Potential Phosphorylation Sites on AAAD.

<table>
<thead>
<tr>
<th>Protein Kinase</th>
<th>Motif</th>
<th>Potential Site(s) on AAAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP-dependent protein kinase</td>
<td>RXS<em>X, RRXS</em>X</td>
<td>Ser 220, 336</td>
</tr>
<tr>
<td></td>
<td>KRXXS*X</td>
<td>Thr 320</td>
</tr>
<tr>
<td>Calmodulin-dependent protein kinase II</td>
<td>XRXXS*X</td>
<td>Ser 359</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>XRXXS*XRX</td>
<td>Ser 359</td>
</tr>
<tr>
<td>Proline-directed protein kinase</td>
<td>XS*PX</td>
<td>Ser 73, 108</td>
</tr>
</tbody>
</table>

The phosphate acceptor site (serine or threonine) is indicated $S^*$. Less essential residues are marked $X$, and determinant residues are shown underlined. $R$=arginine, $S$=serine, $K$=lysine, $P$=proline. Protein kinase recognition sequence motifs from Kemp and Pearson (1990).
Clearly AAAD activity is regulated. Evidence presented in this document demonstrates that forskolin and cAMP analogues (which activate PKA) and phorbol esters (which activate PKC) increase AAAD activity in vivo. The observed increase in AAAD activity occurs rapidly and begins to decline after about 30 minutes. Together with observations from other laboratories, these observations suggest that at least two mechanisms may be responsible for the rise and fall of AAAD activity: an early regulatory mechanism involving activation of AAAD, and a late regulatory mechanism involving induction of the enzyme. Regulatory phosphorylation is one possible mechanism for the early changes. Since both cAMP analogues and phorbol esters can elevate AAAD, there may be more than one kinase involved. This would be similar to the modulation of TH in the brain, short-term activation by phosphorylation and long-term modulation by induction (Joh et al., 1978, Fossom et al., 1992). Since little research had been directed to AAAD regulation and there was evidence for parallel forms of regulation, TH was used as a model system for approaching the study of the regulation of AAAD (both short- and long-term) as far as drugs, concentrations, exposure times to be used.
F. Parkinson’s Disease and the MPTP-Lesioned Animal Model

PD is a chronic, progressive neurodegenerative disorder. Its symptoms are 1) tremor at rest, 2) increase in muscle tone or rigidity that often has a cogwheel- or ratchet-like characteristic, 3) difficulty in the initiation of movement and paucity of spontaneous movements (akinesia), and 4) slowness in the execution of movement (bradykinesia) (Côté and Crutcher, 1991). The pathological substrate of PD is a degeneration of dopaminergic neurons in the substantia nigra. The cause of this degeneration is unknown in the majority of cases (idiopathic). Parkinsonism can develop secondarily to cerebrovascular or other CNS diseases, encephalitis lethargica, exposure to toxins (carbon monoxide, manganese) and neuroleptic drugs.

The preferred treatment for PD is administration of L-DOPA, since it can ameliorate all of the clinical features of parkinsonism (Yahr, 1975). Exogenously administered L-DOPA is converted to DA by AAAD and the beneficial effects of this therapy are apparently associated with the activation of D_2 receptors (Boyce et al., 1990). Unfortunately, L-DOPA loses efficacy with time, either because of pharmacokinetic/pharmacodynamic changes or/and because of the fact that the dopaminergic neurons continue to degenerate. Other drugs such as bromocryptine by acting directly at dopaminergic receptors (Parkes et al., 1976), deprenyl by inhibiting MAO activity (Riederer and Youdim, 1986), or benztropine by blocking muscarinic receptors...
(Aminoff, 1995) can be employed alone or in conjunction with L-DOPA to enhance and/or maintain therapeutic effect.

L-DOPA therapy has both peripheral and central side effects the most serious of which are psychotic episodes and dyskinesias (Birkmayer et al., 1983). Fluctuations in response develop in a substantial number of patients with prolonged use of L-DOPA (Yahr, 1993). Fluctuating responses begin with a "wearing off" phenomena, in which hypokinesia returns at the end of the dosage period. A random, non-dose related "on-off" phenomenon also develops (Clough et al., 1984). Although this effect does not appear to be dependent on the concentration of L-DOPA in the blood, there is a sudden loss of the anti-PD effects, with rigidity and hypokinesia which can last hours, then a rapid switch back "on". During the off phase, it appears that the DA receptors are available for stimulation, but are not receiving sufficient DA (Clough et al., 1984). Both the wearing off and on-off phenomena will sometimes be ameliorated by a drug holiday (a temporary supervised withdrawal from L-DOPA therapy) or an adjunct therapy (Direnfeld et al., 1978).

In 1979, it was discovered that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produced the symptoms of PD in humans (Davis et al., 1979). Since then, MPTP has been used for developing animal models for PD, including monkeys and mice (Burns et al., 1983, and Heikkila et al., 1984). The speculated mechanism for MPTP toxicity is shown in Figure 7.
Figure 7. Hypothesized mechanisms of neurotoxicity of MPTP, which produces parkinsonism in primate species. From Cooper, Bloom and Roth (1991).
MPTP is converted to MPP⁺ by MAO B in glial cells (Heikkila et al., 1984), and taken up into dopaminergic neurons by the DA uptake (Javitch et al., 1985). Inhibitors of MAO B (Bradbury et al., 1986) and DA uptake (Ricaurte et al., 1985) prevent MPTP toxicity. Inside the neuron, it is thought to be concentrated in the mitochondria (Ramsay et al., 1986) and block the electron transport at complex I, reducing ATP levels (Chan et al., 1991) and generating free radicals (Rossetti et al., 1988 and Cleeter et al., 1992). After depletion of protective antioxidants, superoxide, hydrogen peroxide, and hydroxyl radicals are formed which can cause membrane lipid peroxidation, oxidative damage to DNA and RNA, and inactivation of vital enzymes (Kopin and Markey, 1988). Apparently the damage caused by the free radicals and the depletion of energy (ATP) eventually leads to dopaminergic cell death (Bates et al., 1994).

MPTP lesions have been shown to reduce both AAAD and TH activities (Mogi et al., 1988, Hadjiconstantinou et al., 1990) and reduce DA uptake and levels of DA and its metabolites (Heikkila et al., 1984, Hadjiconstantinou et al., 1988). To compensate for the loss of activity the surviving dopaminergic neurons increase the synthesis and release of DA as indicated by enhanced DA turnover and increased levels of the DA metabolites, DOPAC and HVA (Hadjiconstantinou et al., 1988). Richard and Bennett (1994) observed increases in fractional DA synthesis (ratio of DOPA/DA) with increasing DA depletion also suggesting an elevation of the DA synthetic capacity per surviving DA nerve terminal.
TH activity increases with time after MPTP lesion (Hadjiconstantinou et al., 1990). In 6-OHDA lesioned rats, TH mRNA of substantia nigra neurons has been found elevated 20 - 30% 6 months after the lesion (Blanchard et al., 1995). Similarly, the TH protein content is increased in the striatal terminals of these animals. Other compensatory responses may also change the regulation of DA synthesis in the striatum after denervation. For instance, although overall TH protein levels decline due to loss of dopaminergic neurons, the homospecific activity (activity per enzyme protein) is significantly increased in parkinsonian brains (Mogi et al., 1988).

In MPTP treated mice, modulation of AAAD also appears to change. AAAD activity appears to normalize faster than TH during recovery (Hadjiconstantinou et al., 1990), and the response of AAAD to DA receptor antagonists is supersensitive, e.g. lower doses of DA receptor antagonists are required to elevate AAAD in the striatum of the lesioned than in normal mice (Hadjiconstantinou et al., 1993). The development of DA receptor supersensitivity after the lesion (Weihmuller et al., 1990) is probably responsible for this increased response of AAAD.

There is still some argument over the site of decarboxylation of L-DOPA to DA in the striatum after lesions with MPTP or 6-OHDA. AAAD in the striatum is localized primarily within the nigrostriatal dopaminergic nerve terminals. Tashiro et al., 1989 reported the presence of few striatal neurons showing AAAD-like immunoreactivity in the rat, while other
researchers have found neither evidence of striatal neurons with AAAD-immunoreactive protein nor the presence of AAAD RNA by in situ hybridization (Kang et al., 1992, Eaton et al., 1993). Serotonergic and noradrenergic neurons projecting to the striatum may also contribute to striatal decarboxylating activity (Melamed et al., 1980). Cultured rat striatal glial cells express the AAAD gene (Li et al., 1992) and cultured mouse striatal astrocytes can decarboxylate L-DOPA (Juorio et al., 1993). Studies with kainic acid lesions of the striatum (Melamed et al., 1980) or 6-OHDA lesions of the nigrostriatal tract in combination with kainic acid lesions of the striatum (Hefti et al., 1981) have showed a small decrease (~20%) in the decarboxylation of exogenous L-DOPA without affecting striatal TH activity. These observations indicate that DA formation from exogenous L-DOPA within the striatum occurs mainly, but not exclusively, within DA terminals. Some DA formation may occur within kainic acid sensitive striatal interneurons or efferent neurons.

Modulation of AAAD activity by various pharmacological agents could play an important role in treating PD. In MPTP lesioned mice, Hadjiconstantinou et al. (1993) showed that striatal AAAD activity can be increased to normal levels by treatment with D_1 or D_2 antagonists. Modulation of AAAD activity occurring physiologically or by pharmacological agents could either increase or decrease the effectiveness of the L-DOPA therapy.
G. Specific Aims and Significance of the Project

The specific aims are:

1) **To assess the involvement of second messenger systems and protein kinases in the regulation of AAAD activity in vivo.**

   TH activity is modulated by a number of second messenger systems producing both changes in its phosphorylation state and in its level of expression (Masserano et al., 1983 and Zigmond et al., 1989). Although it was assumed that AAAD activity was not modulated, similar regulation seems likely for AAAD since examination of its amino acid sequence for kinase-specific motifs revealed several serine and threonine residues that could serve as phosphorylation sites. Does stimulating or mimicking the activation of PKA and PKC signaling pathways modulate AAAD activity in the striatum and midbrain (region containing dopaminergic cell bodies in the substantia nigra)? Do other second messengers have effects on AAAD activity?

2) **To examine the temporal relationship of the changes in AAAD activity to those of TH activity and DA metabolism.**

   Although AAAD was never thought to regulate catecholamine synthesis, the fact that the enzyme is regulated by mechanisms similar to the rate-limiting enzyme, TH, suggests that the relationship between the two
enzymes must be reexamined. The *in vivo* transient activation of TH by PKC- and PKA-dependent pathways was studied and compared with AAAD activation. Differences in time course between TH and AAAD activation may suggest that AAAD regulation is physiologically significant and perhaps it may become limiting under certain conditions. It is thought that changes in DA metabolites probably reflect more closely changes in synthesis than changes in the release of DA (Zetterstrom et al., 1988, and Westerink et al., 1989) although release and reuptake of DA may both be changed by manipulations of kinase/phosphatase activities. If the observed changes in TH and AAAD activity are physiologically significant then they may be reflected in the levels of DA or its metabolites, DOPAC and HVA. The temporal relationship between TH, AAAD and DA might provide useful clues also regarding the role of AAAD in the regulation of DA synthesis. Does it become rate-limiting under stimulated conditions?

3) *To examine the effects of dopaminergic denervation on second messenger-mediated changes in TH and AAAD activities.*

MPTP-induced dopaminergic lesions are known to change the striatal response to a number of pharmacological manipulations, through the development of DA receptor supersensitivity or changes in coupling to second messenger systems. Therefore, it is important to see if the lesion can alter the response of AAAD and TH to pharmacological manipulation. Does
the response of AAAD and TH to activation of PKA or PKC become
supersensitive or blunted? If yes, does it effect DA metabolism? What might
this mean for treatment of PD? An increased understanding of the
intracellular mechanisms regulating AAAD activity might suggest
alternative or improved therapies for PD.
CHAPTER I
MATERIALS AND METHODS

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. Disodium dihydrogen EDTA is from G.F. Smith Chemical Co. (Columbus, OH). Na$_2$CO$_3$ and Darco G-60 charcoal are from J.T. Baker Co. (Phillipsburg, NJ). Copper disodium EDTA, sodium octyl sulfate and X-OMAT AR film are purchased from Kodak (Rochester, NY). HPLC grade MeOH and Triton X-100 are from Fischer Scientific (Fairlawn, NJ). $^3$H-tyrosine, $^3$H-H$_2$O, $^{32}$P-dCTP and the nick translation kit are from Amersham (Clearwater, IL). N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), phorbol-12,13-myristic acid, 4-$\alpha$-phorbol-12,13-myristic acid, forskolin, chelerythrine, okadaic acid and pentylenetetrazol are from Research Biochemicals Inc. (Natick, MA). Phase II ODS 3 micron column is from BAS (W. Lafayette, IN). Male Swiss-Webster mice, weighing 25 - 30 g, are obtained from Harlan (Indianapolis, IN).
DRUG ADMINISTRATION

A. MPTP treatments

Male Swiss-Webster mice (25 - 30 g body weight) were given 7 daily doses of MPTP (30 mg/kg body weight) by intraperitoneal (ip) injection, then allowed 2 weeks to recover before use in experiments.

B. Intracerebroventricular (icv) injections

Drugs (PMA, 4-α-PMA, forskolin, 8-Br-cAMP, chelerythrine, or okadaic acid) were delivered to the striatum and midbrain in vivo by icv injection. Control or MPTP-treated male Swiss-Webster mice were given diethylether or isoflurane anesthesia. Once anesthetized, an incision was made in the scalp to expose the skull. The injection site was on the bregma, 1 mm lateral to the midline and 2 mm below the surface of the skull. The drug, dissolved in DMSO or artificial CSF, was delivered in a volume of 2 μl by a Hamilton syringe. Following the injection, a surgical staple was used to shut the incision, and the mouse was returned to its cage for recovery. Mice were killed by decapitation at varying times (2 -180 min) after injection.

C. Cycloheximide treatments

Male Swiss-Webster mice (25 - 30 g body weight) were given cycloheximide, 10 mg/kg body weight, in H₂O by ip injection, 1 hr prior the icv administration of forskolin or PMA. This dose was taken from the
literature and has been used routinely in our laboratory for inhibition of protein synthesis (Hadjiconstantinou et al., 1988 and 1993).

D. Pentylenetetrazol treatments

After performing a dose-response study to visually determine the extent of seizure activity following injections of various doses of pentylenetetrazol, 2 doses were chosen for studies of the effects of seizures on AAAD activity. Male Swiss-Webster mice (25 - 30 g body weight) were given either 25 or 75 mg/kg body weight pentylenetetrazol, in H₂O, by ip injection. Mice were killed by decapitation 30 min after injection.

AROMATIC L-AMINO ACID DECARBOXYLASE ASSAY

A. Materials

1. Homogenization Buffer: 0.25 M sucrose
2. Assay Buffer: 50 mM sodium phosphate, pH 7.2, 100 μM pargyline, 100 μM EDTA (disodium, dihydrogen), 170 μM ascorbate, 1 mM β-mercaptoethanol, 500 μM L-DOPA, 10 μM pyridoxal 5'-phosphate
3. Stop solution: 0.525 N HClO₄, 2.5 μM 3,4-dihydroxybenzylamine (IS)
4. DA standards: serial 1:1 dilutions to yield 1.2, 0.6, 0.3, 0.15 and 0.075 nmol DA in 20 μl 0.2 N HClO₄.
5. Tris-HCl 60.55 g Trizma base/1 l H₂O, pH to 8.6 with HCl
6. 0.2 N HClO₄ with 1 mg/100 ml sodium bisulfite

B. Method

A modification of the method of Nagatsu et al. (1979) was used for assaying of AAAD activity (Hadjiconstantinou et al., 1989). Freshly dissected striatal or midbrain tissue is homogenized in 3 ml 0.25 M sucrose. If striatum was to be used for both AAAD and DA, DOPAC and HVA measurements, it was homogenized in 200 µl 0.25 M sucrose and 100 µl was further diluted with 1.4 ml 0.25 M sucrose for the AAAD assay. Twenty µl of the homogenate is added to 400 µl of the assay buffer and incubated in a shaking water bath at 37°C for 20 min. Each sample is assayed in duplicate. The reaction is stopped by addition of 80 µl stop solution and placing on ice. Twenty µl of the standards are added to 400 µl assay buffer and 80 µl stop solution. The reaction mixtures and standards are transferred to Eppendorf tubes containing 20 mg activated alumina and 1 ml Tris-HCl is added before the tubes are manually shaken for 15 min. The supernatant is aspirated off, and the pellet is washed with water before adding 120 µl 0.2 N HClO₄ and vortexing. The tubes are centrifuged at 15,000 x g for 5 min and the supernatant is transferred to small vials for HPLC. HPLC method is discussed in a following section.
C. Calculations

Peak heights for DA and IS are measured from HPLC chromatographs and the ratio of DA/IS is calculated. Using linear regression of the quantity of DA in the standards versus the ratio DA/IS, a standard slope is calculated for quantitating DA.

\[
\frac{DA}{IS}/\text{slope} = \text{nmol DA}
\]

mg prot is calculated after Lowry assay on homogenates (see following section).

nmol DA/mg prot is calculated and the duplicates for each sample are averaged together.

TYROSINE HYDROXYLASE ASSAY

A. Materials

1. Homogenization Buffer: 10 mM Tris Acetate, pH 7.0, 100 μM β-mercaptoethanol, 0.1% Triton-X100
2. Assay Buffer: 40 mM sodium acetate, pH 6.0, 0.1 mg/ml catalase, 1 mM ferric ammonium sulfate, 50 μM DL-6-methyl-5,6,7,8-tetrahydropterine, 200 μM tyrosine (3H-tyrosine, 50 μCuries 3H/μmol tyrosine)
3. 3H-H2O Standard: 1 μl 3H-H2O in 200 μl 1 mM tyrosine
4. 7.5% charcoal in 0.01 N HCl
B. Method

This is a modification of the method of Reinhard et al. (1986). Freshly dissected striatal tissue is homogenized in 170 μl homogenization buffer. Immediately after the preparation of the homogenates, 30 μl of the homogenate is added to 70 μl assay buffer and incubated at 37°C in a shaking waterbath for 20 min. Each sample is assayed in duplicate. The reaction is stopped by the addition of 1 ml 7.5% charcoal and transferred to ice. Blanks without tissue are prepared as follows: 3H-tyrosine in assay buffer + 30 μl H₂O, and 3H-H₂O in assay buffer + 30 μl H₂O. This is to determine the amount of 3H-tyrosine not adsorbed to the charcoal in a later step and the recovery of 3H-H₂O. Samples are centrifuged at 16,000 x g for 10 min and the supernatant is transferred to fresh tubes, spun again at 16,000 x g for an additional 10 min. The supernatant is transferred to scintillation vials and 5 ml Scintiverse cocktail is added. Standards for total radioactivity are prepared by putting 20 μl 3H-tyrosine or 3H-H₂O directly into scintillation vials with Scintiverse for counting.

C. Calculations

From the 3H-tyrosine and 3H-H₂O blanks and total 3H-tyrosine and 3H-H₂O dpms, constants are calculated.

correction = dpm 3H-tyrosine blank

constant = 20 nmol tyrosine/dpm total 3H-tyrosine
recovery = dpm $^3$H-H$_2$O blank/dpm total $^3$H-H$_2$O

dpm sample - correction = corrected dpm

mg prot is calculated after Lowry assay on homogenates (see following section).

nmol/mg prot = (corrected dpm x constant)/(mg prot x recovery x 2)

The duplicate nmol/mg prot values are averaged together for each sample.

DETERMINATION OF DOPAMINE AND METABOLITE CONTENT

A. Materials

1. 0.2 N HClO$_4$ with 1 mg sodium bisulfite/ 100 ml
2. 0.5 N HClO$_4$ with 1 mg sodium bisulfite/ 100 ml
3. Standards: Individual DA, DOPAC, and HVA 10 mM stock solutions in 0.2 N HClO$_4$ with 1 mg/100 ml sodium bisulfite are kept frozen. 150 μl DA, 30 μl DOPAC, and 40 μl HVA stocks are combined in 10 ml 0.2 N HClO$_4$, and 100 μl of this is diluted with an additional 900 μl 0.2 N HClO$_4$ (standard A), then serial 1:1 dilutions are made to yield standards B through G for calculation of standard curve. When 25 μl of the standards are injected on HPLC, the amounts of catecholamines loaded on the column are as follows:

- DA: 375, 187.5, 93.8, 46.9, 23.4, 11.7 and 5.9 pmol
- DOPAC: 75, 37.5, 18.8, 9.4, 4.7, 2.3, and 1.2 pmol
- HVA: 100, 50, 25, 12.5, 6.3, 3.1, and 1.6 pmol
B. Method

If starting with a whole striatum, either fresh or frozen, sonicate in 300 μl 0.2 N HClO₄. If using striatal homogenate from AAAD assay (1 striatum in 200 μl 0.25 M sucrose), combine 100 μl of the homogenate with 50 μl 0.5 N HClO₄ and sonicate. Centrifuge at 15,000 x g for 5 min and transfer supernatant to small HPLC vials. HPLC method is discussed in a later section.

C. Calculations

DA, DOPAC, and HVA peak heights are measured for each sample and standard from HPLC chromatographs. Using linear regression, a standard slope is calculated from quantity of each standard versus peak height.

\[
\text{sample peak height} / \text{slope} = \text{pmol DA, DOPAC, or HVA}
\]

mg prot per sample is calculated from Lowry protein assay.

Values for DA, DOPAC and HVA content are expressed as pmol/mg prot.

HPLC METHOD

A. Materials

1. Citric Acetate mobile phase: 17.6 mM Citrate pH adjusted to around 4.0 with sodium acetate and degassed by application of a vacuum. MeOH (5 - 15%) and sodium octyl sulfate (20 - 60 mg/l) are added before use on column.

2. Column: Pharmacia's Spherisorb ODS-2 5 micron, 4 X 100 mm or BAS's Phase II ODS 3 micron, 3.2 x 100 mm.
B. Method

In the citric acetate buffer, the concentration of MeOH and sodium octyl sulfate, and the pH varied to achieve good separation in a reasonable length of time. Conditions depended on the column used. Flow rate was held at ~1 ml/ min. Compounds of interest are detected by electrochemical detection with a carbon paste or glassy carbon electrode. The potential is held at 0.5 V for detection of DA and IS from AAAD assays and at 0.6 V for DA and metabolite detection. Peak heights are measured from chromatographs.

PROTEIN ASSAY

A. Materials
1. Reagent 1: 189 mM Na\textsubscript{2}CO\textsubscript{3}, 0.58 mM Copper Disodium EDTA in 0.1 N NaOH
2. Reagent 2: 1:1 Folin & Ciocalteau's phenol reagent:H\textsubscript{2}O
3. 1 mg/ml Bovine Serum Albumin (BSA) in H\textsubscript{2}O

B. Method

This is the method of Lowry et al. (1951). Standards are prepared containing 5, 10, 15, 20 and 25 μg BSA in a total volume of 100 μl. Aliquots of homogenates from AAAD or TH assays or from DA and metabolite content determinations were generally diluted into a volume of 100 μl with H\textsubscript{2}O as
follows: 20 μl striatal homogenate from AAAD assay + 80 μl H₂O, 2 μl of striatal homogenate from TH assay + 98 μl H₂O. One ml Reagent 1 is added to each tube and vortexed. After sitting at room temperature for 30 min, 100 μl Reagent 2 is added and tubes are vortexed. Fifty min later, the absorbance at 700 nm is read on the spectrophotometer.

C. Calculations

Using linear regression of Absorbance versus μg BSA, a standard slope is calculated. Sample absorbance/slope = μg prot

μg prot x dilution factor x 1000 = mg prot in assay

The dilution factor for striatal homogenates from TH assays is 15. There is no dilution factor for striatal or midbrain homogenates used for AAAD assays.
CHAPTER II

RESULTS

Establishing Assay Conditions and Controls

A. Establishing Appropriate Controls.

ICV administered compounds: PMA, 4-α-PMA, forskolin, 8-Br-cAMP, chelerythrine, and okadaic acid, were dissolved in DMSO or in artificial CSF. Control animals were injected with equal volumes of the above vehicles, DMSO or artificial CSF, to see whether they affected enzyme activity. There was no significant difference in AAAD activity between non-anesthetized, non-injected mice; anesthetized mice; or mice given injections of either DMSO or artificial CSF (32 ± 1, 33 ± 1, 32 ± 1 and 33 ± 1 nmol DA/mg prot/20 min ± SEM, respectively, n= 4 - 5 mice per group).
B. Conditions for Assaying TH Activity at Subsaturating Concentrations of Cofactor.

Because activation of TH is evident only at subsaturating conditions for its cofactor and is expressed as changes in the $K_m$ for the cofactor (Masserano and Weiner, 1983), kinetic analysis was performed to establish the concentration of the cofactor DL-6-methyl-5,6,7,8-tetrahydropterine (MTHP) to be used for the experiments. Increasing concentrations of MTHP were used while the substrate, L-tyrosine, was held constant (200 μM) (Figure 8). Under normal conditions, the apparent calculated $K_m$ for MTHP was 283 μM. Based on the $K_m$ value, the concentration of 50 μM MTHP was chosen for the TH activity assay because the enzyme is subsaturated with respect to cofactor. To ascertain that activation of TH could be measurable at this concentration of cofactor, we used the $D_2/D_1$ antagonist, haloperidol, as a positive control to increase TH activity. Mice were given 5 mg/kg haloperidol, ip, and sacrificed 1 hr later. Haloperidol treatment yielded significant increases in TH activity (49% over controls), comparable to increases seen in other laboratories (Fine et al., 1986) at that time point.

C. AAAD Conditions

Based on previous experiments in the laboratory (Hadjiconstantinou et al., 1989, and Hadjiconstantinou and Neff, 1990), striatal and midbrain AAAD
Figure 8. TH activity in mouse striatum with varying concentrations of cofactor. Insert shows Hofstee plot used to calculate kinetic parameters.
activity was routinely assayed under saturated conditions for both the
substrate L-DOPA and the cofactor pyridoxal phosphate.

Studies in Normal Mice

A. Effects of Forskolin on AAAD Activity

Forskolin directly activates adenylate cyclase and increases intracellular

cAMP levels. The newly generated cAMP binds to the two regulatory
subunits of PKA and causes them to dissociate from the two catalytic
subunits, allowing the catalytic subunits to phosphorylate substrate proteins.
Forskolin, 2 nmol in 2 μl DMSO, was administered by icv injection to mice
under anesthesia. At timed intervals - 2, 5, 10, 15, 30, 45, 90 and 180 min after
the injection, mice were killed and the striatum and midbrain dissected out.
Forskolin treatment transiently increased AAAD activity. In the striatum,
AAAD activity was significantly elevated by 2 minutes, and remained
significantly greater than basal activity through the 30 min time point (Figure
9). The maximum increase (30% over control) was seen at 5 min, and activity
returned to basal by 90 min. Three hrs after treatment, AAAD activity
remained at basal levels. In the midbrain, AAAD activity was found already
significantly elevated by 15 min (43% above control), which was the earliest
time point examined in this tissue. Activity remained elevated through 30
Figure 9. Rise of AAAD activity in the mouse striatum following forkolin (2 nmol, icv). Animals were killed at the intervals indicated, and AAAD activity assayed. n = 5-10. * p < 0.05 compared with zero-time.
min and declined to basal levels by 90 min and remained there through the 180 min time point (Figure 10).

8-Br-cAMP, a non-hydrolyzable analogue of cAMP, can enter the cells and act as a second messenger to modulate activity of cAMP-dependent processes, including activation of PKA. This compound was used to determine if the forskolin mediated effects on AAAD activity were dependent on its ability to activate adenylate cyclase and increase cAMP levels in the neurons. ICV administration of 8-Br-cAMP enhanced AAAD activity in both the striatum and midbrain in a dose-dependent fashion (Table 2). The lower dose (100 nmol in 2 μl artificial CSF) gave an increase similar to that seen after forskolin treatment. The higher dose of 8-Br-cAMP (200 nmol in 2 μl artificial CSF) increased activity by 53 - 55% over control values 30 min after treatment.

Three to 4 separate determinations of the kinetic parameters- $V_{max}$, and the $K_m$ for L-DOPA and for pyridoxal 5'-phosphate were made for the striatum and midbrain 30 min after icv administration of 2 nmol forskolin in 2 μl DMSO, using Hofstee plots. For these studies, the concentration of either substrate or cofactor was held at saturating concentrations, while the concentration of the other compound was varied. Otherwise, the AAAD assay was as described in the Materials and Methods section. These studies revealed a significant increase of the apparent $V_{max}$ in both the striatum (33%) and midbrain (54%) with little change of the apparent $K_m$ for L-DOPA or
Figure 10. Rise of AAAD activity in mouse midbrain following forkolin (2 nmol, icv). Mice were killed at indicated times after the injection and AAAD activity was assayed. n = 5 - 10. * p < 0.05 compared with zero-time.
Table 2. 8-Br-cAMP Increases AAAD Activity in Striatum and Midbrain.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Striatum</th>
<th>Midbrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>33 ± 1</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>8-Br-cAMP (100 nmol)</td>
<td>49 ± 1*</td>
<td>40 ± 1*</td>
</tr>
<tr>
<td>8-Br-cAMP (200 nmol)</td>
<td>51 ± 4*</td>
<td>49 ± 1*</td>
</tr>
</tbody>
</table>

8-Br-cAMP was injected icv into mice, and AAAD activity in striatum and midbrain assayed 30 min later. n = 5. * p < 0.01 compared with vehicle group.
pyridoxal 5'-phosphate (Table 3). Activation of AAAD by forskolin does not appear to involve modification of the affinity of the enzyme for either the substrate or cofactor.

Cycloheximide is a protein synthesis inhibitor and would be expected to block or reduce increases in enzyme activity if they were the result of synthesis of new enzyme molecules. Cycloheximide (10 mg/kg body weight, in H2O) was administered by ip injection 1 hr before icv injection of 2 nmol forskolin in 2 µl DMSO. Table 4 shows that cycloheximide pretreatment had no effects on either control or forskolin-stimulated AAAD activity indicating that de novo synthesis of AAAD is not involved at this time point.

Another, less well studied second messenger is cGMP, which is produced following activation of guanylate cyclase. Roskoski and Roskoski (1987) used 8-Br-cGMP or sodium nitroprusside (which activates guanylate cyclase to elevate intracellular cGMP levels) to increase TH activity in rat striatal synaptosomes and in PC12 cells. Since TH activation has been used as a model system for investigating conditions for modulation of AAAD activity, we tested the possibility that cGMP might activate AAAD as well. ICV administration of 100 nmol 8-Br-cGMP had no effect on AAAD activity 30 min after administration in either the striatum or midbrain (striatum: control 33 ± 1, 8-Br-cGMP 33 ± 2 nmol/mg prot/20 min ± SEM, midbrain: control 31 ±1, 8-Br-cGMP 32 ± 1 nmol/mg prot/20 min ± SEM, n = 5).
Table 3. Kinetics of AAAD Following Forskolin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (nmol/ mg prot/ 20 min ± SEM)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;(µM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L-DOPA</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>33 ± 1</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Forskolin</td>
<td>44 ± 1*</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>31 ± 1</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Forskolin</td>
<td>48 ± 2*</td>
<td>24 ± 1</td>
</tr>
</tbody>
</table>

Mice were treated with forskolin (2 nmol, icv) and killed 30 min later. AAAD activity in striatum and midbrain were assayed. Apparent V<sub>max</sub> and K<sub>m</sub> values were estimated from Hofstee plots. n = 3 - 4 experiments with 3 animals per group. * p < 0.01 compared with vehicle group.
Table 4. Forskolin Enhances Striatal AAAD Activity in the Presence or Absence of Cycloheximide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAAD Activity (nmol/mg prot/20 min ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Forskolin</td>
<td>50 ± 2*</td>
</tr>
<tr>
<td>Forskolin + Cycloheximide</td>
<td>49 ± 1*</td>
</tr>
</tbody>
</table>

Mice were treated with cycloheximide (10 mg/kg, ip) 1 hr before administration of forskolin (2 nmol, icv) and killed 30 min later and AAAD activity was assayed. n = 5 - 7. * p < 0.01 compared with vehicle group.
B. Effects of Forskolin on TH Activity

Since it is well-known that cAMP and PKA can transiently activate TH, it was expected that this would be observable after exposure to forskolin \textit{in vivo}. A time course study was done in the striatum, looking at 2, 5, 10, 15, 30, 45, 90 and 180 min after the injection of forskolin. Injection of 2 nmol forskolin in 2 \( \mu l \) DMSO, icv, resulted in a rapid and shortlived increase of TH activity (significant at the 2 min time point), which peaked at 5 min after the treatment (50\% above basal values) and returned to baseline levels by 15 min (Figure 11). Interestingly, there was a second significant increase of TH activity occurring at 90 min after treatment. Three hrs later enzyme activity was still elevated. The duration of this second increase in TH activity is not presently known since later times were not studied.

Comparison of the temporal changes of the AAAD and TH (Figure 12) that both enzyme activities rose in parallel and peaked at about 5 min. However, the early rise of TH by forskolin is more transient than that of AAAD. Furthermore, AAAD activity remained significantly elevated up to 30 min, while TH activity had already returned to baseline. AAAD activity did not return to baseline until 90 min after injection of forskolin. TH activity increased again at the later time points, while AAAD activity remained at baseline levels.
Figure 11. Time course of striatal TH activity after forskolin (2 nmol, icv). Animals were killed at the indicated times and TH assayed. n = 5 - 11. *p < 0.01 compared with zero-time.
Figure 12. Comparison of changes in striatal AAAD and TH activities after forskolin (2 nmol, icv).
C. Effects of Forskolin on DA Metabolism

Half of the striatal homogenate prepared for the AAAD activity assay was used for determination of DA and acidic metabolite levels in each animal. Basal levels of DA, DOPAC and HVA are 454 ± 10, 53 ± 1 and 37 ± 1 pmol/mg prot ± SEM, respectively. In general, icv administration of forskolin brought about a small and biphasic increase of DA, DOPAC and HVA content in striatum. After forskolin there was a small (about 14-16% over control) but significant increase of striatal DA at 10 and 15 min. DA returned to normal levels by 45 min, increased again by 90 min (15% over control) and normalized by 3hrs (Figure 13). The rise of DOPAC preceded that of DA and HVA (Figure 13). There was an early increase, between 20 and 30% over control, during the first 5 to 30 min, and return to basal levels at about 45 min. By 90 min, DOPAC levels were again increased (13% over control), and returned to basal levels by 3 hrs. HVA content increased (32 to 34% over control) between 10 to 30 min following forskolin injection, returned to basal at 45 min, rose again at 90 min and normalized at about 3 hrs after the treatment (Figure 13).

D. Effects of PMA on AAAD Activity

PMA is a phorbol ester capable of crossing cell membranes and directly activating PKC. PMA increased AAAD activity in a dose-dependent manner
Figure 13. Effect of forskolin (2 nmol, icv) on striatal DA, DOPAC and HVA. Animals were killed at indicated times and striatal DA, DOPAC and HVA content determined. Basal values are 53 ± 1, 454 ± 10 and 37 ± 1 pmol/mg protein for DOPAC, DA, and HVA, respectively. Data are means ± SEM as % of respective control (n = 8 - 12). * p < 0.01 compared with vehicle group.
in the mouse striatum (Figure 14), when studied 30 min after the treatment. From this study, the calculated maximal activation of the enzyme is 45 nmol/mg prot/20 min and the ED$_{50}$ is 0.51 nmol PMA. These initial experiments showed that 2 nmol PMA given by icv injection yielded a maximal increase in AAAD activity. This dose, delivered in 2 μl DMSO, was used for the studies to follow.

ICV administration of PMA induces seizures in mice (Smith and Meldrum, 1992), and varying degrees of abnormal motor activity, such as barrelling were observed in some mice in these studies. Despite the fact that there was no difference in AAAD activity between PMA-treated mice with increased or normal motor activity, a study was performed to control for possible effects of clinical or subclinical seizure activity on AAAD activity. Pentylenetetrazol was given by ip injection in 2 doses: a low dose (25 mg/kg), which did not produce clinical seizures, and a high dose (75 mg/kg), which induces clinical seizures, and AAAD activity was assayed 30 min later. AAAD activity of the striatum and midbrain was not effected at either dose (Table 5).

PMA has been shown to have several non-PKC mediated effects on cellular function (Blumberg, 1980). To ascertain that the observed effects on AAAD activity were PKC-dependent, the compounds 4-α-PMA and chelerythrine were used. 4-α-PMA is an inactive analogue of PMA, which does not activate PKC, and is frequently used as a negative control in
Figure 14. PMA increases striatal AAAD activity in a dose-dependent manner. Varying amounts of PMA were given by icv injection in 2 μl DMSO and AAAD activity assayed 30 min later. Basal AAAD activity was 33 ± 2 nmol/mg prot/20 min ± SEM. n= 4 - 5.
Table 5. Pentylenetetrazol Has no Effect on AAAD Activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Striatum</th>
<th>Midbrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>33 ± 1</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Pentylenetetrazol (25 mg/kg)</td>
<td>30 ± 2</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>Pentylenetetrazol (75 mg/kg)</td>
<td>30 ± 2</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

Pentylenetetrazol was given by ip injection at either a low dose (25 mg/kg, seizures not observed) or high dose (75 mg/kg, producing clinical seizures). AAAD activity was assayed in the striatum and midbrain 30 min later. n = 8 - 10.
experiments looking at non-kinase mediated phorbol ester effects. 4-α-PMA had no effect on AAAD activity (Table 6). Chelerythrine is a selective inhibitor of PKC with an IC$_{50}$ = 0.66 μM. When coadministered with PMA, chelerythrine (0.132 nmol, icv) blocked the PMA-mediated increase in AAAD activity (Table 6). Chelerythrine had no effect when administered alone at that dose.

To examine the temporal course of the PMA-induced increase of AAAD activity, mice were killed 5, 10, 15, 30, 45, 90 and 180 min after an icv PMA injection. While AAAD activity increased very rapidly (within 2 min) after forskolin treatment, there was at least a 10 min lag before PMA-mediated increases in AAAD activity were observed. Significant increases of enzyme activity were seen as early as 15 min after PMA treatment (Figure 15). The maximal increase, 33% over control, was seen at 30 min, and activity returned to basal levels by 90 min. At 180 min after the injection, AAAD activity remained at basal levels. A similar time course and magnitude (27% above control) of change was seen in the midbrain (Figure 16).

Since the increased AAAD activity could potentially be due to a change in the level of AAAD protein expressed, the protein synthesis inhibitor, cycloheximide, was administered 10 mg/kg body weight, ip, 1 hr prior to the icv injection of PMA to see if inhibition of protein synthesis could block the PMA-induced increase in AAAD activity. Cycloheximide had no effect on
Table 6. Chelerythrine Blocks the PMA-Mediated Increase in Striatal AAAD Activity, while 4-α-PMA Has No Effect on AAAD Activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAAD Activity (nmol/mg prot/20 min ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>4-α-PMA</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>PMA</td>
<td>52 ± 4*</td>
</tr>
<tr>
<td>PMA + Chelerythrine</td>
<td>37 ± 2</td>
</tr>
</tbody>
</table>

Two nmol PMA, 2 nmol 4-α-PMA, or 0.132 nmol chelerythrine were injected icv individually or in combination. Animals were killed 30 min later and AAAD activity assayed in the striatum. n = 5 - 10. * p < 0.01 compared with vehicle group.
Figure 15. AAAD activity increases in mouse striatum after PMA (2 nmol, icv). Animals were killed at the indicated times and AAAD activity assayed. n = 9 - 13. * p < 0.01 compared with zero-time.
Figure 16. AAAD activity increases in mouse midbrain after PMA (2 nmol, icv). Animals were killed at the indicated times and AAAD activity assayed. n = 6 - 9. * p < 0.01 compared with zero-time.
either control or PMA-stimulated AAAD activity in either the striatum or midbrain (Table 7).

Another possible explanation for the increase in AAAD activity after PMA could be a change in the affinity of the enzyme for either substrate or the cofactor. Three to 4 separate determinations of the kinetic parameters- \( V_{\text{max}} \) and the \( K_m \) for L-DOPA and for pyridoxal 5'-phosphate were made for the striatum and midbrain 30 min after icv administration of 2 nmol PMA in 2 \( \mu l \) DMSO. For these studies, the concentration of either substrate or cofactor was held at saturating concentrations, while the concentration of the other compound was varied. Otherwise, the AAAD assay was as described in the Materials and Methods section. Kinetic studies revealed a significant increase in the apparent \( V_{\text{max}} \) with no changes in the apparent \( K_m \) for either L-DOPA or pyridoxal-5'-phosphate (Table 8).

E. Effects of PMA on TH Activity

Since TH activity has been shown to be increased by phorbol esters in \( in \ vitre \) preparations (Onali and Olianas, 1987) a series of experiments were designed to investigate the temporal changes in TH activity after PMA \( in \ vitre \), and to see how they might correlate with changes in AAAD activity. A time course study was done, looking at 2, 5, 10, 15, 30, 45, 90 and 180 min after
Table 7. PMA Enhances AAAD Activity in Mouse Striatum in the Presence or Absence of Cycloheximide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAAD Activity (nmol/mg prot/20 min ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>PMA</td>
<td>53 ± 3*</td>
</tr>
<tr>
<td>PMA + Cycloheximide</td>
<td>47 ± 1*</td>
</tr>
</tbody>
</table>

Animals were treated with cycloheximide (10 mg/kg, ip) 1 hr before administration of PMA (2 nmol, icv) and were killed 30 min after PMA treatment. n = 5 - 10. * p < 0.01 compared with vehicle group.
Table 8. Apparent Kinetics of AAAD Following PMA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$V_{\text{max}}$ (nmol/ mg prot/ 20 min ± SEM)</th>
<th>$K_{\text{m}}$ (uM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L-DOPA</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>33 ± 1</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>PMA</td>
<td>45 ± 2*</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>32 ± 1</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>PMA</td>
<td>45 ± 1*</td>
<td>27 ± 3</td>
</tr>
</tbody>
</table>

Mice were treated with PMA (2 nmol, icv) and killed 30 min later. AAAD activity in striatum and midbrain were assayed. Apparent $V_{\text{max}}$ and $K_{\text{m}}$ values were estimated from Hofstee plots. n = 3 - 4 experiments with 3 animals per group. * p < 0.01 compared with vehicle group.
the icv injection of PMA. TH activity was increased after administration of PMA in a time-dependent manner (Figure 17). Administration of PMA resulted in a more gradual and longer increase in striatal TH activity than seen with forskolin. TH activity was increased as early as 5 min, peaked at 15 to 30 min (40 - 55 % above control), and gradually returned to baseline by 90 min after injection, where it remained through the 180 min time point.

Figure 18 compares the time-course of the changes of TH and AAAD after PMA. As seen, after PMA injection, TH activity rises more quickly than AAAD. The first significant time point for TH is at 5 min and for AAAD is not until 15 min. The maximal activation for both enzymes is at 30 min. The decline in activity towards baseline is faster for TH than AAAD.

F. Effects of PMA on DA Metabolism

As a general observation, PMA increased the content of DA, DOPAC and HVA in the striatum (Figure 19). DA and DOPAC followed a similar pattern, increasing between 5 and 30 min after PMA treatment, returning to basal between 45-90 min and rising again at 3hrs after the treatment. The observed increases for DA were about 13% to 25% over control and for DOPAC 16% to 32% over control. The increases in HVA were larger (22% to 58% over control) and more enduring. HVA increased gradually and reached
Figure 17. TH activity increases in response to PMA (2 nmol, icv). Mice were killed at indicated times and striatal TH activity was assayed. n = 7-13. * p < 0.01 compared with zero-time point.
Figure 18. Striatal AAAD and TH activities following icv injection of 2 nmol PMA. Mice were killed at indicated times after injection and enzyme activities were assayed.
Figure 19. Effect of PMA (2 nmol, icv) on striatal DA, DOPAC and HVA. Animals were killed at indicated times and striatal DA, DOPAC and HVA content determined. Data are means ± SEM as % of respective control n = 7 - 13. Basal values are 51 ± 1, 531 ± 7 and 36 ± 1 pmol/mg protein ± SEM. *p < 0.01 compared with vehicle.
significance at about 15 min after PMA. It peaked by about 30 min and remained elevated for the duration of the studies for up to 3 hrs.

G. Effects of Okaidic Acid on AAAD Activity

Okadaic acid, a serine/threonine-selective protein phosphatase 1 and 2A inhibitor, was used to explore whether endogenous kinase basal activity could be sufficient to increase AAAD activity. By inhibiting phosphatase(s) activity, the phosphorylating effect of endogenous kinase(s) would effectively be potentiated, since the counterbalancing phosphatase activity would be eliminated or reduced. Okadaic acid (0.2 nmol in 2 μl artificial CSF) increased AAAD in striatum and midbrain by about 30 min after the treatment, the earlier time studied (Table 9). The co-administration of a dose of forskolin or PMA that maximally increased AAAD together with okadaic acid did not further enhance AAAD activity when studied at 30 min (Table 10).

Studies in MPTP-Lesioned Mice

A. Setting Conditions for MPTP Lesion Studies.

Two different lesioning paradigms are used in our laboratory. One induces an about 80+% lesion of dopaminergic neurons, and the other, a
Table 9. Okadaic Acid Increases AAAD Activity in the Striatum and Midbrain

<table>
<thead>
<tr>
<th>Time</th>
<th>Striatum (nmol/mg prot/20 min ± SEM)</th>
<th>Midbrain (nmol/mg prot/20 min ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>32 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>Okadaic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>39 ± 1*</td>
<td>40 ± 1*</td>
</tr>
<tr>
<td>45 min</td>
<td>34 ± 2</td>
<td>39 ± 2*</td>
</tr>
<tr>
<td>90 min</td>
<td>30 ± 1</td>
<td>37 ± 1</td>
</tr>
</tbody>
</table>

Okadaic acid (0.2 nmol in 2 μl of artificial CSF was administered icv and AAAD activity assayed at the indicated times. (n = 5 - 9). *p < 0.01 compared with vehicle group.
Table 10. Inhibition of Phosphatases does not Effect the PMA or Forskolin Mediated Increase in AAAD Activity in Striatum and Midbrain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Striatum</th>
<th>Midbrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>34 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>Okadaic Acid</td>
<td>39 ± 1*</td>
<td>40 ± 1*</td>
</tr>
<tr>
<td>PMA</td>
<td>44 ± 1*</td>
<td>44 ± 1*</td>
</tr>
<tr>
<td>PMA+Okadaic Acid</td>
<td>43 ± 1*</td>
<td>40 ± 1*</td>
</tr>
<tr>
<td>Forskolin</td>
<td>43 ± 2*</td>
<td>44 ± 1*</td>
</tr>
<tr>
<td>Forskolin+Okadaic Acid</td>
<td>39 ± 2*</td>
<td>42 ± 2*</td>
</tr>
</tbody>
</table>

PMA (2 nmol), forskolin (2 nmol) or okadaic acid (0.2 nmol) were injected icv alone or in combination and AAAD activity was assayed in striatum and midbrain 30 min later. n = 5 - 8. *p<0.01 compared with vehicle group.
more moderate lesion, with a loss of 50 - 60% of dopaminergic neurons (Weihmuller et al., 1988) as measured by decreases in DA and DOPAC levels. In addition to the size of lesion, another consideration was the time after lesioning that you look for enzyme modulation, e.g. immediately after the lesion, or after a period of time that allows for stabilization of the lesion and development of compensatory changes. Using animals with large or moderate lesions and looking immediately after the completion of the treatment with MPTP, no changes in AAAD activity were observed 30 min after PMA or forskolin treatments (data not shown). Using a moderate lesion, produced by a 7 day course of MPTP injections followed with a 2 week recovery period, changes in TH and AAAD enzyme activities were observed. These results are discussed below.

B. Effects of Forskolin on AAAD, TH and DA Metabolism in the Striatum of the MPTP-Treated Mouse

The MPTP regimen used reduces AAAD activity to 51% of that seen for normal mice (17.0 ± 0.3 nmol/mg prot/20 min ± SEM for MPTP-treated mice versus 33.0 ± 0.5 nmol/mg prot/20 min ± SEM for non-lesioned mice). Forskolin had more pronounced effects on AAAD activity in the MPTP-lesioned striatum that in the non-lesioned striatum. The early changes of AAAD activity after forskolin treatment appear to follow a similar time
course in MPTP-lesioned mice and in normal mice, with significant increases in activity observed from 2 to 30 min after injection (Figure 20). However, the magnitude of the response was increased. In the lesioned animals, the maximal increase was 47% over MPTP control at 10 min compared to 30% over control in normal mice at the same time point (Figure 21). Interestingly, there is a second increase at 90 min in the MPTP-treated striatum that was not seen in normal mice (Figure and 21).

MPTP decreased TH activity in striatum to about 45% of normal (0.10 ± 0.01 nmol/mg prot/20 min ± SEM for MPTP-treated mice versus 0.22 ± 0.01 nmol/mg prot/20 min ± SEM for normal mice). As shown in Figure 22, in the MPTP-treated mouse, forskolin caused a significant increase in TH activity by 5 min, it reached a maximum of 110% over MPTP control by 15 min and remained significantly elevated at 45 min after forskolin treatment. Ninety min after forskolin treatment, TH activity had returned to baseline in the MPTP-lesioned striatum, but appears to be increasing again by 180 min (40% over baseline). Both the magnitude and the time course of the TH response to forskolin were altered in the striatum of the MPTP-lesioned mice (Figure 23). Following MPTP-lesioning, TH activity doubles at its maximal activation and is elevated from 5 to 45 min in comparison with normal mice where activity is 1 1/2 time the basal and activation is more transient, lasting only from 2 to 10 min.
Figure 20. Time course of striatal AAAD activity after forskolin (2 nmol, icv) administered to MPTP-lesioned mice. Animals were killed at indicated times and AAAD activity was assayed. Dashed lines indicate normal control (32 ± 1 nmol/mg prot/20 min) and MPTP control (17 ± 1 nmol/mg prot/20 min). n = 7 - 14. * p < 0.01 compared with zero-time.
Figure 21. Striatal AAAD activity after forskolin (2nmol, icv) in normal and MPTP-lesioned mice expressed as % of respective control. Basal values are 32 ± 1 and 17 ± 1 nmol/mg prot/20 min, respectively.
Figure 22. Striatal TH activity increases in MPTP-lesioned mice in response to icv forskolin injection (2 nmol in 2 μl DMSO). Mice were killed at indicated times after injection and striatal TH activity was assayed. Dashed lines are for normal (0.22 ± 0.01 nmol/mg protein) and MPTP-lesioned (0.10 ± 0.01 nmol/mg protein/20 min) TH activities (n = 7-13). * p < 0.01 compared with zero-time point.
Figure 23. Striatal TH activity after forskolin (2 nmol, icv) in normal and MPTP-lesioned mice expressed as % of respective control. Basal values are 0.22 ± 0.01 and 0.10 ± 0.01 nmol/mg prot/20 min, respectively.
The levels of DA and its acidic metabolites were reduced by MPTP lesioning. Basal levels for DA, DOPAC and HVA were 219 ± 8, 28 ± 1, and 22 ± 1 pmol/mg prot ± SEM, respectively in the MPTP-lesioned mice. This reduction by lesioning varied: DOPAC content was 54% of non-lesioned controls; DA was reduced to 40%; and HVA was only reduced to 60% of non-lesioned controls.

Overall, dopaminergic denervation appears to alter the magnitude of response of DA and DOPAC to forskolin and not the temporal pattern of the change (Figure 24). DA content was significantly elevated between 10 and 30 min, with the maximal increase 52% greater than control at the 30 min time point. DA content was increased again by 90 min, and declining by 180 min (37% and 31% over control). DOPAC levels were significantly elevated at 10 and 30 min, with the maximal increase 30 min after injection of forskolin. The maximal response was also greater, about 50% over respective basal values instead of 33% as in non-lesioned mice. HVA was found to be most greatly affected by forskolin. The response was earlier, by 2 - 5 min, and greater, maximal increase about 82% over the MPTP control values by 30 min after the treatment.
Figure 24. Forskolin (2 nmol, icv) increases striatal DA, DOPAC and HVA in MPTP-lesioned mice. Animals were killed at indicated times and striatal DA, DOPAC and HVA content determined. Data are means ± SEM as % of respective control (n = 8 - 12). Basal values for DOPAC, DA and HVA are 28 ± 1, 214 ± 7, and 22 ± 2 pmol/mg prot, respectively. * p < 0.01 compared with vehicle.
C. Effects of PMA on AAAD, TH and DA Metabolism in the Striatum of the MPTP-Treated Mouse

Treatment with MPTP appears to abolish the PMA-induced increase of AAAD activity in the striatum (Figure 25). No changes were found in AAAD activity at any of the time points examined. Similarly, the response of TH to PMA was blunted, with the only significant increase, about 54% over baseline, found at 15 min after PMA administration (Figure 25). The magnitude of this response is similar to that observed in unlesioned mice.

With respect to dopamine metabolism in striatum, the MPTP lesion eliminated the response of DA to PMA. DA content was below basal levels at all time points studied (Figure 26), although they did not reach significance. Both the magnitude and the pattern of response to PMA of DOPAC were not altered by MPTP (Figure 26), with the exception being the 30 min time point where DOPAC was increased about 71% over the MPTP control level and was significantly more increased than any changes in unlesioned mice. The early phase of the rise of HVA after PMA was maintained in the MPTP-treated animals and the response of the metabolite at the 30 min point (123% over the MPTP control values) was significant different from the unlesioned controls. However, MPTP treatment blunted the PMA-induced increases in the HVA seen in unlesioned animals during the later times of the study, 45-180 min (Figure 26).
Figure 25. AAAD and TH activities in MPTP-lesioned mouse striatum after icv PMA injection (2nmol in 2 μl DMSO). Animals were killed at indicated times after PMA injection and enzyme activities were assayed. Activity is expressed as % of basal activity ±SEM (AAAD 17 ± 1 nmol/mg prot/20 min, TH 0.11 ± 0.01 nmol/mg prot/20 min) in MPTP-lesioned mouse. n = 6 - 11
Figure 26. Effect of PMA (2 nmol, icv) on striatal DA, DOPAC and HVA in MPTP-lesioned mice. Animals were killed at indicated times and striatal DA, DOPAC and HVA content determined. Data are means ± SEM as % of respective control. n = 5 - 13. Basal values for DOPAC, DA and HVA are 28 ± 1, 253 ± 23, and 26 ± 1 pmol/mg prot, respectively. *p < 0.01 compared with vehicle.
CHAPTER III
DISCUSSION

Studies in Normal Mice

A. Forskolin and PMA-Induced Changes in AAAD Activity

Forskolin is a powerful activator of adenylate cyclase and results in increased generation of cAMP. Forskolin, through its effect on adenylate cyclase, transiently increased AAAD activity in the striatum and midbrain after icv injection with similar time courses and degree of activation. That the increase of AAAD activity was dependent on the generation of cAMP was confirmed by the ability of the cAMP analogue 8-Br-cAMP to mimic the forskolin effect.

Kinetic studies 30 min after forskolin administration showed an increase in the apparent $V_{\text{max}}$ with no change in the apparent $K_m$ for either L-DOPA or pyridoxal-5'-phosphate. The activation of AAAD occurred without a change in affinity of the enzyme for substrate or cofactor. One possible explanation would be an increase in the total amount of enzyme present.
This possibility was eliminated when it was shown that the forskolin-mediated increases in AAAD activity 30 min after injection were independent of protein synthesis in the experiments with cycloheximide. Alternatively, under basal conditions, the enzyme pool may be composed of a mixture of more and less active (or inactive) forms of AAAD, and PKA activation increases the ratio of the active form to the less active form, leading to an increase in the apparent $V_{\text{max}}$. Phosphorylation of AAAD by PKA is a possible mechanism for the activation of AAAD. This notion is supported by the presence of potential phosphorylation sites (serine residues 220 and 336) in the deduced AAAD amino acid sequence (Kang and Joh, 1990, our laboratory) and the relatively fast changes in enzyme activity seen after forskolin. Furthermore, two charge isoforms of AAAD have been identified which may be the consequences of post-translational modification (Park et al., 1992), perhaps phosphorylation. Under this scenario, the observed changes in $V_{\text{max}}$ suggest that under basal conditions AAAD is partially phosphorylated. This notion is further supported by the fact that the AAAD increase after forskolin or 8-br-cAMP never exceeded 30% over control. In agreement with our contention that forskolin phosphorylates AAAD in vivo is the work of Berry et al. (1995, submitted) in our laboratory demonstrating phosphorylation of AAAD in synaptosomal preparations of striatum after the addition of the catalytic subunit of PKA.
PMA is a potent activator of PKC. PMA, but not the inactive analogue 4-α-PMA, apparently activated PKC and increased AAAD activity in the striatum and midbrain after icv injection. In addition, when chelerythrine, a selective PKC inhibitor (Herbert et al., 1990), was co-administered with PMA, the PMA-mediated enhancement of enzyme activity was blocked, also suggesting that the response is associated with activation of PKC. As opposed to forskolin, the PMA-induced increase in AAAD was relatively delayed. However, similar to forskolin, PMA increased the apparent $V_{\text{max}}$ of the enzyme. Following the same rationale as with forskolin, PMA by activating PKC might cause phosphorylation of AAAD. Indeed, the deduced amino acid sequence of AAAD (Kang and Joh, 1990, Gudehithlu et al., 1992) contains a potential site for phosphorylation by PKC.

As mentioned above PMA increased the apparent $V_{\text{max}}$ of the enzyme while having no apparent effect on the $K_m$ for either substrate or cofactor. The $V_{\text{max}}$ calculated here was in agreement with that from the PMA dose-response curve. Although there was an increase of $V_{\text{max}}$, the increase was not dependent on protein synthesis as the protein synthesis inhibitor cycloheximide did not block the response to PMA. The increased $V_{\text{max}}$ and the absence of a cycloheximide effect are similar to those seen after forskolin treatment. Once again, the total amount of enzyme does not change, rather the existing enzyme molecules become more active. Whether or not this is due to direct phosphorylation of AAAD by PKC is unknown.
It was also noted that the increase in AAAD activity with PMA treatment at 30 min was more pronounced in the striatum than in the midbrain. This divergence may be due to differences of PKC content or subtype and/or responsiveness in the two regions, different regulation of the enzyme in the cell bodies versus neuronal terminals or differences in the subcellular localization of PKC in the two regions.

PMA has been shown to have convulsant properties when administered icv in mice (Smith and Meldrum, 1992) and we observed convulsions in some of our mice. Seizures result from excessive neuronal activity and it is known that TH activity in the striatum is tied to changes in neuronal activity (Chowdhury and Fillenz, 1988), which means that seizures could change dopaminergic neuronal activity and DA synthesis. With that serving as the model, it was necessary to investigate whether the observed changes of AAAD activity with PMA were secondary to subclinical or clinical seizure activity. Pentylenetetrazol is a drug commonly used to induce seizures. The mechanism by which it causes seizures is not clear, however it is known that it does not alter cAMP levels in the striatum (Ferrendelli and Kinscherf, 1977). Neither the high nor low dose of pentylenetetrazol had significant effects on AAAD activity in striatum or in midbrain, indicating that the PMA effect on AAAD is not secondary to observed seizure activity.

When comparing changes in AAAD activity produced by forskolin or PMA treatments, it appears that the maximal activation with each treatment
is similar. This is also evidenced by the apparent $V_{\text{max}}$ values being nearly identical (forskolin $44 \pm 1$, PMA $45 \pm 2$ nmol/mg prot/20 min). The major difference is in the time course of activation. AAAD activity is increased more rapidly by forskolin treatment, peaking at 5 min, while there is a 10 min lag before activation by PMA is observed. AAAD activity after PMA treatment also peaks later, at 15 - 30 min. Activity is declining after 30 min with both treatments, and AAAD activity is at basal levels by 90 min. It is possible that differences lie in the balance between activation states of the particular kinases and phosphatases involved, or in the potential involvement of direct activation by phosphorylation versus indirect activation through feedback polysynaptic loops.

It appears that activation of 2 pathways involving protein kinases (PKA and PKC) increase AAAD activity. Additional support for regulatory phosphorylation modulating AAAD activity comes from the studies with the protein phosphatase inhibitor, okadaic acid. Okadaic acid administered alone increased AAAD activity 30 min after injection. For a protein to be regulated by phosphorylation, the activities of the protein kinase(s) and protein phosphatase(s) acting on a particular site must be in equilibrium; otherwise, the protein would be either fully phosphorylated or completely dephosphorylated (Hunter, 1995). The observation that phosphatase inhibition alone augments the enzyme activity supports the hypothesis that phosphorylation is involved. Since inhibition of phosphatases increases
AAAD activity, endogenous kinase and phosphatase activities sustaining some basal level of phosphorylation (of AAAD?) appear to normally regulate AAAD activity. Whether phosphorylation is direct or indirect remains to be determined.

Under the treatment conditions used, the observation that combined okadaic acid/PMA or okadaic acid/forskolin treatment did not increase AAAD activity above either treatment alone suggests either complete activation of the enzyme by the individual treatments or that they target the same site. Comprehensive time- and dose-response studies would be necessary to demonstrate the relationship between the two competing processes. The balance between protein kinase and opposing protein phosphatase activity would also be critical in determining the extent and duration of the response.

8-Br-cGMP was without effect on AAAD activity, although it has been shown to increase TH activity in PC12 cells and rat striatal synaptosomes (Roskowski and Roskowski, 1987). The proposed mechanism was that cGMP-dependent protein kinase was activated, which either directly phosphorylated TH, or led to the activation of PKA, which then phosphorylated and activated TH. There is no potential cGMP-dependent protein kinase recognition motif on AAAD, so this result was not unexpected.
B. Forskolin and PMA-Induced Changes in TH Activity

There is a plethora of evidence suggesting that TH is phosphorylated by PKA resulting in activation of the enzyme (Zigmond et al., 1989). In this study, it was demonstrated that forskolin is capable of increasing TH activity in a biphasic manner. There is an early rapid and transient increase in enzyme activity seen under cofactor subsaturating conditions, and a later rise. The kinetics of the delayed rise were not studied. In the early phase, the maximal increase in TH activity is at 5 min and the change is no longer significant at 15 min. The rapid increase in activity produced by forskolin is similar to *in vitro* studies using rat striatal tissue and in chromaffin cells (Haycock and Haycock, 1991, and Haycock 1993). The late increase of TH might represent cAMP-dependent enhanced transcription. The TH gene contains a CREB binding site and cAMP has been shown to modulate TH gene transcription (Kim et al., 1994).

TH and AAAD activities rise in parallel after forskolin treatment. However, AAAD remains activated for a longer time and returns to basal activity more slowly than TH, which returns to basal levels quickly. The temporal differences in the activation/deactivation of the two enzymes might be due to the fact that only 80% of striatal AAAD is colocalized with TH in dopaminergic neurons and that AAAD in other neurons (serotonergic, adrenergic) or glia cells might respond differently to forskolin treatment.
Alternatively, the temporal differences in activation/deactivation would arise from either differential access of PKA to the two substrates, AAAD being mostly cytosolic and TH particulate, and/or from differing rates of dephosphorylation, different affinities of a single phosphatase for TH and AAAD, or that multiple phosphatases are involved.

If TH is rate-limiting, what physiological significance would the prolonged elevation of AAAD activity relative to TH after forskolin treatment have? The initial parallel rise in activity may serve to keep TH activity rate-limiting for the synthesis of DA. The longer activation of AAAD may allow the enzyme to have some role in the regulation of DA synthesis after TH deactivation to maintain high intracellular DA levels thus responding to the increased metabolic demands due to enhanced neuronal function. Such a mechanism presumably will prevent neuronal depletion of DA due to accelerated release and catabolism of the amine. Indeed, as will be discussed later, the changes in DA metabolism after forskolin follow more closely the temporal changes of AAAD activity. Alternatively, the prolonged increase may be more important for the synthesis of trace amines, including phenylethylamine (Dyck et al., 1983), which has been shown to modulate DA synthesis and release in rat striatal synaptosomes (Roberts and Patrick, 1979) and in rabbit brain (Borison et al., 1975). Iontophoretically applied phenylethylamine has also been shown to potentiate neuronal responses of
caudate neurons to DA or to electrical stimulation of the substantia nigra (Paterson et al., 1990).

Phorbol esters and PKC have been shown to increase TH activity in the superior cervical ganglion (Wang et al., 1986) and striatal synaptosomes (Onali and Olianas, 1987), and to increase phosphorylation of TH in vivo (Haycock and Haycock, 1991). In agreement with the above findings is our study, where activation of PKC by icv PMA transiently increased TH activity in the mouse striatum in a time-dependent manner. Under subsaturating cofactor conditions, enzyme activity rose as early as 5 min, peaked at 30 min and returned to basal levels by 45 min after a PMA injection. Although there is evidence that TH is phosphorylated by PKC in vitro, this phosphorylation did not result in enzyme activation (Funakoshi et al., 1991). So, it is not clear whether the TH increase observed in the striatum after PMA is due to phosphorylation, or that another mechanism(s) is involved. The finding that TH activity rose more slowly after PMA than after forskolin suggests that activation and participation of other mechanisms is involved. It has been suggested that PKC may contribute to the activation of TH indirectly, by promoting the action of PKA (Funakoshi et al., 1991).

ICV administration of PMA transiently increased the activity of AAAD and TH. The time courses for activation of TH and AAAD by PMA are similar in the rapidity of the rise of activity but differ in the rate of deactivation of each enzyme, with TH being more rapidly deactivated. TH
activity had returned to baseline by 45 min, while AAAD activity was still declining to baseline, having reached it by 90 min. The mechanism(s) involved in the activation of AAAD by PMA are not presently known. Although the amino acid sequence of AAAD contains a potential site for PKC-dependent phosphorylation, there is no information on phosphorylation of AAAD by PKC \textit{in vitro}. Alternatively, it is possible that PKC does not phosphorylate AAAD directly and that the observed enzyme increase is secondary to changes in the activity of dopaminergic neurons, and/or to postsynaptic feedback neuronal circuits. An intriguing explanation of the data is cross-talk between PKA and PKC pathways whereby PKC phosphorylates adenylate cyclase and activates PKA (Yoshimasa et al. 1987), which in turn activates AAAD. Regardless of the mechanism(s) involved, it is obvious that activation of TH is accompanied by activation of AAAD, stressing once again the possible role of AAAD for DA synthesis and maintenance.

Both PMA and forskolin treatments seem to produce the same degree of activation of TH under the conditions used, with differing time courses. PMA activation of TH was gradual, rising over the first 30 min and returned to baseline by about 90 min. In contrast, transient changes in TH activity with forskolin treatment were very rapid. Activity peaked at 5 min and returned to baseline by 30 min. Different pharmacokinetics might explain the observed differences. Given that both drugs were given icv in the same vehicle and
assuming similar tissue penetration we feel that the drug effect is minimal. The most plausible interpretation of the findings is that PKC-dependent phosphorylation of TH in vivo does not result in activation of the molecule, as seen in vitro (Funakoshi et al., 1991). Other mechanisms activating TH, for example PKA activation, which also phosphorylates TH at serine 40 (Joh et al., 1978 and Funakoshi et al., 1991) are required. Activation of other intermediate signals might explain the slower activation of TH after PMA. An interesting finding that further differentiates the effects of forskolin from those of PMA is the late increase of TH activity seen 90 min after forskolin treatment which was not found after PMA administration. As discussed above, the late increase in TH activity may be due to cAMP-mediated transcription of the TH gene.

Both the forskolin- and PMA-induced activation of AAAD were of about the same magnitude, however, as with TH, the time course of activation differed. Again AAAD activity rose more quickly after forskolin, while the activation of the enzyme by PMA was more gradual. As discussed above, differences in the mechanisms involved in the activation of AAAD by PKA and PKC might account for these observations.

From these studies, it appears that both TH and AAAD in striatum rise and peak in parallel, rapidly after forskolin and gradually after PMA. An explanation for this observation might be that AAAD is co-modulated with TH to ensure the demand-dependent synthesis of DA.
C. Forskolin and PMA-Induced Changes of DA Metabolism

In an attempt to determine whether DA metabolism changes after the assumed manipulations of PKA and PKC, the content of DA, DOPAC and HVA were estimated in the striatum. As it was anticipated, DA and its acidic metabolites increased after both forskolin and PMA. The observed increases were of small to moderate magnitude, and in general, the increases of DA and DOPAC preceded those of HVA. It should be noted, however, that the increases of HVA content were much bigger than those of DA or DOPAC. Studies in the striatum by Westerink (1979), observing time-response curves of striatal DOPAC and HVA concentrations during several pharmacological treatments that modulate DA synthesis, produced consistent temporal changes in metabolites, with DOPAC levels increasing before HVA levels.

After forskolin treatment, the increases in DA and its metabolites were, generally, observed after TH activity had peaked, but AAAD activity was still elevated. After returning to control levels, DA, DOPAC and HVA levels were elevated again at 90 min, when TH activity had increased for a second time. Santiago and Westerink (1990) using in vivo microdialysis showed that intrastriatal infusion of 8-Br-cAMP, forskolin or the phosphodiesterase inhibitor, iso-butyl-methyl-xanthine, produced increases in the extracellular levels of DA, DOPAC and HVA. Although the pattern of the temporal changes of DA and its metabolites follows those of AAAD
activity, the fact that PKA activates TH (Joh et al., 1978), AAAD (Young et al., 1993 and 1994) and facilitates DA release (Bowyer and Weiner, 1989) makes it difficult to draw conclusions about the importance of modulation of AAAD activity for control of DA synthesis.

It is possible that the prolonged activation of AAAD is required to replenish and maintain the intracellular levels of DA, as accelerated release and catabolism, as evidenced by elevated content of HVA and DOPAC, might deplete the releasable pools of the neurotransmitter.

Elevations of DA and its metabolites, DOPAC and HVA, were also seen in the striatum after icv administration of PMA. Again, increases of DA and DOPAC preceded the increases of HVA as observed after forskolin treatment. DA and DOPAC content increased as early as 5 min after PMA treatment, while HVA rose gradually and reached significant levels by 15 min. As with forskolin treatment, the increases of DA and its metabolites were small to moderate, with that of HVA been the largest in magnitude and longer lasting. The temporal changes of DA and DOPAC after PMA follow those of TH and returned to control values by 90 min. The pronounced prolonged rise of HVA suggests facilitation of the extracellular metabolism of DA. This might be due to enhanced release of DA and or a decrease in DA reuptake. Indeed, phorbol esters have been shown to increase DA release (Chandler and Leslie, 1989), and to decrease DA uptake (Copeland et al., 1994) in striatal synaptosomes. Both these changes could cause increased extraneuronal
conversion of released DA to HVA. Drugs which alter dopaminergic neuronal activity and enhance release of DA are attended by increased levels of metabolites, particularly HVA (Roth et al., 1976). Because PMA, presumably via activation of PKC, alters multiple sites on the metabolic pathway of DA, it is difficult to draw conclusions about the role of TH or AAAD in the observed changes. However, data from the PMA studies are in general agreement with those from the forskolin studies, and point towards a role for AAAD in the homeostasis of intracellular DA.

**Studies in MPTP-Lesioned Mice**

The day following the completion of MPTP treatment, no changes in AAAD activity were observed with icv forskolin or PMA injections (data not shown). MPP⁺ has been shown to inhibit TH activity (Nagatsu, 1990) and reduce its phosphorylation (Kiuchi et al., 1988). MPP⁺ has also been shown to inhibit AAAD activity (Naoi et al., 1988). Following MPTP injection, MPTP and its metabolites are still being excreted by the mice 24 to 48 hrs later. It is possible that inhibition of AAAD by MPP⁺ is why no changes were observed in AAAD activity one day after finishing the MPTP injections. By waiting 2 weeks after lesioning, any complications of direct inhibition of the enzymes is remote. Moreover, there has been time for damaged neurons to die, and for the system to adjustment to the lesion.
In our model, MPTP causes a 50% lesion as measured by decreases in DA, DA uptake (Hadjiconstantinou et al., 1988), TH and AAAD (these studies) in striatum, and loss of TH-immunoreactive cells in substantia nigra (Eaton et al., 1993). There are a number of compensatory changes made in the striatum after MPTP-lesion. TH and AAAD activity gradually recover (Hadjiconstantinou et al., 1990), DA receptor numbers increase (Weimuller et al., 1990), and DA turnover accelerates (Hadjiconstantinou et al., 1988). mRNA levels for glutamate decarboxylase (Sauer et al., 1995) and preproenkephalin (Gudehithlu et al., 1991) are increased, while mRNA for Substance P is decreased (Pérez-Otaño et al., 1992).

A. Forskolin Effects on AAAD and TH Activity

In MPTP-treated animals allowed 2 weeks to recover, TH activity is 45% and AAAD activity is 53% of that seen in unlesioned animals. The response of striatal AAAD and TH to forskolin was amplified by MPTP lesions. The time course of the increases in AAAD activity remained the same in lesioned animals as in normals. However, the magnitude of the response was increased in MPTP lesioned mice to ~60% of MPTP lesioned control as compared to ~30% of normal control at maximal activation. There was also a second increase at 90 min that was not seen in unlesioned mice. Activation of TH by forskolin was of a greater magnitude (110% over basal
compared to 67% in lesioned and normal animals, respectively), but slightly delayed in onset and longer in duration in the striatum of MPTP-treated animals. The second increase of TH was also delayed, starting at 180 min post-treatment instead of at 90 min.

The increased magnitude of both TH and AAAD activity may reflect development of supersensitivity at the second messenger level. Hossain and Weiner (1993) have shown increases in basal and stimulated striatal cAMP levels which persist for 2 to 3 months after unilateral 6-OHDA lesions. If similar changes occur within the dopaminergic terminals after MPTP, this would explain the greater activation of the enzymes in the MPTP-lesioned mice. Alternatively, altered enzyme protein and post-translational modification might account for the supersensitive response.

B. PMA Effects on AAAD and TH Activity

While the response of AAAD and TH to forskolin was potentiated in the striatum of MPTP-lesioned mice, responses of both enzymes to PKC activation by PMA are blunted or eliminated compared to normal mice. Whereas TH activity is significantly elevated from 5 to 30 min and increased gradually to a maximum of ~50% over control in unlesioned mice, TH activity was only significantly elevated, ~23% over MPTP control, at the 15 min time point. This increase is not easily explained. AAAD activity was
unchanged by PMA treatment in MPTP lesioned mice. The loss of response may be due to decreased levels of PKC in the striatum and substantia nigra. It has been shown that phorbol ester binding, a measure of PKC levels, is significantly reduced in the substantia nigra, caudate putamen, and pallidum of brain from PD patients (Nishino et al., 1989). It is possible that MPTP has a deleterious effect on PKC or disrupts the pathway that brings about activation of TH and AAAD via PMA. Alternatively, the enzyme protein synthesized in the remaining dopaminergic neurons after a MPTP-lesion may undergo different post-translational modifications.

C. Forskolin and PMA Effects on DA Metabolism

Following MPTP lesion, DOPAC and HVA levels were reduced to a lesser extent (54% and 64% of non-lesioned mice, respectively) than DA concentrations (40% of non-lesioned mice). The ratio of metabolites (DOPAC + HVA) to DA is greater in the striatum of the MPTP-lesioned animals than in normal mice (~1.6 times increased) suggesting an increase in DA turnover in the surviving neurons. The smaller decrease in HVA relative to DOPAC may also be indicative of the loss of DA uptake sites which may shift the primary route of DA inactivation from reuptake and subsequent conversion to DOPAC to that of extraneuronal conversion by COMT to HVA (Altar et al., 1987).
Hefti et al. (1985) were able to increase the ratio of DA metabolites (DOPAC + HVA) to DA in the 6-OHDA-lesioned striatum with haloperidol or morphine treatments, which increase the firing rate and DA metabolism, showing that DA synthesis is already increased by the lesion, but there can be further stimulation. These studies show that enzyme activity and DA metabolism can also be further increased in MPTP lesioned mice after forskolin treatment. Although the overall pattern of temporal changes remained the same, striatal levels of DA, DOPAC and HVA were elevated to a greater extent in the MPTP-treated than in the normal mice treated with forskolin. The ratio of (DOPAC + HVA) to DA was further increased in lesioned mice after forskolin treatment (~2.2 times at maximum) indicating further enhancement of the DA turnover. Thus the supersensitive response of TH and AAAD to forskolin in the denervated striatum is accompanied by a similar supersensitive response of DA metabolism.

The response of DA and its metabolites to PMA was blunted in the striatum of the MPTP-treated animals. The content of DA remained below control levels during the 3 hrs of the studies, while the content of DOPAC fluctuated above the control level without reaching statistical significance, except at 30 min. HVA levels, however, increased indicating enhanced extraneuronal metabolism, probably due to PMA-induced increase in DA release. If this interpretation is correct, then the results suggest that the PMA-induced activation of TH and AAAD and the PMA-induced increase of DA
release are mediated via two separate mechanisms, one sensitive to MPTP-lesioning and another that is not.
CONCLUSIONS

In conclusion, icv administration of forskolin, cAMP analogues, PMA and phosphatase inhibitors increase AAAD activity in the striatum and midbrain of mice. These results suggest that phosphorylation and dephosphorylation of AAAD may normally modulate AAAD activity. Modulation of AAAD activity may allow it to be rate-limiting for DA synthesis under certain conditions. Alternatively, it may serve to keep TH activity rate-limiting or it may be important for regulation of trace amine synthesis, thereby indirectly influencing dopaminergic function.

While in normal mice TH and AAAD activities are regulated roughly in parallel by PKC and PKA, there is differential regulation by the second messengers (PKC and cAMP) in lesioned mice. The response of both TH and AAAD to forskolin is potentiated in MPTP lesioned mice, while the response of TH and AAAD to PMA is blunted. This may indicate that lesions differentially effect the second messenger systems involved or that different processes initiated by the second messengers result in AAAD and TH activation.
AAAD activity is regulated *in vivo* by neuronal activity (Hadjiconstantinou et al., 1988), via neurotransmitter receptors (Hadjiconstantinou et al., 1993, Zhu et al., 1992) and second messenger-dependent pathways (Young et al., 1993 and 1994). Both long-term protein synthesis-dependent (Hadjiconstantinou et al., 1993, Hadjiconstantinou et al., 1988) and transient protein synthesis-independent mechanisms (Young et al., 1993 and 1994, Zhu et al., 1992) appear to be involved in enzyme regulation.
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