MASTER

Serotonin, adenosine and their role in the NO-cGMP pathway in the frontal rat brain

van Abeelen, J.C.M.

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Serotonin, adenosine and their role in the NO-cGMP pathway in the frontal rat brain

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Oktober 1997

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Vakgroep Neuropsychologie & Psychobiologie

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Afstudeer begeleider: Dr. J. de Vente (UM)
Preface

This report describes the study I performed during my internship of 9 months at the faculty of medicine of the university of Maastricht, department of Psychiatry and Neuropsychology. Since the beginning of my study at the university of Eindhoven I was interested in research and development. Later on in my study, although not part of my curriculum, I became more and more interested in biochemistry and etiology of diseases. The study conducted during my internship matched these interests perfectly and, although at times frustrating, motivated me to pursue a career in science.

During my stay at this department I became very interested into the world of Neuroscience and I learned a lot about the anatomy, physiology and biochemistry of the brain. This was not in the least due to my colleagues on the lab-floor, who were very helpful in answering the questions I had and assisted me in the diverse techniques I learned during my stay. Particularly the assistance and help of Marjanne is greatly acknowledged. Special thanks is given to: Wiel, for conducting the surgical lesions; Wilma, for letting me stay at her apartment. Last but not least Dr. Jan de Vente is thanked for his theoretical insight, professional guidance and help in reviewing this report.

John van Abeelen
Maastricht, October 1997
Summary

In the central nervous system disturbed serotonin (5-HT) metabolism and nitric oxide (NO) production are said to be involved in the etiology of several neurodegenerative diseases. Therefore, a possible relation between the presence of the nitric oxide producing enzyme NOS in the 5-HT system and the malfunctioning of these systems in neurodegenerative diseases have to be examined. We studied this by means of uni-lateral elimination of the 5-HT system in the frontal rat brain with 5,7-dihydroxytryptamine (5,7-DHT) and we looked if cyclic-3',5'-guanosine monophosphate (cGMP), nitric oxide synthase (NOS) and 5-HT were affected by this lesioning. Frozen sections of rat frontal brain slices were immunostained for 5-HT, cGMP and NOS, also measurements in cGMP content and NOS activity in frontal cortex and caudate putamen were performed in uni-lateral lesioned rats. 5-HT-immunostaining disappeared completely at the lesioned side. No differences in NOS- and cGMP-immunostaining could be observed between lesioned and non-lesioned side. For the measurements of cGMP content and NOS activity the same result was found, no differences between lesioned and non-lesioned side could be measured. No co-localization could be found for 5-HT and cGMP and cGMP and NOS. In the cortex 5-HT and NOS were partly co-localized as opposed to the striatum were no colocalisation could be found between 5-HT and NOS.

In addition, Miller and Hoffman (1994) proposed a relationship between adenosine induced cGMP production and 5-HT re-uptake into the presynaptic terminals. Therefore, we studied the effects of the adenosine analogue N-ethylcarboxamidoadenosine (NECA) on cGMP accumulation in slices of the frontal brain of untreated rats. cGMP immunoreactivity after stimulation with NECA appeared in astrocytes in the dorsal septum, lateral septum and cingulate cortex. The process of adenosine receptor induced cGMP production was independent of NOS activity so cGMP production did not involve the soluble guanylate cyclase system.
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<th>Description</th>
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<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CC</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine-3',5'-monophosphate</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPu</td>
<td>caudate putamen</td>
</tr>
<tr>
<td>5,7-DHT</td>
<td>5,7-dihydroxytryptamine</td>
</tr>
<tr>
<td>EHNA</td>
<td>erythro-9-(2-hydroxy-3-nonyl)adenine</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial NOS</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamate decarboxylase</td>
</tr>
<tr>
<td>GNC(s)</td>
<td>guanylate cyclase(s)</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>G-proteins</td>
<td>GTP-binding proteins</td>
</tr>
<tr>
<td>5HIAA</td>
<td>5-hydroxyindolacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible NOS</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactivity</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N6-nitro-L-arginine methyl ester hydrochloride</td>
</tr>
<tr>
<td>LSD</td>
<td>lysergic acid diethylamine</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide-adenine-dinucleotide phosphate</td>
</tr>
<tr>
<td>NECA</td>
<td>5'-N-ethylcarboxamidoadenosine</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal NOS</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>PDE(s)</td>
<td>phosphodiesterase(s)</td>
</tr>
<tr>
<td>pGNC</td>
<td>particulate guanylate cyclase</td>
</tr>
<tr>
<td>RBL2H3</td>
<td>rat basophilic leukemia cells</td>
</tr>
<tr>
<td>sGNC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SSRI</td>
<td>specific serotonin (re-)uptake inhibitors</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>triton X-100 containing tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
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</table>
General introduction

Neurochemical findings of disturbed serotonin metabolism in a number of neurological diseases have given serotonin a potential importance in the etiology of diseases. For example, Parkinson's disease is associated with significant losses of serotonin and its major metabolite 5-hydroxyindolacetic acid (5HIAA) from several brain regions. Reduced levels of serotonin have also been measured in post mortem Huntington's disease brains (Dray 1981). Given the pivotal roles that 5-HT plays in a variety of central nervous system (CNS) functions and diseases, and monoaminergic neurons frequently contain co-transmitters, it is of interest to know whether nitric oxide synthase (NOS) is part of the transmitter repertoire of these cells.

NOS is a nitric oxide (NO) producing enzyme and NO is thought to have a range of actions in the CNS, and, by virtue of its chemical reactivity, is suspected to play a role in several neurodegenerative diseases. Therefore a possible relationship between the presence of NOS in certain transmitter systems, like the 5-HT system, and the malfunctioning of these systems in neurodegenerative diseases has to be examined.

Secondly, in the frontal rat brain a large amount of cyclic guanosine-3', 5'-monophosphate (cGMP) is produced when stimulated with NO. The significance of this cGMP production is still not clear. The neuroanatomical relationship between NO-producing, cGMP synthesizing and 5-HT containing neurons was not known, and this was the first research question to be answered.

Recently a group of investigators proposed a relationship between adenosine and a cGMP mediated 5-HT re-uptake (Miller and Hoffman 1994). They established this relationship in rat basophilic leukemia cells (RBL 2H3), which is a model system with cellular components similar to those found in neuronal transmission. They proposed that this interconnection between adenosine and 5-HT re-uptake could also occur in the rat brain. In view of the role of the 5-HT re-uptake mechanism in the etiology and treatment of depressive disorders, a clear understanding of this mechanism is important. It is not known whether such a mechanism is operative in the frontal rat brain. This was one of the reasons to look for an effect of adenosine on cGMP metabolism. A second reason for looking at adenosine and cGMP production is
because of a study conducted by this group (DeVente et al, manuscript in preparation). In this study they looked at different types of phosphodiesterase (PDE) inhibitors (compounds that inhibit the hydrolysis of cyclic nucleotides) and their effect on cGMP production in the hippocampal brain area of the rat. Two of those PDE inhibitors showed a cGMP accumulation which was not expected on the score of their described specificity. It appeared that both inhibitors also had an effect on the extracellular adenosine metabolism. The two PDE inhibitors erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) and dipyridamole, respectively an adenosine deaminase inhibitor reported to be a selective PDE II inhibitor and an adenosine re-uptake inhibitor selective towards cGMP-specific PDE V, showed much more cGMP positive fibers after stimulation with NO than other selective PDE inhibitors. Because of this increased signal it was speculated that adenosine might be involved in this rise in cGMP. We studied the role of adenosine on cGMP accumulation using 5'-N-ethylcarboxamidoadenosine (NECA), a non-selective adenosine receptor agonist.
1. Neurochemicals in the frontal rat brain

1.1. Introduction

For a better understanding of this study some theory will be given. In keeping with the objectives of this study, I will not attempt to be comprehensive. First a brief description of the frontal rat brain will be given and one special type of brain cell will be discussed in some detail. Second, the neurochemical substances adenosine and serotonin will be introduced. Furthermore the NO-cGMP pathway is explained and its role in the central nervous system is discussed. Finally a detection method is explained which is often used in biochemical identifications of cellular components and on which this study is partly based.

1.2. Brief description of the frontal rat brain

The frontal rat brain can be divided into two major areas, the cortex and the basal ganglia (see figure 1 and 2). The basal ganglia is a complex neuronal net that transports and connects signals originating from the cerebral cortex. In terms of structure, the basal ganglia are formed by a deep and voluminous nucleus on each side of the cerebral hemispheres (striatum, formed by the caudate putamen and pallidum). The basal ganglia are highly innervated by other brain regions and a great
diversity of neurotransmitters (like serotonin) and neuromodulators (like adenosine) can be found in these pathways. Their functions include memory aspects, programation, selection and execution of motor movements. Furthermore, the striatum contains one of the highest contents of soluble guanylate cyclase and NOS compared to other brain areas.

1.3. Astrocytes

The main function of the brain is to process information by conveying complex electrical signals. These electrical signals are processed by excitable cells: the neurons. Surprisingly, up to half of the brain's volume does not consist of excitable but of non-excitable cells. The largest class of non-excitable cells is that of the neuroglia. The most abundant of the glial cells are those known as astrocytes (Kimelberg and Norenberg 1989). Astrocytes have key roles in normal physiology, in brain development and in pathology of the nervous system. It is known that astrocytes play an important role in the metabolism of some neurotransmitters. In order for the neuronal network to function smoothly, these transmitters, after being released into the synaptic cleft (the gap between neurons), must be removed. Some of the removed transmitter molecules are carried into astrocytes. Astrocytes also play a role in controlling the ionic composition of the interstitial fluid around neurons.

1.4. Serotonin

Serotonin, a substance with vasoconstrictive properties, was first isolated by Page, Rapport and Green out of bloodserum in 1948. It was called serotonin because of its source, serum, and its activity, constriction. Serotonin was chemically defined as 5-hydroxytryptamine, 5-HT.
The presence of serotonin in the brain was demonstrated later by Twarog and Page in 1953.

The structural similarities between serotonin and the hallucinogenic lysergic acid diethylamine (LSD) led to the logical hypothesis that 5-HT might be involved in the pathogenesis of some psychiatric disorders. Also the study and use of antidepressants, like tricyclics and the monoamine oxidase inhibitors, was followed by the serotonin hypothesis of depression, because their modes of action is supposed to be based on the increased availability of monoamine neurotransmitters like noradrenaline and serotonin. The foregoing brought a very large attention on the function of serotonin as a neurotransmitter (Van Kempen 1994, Jacobs 1994).

As a vasoconstrictor 5-HT is present in considerable amounts in the body, only 1-2% is present in the brain. Under normal circumstances the cerebrospinal fluid contains very low 5-HT concentrations. Serotonin cannot cross the blood-brain barrier and is synthesized locally in the brain from the amino acid L-tryptophan. Once tryptophan is carried into the brain and into certain neurons, it is converted into serotonin by two enzymatic steps and thereafter stored in synaptic vesicles. After release from the presynaptic terminal serotonin is mainly inactivated by re-uptake into the presynaptic nerve terminals, and thereafter re-stored in the synaptic granules or metabolized. This re-uptake mechanism led to the introduction of the group of so called specific serotonin (re-)uptake inhibitors (SSRI's), like prozac®, used as antidepressants in psychiatry (Kempen 1994, Jacobs 1994). Besides depression serotonin is said to be involved in the pathophysiology of many other disorders like anxiety, psychosis, and aggression. It also plays a role in learning and memory and disturbances in sexual behavior and sleep (Jacobs 1994).
5-HT predominantly serves as an inhibitory neurotransmitter in the brain. Signals are transmitted via both conventional junctional synapses and non-direct contacts. Most of the serotonergic cell bodies are found in the raphe nucleus in the brainstem and these cell bodies project through major ascending and descending projection systems to many brain areas. The 5-HT system can be subdivided into two major parts: a rostral part which projects mainly to the frontal brain areas and caudal part with important projections to the spinal cord (see Fig. 3). Both parts have projections to areas within the brainstem and the cerebellum (Olivier 1994).

In this study we used lesioned rats for our experiments. Lesions were put in the left medial forebrain bundle (mfb, see Fig. 3). This means that the serotonergic innervation to the caudate putamen (CPu) and the cortex is completely destroyed on the left side of the brain.

Recently, evidence has accumulated that the regulation of neurotransmitters between the synapses could be mediated by NO. NO might regulate neurotransmitter release through activation of cGMP-dependent protein phosphorylation cascades (Dawson and Dawson 1995), or NO might inhibit re-uptake of some transmitters in the brain (Pogun and Kuhar).

1.5. Adenosine

The profound hypotensive, sedative, antispasmodic, and vasodilatory actions of the nucleoside adenosine were first recognized over 7 decades ago (Daly 1982). During the intervening years much research has been conducted and the biological roles of adenosine have increased considerably. A lot of attention has been given to its cardiovascular effects which leads to vasodilatation and hypotension. In the CNS adenosine is an important inhibitory neuromodulator (Sweeney 1997). It perhaps represents a general regulatory substance, since
no particular cell type or tissue appears uniquely responsible for its formation. Its effects are mediated by membrane bound adenosine receptors. Up till now 4 types of receptors have been characterized, and classified into A1, A2a, A2b, and A3 receptors (Collis and Hourani 1993, Fredholm 1994, Palmer and Stiles 1995, Sweeney 1997). Through these different receptors adenosine can affect a variety of physiological functions. All the adenosine receptors are linked to GTP binding proteins (G-proteins), and the main target is the enzyme adenylate cyclase, the enzyme that produces cyclic adenosine-3’5’-monophosphate (cAMP). The adenosine receptors can either inhibit (via A1 or A3 subtype) or stimulate (via A2a or A2b) adenylate cyclase activity. Other targets for the adenosine receptors are guanylate cyclase (Kurtz 1987, Levy et al 1991), ion channels and phospholipases (Sweeney 1997).

The extracellular concentration of adenosine is regulated by several mechanisms. The major route of formation of adenosine involves hydrolysisation of ATP, ADP, and AMP (Adenosine-, Tri-, Di-, Monophosphate, respectively). Adenosine formation increases considerably under high energy demands and can occur both extracellularly and intracellularly. A variety of routes for inactivation of adenosine are present in cells. These includes enzymes such as adenosine deaminase and adenosine kinase, and uptake processes back into the cells. Because of these inactivation processes we did not use adenosine in our study to look for a response on 5-HT re-uptake. In stead we used the non-selective adenosine receptor agonist NECA. The advantage of such a compound is that it acts practically the same as adenosine, it binds to and activates all the adenosine receptors, but it will not be inactivated like adenosine.
Fig. 4. Simplified scheme depicting how the retrograde messenger nitric oxide is produced and how it stimulates soluble guanylate cyclase to produce cGMP. Release of glutamate activates the ionotropic glutamate NMDA receptors. This causes Ca\textsuperscript{2+} influx via Ca\textsuperscript{2+} channel of the NMDA receptor. The increase in Ca\textsuperscript{2+} activates NO synthase. NO then diffuses randomly through cytosol and membranes and binds with very high affinity to the haem group of the soluble guanylate cyclase. Furthermore phosphodiesterases (PDE) exists that produces a rapid decrease in concentration of cGMP after activation of guanylate cyclase and the following increase in concentration of cGMP.

1.6. NO-cGMP signaling pathway

1.6.1. Introduction

Nitric oxide, a poisonous, chemically unstable gas was first recognized as a messenger molecule in the CNS in 1988 (Garthwaite). The discovery that NO functions as a signaling molecule in the brain opened a new dimension in the concept of the classical picture of chemical transmission, where information is passed between neuronal elements at discrete loci (synapses) and in one direction. During the past few years, much information on the enzymology and molecular characteristics of NO synthesis has accumulated. In the CNS, as can be seen in figure 4, production of NO occurs when Ca\textsuperscript{2+} flows into a cell due to activation of a glutamate receptor. This increase in intracellular Ca\textsuperscript{2+} activates the enzyme nitric oxide synthase (NOS) which converts L-arginine, in the presence of molecular oxygen, into NO and the co-product L-citrulline. NO in turn activates the soluble isoform of the enzyme guanylate cyclase which converts guanosine triphosphate (GTP) into cyclic guanosine-3',5'-

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monophosphate (cGMP). This so called second messenger then mediates its biological action. The exact role of cGMP in the brain has not yet been established, but research on its biological action is accumulating. When cGMP has played its part in the signal transduction, it has to be broken down rapidly. Therefore phosphodiesterases exist which produce a rapid decrease in the concentration of cGMP.

The amount of information given in this introduction will be elaborated in the next paragraphs for a better understanding of this pathway. As in all signal transduction pathways different isoforms exist of the diverse enzymes given here. Many enzyme isoforms are activated in the same way but some are activated in a completely different manner. Also the biological role of NO will be discussed and the clinical or experimental relevance of some enzyme inhibitors are given.

1.6.2. Biological role of NO

Early studies of NO suggested that the compound was anything but beneficial. NO is a toxic, labile free radical that is extremely reactive. Its half-life is a few seconds and then it is converted by oxygen and water into nitrates and nitrites. Years of research led to a series of discoveries, revealing the major biological roles of nitric oxide. It enables macrophages to kill bacteria and tumor cells and it is produced by endothelial cells to mediate blood vessel relaxation through neurotransmitter release (Snyder and Bredt). It also plays a role in neuronal adaptation (Brenman and Bredt) and regulation of neurotransmitter re-uptake (Pögün and Kuhar) and release (Kendrick et al.). Nitric oxide simultaneously serves as a messenger for neurons, much like a neurotransmitter, in the brain and other parts of the body (See figure 5) (Resink et al.)
NO, more than any other biological molecule, depends on its small size, reactivity, and diffusability to exert its biological effects, rather than its molecular shape like the conventional neurotransmitters (Dawson and Dawson 1995). Conventional neurotransmitters are enzymatically synthesized, stored in synaptic vesicles, and released by exocytosis from synaptic vesicles during membrane depolarization. They act on membrane-associated receptors to mediate their biological action. This is subsequently followed by inactivation through re-uptake mechanisms or enzymatic degradation. NO, on the other hand, does not use any of these means for control of its biological action. Instead, NO is synthesized on demand by the enzyme NO synthase. Thus, the control of NO synthesis is the key to the regulation of its activation.

On the other hand NO can be considered as a neurotoxin. As a common air pollutant and a component of cigarette smoke, NO is a toxin for lung tissue. Endogenous formed NO can inhibit several enzymes of the mitochondrial electron transport complex, the citric acid cycle enzyme, and the rate limiting enzyme in DNA replication. Alternatively, NO can react with the superoxide anion, $O_2^-$, to form the peroxynitrite anion (ONOO-) which is an extremely reactive molecule with potent oxidant properties.

NO is clearly an unique biological effector molecule. The primary stimulus for NO synthesis in central neurons is activation of the NMDA-type glutamate receptors (as shown in Fig. 4). Under conditions of normal activation, NO is a neuronal messenger molecule. Overactivity of
NMDA receptors is implicated in the pathogenesis of several neurodegenerative disorders, including stroke, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Brenman and Bredt).

1.6.3. Nitric oxide synthases

Up to now the existence of 3 distinct isoforms of NOS have been recognized, neuronal NOS (nNOS or NOS-I), endothelial NOS (eNOS or NOS-III), and inducible NOS (iNOS or NOS-II) (Dawson and Dawson 1996, Kerwin et al. 1995), each of them having restricted anatomical and cellular distributions and distinct regulatory features. nNOS is the major isoform in the brain and probably accounts for the majority of physiological processes attributed to NO in the nervous system. eNOS is localized in the endothelial cells in blood vessels where it mediates blood vessel relaxation. iNOS, under normal conditions, does not appear to be expressed in the nervous system (Dawson and Dawson, 1996).

Both the neuronal and the endothelial isoforms are constitutively expressed and are briefly activated by increases in intracellular calcium binding to calmodulin (Bredt and Snyder, 1990). On the other hand the inducible isoform is expressed in response to immunological stimuli such as cytokines and lipopolysaccharide and its activity is independent of intracellular Ca^{2+}. Once the iNOS protein is produced, it keeps on synthesizing NO. Thus, regulation of iNOS primarily occurs at the level of gene transcription, whereas regulation of nNOS and eNOS occurs by controlling the levels of various necessary cofactors and substrates within the cell (Dawson and Dawson 1996, Kerwin et al. 1995).
All three NOS isoforms catalyze the oxidation of L-arginine to NO and L-citrulline using reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as the source of electrons, O₂, haem and some other co-factors (Kerwin et al. 1995, Marletta 1993). A general scheme for the biosynthesis of NO from L-arginine is given in figure 6.

Inhibition of NOS is thought to be therapeutically important in certain clinical situations, and for this reason isoform selective inhibitors are being designed. Up to now several inhibitors of NOS have been developed. Unfortunately, the majority of currently available NOS inhibitors are relatively non-selective, rendering them essentially useless for most clinical applications (Resink et al 1996). In this study N⁶-G-nitro-L-arginine methyl ester hydrochloride (L-NAME) was used, which is a potent inhibitor of all the NOS isoforms (Pfeiffer et al. 1996).

1.6.4. Signal transduction by guanylate cyclases

Guanylate cyclases (GNCs) are enzymes which are responsible for the synthesis of cyclic GMP. There is a growing family of guanylate cyclase (GNC) enzymes known. At present two main groups of isoforms can be distinguished: a particulate form (pGNC) that is membrane bound and a soluble form (sGNC). The particulate isoform is activated by peptide hormones through cyclase coupled receptors. In mammals, the natriuretic peptides (atrial natriuretic peptide, ANP; brain natriuretic peptide, BNP; C-type natriuretic peptide, CNP) are important
ligands for activating pGNC. On the other hand, only the soluble isoform of GNC is activated by NO. NO reacts with the haem moiety of the sGNC to yield a NO-haem complex which is responsible for enzyme activation (Murad, 1994; Knowles et al., 1989; Chinkers and Garbers, 1991).

Activation of the GNCs leads to an increase in cGMP levels. The diverse targets where cGMP then acts remains to be defined. However, there are cGMP-dependent protein kinases, cGMP-dependent phosphodiesterases (Dawson et al., 1992) as well as cGMP dependent cation channels (Zufall et al., 1997) which can be a target for cGMP. Difficulties in assigning a role for cGMP in the CNS partly relate to the physically and biochemically different forms of GNC which are present in the CNS.

Recently a selective inhibitor of sGNC has been found (Garthwaite et al., 1995). The compound 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) potently and selectively inhibits NO-stimulated GNC activity. It does not affect NOS activity, nor does it affect the activity of pGNC or adenylyl cyclase. This substance has proven to be useful in investigating the NO-cGMP pathway. Kim and Burstyn (1994) have identified an endogenous inhibitor of the sGNC from bovine lung. This finding of an endogenous inhibitor provides evidence of a new pathway of cGMP regulation. This inhibitor may play a role in the suppression of cGMP levels under activated or unactivated conditions.
1.6.5. Cyclic nucleotide phosphodiesterases

The length and magnitude of the second messenger response of cAMP (another second messenger, which actions or mechanism will not be discussed in this thesis) and cGMP is regulated by phosphodiesterases (PDEs). PDEs are responsible for degrading cGMP and cAMP and thereby turning off the second messenger signal. It has become appreciated that the process of cyclic nucleotide degradation can be catalyzed by one, or by a combination of a large number of different PDE isoenzymes. Current evidence suggests that at least seven different families of PDE isoenzymes exist in higher eukaryotes. These subfamilies, identified by Roman numerals, are distinct in substrate specificity and inhibitor profile. Although all PDEs hydrolyze both cAMP and cGMP to some extent, large differences in affinity for the nucleotides are found between the different enzymes.

A list of these families is shown in table 1.

<table>
<thead>
<tr>
<th>PDE isoenzyme family</th>
<th>Descriptive name</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>CaM-dependent</td>
</tr>
<tr>
<td>II.</td>
<td>cGMP-stimulated</td>
</tr>
<tr>
<td>III.</td>
<td>cGMP-inhibited</td>
</tr>
<tr>
<td>IV.</td>
<td>cAMP-specific</td>
</tr>
<tr>
<td>V.</td>
<td>cGMP-specific</td>
</tr>
<tr>
<td>VI.</td>
<td>Photoreceptor</td>
</tr>
<tr>
<td>VII.</td>
<td>HPC1</td>
</tr>
</tbody>
</table>
The CaM-stimulated cyclic nucleotide phosphodiesterases (PDE I family) constitute a genetically diverse and expanding family of enzymes that catalyze both cAMP and cGMP hydrolysis. Calmodulin (CaM, a coenzyme) in the presence of Ca\(^{2+}\) stimulates the activity of these isoenzymes several-fold. At present, at least six different CaM-dependent PDE isoforms are thought to exist (Sonnenburg and Beavo). Because the total amount of CaM does not change rapidly in the cells, most investigators think that regulation of the enzyme in vivo is most likely due to changes in Ca\(^{2+}\), which in turn determines the amount of active Ca\(^{2+}\)-CaM complex available. The PDE type I family or Ca\(^{2+}\)/Calmodulin-phosphodiesterases is the predominant form in mammalian cells. The cGMP-stimulated phosphodiesterase (PDE II family) also hydrolyses both cAMP and cGMP. The cGMP-stimulated PDEs are unique in that they contain a noncatalytic binding site having high specificity for cGMP. When cGMP binds to this site, a large increase in activity occurs under normal substrate conditions.

Members of the cGMP-inhibited family (PDE III family) share the property of having a relatively high specificity for cAMP as a substrate. They are unique compared with other PDEs in that they have high affinity for cGMP but it hydrolyzes cGMP poorly, thereby conveying specificity for cAMP. Because cGMP binds tightly to the enzyme but is hydrolyzed poorly, many investigators now think that an important physiological role for cGMP is to act as an inhibitor of cAMP hydrolytic activity by this PDE. The PDE IV family is currently the largest. These cAMP-specific isoenzymes mainly have cAMP as the substrate and selectively hydrolyze cAMP in many tissues. The PDE V family has traditionally been termed the cGMP-specific PDE or the cGMP-binding PDE. These names are based on the substrate specificity of the enzyme and on the fact that it contains a high-affinity noncatalytic cGMP binding site. It has been thought of mostly as a regulator of cGMP function.

The photoreceptor PDEs of the PDE VI family play a very important role in vision and is probably the best understood of any of the PDE families. The photoreceptor enzymes are highly enriched in the outer segments of the retinal photoreceptor neurons and mainly have cGMP as the substrate. The PDE VII family, at this time, has only one described member and the physiological role of this enzyme is completely unknown until now. A more detailed description of phosphodiesterases can be found in Sonnenberg and Beavo; Beavo and Reifsnyder; and Beavo.
1. Neurochemicals in the frontal rat brain

1.6.6. Selective inhibitors for PDE isoenzyme families

The recent understanding that many different isoenzymes of PDEs representing 7 families exist in humans and animals greatly increased the search for selective inhibitors for therapeutic interventions. By using PDE inhibitors the second messengers cAMP and cGMP will not be hydrolyzed, or at least more slowly, resulting in a higher response and a longer duration of the second messenger signal.

In principle, one should be able to inhibit one family of PDEs (or better yet a small subset of the family) and achieve much more selective results. The first generation of inhibitors of PDE turned out not to be isoenzyme specific and showed a lot of toxic side effects at doses that are very close at therapeutic levels. The second generation of drugs that are specific for individual PDE families do appear to be much better both in terms of therapeutic efficacy and also decreased number of side effects. In table 2 a few PDE inhibitors are given that were used in this study. A list of many of the more commonly used PDE inhibitors that are selective for individual PDE families can be found in Beavo (1995).

Table 2: Selective inhibitors for PDE isoenzyme families used in this study

<table>
<thead>
<tr>
<th>PDE Family</th>
<th>Inhibitor</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE 1</td>
<td>Vinpocitine</td>
<td></td>
</tr>
<tr>
<td>PDE 2</td>
<td>EHNA</td>
<td>also adenosine deaminase inhibitor</td>
</tr>
<tr>
<td>PDE 5</td>
<td>Zaprinast</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dipyridamole</td>
<td>also adenosine re-uptake inhibitor</td>
</tr>
<tr>
<td>PDE 6</td>
<td>Zaprinast</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dipyridamole</td>
<td></td>
</tr>
<tr>
<td>Nonselective</td>
<td>IBMX</td>
<td>also adenosine receptor antagonist</td>
</tr>
</tbody>
</table>

EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; IBMX, 3-isobutyl-1-methylxanthine
1.7. Immunocytochemistry

Immunocytochemistry is a staining technique for the detection of various substances in biological tissues. For a better understanding of this method, it is necessary to have a basic knowledge of the building blocks of immunology (antigens and antibodies). An antibody is a serum protein that is formed in response to exposure to an antigen, a substance foreign to the host, and reacts specifically with that antigen to form immune complexes either in the body or in the laboratory.

In order to produce an antibody for laboratory use, it is first necessary to purify an antigen. A source for the antigen such as serum, urine or tissue is subjected to various purification techniques, including precipitation, chromatography and electrophoresis. The purified antigen is then injected into an animal of different species than that of antigen source. The animal will then identify the antigen as a foreign substance and in response to that it will produce an antibody directed specifically against it. Repeated injections are given regularly in order to

Fig. 8. Sketch depicting how substances can be visualised by immunocytochemistry. A primary antibody binds to the antigen. The antigen/primary antibody complex is thereafter visualized by a secondary antibody, linked to a fluorescent label.
promote a consistent antibody production. After several bleedings are pooled the antibody is recovered from the blood and contaminants present will be removed. This is usually accomplished by either liquid or solid phase antigen absorption techniques.

For detection purposes, the antibodies are often linked to some type of marker molecule. This can be a fluorescent label such as fluorescein or rhodamine, or an enzyme such as alkaline phosphatase or horseradish peroxidase. In this study we used fluorescent conjugated antibodies for the staining of the various substances.
2. Materials and methods

Animals

Young adult male Lewis rats were used. The animals weighed 180-200 grams at the start of the experiment, and were housed under standard conditions, with food and water present ad lib. All animal experiments were approved by the local Committee on Animal Welfare.

5,7 Dihydroxytryptamine lesion

Rats were injected 2 hours prior to the operation with desmethylimipramine (25 mg/kg i.p.) to protect other catecholamine systems from the neurotoxin 5,7-dihydroxytryptamine (5,7 DHT). Rats were anesthetized with Rompun (4 mg/kg s.c.) and Vetalar (50 mg/kg i.m.), thereafter immobilized in a stereotaxic instrument (A -4.4; L +1.1; V +1.8) according to Paxinos and Watson (1982). 2 µl 5,7 DHT (4 mg/ml) in saline, containing 0.02 % ascorbic acid, was slowly infused at a rate of 0.5 µl/min in the medial forebrain bundle (mfb) by means of a motor driven infusion pump (SP210iw Syringe pump, WPI). Lesions were performed unilateral at the left side of the brain. The animals were killed by decapitation 2 weeks after lesion and brains were processed for immunocytochemistry, NOS and cGMP measurement.

Tissue preparation of 5,7-DHT lesioned rats

Lesioned rats were decapitated, brains were removed from the skull and immediately placed in ice-cold aerated Krebs-Ringer bicarbonate solution containing (mM): NaCl 121.1; KCl 1.87; KH₂PO₄ 1.17; MgSO₄ 1.15; NaHCO₃ 24.9; CaCl₂ 1.2; glucose 11.0; (pH 7.4). Brain tissue was glued onto a plastic table and transversal 300 µm slices were cut, while submerged in
aerated Krebs (at 4 °C), using a 752M Vibroslicer® (Campden Instruments). Slices were transferred in incubation wells containing aerated Krebs-Ringer solution and, unless stated otherwise, a PDE inhibitor (0.1 mM IBMX or 10 µM Zaprinast) was included from the start of the experiment. Incubation time before adding drugs was 30 min. When included, the NO donor sodium nitroprusside (SNP; Fluka) was present for another, final, 10 minutes. Incubation wells were placed on a waterbath (37 °C) and continuously aerated with 5% CO₂ in O₂. The incubations were terminated by adding ice-cold fixative solution (final concentration 4% paraformaldehyde, 0.1 M Phosphate buffer, pH 7.4). After 15 minutes the slices were transferred into fixative solution containing 4% paraformaldehyde, 10% sucrose, 0.1 M Phosphate, pH 7.4. Fixation was continued for 1½ hour, followed by a 30-min. wash in ice-cold 0.1 M Phosphate buffer (pH 7.4) containing 10% sucrose for cryoprotection. Slices were frozen and 10 µm cryostat sections were cut and thawed onto chrome-alum/gelatin-coated slides and stored in -20 °C or processed for immunocytochemistry.

Tissue preparation for adenosine experiments

Preparation of brain slices was done in a different manner as described previously. Untreated rats were anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg, Sanofi Sante B.V.), decapitated, and the brains were quickly removed from the skull. The brain was immediately placed in ice-cold aerated Krebs-Ringer bicarbonate solution containing (mM):

NaCl 121.1; KCl 1.87; KH₂PO₄ 1.17; MgSO₄ 1.15; NaHCO₃ 24.9; CaCl₂ 2; glucose 11.0; (pH 7.4). Brain tissue was glued onto a plastic table and transversal 400 µm slices were cut (at 4 °C) using a 752M Vibroslicer® (Campden Instruments). Slices were transferred into incubation wells and continuously perfused with fresh aerated Krebs buffer (flow rate of 3.5 ml/min) for 45 minutes at 37 °C. After the pre-incubation, the slices were perfused with aerated Krebs buffer (37 °C) containing a PDE inhibitor (IBMX, Zaprinast, Dipyridamole; (Sigma); EHNA, Vinpocitin; (RBI)) for 30 min and for an additional 10 min the slices were perfused with an adenosine analogue (NECA; RBI). To assess if cGMP production was due to NOS activity some slices were processed in the presence of 1 mM L-NAME. The incubation was stopped by transferring the slices into ice-cold fixative solution, containing 4%
paraformaldehyde, 0.1 M phosphate buffer, pH 7.4. After this fixation step the slices were processed as described before. The PDE I inhibitor Vinpocetine, which was used in a concentration of $10^{-4}$ M, came out of solution and it is not sure what the real concentration during the experiment was. Recent experiments, conducted at this group, with Vinpocetine showed a solubility of $2 \times 10^{-5}$ M. The PDE inhibitors used were first dissolved in DMSO before adding to the incubation buffer. DMSO concentration in all experiments was 1% (v/v).

**Immunocytochemistry**

Sections were air-dried for 20 minutes, washed 3 x 5 minutes in Tris-buffered saline (TBS; pH 7.6). Thereafter sections were incubated with sheep anti-cGMP (1:4000) in TBS containing 0.3% (v/v) Triton X-100 (TBS-T), overnight at 4 °C. After incubation, the slides were washed 3 x 15 minutes, respectively in TBS, TBS-T, TBS and incubated for 1 hour (at room temperature) with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep antiserum (Calbiochem) at a 1:30 dilution in TBS-T, to visualize the cyclic-GMP immunoreactivity. Finally the slides were washed again 3 x 15 minutes as described above and mounted in TBS-glycerol (1:2). For double immunostaining the following procedure was used: Sheep anti-NOS (1:750) was diluted in Rabbit anti-cGMP (1:300 in TBS-T) and processed as stated above. For visualizing the immunoreactivity of cGMP, the secondary antibody Donkey anti-Rabbit Cy3 (1:800 in TBS-T) was used and incubated at room temperature for 1 hour. Thereafter the slides were washed 3 x 15 minutes in TBS, TBS-T, TBS, respectively and incubated for 1 hour with Rabbit anti-Sheep FITC for visualizing the NOS immunoreactivity. After this incubation the slides were washed again and mounted as previously described. For the visualization of 5-HT a Rabbit anti-5-HT antibody was used (1:1000) and as second antibody the Donkey anti-Rabbit Cy3. In case of double staining for 5-HT and cGMP, the Sheep anti-cGMP antibody was used and processed as described above. To look if the adenosine induced cGMP production was a process controlled by astrocytes, double staining were performed with cGMP (Rabbit anti-cGMP) and GFAP (Mouse anti-GFAP, 1:10). In the cytoplasm of astrocytes GFAP (glial fibrillary acidic protein) can be found. GFAP is found only in astrocytes and has
therefore proved to be an invaluable marker for identifying those cells in tissue samples and in cultures.

**NOS activity assay**

Lesioned rats were decapitated and the brains were quickly removed from the skull. Brain regions were dissected and chopped to pieces. Tissue was transferred into 0.500 ml ice-cold homogenization buffer (50 mM Tris-buffer pH 7.4, 2 µM leupeptin, 200 µg/ml soybean trypsin inhibitor, 40 µg/ml aprotinin, 0.32 M sucrose, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl-fluoride, 1 mM dithiothreitol). Tissues were homogenized in a glass tube with a teflon pestle on ice. The suspensions were centrifuged at 20,000 x g for 20 minutes at 4 °C. The pellet (membrane fraction) was discarded, and the supernatant (cytosolic fraction) was used for NOS activity measurement and protein measurement. Aliquots of the cytosol were added to incubation buffer (50 mM Tris pH 7.4, 0.25 mM NADPH, 5 µM arginine and 0.4 mM Ca²⁺ and ³H-arginine (± 110,000 dpm), 4:5:1(v/v/v), final volume 100 µl. Measurements were done in triplicates. Incubation time was 45 minutes at 30 °C. Incubation was terminated by adding 1 ml of ice-cold HEPES buffer pH 5.5. 600 µl of the incubate was added to 0.5 ml Dowex AG 50 (Na⁺ form), and radioactivity in 550 µl supernatant was counted by liquid scintillation counting. Total citrulline recovered was calculated from specific activity of the ³H-arginine, correcting for counting efficiency, and was expressed as picomoles per milligrams protein. To assess that citrulline conversion was due to NOS activity, parallel samples were processed in the presence of 1 mM L-NAME. In order to see if the 5,7-DHT lesion was successful, a brain slice was processed for immunocytochemistry and stained for 5-HT.
Lesioned rats were decapitated and the brains were quickly removed from the skull. Brain regions were dissected and chopped crosswise at 300 µm and washed three times at 10-minute intervals with aerated Krebs-Ringer solution and 1 mM IBMX at 37 °C. The tissues were dispensed in Eppendorf tubes in a final volume of 0.250 ml, containing 25-500 µg of tissue protein. The tissues were pre-incubated for 20 minutes in the presence of L-NAME (100 µM) or without L-NAME. Thereafter, tissues were incubated in the presence of SNP (0.1 mM) or without SNP for an additional 10 minutes. Incubations were terminated with 30% ice-cold trichloroacetic acid (TCA) (final concentration 5%). Subsequently, the samples were sonicated and centrifuged for 10 minutes at 14,000 rpm. The supernatants were transferred into glass tubes and the protein pellets were dissolved in 300 µl 1 M NaOH at 65 °C for 30 minutes. The protein samples were stored at 4 °C and measured within a week. The supernatant was extracted 4 times with 1 ml of water-saturated diethyl ether. In order to remove residual ether, samples were placed for 30 minutes at 50 °C. Cyclic GMP content was measured using a radioimmunoassay procedure (Brooker et al. 1979) with the cyclic GMP antibody, as described by Steiner et al. (1972). Protein concentration was measured according to Lowry et al. Results were expressed as picomole cGMP per milligram protein. In order to see if the 5,7-DHT lesion was successful, a brain slice was processed for immunocytochemistry and stained for 5-HT.
3. Results

All in vitro slice incubations of 5,7 DHT lesioned rats were performed in the presence of 1mM IBMX to inhibit phosphodiesterase activity. Without IBMX no cGMP immunostaining was observed in the frontal rat brain, except for weak staining of blood vessel walls. So in case of immunostaining or measurements of cGMP under so-called basal conditions, not the actual basal activity is meant, but ongoing cGMP production in the absence of added stimulators or inhibitors of NOS. When we speak about the control side, we mean the side that is not affected by the lesion. Lesions were put in the left medial forebrain bundle (mfb). The mfb’s project ipsilateral which means that the left side is also the lesioned side. Because the lesion was placed uni-lateral, lesioned and control sides were present in the same slice of the frontal rat brain. During the biochemical measurements a slice was processed for immunocytochemistry to establish if the lesion had succeeded.

3.1. Immunocytochemistry of 5,7-DHT lesioned rats

In slices of the rat frontal brain, incubated under basal or stimulated conditions, an intense 5-HT-immunoreactivity (5-HT-IR) in the non-lesioned side could be seen. 5-HT fibers were heavily concentrated in the ventral, medial and caudal regions of the CPu (Fig. 10b). In the cortex a similar fiber network could be seen as compared to the CPu (Fig. 11a and c), also in the ventral part of the cortex the intensity of 5-HT fibers increased. 5-HT fibers in the septum were mainly concentrated in the lateral and medial parts of the septum (not shown). At the lesioned side almost all the 5-HT positive fibers were vanished (Fig.10e), reflecting the success of the lesion. In both lesioned side and control side no cell bodies could be detected.

In case of immunostaining for NOS no observable decrease in density of positive fibers could be detected between the lesioned side and control side (Fig. 10a and d). NOS fibers could be detected in all the regions of the slice with no apparent diversities in density for subregions. Two different types of NOS-positive fibers could be distinguished throughout the slice, very thin fibers with no varicosities and somewhat thicker fibers with lots of varicosities. Also cell

Serpotonin, adenosine and their role in the NO-cGMP pathway
bodies could be detected distributed at random in every region of the frontal brain. However, the NOS immunoreactivity of cell bodies in the CPu is more pronounced compared to the cortical regions.
cGMP-IR in frontal brain under basal conditions resulted in the appearance of a few isolated varicose fibers, which might be observed in any region of the frontal brain slice (Fig. 9a and b), and an intense cGMP staining was found in fiber like structures in the islands of Calleja (not shown). Intense cGMP immunofluorescence was found in the frontal rat brain after stimulation with SNP (Fig 9c). SNP stimulation was apparent in an abundant amount of thin fibers containing numerous varicosities in the CPu, and to a lesser extent in the diverse regions of the cortex (not shown). In addition a small amount of cGMP positive cell bodies could be detected scattered throughout the frontal brain after stimulation with SNP.

In case of double staining for 5-HT and cGMP colocalisation could not be excluded in CPu. This is due to the fact that the cGMP positive fibers are very dense, so no separate fibers could be found which colocalises with 5-HT. In the cortex no colocalisation of cGMP and 5-HT could be detected (not shown).

No cGMP-immunostaining was observed in NOS-positive structures in CPu (Fig. 9c and d) and in the diverse regions of the cortex (not shown). NOS-immunostaining and 5-HT immunostaining were not colocalised in striatal tissue (Fig 10a and b). Tissue of the frontal cortex however showed colocalisation between NOS and 5-HT (Fig 11a, b, c and d).
Figure 9. cGMP immunostaining in tissue slices incubated in vitro in the presence of 1 mM IBMX: a) frontal cortex, unstimulated; b) striatum, unstimulated. cGMP-immunostaining after incubation of striatal slices in the presence of 1 mM IBMX and 0.1 mM SNP is shown in c). This section was double stained for NOS as shown in d). Specificity of the fluorescence filters used is demonstrated by showing the Cy-3 immunofluorescence as in c) through the NIBA FITC filter in e), and the FITC-immunofluorescence as in d) through the Cy-3 filter in f). Antibodies used: a and b) sheep anti-cGMP; c) rabbit anti-cGMP; and d) sheep anti-NOS. Scale bar is 100 μm for all photographs.
3. Results

Figure 10. Absence of colocalisation between serotonin, and NOS. a) and b) show NOS- and serotonin-immunostaining in the same section from a striatal slice. c) shows cGMP-immunostaining in a section from a striatal slice from a 5,7-DHT lesioned brain. d) and e) show NOS and serotonin respectively in the same section from a striatal slice from a 5,7-DHT lesioned brain. Slices were incubated under standard conditions. Antibodies used: a) and d) sheep anti-NOS; b) and e) rabbit anti-5-HT; c) sheep anti-cGMP. Scale bar is 100 μm for all photographs.
3. Results

Figure 11. a) and b) show colocalisation of serotonin and NOS in a section of the frontal cortex, double stained for both substances. c) and d) same as a) and b) but in another area of the frontal cortex and with a smaller magnification. Slices were incubated under standard conditions. Antibodies used: b) and d) sheep anti-NOS; a) and c) rabbit anti-5-HT.
3. Results

Table 3: Cyclic GMP content in control and lesioned brain slices

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Basal</th>
<th>L-NAME</th>
<th>SNP§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex control side</td>
<td>0.88±0.25</td>
<td>0.66±0.15</td>
<td>10.80±1.07*</td>
</tr>
<tr>
<td>Frontal cortex lesioned side</td>
<td>0.75±0.10</td>
<td>0.40±0.09</td>
<td>10.89±1.16*</td>
</tr>
<tr>
<td>CPu control side</td>
<td>1.11±0.14</td>
<td>0.56±0.06</td>
<td>30.87±2.12*</td>
</tr>
<tr>
<td>CPu lesioned side</td>
<td>1.29±0.11</td>
<td>0.62±0.09</td>
<td>30.72±4.46*</td>
</tr>
</tbody>
</table>

Brain slices from different structures were incubated in the presence of 1 mM IBMX. Cyclic GMP values are pmol cyclic GMP/mg protein and are the means ±S.E.M. of 7 rats; each sample was assayed in duplicate. Statistical analysis was performed according to one-way ANOVA, followed by a Student-Newman-Keuls test. 0.1 mM L-NAME was present throughout the preincubation. §1.0 mM SNP was added 10 min before terminating the incubations. *P<0.01 compared to basal levels.

3.2. Biochemical determination of cGMP

Frontal cortex

Biochemical data support the immunocytochemical data. No differences in cGMP content were present in the frontal cortex between the lesioned side and the control side. SNP increased cGMP content significantly (P<0.01) of the control side from 0.88 pmol cGMP/mg protein under basal conditions to 10.80 pmol cGMP/mg protein. A similar significant (P<0.01) increase could be measured for the frontal cortex in the lesioned side. cGMP content under stimulated conditions went from 0.75 pmol cGMP/mg protein to 10.89 pmol cGMP/mg protein. Incubations of the slices with 0.1 mM L-NAME decreased cGMP content to 0.66 pmol cGMP/mg protein in the control side and 0.40 pmol cGMP/mg protein in the lesioned side (see table 3).


3. Results

Caudate-Putamen complex

Again the biochemical data support the immunocytochemical data. Higher amounts of cGMP can be found in CPu compared to frontal cortex under both basal and SNP stimulated conditions. The cGMP content in Caudate-Putamen complex (CPu) in the control side compared to the CPu in the lesioned side showed no differences. Under SNP stimulated conditions the cGMP content in CPu in both lesioned and control side increased thirty-fold, from 1.11 pmol cGMP/mg protein to 30.87 pmol cGMP/mg protein in the control side and 1.29 pmol cGMP/mg protein to 30.72 pmol cGMP/mg protein in the lesioned side. Incubations with L-NAME decreased cGMP content to 0.56 cGMP/mg protein in control side and 0.62 cGMP/mg protein in lesioned side (see table 3).

3.3. Biochemical determination of NOS-activity

Table 4: NOS activity in uni-lateral 5,7-DHT lesioned rats

<table>
<thead>
<tr>
<th>Brain region</th>
<th>NOS activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex control side</td>
<td>71.3±7.4</td>
</tr>
<tr>
<td>Frontal cortex lesioned side</td>
<td>55.5±10.9</td>
</tr>
<tr>
<td>CPu control side</td>
<td>308.5±26.9</td>
</tr>
<tr>
<td>CPu lesioned side</td>
<td>308.9±17.6</td>
</tr>
</tbody>
</table>

NOS activity is expressed as pmol citrulline/mg protein and are means±S.E.M. of 6 rats. Each sample was assayed in triplicate. Statistical analysis was performed according to one-way ANOVA, followed by a Student-Newman-Keuls test. The lesioned side was in all cases the left side of the slice.
Table 5: NOS-activity in control rats

<table>
<thead>
<tr>
<th>Brain region</th>
<th>NOS activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>right frontal cortex</td>
<td>60.8±9.6</td>
</tr>
<tr>
<td>left frontal cortex</td>
<td>61.6±12.6</td>
</tr>
<tr>
<td>right CPu</td>
<td>230.6±34.1</td>
</tr>
<tr>
<td>left CPu</td>
<td>204.4±59.1</td>
</tr>
</tbody>
</table>

NOS activity is expressed as pmol citrulline/mg protein and are means±S.E.M. of 3 rats. Each sample was assayed in triplicate. Statistical analysis was performed according to one-way ANOVA, followed by a Student-Newman-Keuls test.

Frontal cortex

Again confirmation with the immunocytochemical data was found. No differences were present between the lesioned side and the control side. The control side showed a NOS activity of 71.3 pmol citrulline/mg protein and the lesioned side had a NOS activity of 55.5 pmol citrulline/mg protein. Statistical analysis showed that this difference was not significant (see table 4). Incubations with L-NAME completely abolished the activity of NOS (results not shown). In control rats no significant difference between the left or right side of the frontal cortex could be measured (see table 5).

Caudate-putamen complex

NOS activity in CPu is much higher compared to frontal cortex (P<0.01). This is in accordance with the immunocytochemical findings. Again no significant differences between the lesioned side and the control side were measured. The NOS activity in CPu at the lesioned side was 308.9 pmol citrulline/mg protein, and the NOS activity in CPu in control side was 308.5 pmol citrulline/mg protein. Again incubations with L-NAME completely abolished the activity of NOS (results not shown). In CPu no statistical significant difference could be measured in...
3. Results

NOS activity between lesioned and control rats. Again no significant difference could be measured between the left and right side of the CPu in non-lesioned rats.

3.4. Adenosine and cGMP accumulation

A response in cGMP accumulation was found when slices were stimulated with the adenosine analogue NECA, a non selective adenosine receptor agonist. The cGMP accumulation in different experiments varied from day to day in intensity of the cGMP signal but the region where the response was observed was always the same. The cGMP signal appeared in cells in the cingulate cortex (Fig. 12a) and in the dorsal lateral septum (Fig. 13a). No fibers could be detected. Stimulation with different concentrations of NECA resulted in a concentration dependent effect of NECA on cGMP accumulation (results not shown). The minimal effect concentration of NECA was $10^{-5}$ M. Lower concentrations showed no effect at all. Stimulation with higher concentrations gradually increased the cGMP positive signal but did not alter the region of appearance of cGMP positive cells. Studied in time, the effect of $10^{-5}$ M NECA was first apparent after 3 min of stimulation. A maximal effect of $10^{-5}$ M NECA was observed after 10 min; longer incubation times did not increase the cGMP response (not shown).

In an attempt to get a better and a less variable response with NECA various selective PDE inhibitors were tried to upgrade the cGMP signal. However all the inhibitors used, see table 6, had no significant effect on the cGMP signal (Fig. 12c, d, e and f). The concentrations of the PDE inhibitors used in these experiments have been proven effective by various other investigators and in experiments using other stimuli to increase cGMP synthesis.

<table>
<thead>
<tr>
<th>Name inhibitor</th>
<th>Family</th>
<th>Description</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinpocitine</td>
<td>PDE I</td>
<td>CaM-dependent</td>
<td>$10^{-4}$ M</td>
</tr>
<tr>
<td>EHNA</td>
<td>PDE II</td>
<td>cGMP-stimulated</td>
<td>$10^{-5}$ M</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>PDE V</td>
<td>cGMP-specific</td>
<td>$10^{-5}$ M</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>PDE V</td>
<td>cGMP-specific</td>
<td>$10^{-5}$ M</td>
</tr>
</tbody>
</table>

Table 6: PDE inhibitors used
To establish whether the cGMP accumulation induced by NECA was due to the activation of an adenosine receptor, IBMX was used in combination with NECA. Besides being a potent non-selective PDE inhibitor, IBMX is also a potent non-selective adenosine antagonist. Incubations with IBMX alone resulted in some cGMP positive bloodvessels, but no cGMP positive fibers or cells could be detected (Fig. 9a and b). In combination with NECA the same effect could be seen, no cGMP positive fibers or cells were detected (Fig. 13b). IBMX completely antagonized the effect of NECA in the slices, demonstrating its antagonistic property. Because IBMX is also a potent non-selective PDE inhibitor and no cGMP positive fibers or cells could be detected strong evidence has been given that the cGMP accumulation is mediated by the adenosine receptors.

Stimulation with NECA in the presence of the NOS inhibitor L-NAME showed no change in the cGMP positive signal as could be seen with NECA alone (Fig. 12a and b). Double staining for cGMP and GFAP, a substance which can be found in astrocytes, resulted in 100% colocalisation for the cGMP positive cells (Fig. 13c and d, 13e and f) This means that the adenosine stimulated cGMP synthesis is a pathway which plays solely in a subpopulation of astrocytes. Not all astrocytes showed a cGMP response. This means that a special subclass of astrocytes is involved in adenosine induced cGMP production.
Figure 12. cGMP-immunostaining in various regions of the cingulate cortex of the frontal rat brain. All slices were incubated in the presence of 30µM NECA (a) alone, and the presence of b) 1 mM L-NAME; c) 10 µM EHNA; d) 10 µM dipyridamole; e) 20µM vinpocitine; f) 10µM zaprinast. Antibody used: Sheep anti-cGMP.
3. Results

Figure 13. a) and b) cGMP immunostaining in tissue slices incubated in vitro in the presence of 30 µM NECA: a) septum, NECA alone; b) cingulate cortex, NECA in the presence of 0.1 mM IBMX.

c-f show colocalisation of cGMP and GFAP in slices of the cingulate cortex: c) and d) slice incubated in the presence of 30 µM NECA and 0.1 mM L-NAME; e) and f) slice incubated in the presence of 30 µM NECA only.

Antibodies used: a) and b) Sheep-cGMP; c) and e) Rabbit-cGMP; d) and f) Mouse-GFAP.
4. Discussion

4.1. Effects of 5,7-Dihydroxytryptamine lesioning

As part of a larger study (DeVente et al. manuscript in prep.) where it is analyzed whether cGMP is colocalised with NOS, 5-HT, glutamate decarboxylase (GAD) and tyrosine hydroxylase (TH) in the frontal cortex and caudate putamen complex in lesioned rats, this study deals with the relationship between cGMP, NOS and 5-HT in 5,7-DHT lesioned rats. By using the unilaterally 5,7-DHT lesioned rat, we can make a direct assessment of the effect of absence of the 5-HT innervation on cGMP levels and NOS. First, lesions with 5,7-DHT showed no effect on cGMP levels under both basal or SNP stimulated conditions. This finding demonstrates that 5,7-DHT treatment does not alter sGNC activity. Secondly, in 5,7-DHT treated animals no difference in NOS activity could be measured between lesioned side and non-lesioned side. Both ipsilaterally as well as contralaterally the NOS activity in CPu stayed the same in the uni-lateral lesioned brain, therefore it is postulated that 5-HT is not colocalised with NOS in the CPu, at least not in the majority of 5-HT fibers in CPu. If NOS is present in 5-HT fibers in the CPu than an ipsilaterally downfall in NOS activity was expected. This was not the case. Immunostaining for NOS in the CPu of the lesioned brain confirms this postulation. No differences in immunostaining could be found for NOS between lesioned side and control side. In addition, no colocalisation of NOS and 5-HT could be found in the CPu by means of ICC. Although the CPu showed no colocalisation, double staining could be observed in the frontal cortex for NOS and 5-HT. One explanation for this difference in region for colocalisation, could be the different projections of the serotonergic cell bodies in the dorsal raphe nucleus. Johnson and Ma (1993) found that approximately 70% of the serotonergic neurons in the medial subnuclei displayed NADPH diaphorase activity (another method to visualize NOS fibers). Maybe the cells in the medial raphe nuclei, project to the frontal cortical areas whereas the other serotonergic cells in the raphe nuclei, which do not show colocalisation, project to the Cpu.
The major target for NO is the soluble isoform of GNC (Murad), but no cGMP was found in NOS-containing fibers. This data confirms the idea that NO is a retrograde intercellular messenger in the brain, meaning that the NO produced has its function in neighbouring cells. What exactly the function of NO-mediated cGMP production is, is not completely clear but it is conceivable that it involves neurotransmitter release in the striatum. Several researchers have shown that NO, either released from an NO-donor compound or after NMDA-receptor stimulation, influences the release of every major neurotransmitter in the striatum, including serotonin (Guevara-Guzman, Kendrick et al.). Other studies found no evidence for a role of cGMP stimulated release in neurotransmitters.

Colocalisation between cGMP and 5-HT in the CPu remains elusive because cGMP immunoreactivity was very dense. It was not clear if serotonergic fibers contained cGMP because no separate cGMP fibers could be detected. Biochemical data however showed no differences in cGMP levels between lesioned side and non-lesioned side in both CPu and frontal cortex. This strongly suggests that 5-HT and cGMP cannot be found in the same fibers in CPu and frontal cortex. Immunocytochemistry in the frontal cortex showed no colocalisation for 5-HT and cGMP. These findings and the fact that cGMP is a intracellular messenger suggests that cGMP is not directly involved in 5-HT release, at least not within the serotonergic fibers.

4.2. Adenosine and cGMP accumulation

The objective of this study was to find out if adenosine plays a role in the alteration of the intracellular level of cGMP in the frontal rat brain. Another and maybe more important reason was to look if the elevation of cGMP levels could be a way of signal transmission through which adenosine could induce 5-HT re-uptake. We used a qualitative method, by means of immunocytochemistry (ICC), to visualize the response of the adenosine analogue NECA. The reasons for using ICC, instead of quantitative method, are several. First of all, we had no idea if a response could be expected, and if so where this response would take place. Secondly, after a pilot experiment, we came to the conclusion that the response was very small and it is not sure
4. Discussion

If this elevation of cGMP can be measured within the boundaries of the sensitivity of the assay. The final reason for not using a quantitative measurement was the reason that the response varied from experiment to experiment. We first wanted to look for a way which would elevate the cGMP response and which subsequently would limit the variability between experiments, before starting with quantitative measurements. Due to limited time, this investigation only used the ICC.

This study clearly shows that activation of an adenosine receptor leads to an elevation in cGMP levels in a special group of astrocytes located in the cingulate cortex and in the dorsal-lateral septum. Which subclass of astrocytes are involved in this signal transmission is not apparent. All four subtypes of adenosine receptors are expressed in brain and on astrocytes (Janigro et al 1996, Collis and Hourani 1993, Palmer and Stiles 1995). Which type of adenosine receptor A₁, A₂a, A₂b or A₃ is involved in the stimulation of cGMP synthesis is not yet clear. Further experiments with receptor selective agonists and antagonist have to be done to clarify this question.

There exist at least two different enzymes which produce cGMP on activation, a particulate bound GNC and a soluble GNC. These enzymes are different in respect to their physical characteristics and different in regard to their localization (De Vente and Steinbusch 1990). Both forms have been described to elevate cGMP levels in astrocytes (De Vente et al. 1990). When brain slices are stimulated with ANF, an activator of pGNC, cGMP positive cells appear in the various regions (De Vente et al. 1989), but also in the same regions as when the slices are stimulated with an adenosine analogue. ANF responding cells were found, among other regions, in a high number in the lateral septum and to a lesser extent in the cingulate cortex. Because the NOS inhibitor L-NAME had no effect on the appearance of cGMP positive cells during stimulation with NECA it is clear that the signal transmission through the adenosine receptor does not involve the activation of NOS. It is reasonable to assume that then sGNC also is not of importance in this pathway. The only other way in which the adenosine receptor can work is via the particulate isoform of GNC. Taken together, our findings suggest that the effect of adenosine agonist NECA on cGMP production is either mediated through an
adenosine receptor/pGNC complex or through an adenosine receptor coupled to the ANF receptor/pGNC complex.

Several studies show that adenosine can serve as an endogenous signal to trigger NO release from cortical astrocytes through calcium influx and subsequent activation of nNOS (Janigro et al 1996, Delumeau et al 1991). To my knowledge no investigations have been done which show an adenosine induced cGMP elevation in brain. Although several investigations have connected adenosine receptor activation directly to cGMP elevation (Kurtz 1987, Miller and Hoffman 1994, Nikodijevic and Klein 1989), most of those investigations were in vitro studies with non-neuronal cell cultures. Kurtz showed, in cultures of vascular smooth muscle cells, that adenosine raises intracellular cGMP levels by the stimulation of a membrane bound guanylate cyclase. This finding of a rise in cGMP levels through a particulate isoform of GNC is in accordance with our findings. He suggested that the effect of adenosine on cGMP is mediated by A₁-type cell surface receptors. This suggestion was based on the order of specificity of non-selective adenosine agonist which he used, because highly specific agonist were not available in those days. Also the adenosine receptors then, were only divided in A₁ and A₂ receptors. Nowadays the existence of four kinds of receptors are known, so it is not clear which adenosine receptor is involved in the cGMP synthesis in that study.

Another study, on which our own investigation was partly based, was conducted in rat basophilic leukemia cells (RBL 2H3). RBL 2H3 cells provide a model system with cellular components similar to those involved in synaptic neurotransmission. They demonstrated that a A₃ receptor regulates the 5HT transporter via NO and cGMP. They proposed the following pathway: Activation of an A₃ receptor leads to an increase in calcium levels which would activate NOS. Subsequent production of NO would lead to the activation of cGMP production by stimulating soluble guanylate cyclase. cGMP would then activate cGMP-dependent kinase which would then phosphorylate either the 5-HT transporter directly or some other closely associated protein.
The proposed pathway via soluble GNC completely disagrees with our own findings. In our study the response to receptor activation was not blocked by the NOS inhibitor L-NAME, meaning that the pathway is not mediated by NO and subsequent not by soluble GNC. Consequently the proposed signalling mechanism, adenosine regulated 5-HT re-uptake through a NO-cGMP pathway, described by Miller and Hoffman cannot be the same as in our study. Whether or not adenosine receptor activation is involved in 5-HT re-uptake in brain, is not yet apparent and more experiments have to be done to clarify this question.

The PDE inhibitors used in this experiment had no effect on the cGMP signal, so it is not yet clear which PDE is active in the astrocytes which reacted to NECA. The concentration of the PDE inhibitors used in this study were sufficient as proved by other researchers. The PDE inhibitors which also play a role in the extracellular adenosine metabolism, EHNA and dipyridamole, have also no effect on the cGMP signal. A reason for this can be that the experiments were conducted with a constant flow of fresh buffer, resulting in a constant removal of endogenous formed adenosine. In the experiments on hippocampal slices (De Vente

**Fig. 14. Adenosine induced uptake of 5-HT as proposed by Miller and Hoffman (1994).** Adenosine A<sub>3</sub> receptor activation leads to an increase in intracellular Ca<sup>2+</sup> levels. Ca<sup>2+</sup> activates nitric oxide synthase (NOS), which in turn will produce nitric oxide. NO then stimulates the soluble form of guanylate cyclase to produce cGMP. cGMP subsequently activates a protein kinase (PK). This PK will then activate the 5-HT transporter through a phosphorylation reaction.
et al. Manuscript in prep.) the incubations were done by means of static incubations, meaning that no fresh buffer was supplied during the experiments. As a consequence, endogenous formed adenosine was not removed, resulting in a high concentration of adenosine and more cGMP production.

The functional implication of the adenosine stimulated cGMP production is not clear. The concentration of NECA necessary to induce a rise in cGMP is high. Similar concentrations of adenosine (up to $10^{-4}$ M) can be found in the ischemic or hypoxic brain (Sweeney 1997). These high concentrations of adenosine are neuroprotective against damage induced by ischemia or hypoxia. Maybe the rise in cGMP has a function in the neuroprotective effects of adenosine by means of cGMP dependent ion channels or cGMP dependent phosphodiesterases. Recently it was found that rats which showed increased sensitivity to ischemia had a reduced number of ANF receptors in the brain (Jeffs et al 1997). This finding is in line with the hypothesis that the adenosine receptor is related to the ANF-receptor in one way or the other.
5. Conclusions

In conclusion we found that there is no neuroanatomical relationship between NO-producing and cGMP-synthesizing neurons in the frontal rat brain in the sense that both were separately present within different neurons. For 5-HT and cGMP the same answer can be given. No cGMP could be found in the serotonergic fibers in the frontal rat brain. Serotonergic fibers, that originate from the medial dorsal raphe nucleus, which are known to contain NOS, project to the frontal cortex. The serotonergic fibers from other parts of the dorsal raphe, which show no colocalisation, project to the CPu.

For the proposed relationship between adenosine and 5-HT re-uptake in the brain the following conclusions can be made. Adenosine has an effect on cGMP production in a special subclass of astrocytes. This cGMP production is independent of NOS activation and subsequently does not involve the sGNC system. This finding completely disagrees with the proposed pathway. We have no evidence to confirm or to reject the hypothesis that adenosine is involved in the re-uptake of 5-HT.
6. Future studies

In case of co-localization of NOS, cGMP and 5-HT the only way to be sure if co-localization can be excluded or confirmed is by means of electron microscopy. Immunocytochemistry is a proper and relatively simple method to look for co-localization but when fibers are becoming very dense, as for example could be seen in CPu for cGMP immunostaining, then no statement can be made whether co-localization is present. So to be sure if co-localization is present you have to look at a subcellular level. Pilot experiments with electron microscopy are already in progress.

In case of the adenosine stimulated cGMP accumulation, and the possible 5-HT re-uptake, much more experiments have to be performed to unravel this cellular mechanism. First experiments with different selective adenosine agonist and antagonist have to be conducted in order to find out which adenosine receptor is involved in this mechanism. Secondly experiments have to be done to find out which PDE is active in the adenosine analogue responding astrocytes. When it is clear which PDE is active, specific PDE inhibitors can be used to elevate the cGMP response of adenosine. Furthermore, experiments with ODQ, a sGNC inhibitor, have to be performed to be sure that the adenosine induced cGMP production is not dependent on sGNC. Experiments with a ANF-receptor antagonist have to be conducted to confirm the hypothesis that the adenosine induced cGMP production is a process dependent on the particulate isoform of the GNC. In order to find out if adenosine is involved in 5-HT re-uptake double staining for cGMP and 5-HT have to be performed after stimulation of the slice with a adenosine analogue.
7. Literature

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