DOPAMINERGIC AMACRINE CELLS IN THE CHICKEN RETINA

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge, this thesis contains no material previously published or written by another person, except when due reference is made in the text.

Thesis submitted for the degree of
Doctor of Philosophy

by

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May 1986
I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge, this thesis contains no material previously published or written by another person, except when due reference is made in the text.
Animal code.

All experiments were carried out according to the Australian N.H. & M.R.C. "Code of practice for the care and use of animals for experimental purposes" and were covered by ANU Animal Welfare Committee protocol numbers: RBB 29/84 and RBB 8/82.
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Special thanks to Mary, for reminding me of, and Tim for rearranging, my priorities in life.

I gratefully acknowledge the receipt of an ANU PhD scholarship.
### Abbreviations

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<th>Description</th>
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<tr>
<td>AADC</td>
<td>aromatic-L-amino acid decarboxylase</td>
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<tr>
<td>AChE</td>
<td>acetylcholine esterase</td>
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<tr>
<td>cAMP</td>
<td>adenosine-3',5'-cyclic-monophosphate</td>
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<tr>
<td>CAT</td>
<td>choline acetyltransferase</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-0-methyl transferase</td>
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<tr>
<td>DBH</td>
<td>dopamine-beta-hydroxylase</td>
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<tr>
<td>DDC</td>
<td>DOPA decarboxylase</td>
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<tr>
<td>5,6-DHT</td>
<td>5,6-dihydroxytryptamine</td>
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<td>5,7-DHT</td>
<td>5,7-dihydroxytryptamine</td>
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<tr>
<td>DMPH4</td>
<td>6,7-dimethyl-5,6,7,8-tetrahydropterine</td>
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<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
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<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DOPEG</td>
<td>3,4-dihydroxyglycol</td>
</tr>
<tr>
<td>ED</td>
<td>embryonic day</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine-tetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(beta-aminoethylether)N,N-tetra-acetic acid</td>
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<tr>
<td>ERG</td>
<td>electroretinogram</td>
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<tr>
<td>fEPSP</td>
<td>fast excitatory post-synaptic potential</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography - mass spectroscopy</td>
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<td>GS</td>
<td>glutamine synthetase</td>
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<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>HPLC-ED</td>
<td>high performance liquid chromatography - electrochemical detection</td>
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<tr>
<td>5-HT</td>
<td>serotonin</td>
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<tr>
<td>5-HTP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
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<tr>
<td>INL</td>
<td>inner nuclear layer</td>
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<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
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<td>MA</td>
<td>metadrenaline</td>
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<td>MAO</td>
<td>monoamine oxidase</td>
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<tr>
<td>MOPEG</td>
<td>4-hydroxy-3-methoxyphenylglycol</td>
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<tr>
<td>MTA</td>
<td>4-hydroxy-3-methoxy-phenylethylamine, (3-methoxytyramine)</td>
</tr>
<tr>
<td>NMA</td>
<td>normetadrenaline</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxodopamine</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
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<tr>
<td>PH</td>
<td>post-hatch</td>
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<tr>
<td>PN</td>
<td>post-natal</td>
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<tr>
<td>PNMT</td>
<td>phenylethanolamine-N-methyl transferase</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sEPSP</td>
<td>slow excitatory post-synaptic potential</td>
</tr>
<tr>
<td>sIPSP</td>
<td>slow inhibitory post-synaptic potential</td>
</tr>
<tr>
<td>SCG</td>
<td>superior cervical ganglion</td>
</tr>
<tr>
<td>TCA</td>
<td>tri-chloroacetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>VEP</td>
<td>visual evoked potential</td>
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A radioenzymatic assay for tyrosine hydroxylase (TH) activity was optimized for measuring the low activities in retinal homogenate samples. The sensitivity of the assay was 0.05 mg protein, and 0.10 mg protein with sub-saturating pterin substrate concentrations. The assay was linear between 0.05-0.45 mg protein, at incubation times up to 45min.

The apparent kinetic properties of chick retinal TH in vitro were $K_m$,tyr $57 \pm 1 \mu M$ (n=6), $K_m$,6,7-dimethyl-5,6,7,8-tetrahydroppterine(DMPH₄) $200 \pm 20 \mu M$ (n=6), and $V_{max}$ $6 \pm 1$ pmol/min.mg protein (n=16). TH activity was inhibited by 3-iodotyrosine.

An ion-pair reverse phase high performance liquid chromatograph, coupled to an electrochemical detector (HPLC-ED) was set up. With this system it was possible to separate (in order according to retention times) 3,4-dihydroxyglycol (DOPEG) adrenaline, dopamine, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 5-hydroxytryptophan (5-HTP), and serotonin, within a 20min chromatographic run. 4-Hydroxy-3-methoxyphenylglycol (MOPEG) - noradrenaline and 3,4-dihydroxyphenylalanine - 3,4-dehydroxyphenylacetic acid (DOPA - DOPAC) were not separated. DOPEG, MOPEG/noradrenaline, adrenaline, and DOPA/DOPAC did run in a crowded region of the chromatogram of a retinal sample.
With cation-exchange HPLC-ED, adrenaline, noradrenaline, and dopamine were separated from each other, from the void volume, and not masked by peaks of unknown origin of a retinal sample.

With cation-exchange HPLC-ED, dopamine, but no noradrenaline or adrenaline was detected in retinal homogenates. The detection limit for the analysis method was 0.02 nmol/g wet weight for these compounds.

With ion-pair reverse phase HPLC-ED retinal levels of dopamine were determined to be 0.28-0.51, of serotonin 76-110, and of HVA 200 pmol/g wet weight, the exact values depending upon previous exposure to light. A combined DOPA/DOPAC peak, and sometimes 3-methoxytyramine (MTA) could be detected in retinal homogenates.

Isolated chicken retina demonstrated a high-affinity $^3$H-dopamine accumulating activity. The dopamine-accumulation was temperature- and sodium-dependent. The affinity for dopamine was $K_m$ 590 ± 80 nM (n=4) and the capacity was $V_{max}$ 140 ± 20 fmol/min.mg protein.

The accumulated dopamine could be released by superfusion with high-potassium (60 mM) containing physiological buffer. During 12min exposure to high-potassium the efflux was increased by 70%. An increase of only 10% in efflux was observed when the calcium had been replaced with ethyleneglycol-bis(beta-aminoethylether)N,N-tetraacetic acid (EGTA).

After 15min or 24h exposure to light, retinal levels of dopamine were 30±3 vs 16±2 pmol in animals kept in the
dark for 24h, combined levels of DOPAC and DOPA were 120±20 vs 23±2 pmol, MTA levels were slightly increased, but still around the detection limit of 300 pmol, and HVA levels remained unaffected, 200±40 pmol.

Exposure to light for 15min or 24h had no effect on the TH capacity of chicken retina in vitro. After 15min or 24h exposure to light, TH activity assayed with sub-saturating concentrations for the cofactor were 2.7±0.2 and 2.2±0.1 pmol/min.mg protein respectively, vs 1.58±0.08 in dark-adapted retinas, indicating an increased affinity of TH for the pterin cofactor.

With monoamine histofluorescence microscopy, faint catecholamine-containing neurons were detected. The fluorescence of these neurons was greatly enhanced by preloading the eye with 250 pmol noradrenaline for 4h. After this treatment light-microscopic details of the morphology became detectable. The same type of neurons was TH-immunoreactive. The cell bodies of these neurons were localized in the inner nuclear layer (INL), at the border with the inner plexiform layer (IPL). The cell bodies were relatively large, an estimated 10 um in diameter.

After preloading with noradrenaline, bright varicose processes were seen, which formed a dense network in sublamina 1 of the IPL, but minor plexuses were seen in sublaminas 3 and 5 as well.

The mosaic of the dopamine amacrine cells was neither random nor highly regular, with a regularity index of 2.9-
3.4. The cell density was $21_{+1}^{+30} \pm 1$ cells/mm$^2$. The highest density ever observed was 48 cells/mm$^2$ in the central area of the retina.

The results under points 2 and 5-12, strongly favour the idea that dopamine is a neurotransmitter in the chicken retina.

High-affinity dopamine uptake, 30 pmol/min.retina, was the first dopamine-related activity detected during development, at the 10th embryonic day (ED10). At ED12, TH became detectable (0.52 pmol/min.retina), whereas dopamine (10.8 pmol/retina) was first detected at ED15. Dopamine uptake activity rose between ED10 and ED12 to 0.35 pmol/min.retina, remained virtually constant until hatch, and rose after hatch to reach mature activity of 9.5 pmol/min.retina at PN10. TH activity kept increasing from ED12 on, but increased most between ED18 and PN4 from 45 to 230 pmol/min.retina. Dopamine levels rose between ED12 and ED18 to 34 pmol/retina, and then remained constant.

By injecting the retina with 200-300 nmole 6-hydroxydopamine (6-OHDA) daily, on 3 subsequent days, retinal TH activities decreased by 75%, retinal dopamine levels decreased by 59%, and retinal dopamine uptake activity decreased by 38%. High-potassium-evoked release of exogenous dopamine decreased by 46%. In 6-OHDA-
treated retinas 40% of the high-potassium-evoked release
was calcium-dependent vs. 84% in control retinas. The
decrease in TH activities was observed up to 31 days after
the last injection of 6-OHDA. The number of dopaminergic
neurons decreased by 46%, and the dopaminergic processes
were clearly disrupted after this 6-OHDA treatment.

The same treatment with 6-OHDA did not significantly
affect retinal wet weights, protein contents, glutamine
synthetase (GS) activities, serotonin levels, and the
levels of taurine, glycine, glutamate, aspartate. Choline
acetyltransferase (CAT) activities and gamma-aminobutyric
acid (GABA) levels were the only entities not related to
dopamine, which were slightly decreased by that 6-OHDA
treatment.

Doses of more than 400 nmol 6-OHDA decreased retinal CAT
activities by 58%, and the levels of taurine by 54%, of
GABA by 68%, and of glutamate by 16%, and increased the
levels of glycine by 51%, aspartate by 48%, tyrosine by
170%, and phenylalanine by 130%.

The results under points 16-18 demonstrate that 3 x 200
nmol 6-OHDA selectively disrupts dopaminergic
transmission.

Intravitreal injections of 130 nmol haloperidol decreased
retinal GABA levels by 24%, 2h after the injection.
Intravitreal injections of 100 nmol dopamine together with
500 nmol pargyline, increased GABA levels by 18%.
Neither haloperidol nor dopamine affected the levels of taurine, glutamate, and glycine, or the activities of GS and CAT, or the protein contents.

The results under point 20 indicates a dopaminergic innervation of GABAergic neurons, probably involving a D₁-receptor.

In urethane anesthetized chickens a maximum pupillary constriction of 16% was observed, compared to a dark-adapted diameter of 3.6 mm observed. In 6-OHDA-treated eyes (see point 19), the same adapting light intensity resulted in a constriction of 26%.

The absolute threshold light intensity for visual evoked potentials (VEPs) was 0.5 log units lower in 6-OHDA-treated retinas, compared to control retinas. In 6-OHDA-treated retinas, the dynamic range was increased by 1 log unit, compared to control retinas. Within the dynamic range of the VEP I/R-function, the VEP was 25 μV larger in 6-OHDA-treated retinas, than in control retinas.

At scotopic light levels the increment threshold of 6-OHDA-treated retinas was increased by 0.3 log units, compared to control retinas. The Weber fraction was 0.72 for treated retinas vs 0.59 for control retinas. At photopic light levels, the Weber fraction was 1.12 in 6-OHDA-treated retinas, vs 1.75 in control retinas.

After exposure to high light intensities (7 log units above VEP threshold, for 10min), the VEP threshold for 6-
OHDA-treated retinas was elevated by 4.4 log units, whereas by 3.6 log units in control retinas. The time constants of the cone-related phase in the recovery to dark-adaptation were 0.44 min\(^{-1}\) in 6-OHDA-treated retinas, vs 0.56 min\(^{-1}\) in control retinas. The time constants of the rod-related phase in the recovery to dark adaptation were 0.21 min\(^{-1}\) in both 6-OHDA-treated and control retinas.

The results under points 23-26 indicate an increase in the light sensitivity, and a change of qualitative properties of the 6-OHDA-lesioned retinas (see point 19), compared to control retinas. These results indicate that the dopaminergic amacrine cells may have a regulatory role in the performance changes of the retina during the transition from scotopic to photopic light conditions.
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References
GENERAL INTRODUCTION. 1.1 Neurotransmitters.

Since communication between neurons is the essence of brain functioning, and since it is now generally accepted that chemical transmission is the predominant, although not the only, form of inter-neuronal communication, knowledge of the chemistry and physiology of neurotransmitters is essential for our understanding of how the nervous system works. An important question in this type of research is which transmitter (or transmitters) is used by a neuron.

How to define a neurotransmitter has been, and still is, a matter of some controversy, but the most fundamental aspect of a neurotransmitter is that its presence in extracellular space must be a message from the neuron in which it originates, to the cell it affects. This rather abstract definition encompasses the following two points:

1. the compound must be released by a neuron,
2. the compound must do something, i.e., the message must be read and responded to.

With respect to the first criterion the following experimental tests can be performed.

(1) If a neurotransmitter has to be released, it should be possible to detect the compound in neurons before release. Thus, the presence of a particular compound in nervous tissue, together with its localization within neurons, are essential for a neurotransmitter function.

(2) The compound has to be acquired by the neuron. So far, all neurotransmitters seem to be synthesized by the neuron itself rather than concentrated there purely by uptake mechanisms. The detection and localization of the synthetic mechanism, provides a further means of
examining neurotransmitter status (see Hokfelt et al., 1984), and also seems to be an essential requirement for a compound to have a neurotransmitter function.

(3) The demonstration that a compound can be released by a physiologically relevant stimulus is a crucial test.

For evoking neurotransmitter release, electrical stimulation of a discrete neuronal tract can be used, but only in fortunate circumstances. This approach cannot be applied in many cases, especially not in many parts of the central nervous system (CNS), where the neurons of interest are small interneurons. A commonly used alternative is biochemical manipulation, mimicking the separate events in the sequence which lead to vesicular neurotransmitter release. By exposing the tissue to high concentrations of potassium (40-70 mM) or calcium ionophores, neurotransmitter release can be elicited. However, these less physiological methods are generally regarded as less convincing.

In general, the calcium dependency of neurotransmitter release is accepted (Katz and Miledi, 1967; see Cooper and Meyer, 1984; Vizi, 1984), and the dependence upon extracellular calcium is often used to distinguish between physiologically relevant and non-specific release. However, it has been suggested that with certain stimuli, calcium could be recruited from intracellular pools (see Vizi, 1984), so that a lack of dependence upon extracellular calcium does not necessarily prove that release is not calcium dependent.

With regard to the second criterion, it is essential that physiological effects of a neurotransmitter are demonstrated. After release, the first stage in this process, at least with all neurotransmitters studied so far, is that the neurotransmitter interacts...
with a receptor which is part of the plasma membrane of the target cell. This interaction must then be transduced into an effect on the target cell. In molecular terms the concept of a receptor-mediated effect can be broken down into recognition and effector sites. (4) Selective binding of a neurotransmitter or a neurotransmitter-analogue can be studied in membrane preparations, and in combination with autoradiography, can be used to localize receptors to different cell types at the cellular level. Useful though these techniques are for defining neurotransmitter-receptor interaction precisely, ligand binding studies do not give information about physiological responses. Therefore, functional assays, where the cellular response to binding is measured, are an essential complement of such studies, and are in fact crucial, since the only definition of a physiologically relevant receptor is that it has the same pharmacological profile as the receptor which mediates the physiological effects of the compound. (5) When receptor activation by a neurotransmitter opens or closes ion channels across the synaptic membrane, this can be measured as changes in membrane potential, synaptic currents, or single channel events. These changes can be measured with extracellular or intracellular recording techniques. Direct effects on membrane electrical properties are often referred to as ionotropic. In general these effects are fast (usec-msec) events following receptor activation.

As opposed to ionotropic effects, other compounds may exert their effects by quite different mechanisms, involving second messenger systems. The intracellular messengers, which include adenosine-3′,5′-cyclic-monophosphate (cAMP), guanosine-3′,5′-cyclic-monophosphate (cGMP), inositol phosphates, and Ca++ (although the influx of the calcium ions may directly change the membrane potential as well), then
initiate a cascade of events, which leads to, or represents, the ultimate response. Second messengers have the capacity to affect electrical properties directly by changing the properties of ion channels, or they may elicit more general metabolic responses. In general, second messenger mediated responses are slower (msec-sec), more graded and more sustained than ionotropic effects. If the effects are purely on the metabolism, then it is a matter of debate whether the compound should be named a neurotransmitter.

In addition to chemical transmission, the more diffuse concept of neuromodulation is often encountered in the literature, as are the even vaguer terms like neuroregulator and neuromediator (see Kupferman, 1979; Schmitt, 1984). Generally, neuromodulation refers to the effect a compound has on the transmission mediated by another compound, in the absence of direct effects of its own. However, when a neuron releases a compound, it does so without the label “neurotransmitter” or neuromodulator”. It is the organization of the plasma membranes and intracellular metabolism of the target cell which determines what the response to the message will be. It is therefore wrong in principle, and confusing in practice, to label a molecule with a name which assumes the type of response which the molecule may cause. It is now clear that all neurotransmitters can evoke a variety of responses, depending upon the post-synaptic cell, and some of these responses may in fact fall across the rather ill-defined border-line between neurotransmission and neuromodulation.

(6) A mechanism for inactivating the neurotransmitter is one of the classical criteria. However, simple diffusion may be a means of inactivation (Werman, 1966). Since any compound capable of diffusing across the synaptic cleft should also be able to diffuse out of the
synaptic cleft, inactivation as a criterion has little discriminatory power. Nevertheless, the presence of specific inactivation mechanisms, like enzymatic metabolism and (re)uptake, lends some credence to the idea that the compound has a transmitter role. Enzyme activities involved in inactivation can be quantified biochemically at the tissue level and detected at the cell level. Uptake systems can be quantified biochemically or localized by autoradiography of the sites of uptake of labelled compounds.

These experimental tests must be applied with caution. Firstly, given the way in which concepts of neurotransmission have changed over the past few years, it would be unwise to be too dogmatic, particularly concerning peripheral criteria such as synthesis, uptake etc. However, the demonstration of release, receptors, and physiological effects seem to be essential to any meaningful concept of neurotransmission. A further need for caution concerns the detection limit inherent in any experimental test, and the consequent problem of false negatives. However, with the enormous improvements with respect to sensitivity and selectivity of detection methods, such as electrochemical detection coupled to high performance liquid chromatography (HPLC-ED), immunohistochemistry, and patch-clamp recording techniques, negative results are more and more reliable indications of the absence of a particular phenomenon. While absolute proof may never be achieved, the results of these experimental tests can give enough circumstantial evidence on the basis of which it can be decided whether the compound is used as neurotransmitter.

Most of these tests can be done at the tissue level, as well as at the cell level. Results obtained at the tissue level in general address the more global question of whether a compound can be a
neurotransmitter, or whether it functions as a neurotransmitter in a particular part of the nervous system. Research of this kind led to the establishment of the classical neurotransmitters, and (although not as established as yet) the neuropeptides, as classes of compounds which can be considered as potential neurotransmitters in parts of the nervous system. With the more sophisticated techniques now available, it has become possible to study at the cell level, the actions of these compounds in a given system. Insight in this area leads us to the core of brain functioning: the communication between individual neurons. In a final rigorous check, results obtained from experimental tests at both levels can be compared quantitatively, for example to check whether endogenous amounts of a compound are indeed sufficient to evoke a response. As yet, this has only been done for cholinergic transmission in the neuromuscular junction, and the fit is still not perfect (see Krnjevic, 1974).

The aim of this thesis is to study a possible role for dopamine as a neurotransmitter in chicken retina. In the following section (Chapter 1.2 dopamine as a neurotransmitter) I will discuss evidence which led to the general conclusion that dopamine is a neurotransmitter in the CNS. After a brief introduction on general aspects of the functional organization of the vertebrate retina (Chapter 1.3 the vertebrate retina), I will review the data supporting the idea that dopamine is a neurotransmitter in vertebrate retina (Chapter 1.4 dopamine in the retina). My own work has focussed upon extending knowledge about dopaminergic neurons in chicken retina. Data concerning biochemical and morphological aspects of the dopaminergic neurons in chicken retina, along with physiological deficits caused by a specific removal of those neurons will be presented.
The catecholamine family is comprised of dopamine, noradrenaline and adrenaline (FIGURE 1.1). These components are formed from the dietary amino acids phenylalanine and tyrosine by the following pathway:

L-tyrosine $\rightarrow$ L-dihydroxyphenylalanine (DOPA) $\rightarrow$ dopamine $\rightarrow$
L-noradrenaline $\rightarrow$ L-adrenaline.

Since the initial isolation of adrenaline in the late 19th century, an extensive literature has accumulated on the localization and function of catecholaminergic transmission in the nervous system. In this section I will summarize aspects of the literature dealing with a transmitter role for catecholamines, especially dopamine. I will focus on methods employed, rather than discussing details of the transmission systems themselves. All the experimental tests relevant to the two criteria outlined in the previous section of the introduction, will be discussed at the tissue level, and then at the cell level.

**Tissue studies.**

**Catecholamine measurement in nervous tissue.**

Fluorimetric assays for catecholamines are based upon the oxidation and cyclization of catecholamines, resulting in the production of fluorophores. The fluorescent properties of the compound depend on the parent catecholamine. With the method described by Carlsson (1959a) dopamine can be distinguished from adrenaline and noradrenaline, but not from DOPA. Since then, numerous modifications of this method have been described (for review, see Taylor, 1979). Sensitivities of most of these methods are better than 10 pmol, but the fluorimetric assays are not entirely specific, so that the individual catecholamines must be separated first.
Figure 1.1: Structures of the catecholamines.
Radioenzymatic methods for the determination of catecholamines, are based on methylation of catecholamines. The labelled catecholamines are then quantified by counting radioactivity. N-Methylation mediated by phenylethanolamine-N-methyl transferase (PNMT) is only suitable for noradrenaline and metabolites, whilst catechol-O-methyl transferase (COMT) catalyzed O-methylation is suitable for all catecholamines and metabolites (for review, see Cuello, 1979). The sensitivities of these assays are comparable with the sensitivity of fluorometric methods, i.e. better than 10 pmol. The radioenzymatic assays themselves are not specific for the different catecholamines, and the individual catecholamines must be separated either before or after methylation.

The sensitivity of catecholamine measurement by gas chromatography coupled to mass spectroscopy (GC-MS) is in the same range as obtained with the fluorimetric and radioenzymatic methods (see Karoum and Neff, 1979). However, GC-MS gives unambiguous answers about the molecular weights, and the structures of the different compounds, which makes GC-MS the most powerful method available for the selective quantification of the different catecholamines. For GC the catecholamines must be derivatized into more volatile compounds (Karoum and Neff, 1979). The major problem with the GC-MS technique is that the equipment required is quite expensive, beyond the budget of most laboratories for routine use.

The most commonly used method nowadays is separation of catecholamine by HPLC, combined with electrochemical detection (HPLC-ED) (Keller et al., 1976; see Adams, 1979; Life Sci. 28: Kissinger et al., 1981; Goldstein et al., 1981; Mefford et al., 1981; Rahman et al., 1981; Watson et al., 1981; Cross and Joseph, 1981). Electrochemical detection is very sensitive for the easily oxidizable catecholamines and metabolites, detection of 100 fmol noradrenaline and dopamine having
been reported (Keller et al., 1976; Adams, 1979). Methods have been described where, with virtually no sample preparation, levels of up to 14 biogenic amines, precursors and metabolites can be determined within a 20 min chromatographic run (Zaczek and Coyle, 1982; Duda and Moore, 1985; Hadfield and Narasimhachari, 1985). In general, the only criterion for identification for a compound is its retention time (Johnson and Stevenson, 1978; Molnar, ed., 1983). This means that it is essential to show that a range of other compounds have different retention times. This aspect of an otherwise very useful method is often neglected.

Dopamine has been shown to be present in the nervous system and some peripheral organs of all vertebrate species investigated (see Holzbaucer and Sharman, 1979). With the refinements in catecholamine detection mentioned above it has become possible to measure levels of the different catecholamines in localized regions of the nervous system. In some areas the high dopamine/noradrenaline ratio suggests that dopamine is important in itself, rather than being a precursor for noradrenaline (see Palkovits, 1979).

**Catecholamine biosynthesis.**

The complete sequence of catecholamine biosynthesis, starting from the dietary amino acid phenylalanine, was correctly predicted by Blaschko (1939) (FIGURE 1.2). However, it was not until 1963, with the availability of radiolabelled tyrosine, that McGeer et al. (1963) demonstrated the existence of a tyrosine hydroxylating capacity of brain, the last link to be established.

Phenylalanine hydroxylase (EC 1.14.16.1) (FIGURE 1.2) has been purified from mammalian liver, and has a molecular weight of 50,000
Figure 1.2: The biosynthesis of catecholamines.

Enzymes: PHE-H, phenylalanine hydroxylase
TH, tyrosine hydroxylase
DDC, DOPA decarboxylase
DBH, dopamine-beta-hydroxylase
PNMT, phenylethanolamine-N-methyltransferase.

Cofactors: BH$_4$, tetrahydrobiopterin
BH$_2$, dihydrobiopterin
SAM, S-adenosyl methionine.
PHENYLALANINE  \[ \text{BH}_4, \text{O}_2, \text{H}_2\text{O} \rightarrow \text{TYROSINE} \]

TYROSINE  \[ \text{BH}_4, \text{O}_2, \text{H}_2\text{O} \rightarrow \text{DOPA} \]

DOPA  \[ \text{pyridoxal phosphate} \rightarrow \text{DOPAMINE} \]

DOPAMINE  \[ \text{ascorbate, O}_2, \text{Cu}^{II}, \text{dehydroascorbate, H}_2\text{O} \rightarrow \text{NORADRENALINE} \]

NORADRENALINE  \[ \text{CH}_3-\text{SAM} \rightarrow \text{ADRENALINE} \]

ADRENALINE
daltons (see e.g. Smith et al., 1984). Using assay conditions under which tyrosine hydroxylase (TH) is not active (pH 7.3), no phenylalanine hydroxylase activities could be detected in brain of rat and guinea pig (Guroff and Abramowitz, 1967). However, it has been shown in vitro that highly purified TH from adrenal medulla hydroxylates phenylalanine as well as tyrosine, provided that biopterin is present as cofactor (Kaufman, 1974). Phenylalanine hydroxylating activity has also been detected in synaptosomes from striatum, hypothalamus, and hippocampus (Leighton and Waggoner, 1981), and in several brain regions in rat in vivo (Bagchi and Zarycki, 1973). Weiner et al. (1974) demonstrated in guinea pig in vivo, that over 90 % of the catecholamines formed in the brain are derived from tyrosine rather than from phenylalanine, and nervous tissue probably depends upon an adequate supply of tyrosine for its catecholamine synthesis (Fernstrom, 1983; Westerink and Wirix, 1983).

TH (EC 1.14.16.2) is the first enzyme in the sequence specific for catecholamine biosynthesis (FIGURE 1.2). It is a mixed function oxygenase, requiring molecular oxygen and a reduced cofactor for catalytic activity. TH hydroxylates tyrosine to produce DOPA, while the pterin cofactor is oxidized and water is released. Biopterin is the presumed natural cofactor for TH (see Gal, 1982).

TH has been purified from several sources and apparently occurs in several molecular weight forms, ranging from 49,000 to 186,000 daltons (see Roth, 1979). The smallest form which still shows catalytic activity has a molecular weight of 34,000 daltons and was obtained by mild tryptic digestion in vitro. The enzyme probably exists in a multitude of polymeric forms, and might be conjugated with non-protein moieties such as RNA. TH is present in soluble form, although the
enzyme readily aggregates, adsorbs to subcellular particles and becomes sedimentable. Differences between TH isolated from noradrenergic and dopaminergic tissues in apparent affinity for the cofactor have been reported (Reis et al., 1975). However, the TH had not been purified to homogeneity, and differences in apparent kinetic properties could as well be due to the variations in constituents of the TH-preparations. Synaptosomes derived from a noradrenergic source (hippocampus, hypothalamus) showed TH activities with kinetic properties comparable to those detected in striatal synaptosomes (Boarder and Fillenz, 1978; Leighton and Waggoner, 1981). So, there is no evidence as yet to support the idea that there are different TH enzymes in the various catecholamine-synthesizing tissues.

TH is the rate-limiting step in catecholamine biosynthesis (Levitt et al., 1965; see Roth 1979) and has been studied as the principal site for regulation of catecholamine biosynthesis. Early studies demonstrated that TH activity was inhibited by catecholamine-like compounds, including DOPA, dopamine, noradrenaline and adrenaline, in vitro (Nagatsu et al., 1964; Karobath, 1971; Waggoner et al., 1980). Kinetic studies have revealed that these compounds compete with the pterin cofactor, as demonstrated by an increase in apparent $K_m$ for the cofactor in the presence of catechol inhibitors (Nagatsu et al., 1972; Ames et al., 1978). Physical competition at the pterin binding site on TH has not yet been demonstrated. In vivo, the observed increase in formation of $^3$H-noradrenaline from $^3$H-DOPA together with a decreased formation of $^{14}$C-noradrenaline from $^{14}$C-tyrosine, after the accumulation of catecholamines by inhibition of monoamine oxidase (MAO), confirm that catecholamine synthesis in the brain is subject to endproduct inhibition (Spector et al., 1967). In addition, it has been shown that dopamine...
taken up by synaptosomes inhibits intrasynaptosomal TH. This suggests that TH activity can also be affected by released and recaptured catecholamines via endproduct inhibition (Waggoner et al., 1980; Maura and Raiteri, 1982).

Substrate availability has been proposed as a factor determining TH activity \textit{in vivo} (for review, see Fernstrom, 1983). During conditions of increased dopamine synthesis, e.g. after treatment with a dopaminergic antagonist, significant drops of tyrosine levels in brain areas rich in dopaminergic innervation have been observed (Westerink and Wirix, 1983). Administration of tyrosine has been shown to stimulate the accumulation of DOPA after the blockade of DOPA decarboxylase (DDC), indicating stimulated dopamine synthesis (Wurtman, 1974; Badawy and Williams, 1982). These results indicate that, especially during high activity, the availability of tyrosine can limit catecholamine biosynthesis. Changes in environmental oxygen levels have also been shown to affect catecholamine synthesis in rat brain, which indicates that TH might not be saturated with respect to its oxygen substrate (Davis, 1975). Whether this represents a physiological site for regulation of catecholamine synthesis has yet to be established. Levels of biopterin have been found to be low in catecholamine synthesizing tissue (Gal et al. 1976; Abou-Donia and Viveros, 1981), although the actual concentrations at the enzyme sites are not known. Addition of tetrahydrobiopterin elevated TH activity in synaptosomes prepared from rat striatum and hypothalamus, but not in synaptosomes from hippocampus (Boarder and Fillenz, 1978). Injection of tetrahydrobiopterin into rat cerebral ventricles has been shown to increase striatal dopamine synthesis (Kettler et al., 1974). These results demonstrate that TH, at least in certain brain areas, is not saturated for the pterin cofactor.
The extent of endproduct inhibition and limits placed on activity by substrate availability, are likely to be further regulated by allosteric modification of TH. TH is a phosphoprotein and its phosphorylation appears to be regulated by cAMP- and calcium/calmodulin-dependent protein kinases (Haycock et al., 1982; Treiman et al., 1983; Nestler et al., 1984; Niggli et al., 1984). It has been demonstrated in various tissues, that activation of TH by phosphorylation-favouring conditions is accompanied by an increased affinity of the enzyme for the pterin cofactor and a decreased affinity for catechol inhibitors (Zivkovic et al., 1974; Lovenberg et al., 1975; Morgenroth et al., 1975; Lazar et al., 1982; Mestikawy et al., 1983; Vrana and Roskoski, 1983; Iuvone, 1984a). Thus the second messenger-regulated phosphorylation of TH could well represent the molecular basis for receptor-mediated modulation of catecholamine biosynthesis (see Chesselet, 1984).

Recently, it has been shown that the methylation of TH from striatum is correlated with comparable changes in the kinetic properties of TH (Mann and Hill, 1983). Since it has also been reported that stimulation of dopaminergic autoreceptors (see Dopamine receptors) increases protein methylation (Wolf and Roth, 1985), this might represent an additional molecular pathway, specific for the autoreceptor mediated feedback control of catecholamine synthesis.

The close relationship between TH activity and the amount of reduced pterin suggests that features of pterin metabolism might serve as a marker for catecholamine synthesis. However, both phenylalanine- and tryptophan hydroxylase also depend upon reduced pterin cofactor (Gal, 1982), and thus biopterin reductase will not indicate catecholamine synthesis selectively.
DDC is the next enzyme in catecholamine synthesis (FIGURE 1.2). DDC catalyses the decarboxylation of DOPA into dopamine and depends upon pyridoxal-5-phosphate as cofactor. It also decarboxylates 5-hydroxytryptophan (5-HTP), the precursor for serotonin, and other aromatic amino acids. DDC activities are more widely distributed than the activities of the other enzymes involved in catecholamine synthesis. Antiserum raised against DDC precipitates 5-HTP decarboxylase activity. Thus, it is likely that the two decarboxylating activities represent the same enzyme. Accordingly, the more general term aromatic amino acid decarboxylase (AADC) (EC 4.1.1.28) is often used (for review, see Sourkes, 1979).

DDC has a high affinity for its substrate DOPA, and *in vivo* its $V_{\text{max}}$ exceeds the capacity of TH to produce DOPA. This explains the virtual lack of free DOPA in catecholaminergic tissue and why very high doses of DDC inhibitors are necessary to block catecholamine synthesis (Nagatsu *et al*., 1964; Jung *et al*., 1979; Westerink, 1985). Alpha-fluoromethyl-DOPA alkylates the decarboxylase at its active site and is an effective inhibitor of AADC activity (Kollonitsch *et al*., 1978). It is not, however, selective for DDC.

Dopamine-beta-hydroxylase (DBH) (EC 1.14.17.1) and PNMT (EC 2.1.1.28) are the next enzymes in catecholamine biosynthesis. DBH hydroxylates dopamine to noradrenaline, which is the substrate for PNMT catalyzed methylation to adrenaline (FIGURE 1.2).

As can be seen from figure 1.2, PNMT activity indicates the presence of adrenergic activity. The absence of PNMT activity together with the presence of DBH is an indication of noradrenergic activity. Dopaminergic areas are characterised by the presence of TH activity alone. The turnover of dopamine seems generally higher than the
turnover of noradrenaline (Bacopoulos and Bhatnagar, 1977). This may explain the observation that highest TH activities are generally found in brain regions containing dopaminergic neurons.

**Cathecholamine release.**

Dopamine is taken up into vesicles by an energy dependent process. The vesicular amine pump concentrates catecholamines within the vesicles, and thus under normal conditions, low concentrations of free catecholamine are present in the cytosolic subcellular fraction (Holz, 1978; see Axelroth, 1971; Horn, 1979).

Most studies of the release of dopamine *in vitro* and *in vivo* have been done on the nigrostriatal dopamine system (for review, see Glowinski *et al.*, 1979). The spontaneous and evoked releases of endogenous dopamine can be detected in superfusates. However, due to the small amounts of dopamine released, most authors have used isotopic techniques which consist of prelabelling the neurons with exogenous radiolabelled dopamine, or with radiolabelled dopamine synthesized from labelled tyrosine *in situ*. Both the spontaneous release of dopamine and the release evoked by various depolarizing agents or electrical stimulation appear to be calcium-dependent and sensitive to tetrodotoxin. These results point towards the presence of a "classical" (see Cooper and Meyer, 1984), depolarization-triggered, calcium-dependent, vesicular release mechanism for dopamine.

It has been shown *in vitro* and *in vivo* that dopamine can also be released from dendrites (see Cheramy *et al.*, 1981). Using tissue slices from the pars reticulata of the substantia nigra of the rat, where dopaminergic dendrites rather than the cell bodies are localized, high-potassium-evoked release of exogenous dopamine has been demonstrated.
(Geffen et al., 1976). In vivo, release of dopamine in the substantia nigra of the cat has been demonstrated using the push-pull cannula method together with continuous labelling of the dopaminergic dendrites with 3H-tyrosine (Nieoullon et al., 1977). The mechanism of dopamine release in the dendrites seems to differ from the mechanism in the nerve terminals, as the dendrites contain very few vesicles. It has been suggested that in the dendrites, dopamine is stored in the smooth endoplasmatic reticulum (see Cheramy et al., 1981). Non-vesicular release of transmitter may be a physiologically important mode of transmitter release (see Cooper and Meyer, 1984; Vizi, 1984), and it is likely that such a release mechanism exists for dopamine.

An indirect method of measuring release of catecholamines is by monitoring catecholamine metabolites. Because of the extracellular localization of the enzyme COMT (see Inactivation of catecholamines), the levels of O-methylated metabolites are likely to reflect the amount of released catecholamine. Decreases in the levels of 3-methoxytyramine (MTA) upon pharmacologically decreasing the release of dopamine, have been reported (Ponzio et al., 1981; Westerink and Spaan, 1982; see Westerink, 1979). However, treatment with benztropine or morphine, which inhibit the reuptake of dopamine, did not reveal detectable increases in levels of MTA (Westerink and Spaan, 1982). It may be preferable to look at the entire spectrum of metabolites, to examine shifts within dopamine pools (Ponzio et al., 1981; Westerink, 1985).

A promising method of measuring monoamine release is by voltammetry (see Stamford, 1985). As with electrochemical detection, the oxidative currents contributed by oxidizable species of compounds in the extracellular fluid are measured at the surface of an electrode. Electrodes with diameters as small as 8 μm obviously have an enormous
potential for anatomical selectivity. The levels of compounds are measured directly in the extracellular fluid. Thus, in contrast to superfusion methods, there is no dilution step diminishing sensitivity. The sampling time can be as short as 5 seconds for a full voltammetric spectrum, or less than one second at a particular potential, illustrating the method's high temporal resolution. Poor selectivity for different compounds is the major technical problem yet to be overcome (Forni and Nieoullon, 1984; Kuhr et al., 1984; for review, see Stamford, 1985).

Dopamine-sensitive adenylate cyclase.

Initiated by observations in the mammalian superior cervical ganglion (SCG) (see *The electrophysiology of dopamine*, FIGURE 1.6) it has been proposed that the physiological action of dopamine in general is mediated by adenylate cyclase (EC 4.6.1.1) (Kebabian et al., 1975; see Miller and McDermed, 1979; Drummond, 1984). Since the first report identifying dopamine-sensitive adenylate cyclase in caudate nucleus as the "dopamine receptor" (Kebabian et al., 1972), dopamine-sensitive adenylate cyclase activities have been described in all areas of the nervous system which receive dopaminergic input (see Miller and McDermed, 1979; Drummond, 1984). It is now widely accepted that all dopamine receptors are coupled to adenylate cyclase, either activating or inhibiting enzyme activity (see *Dopamine receptors*).

Like dopamine, many hormones and transmitters exert their effects on target cells by changing the intracellular levels of cAMP, which acts as a second messenger. This is achieved by binding to specific sites on the cell surface, which then affect the activity of adenylate cyclase. This has been studied particularly in the adrenergic adenylate cyclase
system, where the interaction between the two entities appears to be mediated by a regulatory GTP-binding protein. Two types of regulatory proteins have been isolated: one mediating an activation- and the other an inhibition of the catalytic unit (See Houslay, 1983; Schramm and Selinger, 1984; Sibley and Lefkowitz, 1985).

There are some indications that the dopaminergic adenylate cyclase system is organized in the same way. Solubilization of dopamine receptors from the anterior pituitary revealed the presence of a third protein component, in addition to the transmitter binding site and the catalytic site of adenylate cyclase (Caron et al., 1983; George et al., 1983). Further, GTP in the presence of divalent cations has been found to favour the low affinity state for agonist binding, whilst the affinities for antagonists remained unchanged (Makman et al., 1982; Caron et al., 1983).

In a unifying hypothesis it has been proposed that recognition sites for the various transmitters known to affect adenylate cyclase activity, all interact with shared regulatory- and catalytic units (Cuatrecasas, 1974). This would leave the dopamine recognition site as the only molecular entity selective for the dopamine receptor.

These features of dopamine's physiological effects equip research into dopaminergic transmission with powerful probes. The presence of dopamine-sensitive adenylate cyclase is an important indication that dopamine serves a transmitter role in the area under search. However, the inhibitory effects of dopamine on adenylate cyclase are often difficult to detect (see Dopamine receptors), and a lack of effect of dopamine on cAMP levels does not therefore exclude dopaminergic innervation.
Adenylate cyclase activity can be measured radioenzymatically (Schultz and Mailman, 1984), or indirectly by measuring cAMP accumulation (Greengard et al., 1972). Accurate estimates of cAMP levels in vivo are often difficult to obtain due to pronounced post mortem changes. Killing by microwave irradiation has been reported as the best available method to preserve endogenous cAMP levels (Schneider, 1984; see Bonnet, 1982).

It might also be possible to use cAMP-dependent protein phosphorylation, which is thought to mediate the biological response of the changed levels of cAMP (Greengard, 1978b; Williams, 1979; for review, see Nestler and Greengard, 1983) as a marker for dopaminergic transmission. The presence of one of the numerous phosphoproteins whose phosphorylation is regulated by cAMP-dependent protein kinase (see Nestler et al., 1984) is not in itself indicative of dopaminergic innervation. However, a change of degree of phosphorylation brought on by dopamine (see e.g. Jork et al., 1984; Memo and Hanbauer, 1984) indicates responsiveness to dopamine, and thus possible dopaminergic innervation. Recently a phosphoprotein, DARPP-32, whose phosphorylation is regulated by dopamine via cAMP, has been found specifically in dopamine rich brain tissues of the rat. The protein was not affected by 6-hydroxydopamine (6-OHDA) lesioning, which has been interpreted as indicating that the protein is not found within dopaminergic neurons, but may be in cells post-synaptic to them (Walaas and Greengard, 1984).

**Dopamine receptors.**

Based on binding characteristics, Seeman (1982) proposed that there are four classes of dopamine receptors (TABLE 1.1). However, further studies into the mechanism of receptor-affected adenylate cyclase
Table 1.1: Classification of dopamine receptors

<table>
<thead>
<tr>
<th></th>
<th>D₁</th>
<th>D₂</th>
<th>D₃</th>
<th>D₄</th>
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<td>D₁</td>
<td>D₂</td>
<td>D₃</td>
<td>D₄</td>
</tr>
<tr>
<td>Dopamine EC₅₀</td>
<td>µM</td>
<td>µM</td>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>Spiperone IC₅₀</td>
<td>µM</td>
<td>nM</td>
<td>µM</td>
<td>nM</td>
</tr>
<tr>
<td>Laduron (1982)</td>
<td>dopamine receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kebabian and Calne (1979)</td>
<td>D₁</td>
<td>D₂ low</td>
<td>D₂ high</td>
<td></td>
</tr>
<tr>
<td>Labrie et al. (1983)</td>
<td>D₁⁺</td>
<td>D₂⁻</td>
<td>D₀</td>
<td></td>
</tr>
<tr>
<td>Creese (1985)</td>
<td>D₁ low</td>
<td>D₂ low</td>
<td>D₁ high</td>
<td>D₂ high</td>
</tr>
</tbody>
</table>

The most generally applied classification comprises two types of dopamine receptors (Kebabian and Calne, 1979) (Table 1.1). The system of receptors is linked to the messenger electrical signals, such that against blocking increases appropriate physiological activity. The brain's dopamine
activity revealed that binding of the agonist favours the exchange of GDP for GTP on the regulatory unit. The regulatory unit then dissociates from the receptor site, which leaves the receptor site in a low affinity state (see Schramm and Selinger, 1984). Thus the change in affinity is an integral part of the response, which by definition, only can be elicited by agonist binding. Considering these aspects, the four distinct ligand-binding profiles probably represent two receptors, which in ligand-binding assays, can appear in different states of affinity for agonists and antagonists (see Creese, 1985). Labrie et al. (1983) proposed the use of the effects on adenylate cyclase activity as the only classifying criterion only (TABLE 1.1). DA_+ and DA_- refer in their classification to dopamine stimulated- and inhibited adenylate cyclase activity respectively. They predict the existence of a third type of receptor which would not be linked to adenylate cyclase: DA_0. However, such a receptor has yet to be found, and inherent to its negative definition, it would be difficult to define as being different from the DA_+ or DA_- types. Further, without demonstrated physiological effects, which is essential for the concept of a receptor, the DA_0-type is meaningless. A different position is taken by Laduron (Laduron and Ilien, 1982; Laduron, 1983), who argues that there is only one type of dopamine receptor (TABLE 1.1). He makes this argument by insisting that a receptor should mediate a detectable physiological effect at the whole-body level, and in doing so in fact, he rejects most data obtained in neuroscience studies in general.

The most generally applied classification recognizes two types of dopamine receptors (Kebabian and Calne, 1979) (TABLE 1.1). The D_1-type receptor is linked to the enzyme adenylate cyclase, such that agonist binding increases adenylate cyclase activity. The bovine parathyroid
has been proposed as the tissue containing the prototype receptor (Brown and Dawson-Hughes, 1983). Dopamine binding sites on the mammotrophs of the anterior pituitary initially appeared not to be linked with adenylate cyclase activity, and were referred to as $D_2$-type receptors. However, agonist binding to receptors in the intermediate lobe of the pituitary was found to decrease adenylate cyclase activity (De Camilli et al., 1979a; Kebabian et al., 1983). Binding characteristics did not distinguish these receptors from the $D_2$-type. It is now generally accepted that agonist binding to $D_2$-sites inhibits adenylate cyclase, although such inhibitory effects, as in the mammotroph model, might be difficult to detect, either because of low basal levels of cAMP or interference by parallel stimulation of $D_1$-type receptors.

Thus, the effects of dopamine on adenylate cyclase activity and cAMP levels can be used as a functional assay for the concurrent examination of binding sites and effector sites. The two types of dopamine receptors are distinguished by characteristic differences in the potencies of several agonists and antagonists (TABLE 1.2). The ergot derivatives (e.g. bromocryptine and lisuride) act as agonists for the $D_2$-type receptors, and as potent antagonists for the $D_1$-type. Because the $D_2$-mediated effects are not always easy to measure, the discriminatory capacity of these ergot derivates is often difficult to apply. The $D_2$-selective ligand domperidone is often employed in binding studies to differentiate between the $D_1$- and $D_2$-receptors (Watling and Iversen, 1981). Recently a $D_1$ specific antagonist, SCH23390, has become available for this purpose (Christensen et al., 1984).

There is considerable amount of biochemical and electrophysiological data to support the existence of autoreceptors, or presynaptic receptors, on dopaminergic neurons (see Bunney, 1979; Roth,
<table>
<thead>
<tr>
<th>Receptor Function</th>
<th>Receptors</th>
<th>Agonists/Agonist Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulatory</td>
<td>D1</td>
<td>Apomorphine, SKF-38393, SCH-23390</td>
</tr>
<tr>
<td>Partial agonist</td>
<td>D1</td>
<td>Bromocriptine, Quinpirole</td>
</tr>
<tr>
<td>Antagonist</td>
<td>D1</td>
<td>Haloperidol, (-)-Sulpiride</td>
</tr>
<tr>
<td>Inhibitory</td>
<td>D2</td>
<td>Fluphenazine, Chlorpromazine</td>
</tr>
<tr>
<td>Partial agonist</td>
<td>D2</td>
<td>Domperidone, (-)-Sulpiride</td>
</tr>
<tr>
<td>Antagonist</td>
<td>D2</td>
<td>Haloperidol, (-)-Sulpiride</td>
</tr>
</tbody>
</table>

*relative selectivity of some agonists*

**Table 1.2**: Dopamine receptors

**SKF-38393**: 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine

**SCH-23390**: R(+) 8-chloro-2,3,4,5-tetrahydro-5-methyl-1H-3-benzazepine-7-ol

**3-PPP**: 3-(3-hydroxyphenyl)-N-n-propyipiperidine

Kebabian and Calne's (1979) classification, based on bovine parathyroid and mammotroph of anterior pituitary as loci for prototypic D1 and D2 respectively.

Creese (1985)

(+)-enantiomer weak agonist, and autoreceptor agonist (Hjorth et al., 1981); (-)-enantiomer weak antagonist in rabbit retina (Schorderet et al., 1984); racemic 3-PPP weak antagonist in carp retina for D1 type (Watling et al., 1982).
That is, dopamine agonists have been demonstrated to decrease the firing rate, the release of (exogenous) dopamine, and the dopamine turnover of dopaminergic neurons, in vitro and in vivo. Similar effects have been obtained when nerve activity is blocked with tetrodotoxin, or after lesions of suspected feedback pathways. Some of the effects of dopaminergic drugs on dopamine release and turnover have been detected in synaptosomes. The effects of dopamine agonists on the activity of the dopaminergic neurons, were not accompanied by an increase in cAMP levels, which suggests that the receptor mediating these effects is not the D<sub>1</sub>-type. However, the pharmacological characteristics of the autoreceptors are different to that expected for D<sub>2</sub>-receptors. In particular, 3-(3-hydroxyphenyl)-N-n-propylpiperidine (3-PPP) has been reported to decrease dopamine turnover. Because 3-PPP did not elicit hypermobility or turning-behaviour, stereotypes normally elicited by the activation of postsynaptic dopamine receptors, 3-PPP has been put forward as a selective autoreceptor agonist (TABLE 1.2) (Hjorth et al., 1981). However, it remains to be demonstrated that autoreceptors, so far detected only through their physiological effects, are present as ligand-binding entities on the dopaminergic neurons.

After suppression of dopaminergic transmission, increased sensitivity of dopamine receptors has been observed (Ungerstedt et al., 1975). This physiological supersensitivity is accompanied by an increase in the number of ligand binding sites, rather than by changes in affinity (Von Voigtlander et al., 1973; see Hornykiewicz, 1979). Receptors may appear or disappear from the cell membrane within one minute or less (see Schramm and Selinger, 1984). This poses serious problems in the interpretation of observed binding characteristics after
6-OHDA treatment, or after other manipulations of endogenous dopamine levels (Leff and Creese, 1983; Bacopoulos, 1984).

**Inactivation of catecholamines.**

Methods for disposing of the released catecholamine include uptake by the nerve-endings, breakdown by MAO and COMT, uptake into other cells, and diffusion from the synaptic cleft. The most important mechanism for terminating physiological action of catecholamines is reuptake into the nerve-endings. Catecholamines taken up are concentrated in vesicles and can be re-used as neurotransmitter (Costa and Neff, 1970; see Axelrod, 1971).

Re-uptake of catecholamines is an energy dependent process. The Na\(^+\)- and K\(^+\)-dependency and sensitivity to ouabain *in vitro*, suggest a coupling with the Na\(^+\), K\(^+\)-ATPase activity of the plasma membrane (see Horn, 1979). This distinguishes the pump from the vesicular catecholamine pump, since the latter is Mg\(^{++}\) and ATP-dependent, and is coupled to a proton pump (Holz, 1978). The vesicular pump is specifically inhibited by reserpine, which leads to depletion of the vesicles, and consequently abolishes (vesicular) catecholamine release. The effects of inhibition of the plasma membrane pump (carrier), in contrast, prolongs catecholamine presence in the synaptic cleft, which results in effects related to an increase in catecholaminergic transmission (see Horn 1979).

The carriers on the terminals of the different classes of catecholaminergic neurons are not identical. The dopamine carrier can be specifically blocked with benztropine, whereas high affinity noradrenaline uptake is blocked by desipramine (see Horn, 1979). This property has been employed to increase selectivity in uptake studies (Breese and Taylor, 1971).
The plasma membrane carriers in catecholaminergic cells, although having high substrate affinities, are easily saturated, whereas non-catecholaminergic cells possess low-affinity but high-capacity carrier mechanisms. High concentrations of catecholamines thus lead to accumulation by both catecholaminergic and non-catecholaminergic cells, which diminishes the selectivity of a method using catecholamine uptake as a marker for catecholaminergic neurons. However, catecholaminergic carriers do show an absolute stereochemical selectivity for the L-form of catecholamines (see Horn 1979), which can be exploited to distinguish between catecholaminergic and non-catecholaminergic accumulation.

The catecholamine carriers have a wide range of substrate specificity. This property is exploited in chemical lesioning studies with 6-OHDA, where the carrier transports this catecholamine-analogue inside the nerve terminal, and the resulting accumulation of the cytotoxin kills the cell (see Manipulating dopaminergic transmission and Chapter 6). The wide range of substrate specificity can also be employed to load neurons with more exotic catecholamine congeners than the ordinary 3H- or 14C radiolabelled compounds. 6-[^19F]-Fluorodopamine has been used to study dopaminergic transmission with the aid of 19F-nuclear magnetic resonance in vitro (Diffley et al., 1983), and 6-[^19F]-fluoroDOPA with positron emission tomography in vivo (Calne et al., 1985).

Two main enzymes are involved in the breakdown of catecholamines (FIGURE 1.3). MAO (EC 1.4.3.4) oxidizes catecholamines into the corresponding aldehydes. COMT (EC 2.1.1.6) transforms catechols into O-methylated derivatives. Oxidized catecholamines are not physiologically active, and O-methylated compounds lose virtually all their physiological activity. Thus, these two enzymes play an important role
Figure 1.3: Breakdown of catecholamines.

COMT, catechol-0-methyltransferase
MAO, monoamine oxidase
ADH, alcohol dehydrogenase
DOPAC, 3,4-dihydroxyphenylacetic acid
MTA, 4-hydroxy-3-methoxy-phenylethylamine
    (3-methoxytyramine)
HVA, homovanillic acid
NMA, normetadrenaline
DOPEG, 3,4-dihydroxyphenylglycol
MA, metadrenaline
MÖPEG, 4-hydroxy-3-methoxyphenylglycol.
In catecholamine inactivation, the localization of the two enzymes suggests a different metabolic pathway for different pools of catecholamines. MAO is bound to the inner membrane of mitochondria and oxidizes cytoplasmic catecholamines (see Tipton, 1979). COMT is located primarily on the outer membranes of mitochondria and lysosomes, and hence acts on endogenous production and turnover, 1970; see Goldberg, 1970. Catecholamines (as dopamine) have been isolated from different species (see Tipton, 1977; McCann, 1978). The difference in enzyme isolated from liver appears to be antigenically identical and has been suggested to be a polymorphic form of a single enzyme. Two classes of MAO have been described. MAO-A is specifically inhibited by pargyline and has been suggested to have activity against serotonin and noradrenaline. MAO-B is found to be most specific with respect to dopamine and norepinephrine. MAO-B is active in the different catecholaminergic terminal in central cell or in thepostsynaptic neuron. Insight into the localization of the different forms of MAO with the availability of specific inhibitors, could provide an enormous potential for selective pharmacological manipulation of specific aspects of catecholaminergic transmission.
in catecholamine inactivation. The localization of the two enzymes suggests a different metabolic pathway for different pools of catecholamines. MAO is bound to the outer membrane of mitochondria and oxidizes cytoplasmic catecholamines (see Tipton, 1979). COMT is located primarily on the outer surface of the plasma-membranes of most cells, and hence acts on extracellular catecholamines (Uretsky and Iversen, 1970; Kaplan et al., 1979; see Guldberg, 1979). Levels of O-methylated catecholamines (MTA for dopamine) have been used as a marker for the metabolism of released catecholamine (Ponzio et al., 1981; Westerink and Spaan, 1982; Westerink, 1985). Unfortunately, the extremely fast degradation of MTA by MAO and the consequently low pools of MTA (Waldmeier et al., 1981), limit the utility of this approach in catecholamine turnover studies.

Several forms of MAO have been isolated from different sources (see Tipton, 1979; McCanley 1981). The different isoenzymes isolated from liver appear to be antigenically identical and have been suggested to be polymeric forms of a 425,000 daltons subunit. On the basis of selective inhibition two classes of MAO have been described from rat brain. MAO-A is specifically inhibited by clorgyline and shows highest activity against serotonin and noradrenaline. MAO-B is found to be most effectively inhibited by deprenyl and is less specific with respect to substrate. It is not clear which form of MAO is active in the different catecholaminergic terminals, in glial cells or in the postsynaptic neurons. Insight into the localization of the different forms together with the availability of specific inhibitors, would provide an enormous potential for selective pharmacological manipulation of specific aspects of catecolaminergic transmission.
**Dopaminergic transmission at the tissue level.**

Overwhelmingly, these results reviewed favour the acceptance of dopamine as a neurotransmitter in the nervous system. Dopamine is present in neuronal tissue and its heterogenous distribution suggests that it has a special role confined to particular areas. The same areas possess a mechanism for synthesizing dopamine from tyrosine. Dopamine is also a transient metabolite in the synthesis of noradrenaline, itself a substrate for adrenaline synthesis. Thus there is no enzyme specific for dopamine synthesis. However, the distribution of the specific pattern of enzyme activities, i.e. TH activity but not DBH-, or PNMT activity, does seem to follow the distribution of relatively high dopamine-to-noradrenaline ratios in the nervous system. The sensitivities of the available analytical methods for these entities are very high.

The dopamine present in various parts of the nervous system can be released by a variety of stimuli. Receptors for dopamine have been characterized and appear to be coupled (positively or negatively) to adenylate cyclase. Dopamine, together with other transmitters, changes intracellular levels of the second messenger cAMP, which has been implicated in various cell processes (for review, see Greengard, 1978a; Drummond, 1983). It is generally accepted that dopamine exerts physiological effects, despite the lack of a clear physiological role for dopamine-related cAMP (see *The electrophysiology of dopamine*).

Numerous dopaminergic areas have been defined in the nervous system, but it is essential to go beyond this level to describe which particular cells are dopaminergic, and what effects they exert.
Cellular studies.

Catecholamine histofluorescence.

Histofluorescence techniques for detecting catecholamines (Falck et al., 1962) were the first methods enabling neurons to be visualized on the basis of transmitter content. They are based upon the fact that certain catecholamines and serotonin react with formaldehyde, to form intensely fluorescent reaction products (FIGURE 1.4). If endogenous levels are sufficient to create detectable fluorescence, individual neurons can be examined for their biogenic amine content with this method. The yellowish fluorescence originating from serotonin is easily distinguishable from the greenish fluorescence of the dopamine-noradrenaline derivatives. The method is not useful for distinguishing dopamine- from noradrenaline-containing neurons. As can be seen from its chemical structure, adrenaline with its secondary ammonium (FIGURE 1.1), is not suitable as substrate. Thus the method is not applicable for tracing adrenaline-containing neurons.

Several modifications of the original histofluorescence method have been made (see Berger and Nguyen-Legros, 1979). An important simplification has been described by Furness et al. (1972). In contrast to the Falck method, where dehydrated tissue is exposed to formaldehyde-vapour, tissue is exposed to formaldehyde-solutions during mild fixation. Thus, prior dehydration and careful control of humidity during formaldehyde treatment are avoided with this modification. Bjorklund et al. (1968) devised a microspectrofluorometric method to distinguish between dopamine- and noradrenaline-derived fluorescence. Exposure to HCl-fumes after the development of histofluorescence shifts the excitation optima and emission maxima for dopamine- and noradrenaline derivatives, so that the fluorophores can be discriminated spectrophotometrically.
Figure 1.4: Reactions of dopamine and serotonin with formaldehyde to produce fluorophores (after Falck et al., 1962).
DOPAMINE

3,4-dihydroxyisoquinoline

GREEN

SEROTONIN

3,4-dihydro-β-carboline

YELLOW
The formaldehyde fluorescence technique is only applicable at the light microscopic level, and reveals catecholamine containing cell bodies and varicosities. To study axonal networks or synaptic contacts an electron microscopic stain is essential. Based upon the efficient monoamine accumulating capacity of catecholaminergic neurons, several radiolabelled catecholamines have been used for autoradiographic observations of the neurons. Due to the broadness and overlap of substrate selectivities of the different uptake systems, methods based upon catecholamine accumulation tend to suffer from a lack of selectivity.

Catecholamine immunohistochemistry.

Some of the limitations of the histofluorescence technique have been overcome with the development of antibodies against the enzymes involved in catecholamine synthesis. As with the biochemical localization of the different enzyme activities in nervous tissue, the combination of the presence and the lack of enzyme-immunoreactivities indicate the nature of the catecholaminergic neuron. In general, dopamine-containing neurons are TH-positive, and immunologically negative for DBH and PNMT. Noradrenaline-containing neurons are in addition DBH-positive, and adrenaline-containing neurons are PNMT-positive as well (Hokfelt et al., 1984).

Recently, Foster et al. (1985) described the presence of PNMT-positive neurons in the retina and posterior hypothalamus of rat, which immunohistochemically lacked TH and DBH. Several explanations have been suggested by the authors. (1) Levels of TH and DBH could be too low to detect, which would distinguish these particular PNMT-positive neurons from others on the nervous system. (2) The antibodies used in this
study might not be immunoreactive against TH and DBH in the PNMT-positive cells. This is unlikely, since TH- and DBH-positive neurons were detected in other parts in the preparations, and there is no convincing evidence for the existence of different forms of TH in nervous tissue (see Catecholamine biosynthesis). (3) The enzyme which shows PNMT-like-immunoreactivity might not be involved in the synthesis of adrenaline, which leaves open the question of the source of adrenaline detected in those tissues. An alternative route of noradrenaline synthesis, via tyramine and/or norsynephrine, has been documented in the past (Iversen, 1970), but is unlikely to explain the lack of TH and DBH, since the different routes are merely a difference in the order of reactions, and probably involve the same enzymes. In addition to these potential explanations (Foster et al., 1985), and leaving cross-reactivity of the antibody aside, it might be possible that the PNMT-positive neurons acquire the noradrenaline substrate by accumulation rather than synthesis. The retina could, for example, be provided with noradrenaline via sympathetic innervation of the choroid (see Chapter 1.4).

A comparable anomaly has been described for neurons in the brainstem of the rat, where AADC-immunoreactive neurons lacking immunoreactivity against TH and tryptophan hydroxylase, have been described (Jaeger et al., 1984). In this situation, however, the most plausible explanation is that the detected AADC is not involved in monoamine transmitter synthesis. The presence of AADC-immunoreactivity is not suitable as a selective marker for monoaminergic pathways, because of the general distribution of the enzyme. However, the absence of AADC-immunoreactivity from neurons which were TH-positive in the
dorsal motor nucleus of rat nervus vagus (Jaeger et al., 1984) is a first indication that L-DOPA itself could be a transmitter in some parts of the nervous system.

The general distribution of MAO and COMT in nervous and nonnervous tissue renders immunohistochemistry to these enzymes useless as selective marker for monoaminergic transmission.

Recently an antiserum against dopamine, conjugated to BSA via glutaraldehyde, has been developed (Geffard et al., 1984), which has also been used at the electron microscope level (Onteniente et al., 1984). The major disadvantage of immunohistology against small molecules is the possibility that the antigen may diffuse during (mild) fixation (Geffard et al., 1985).

Cell physiological effects of dopamine.

Most of the mediators of dopamine's physiological effects, receptor sites, adenylate cyclase, cAMP, cAMP-dependent protein kinase and substrates for the protein kinase, can be detected at the cell level and taken into consideration in judging dopamine's transmitter role.

Photoaffinity labelling of receptors with $^3$H-dopamine, where exposure to ultraviolet light results in covalent linkage of the ligand to the receptor, has been used to label receptors for purification purpose (Tanaka et al. 1983, Maeno et al. 1983). This labelling method has not been used for autoradiographic localization. Using the radiolabelled antagonist $^3$H-spiroperidol, dopamine receptors have been detected autoradiographically (see Kuhar et al., 1978).

The selectivity of dopamine-sensitive adenylate cyclase for dopamine seems to be determined by the receptor site only. The detection of regulator and catalytic subunits is therefore not useful as
a selective indicator for dopaminergic innervation. Neither is the presence of the second messenger cAMP, nor the presence of substrate proteins for cAMP-dependent protein kinase, an indication for dopaminergic innervation. However, intracellular cAMP has been detected immunohistochemically (Ariano and Matus, 1981), indicating that changes in intracellular cAMP levels evoked by dopamine can be used to detect cells receiving dopaminergic input. Changes in the degree of phosphorylation of cAMP-dependent protein kinase phosphorylated proteins have also been studied at the cell level (see Levitan et al., 1983).

The recently described phosphoprotein DARPP-32, whose phosphorylation is regulated by dopamine via cAMP-dependent protein kinase, is claimed to be specific for dopaminergic innervated tissues (Walaas and Greengard, 1984). The protein has been isolated (Hemmings et al., 1984), and immunoreactivity against DARPP-32 has been found in nerve terminals in brain areas which receive dopaminergic innervation (Ouimet et al., 1984). It has not been demonstrated, by e.g. double-staining methods at electronmicroscopic level, that those nerve terminals are actually postsynaptic to dopaminergic neurons.

**Manipulating dopaminergic transmission.**

A classical way of studying the function of a system is by removing it, and looking for functional deficits. In looking for false-transmitters to replace noradrenaline, Tranzer and Thoenen (1967) discovered that 6-OHDA (FIGURE 1.5) destroyed the terminal plexus of peripheral noradrenergic neurons (for review, see Kostrzewa and Jacobowitz, 1974). 6-OHDA must be taken up by a neuron to exert its cytotoxic effects, and the selectivity of 6-OHDA for catecholaminergic neurons is determined by the efficient monoamine uptake systems those
Figure 1.5: Chemical structures of some dopaminergic cytotoxins.

6-OHDA, 6-hydroxydopamine
MPTP, N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
MPP⁺, N-methyl-4-phenylpyridinium ion.
Another potentially useful cytotoxin is N-acetyl-β-phenyl-d,l-3,4-
metahydroxyphenylalanine (MPP⁺) (Snoke et al., 1985; Snyder and
Björklund, 1983). This by-product of Parkinson's disease was found to
cause long-lasting Parkinson effects in rodents. The long-term effects
seem to involve destruction of dopaminergic pathways. Apparently, not
MPP⁺ itself, but a metabolized product N-acetyl-β-phenylalanine (MPP⁺),
which is a potent inhibitor of COMT, a class of enzymes correlated with
dopaminergic activity in vivo, protects against MPTP-induced
dopaminergic cell death (Farrell et al., 1990). A beta selectivity for
dopaminergic neurons and affecting the metabolism of MPP⁺-induced
dopaminergic toxicity. This is not due to a non-specific interaction,
selectivity, and mechanism of MPP⁺-induced dopaminergic toxicity, MPP⁺
is not as specific as the 6-OHDA as yet.

6-OHDA
neurons possess. Once inside the neurons, at intracellular levels estimated to be more than 50 mM, 6-OHDA becomes cytotoxic. 6-OHDA generates highly reactive products, such as peroxides, quinone, and free OH-radicals. These products are thought to react nonspecifically with intracellular structures and destroy the neuron. Noradrenergic neurons seem to be more susceptible to 6-OHDA than dopaminergic neurons. However, depending upon the site of administration, and pharmacological pretreatment, such as specifically blocking noradrenergic carriers, more or less specific lesions of noradrenergic or dopaminergic pathways can be obtained. Additional aspects of 6-OHDA induced neurotoxicity will be discussed in chapter 6.

Another potentially useful cytotoxin is N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) (FIGURE 1.5) (see Burks, ed, 1985; Snyder and D'Amato 1985). This by-product of pethidine synthesis was found to cause long lasting Parkinson effects in primates. Its long term effects seem to involve destruction of dopaminergic pathways. Apparently, not MPTP itself, but its oxidized product N-methyl-4-phenyl-pyridine (MPP+), is the active cytotoxic compound (FIGURE 1.5). Specific inhibition of MAO-B, a class of MAO correlated with dopaminergic activity in striatum, protects against MPTP-induced toxicity in mice (Heikkila et al. 1984). A MAO specific for dopaminergic neurons and effective in oxidizing MPTP, may be the key in the so far unexplained specificity of MPTP-induced neurotoxicity. Due to the relative lack of knowledge regarding selectivity, efficacy, and mechanism of MPTP-induced neurotoxicity, MPTP is not as widely used as 6-OHDA as yet.
The electrophysiology of dopamine.

The electrophysiological effects of dopamine in the CNS have been studied predominantly in the nigrostriatal dopaminergic system. Three general approaches have been used: (1) Iontophoretic application of dopamine. (2) Electrical stimulation of the dopaminergic tract, or the tract driving the dopaminergic neurons. (3) Deficits have been examined after lesioning the dopaminergic pathways. Generally, the electrophysiological effects have been monitored by extracellular recording.

The majority of studies (17 out of 20, see Moore and Bloom, 1978) reported a depression of spontaneous firing rates of the neurons after iontophoretic application of dopamine (Krnjevic, 1975; see Moore and Bloom, 1978; York, 1979; Bunney, 1979). The inhibition appeared 2-15 sec after the onset of iontophoresis, which is rather delayed compared to the appearance of effects after iontophoretic application of excitatory amino acids in comparable studies (see Krnjevic, 1974). In addition, the inhibition has been found to persist for minutes after the termination of the ejecting current, which, considering the efficient uptake systems for dopamine (see inactivation of catecholamines), could indicate the involvement of an intracellular second messenger.

In a study of intracellularly recorded actions of iontophoretically applied dopamine, a depolarization within 10 msec of the onset of the iontophoretic current has been reported (Kitai et al., 1976). In this study a very short pulse of relatively high iontophoretic current was used. This could have led to direct depolarization of the neuron, as pointed out by Moore and Bloom (1978).

Single electrical stimuli in the substantia nigra have been found to have depolarizing actions in striatum, sometimes followed by an
inhibitory effect. In contrast, trains of stimuli generally led to inhibitory responses, although some excitatory effects have also been reported. These inhibitory and excitatory effects generally showed long latencies, of 5-25msec (see Moore and Bloom, 1978; York, 1979; Bunney, 1979). A further contribution to the confusion was the observation that after chemical lesioning of the dopaminergic neurons, electrical stimulation of the substantia nigra still inhibited activity recorded in striatum (see Krnjevic, 1974; Moore and Bloom, 1978; York, 1979).

The question remains which of these effects represent the electrophysiological effects, if any, of dopamine. Although the release of endogenous dopamine seems more natural than iontophoretic application, a clear relation between stimulus and effect is difficult to establish due to the involvement of presynaptic control mechanisms of dopamine release (Chesselet, 1984). Such regulatory mechanisms are bypassed in studies where dopamine is applied iontophoretically. A general problem in these studies is that anaesthesia affects, and sometimes even arrests, the firing of the spontaneous firing neurons under study (Bunney, 1979). Most authors seem to agree with the principal idea that dopamine's direct effects are inhibitory (see Krnjevic, 1974; Moore and Bloom, 1978; Bunney, 1979; however, see York, 1979).

Probably due to its complex organization, the studies in the nigrostriatal dopaminergic system do not clearly point to general features of dopamine's electrophysiology. Without clear insight into the electrophysiological effects of dopamine in a system, one can hardly check for a mediatory role of cAMP in that system. However, the observations of the delayed onset and prolonged nature of dopamine's effects in all studies, do not indicate a direct effect of dopamine on
ion channels, but are more readily compatible with the involvement of a second messenger system.

Major contributions to the electrophysiological analysis of dopamine's actions have also come from studies of the SCG (FIGURE 1.6). Two clear types of physiological action of dopamine have been established in the SCG (see Libet, 1979). (1) Dopamine acts directly as the transmitter which evokes a slow inhibitory postsynaptic potential (sIPSP). (2) Dopamine produces a long lasting increase in the slow excitatory postsynaptic response to acetylcholine mediated by muscarine receptors (sEPSP).

Since dopamine-sensitive adenylate cyclase activity has been demonstrated in the SCG (Kebabian and Greengard, 1971), this ganglion has been studied extensively to demonstrate a mediatory role for cAMP in dopamine's electrophysiological effects. It has been reported that superfusion with monobutyryl-cAMP elicited a hyperpolarizing response in rabbit SCG (McAfee and Greengard, 1972). However, these results could not be confirmed despite several attempts, and have been suggested to be an artifact (see Libet, 1979). The modulatory action of dopamine on the sEPSP, in contrast, has been mimicked by superfusion with butyryl-cAMP and intracellular injection of cAMP (see Libet, 1979).

Thus, although it still must be established whether cAMP mediates the direct effects of dopamine, the studies in the SCG have convincingly revealed that the dopamine-elicited increase in cAMP levels does affect the electrophysiology of a neuron, and accounts for one of dopamine's post-synaptic effects.

It has been proposed that cAMP, by activating a specific protein kinase (cAMP-PK), regulates the phosphorylation of certain proteins, which leads to the final biological response (see Greengard, 1978b;
Figure 1.6: Schema of synaptic transmission in the rabbit superior cervical ganglion (modified from Clark, 1985).

AC, adenylate cyclase
fEPSP, fast excitatory post-synaptic potential
sIPSP, slow inhibitory post-synaptic potential
sEPSP, slow excitatory post-synaptic potential.

Acetylcholine released by the preganglionic fibers activates nicotinic acetylcholine receptors on the noradrenergic postganglionic neurons, generating fEPSPs. In addition, released acetylcholine activates a dopaminergic interneuron. The dopamine released from this neuron increases cAMP levels in the noradrenergic neuron via D1-receptors. The cAMP increases the amplitude of the sEPSP, originating from muscarinic receptor activation by the preganglionic nerve fibers. It has been suggested that cAMP also mediates the generation of a sIPSP, but this has not been demonstrated conclusively (Libet 1979).
MUSCARINIC RECEPTOR
LINKED TO GUANYLATE CYCLASE
NICOTINIC RECEPTOR

postganglionic neuron.

dopaminergic interneuron

DOPAMINE RECEPTOR LINKED TO ADENYLA
TE CYCLASE

after DOPAMINE

mV

MUSCARINIC RECEPTOR

preganglionic cholinergic neuron

fEPSP sIPSP sEPSP
Williams, 1979; Drummond, 1983). Recent studies have provided direct evidence for a causal relationship between cAMP-dependent protein phosphorylation and electrophysiological responses in excitable cells (see Siegelbaum and Tsien, 1983; Levitan et al., 1983; Nestler and Greengard, 1983). It has been demonstrated in voltage-clamped molluscan neurons and mammalian heart cells, that injection of the catalytic subunit of cAMP-PK mimics the effects of the first messenger on membrane conductance properties, and that injection of a protein kinase inhibitor blocks these effects of the first messenger. In cell-free membrane patches some of these results have been documented at the single channel level (Green and Gillette, 1983; Shuster et al., 1985).

Thus, the intracellular messenger cAMP is now known to be able to regulate ionic currents by phosphorylating either the ion channel protein directly, or a membrane associated protein which regulates the channel activity. These events have not been demonstrated yet in systems where dopamine-sensitive adenylate cyclase contributes to the regulation of intracellular cAMP levels.

All receptors which mediate dopamine's physiological effects, have been found to be linked with adenylate cyclase so far, and the electrophysiological effects of dopamine are compatible with a second-messenger mediated response. Although cAMP could not be demonstrated convincingly to elicit the sIPSP in the SCG, no system has been described where dopamine's effects are not accompanied with changes in cAMP levels. An increasing amount of evidence accumulates, demonstrating electrical consequences of changed intracellular levels of second messengers. Thus, it seems very likely that cAMP mediates the physiological effects of dopamine in general.
**Dopamine as a neurotransmitter.**

In addition to the clear indications obtained at the tissue level, the results of the various cellular studies strongly support a transmitter role for dopamine.

Although the electrophysiological effects of dopamine are not fully understood, this should not be interpreted as a lack of compliance with the second criterion for a neurotransmitter. Dopamine does evoke a clear response in its target cells in the form of changed levels of intracellular cAMP. Dopamine-sensitive adenylate cyclase, which is the entity which receives and transduces the message in dopaminergic transmission, has been well characterized biochemically and pharmacologically. Thus, the physiological effects of dopamine, essential indeed for its acceptance as a neurotransmitter, have been demonstrated in a variety of systems. However, the physiological role of the cAMP levels affected by dopamine, are as yet not clear for any system.

A neuron is distinguished from other cell types by its electrical features, but it could be argued that this is no reason to demand that a neurophysiological response must involve changes in conduction properties. However, cAMP has been demonstrated to regulate the conductive properties of ion channels in some circumstances, and there is no reason to expect that cAMP involved in dopaminergic transmission cannot regulate transmembrane ion movements.

In looking for the function of the changes in cAMP levels affected by dopamine electrophysiologically, several technical problems have to be overcome. In general, second messenger mediated responses involve several enzymatic steps, which are generally slower and of a more graded nature than the conductive changes accompanying an ionotropic
response. This poses problems for the electrophysiological detection of changes induced by dopamine. Another problem is that the actions of several other transmitters may converge at the level of the second messenger cAMP. In addition, different second messengers influence each other, and further, the intracellular substrates for responses regulated by second messengers are often not uniquely linked to a single second messenger system. Thus it is clear that in a complex neuronal network like the nigro-striatal pathway, it will be difficult to relate a response observed with classical electrophysiological techniques to dopamine.

Due to these aspects of the cellular dopaminergic response, it is essential to choose a relatively simple neuronal network to study the role of dopamine in that network. The SCG exhibits a clear dopamine effect, and has proven to be an extremely useful model to unravel the molecular events underlying dopamine's modulatory role. The vertebrate retina, with its confined, and relatively well-described processing capacities, might become the next model system where the role of dopaminergic transmission within a processing network could be revealed.
GENERAL INTRODUCTION. 1.3 The vertebrate retina.

The optical system of the eye projects an image of the visual world onto the retina, where closely packed photoreceptors feed into the neuronal network of the retina. Photoreceptors transduce electromagnetic radiation into membrane potentials. An important feature of vertebrate photoreceptors is that they are depolarized in the dark. Light activation hyperpolarizes them and diminishes their transmitter release. The output of the photoreceptors is relatively simple, only coding for light intensity and color. Measurements of ganglion cell responses have, however, revealed that the output signal of the retina are diverse and complex, encoding information about complex temporal and spatial relationships.

On the basis of light and electron microscopic studies, Dowling and Boycott (see for review and detailed references Dowling and Dubin, 1984) have described the general synaptic organization of the vertebrate retina. In transverse section, the vertebrate retina shows a well-defined laminar organization of cells and their processes (FIGURE 1.7). Cell bodies of the photoreceptors form the outer nuclear layer (ONL). In the outer plexiform layer (OPL), photoreceptors make synaptic contacts with bipolar cells and horizontal cells. The cell bodies of horizontal cells, bipolar cells, and amacrine cells are layered within the inner nuclear layer (INL). The bipolar cells are the only cell type which pass information from the OPL to the inner plexiform layer (IPL), so all visual information is channelled through the bipolar cells, except in catfish retina, where horizontal cell processes contact amacrine cells (Sakai and Naka, 1985), suggesting a form of information transfer which by-passes the bipolar cells. In the IPL, the processes
Figure 1.7: Schema of synaptic connections in the vertebrate retina (modified from Dowling, 1970).

PRL, photoreceptor layer  c, cone
ONL, outer nuclear layer   r, rod
OPL, outer plexiform layer h, horizontal cell
INL, inner nuclear layer  b, bipolar cell
IPL, inner plexiform layer a, amacrine cell
GCL, ganglion cell layer  g, ganglion cell.
OFL, optic fibre layer
Light enters the retina from the bottom.
of bipolar cells, amacrine cells, and ganglion cells make synaptic contacts. Cell bodies of the ganglion cells and displaced amacrine cells form the innermost cellular layer of the retina. Ganglion cells send an unmyelinated axon over the inner surface of the retina. These axons leave the retina at the optic disc, become myelinated, and form the optic nerve. Supporting cells of the retina, the Muller glial cells, extend through all layers.

This morphological model suggests a straight-through pathway from photoreceptors via bipolar cells to ganglion cells, with lateral interaction in both plexiform layers, mediated by horizontal cells in the OPL and by amacrine cells in the IPL. In some species, relatively few direct bipolar-to-ganglion cell synapses, and a high frequency of bipolar-to-amacrine and amacrine-to-ganglion cell synapses are observed. In such so called complex retinas (see Dowling and Dubin, 1984) amacrine cells seem to be an integral part of many straight-through pathways.

Based on intracellular recordings in lower vertebrates, Werblin and Dowling (1969; see Dowling and Dubin, 1984) developed a physiological model (FIGURE 1.8), which may explain how the OFF-response of the photoreceptor can be transformed into ON, ON-OFF, and OFF responses measured at the ganglion cell level. The model also provides an explanation for the observed antagonistic centre-surround receptive field organization detected at the bipolar cell level. The pattern of synaptic contacts between the major cell types suggested in the electrophysiological model, is consistent with the principal contacts seen in electronmicroscopic studies. Thus the simple electrophysiological model correlates well with the simple morphological model.
A flash of light is presented to the photoreceptor on the left, whilst the photoreceptor on the right is continuously illuminated by a dim background. The horizontal cell mediates the antagonistic response in the right bipolar cell, resulting in the OFF-response of the right-hand ganglion cell. The amacrine cell responds transiently at both the onset and cessation of illumination anywhere in its receptive field, and drives the ON-OFF ganglion cell in the middle.
However, it appears that even the generation of the single ON, OFF, and ON-OFF responses are not correctly described by the electrophysiological model of Wettlin and Dowling. The major shortcomings in this two-receptored model, the ganglion cells are only driven by excitatory inputs. Increased depolarization input from an ON- or OFF bipolar cell to the ganglion cell, response originated from an excitatory input. In the ganglion cell, excitatory and inhibitory inputs. The shift in retinal ganglion cell activity in the ganglion cell. In the "push-pull model" of insects (Bark et al., 1982) based on intracellular recordings, the "amphibian" cell as well as the bipolar cell is driven by excitation and conventional receptive field organization.

The receptive fields of the retina have temporal and spatial properties. The "selective receptive field" detection, of ganglion cell activity has not clear as yet. There are two major reasons for suspecting that the amphibian cell is involved in above complex processes. Firstly, these complex receptive fields cannot be detected at the bipolar cell level, suggesting that they are formed by interactions within the L2B. Secondly, the morphological complexity of the retina shows selectivity correlated with the occurrence of non-conventional receptive fields. For example, mouse retinae, which are complex retinae characterized by a high frequency of similar-to-amphibian and non-reactive-to-ganglion cell symptoms, have a high proportion of complex receptive fields at the ganglion cell
However, it appears that even the generation of the simple ON, OFF, and ON-OFF responses are not correctly described by the electrophysiological model of Werblin and Dowling. The major shortcoming is that in the proposed model, the ganglion cells are only driven by excitatory input: increased excitatory input from an ON- or OFF bipolar cell, results in the ganglion cell ON- and OFF response respectively, whilst the ganglion cell ON-OFF response originates from an excitatory drive from an amacrine cell. Instead it appears that the ganglion cells in fact receive both inhibitory and excitatory input. The shift in relative degree determines the activity of the ganglion cell. In the cat, this excitatory and inhibitory input is thought to come from pairs of bipolar cells (see Sterling et al., 1986), whilst in the "push-pull" model, as described by Belgum et al. (1982) based on intracellular recordings in mudpuppy retina, the amacrine cells as well as the bipolar cells, play a major role in the conventional receptive field organizations of the ganglion cells.

The synaptic basis for the more complex spatial and temporal properties, such as edge detection and direction-selective motion detection, of ganglion cell receptive fields is not clear as yet. There are two major reasons for suspecting that the amacrine cells are involved in these complex processes. Firstly, these complex receptive fields cannot be detected at the bipolar cell level, suggesting that they are formed by interactions within the IPL. Secondly, the morphological complexity of the retina across species correlates well with the occurrence of non-conventional receptive fields. For example avian retinas, which are complex retinas characterized by a high frequency of bipolar-to-amacrine and amacrine-to-ganglion cell synapses, have a high proportion of complex receptive fields at the ganglion cell
level (see Holden, 1982). Cat retina, in contrast, has less amacrine cells and a high frequency of direct bipolar-to-ganglion cell synapses. Although the cat has a comparable variety in receptive field types at the ganglion cell level and a comparable diversity of amacrine cells compared to avian retina, both the number of amacrine cells and the frequency of occurrence of complex ganglion cell receptive fields are much lower than in avian retinas (see Sterling, 1983). Thus, insight into detailed aspects of the amacrine cells seems to be essential for a full understanding of the mechanisms underlying the processing capacities of the retina.

The cell bodies of the amacrine cells are generally located in the inner half of the INL. Their processes are found in the IPL, often organized in certain horizontally confined areas. As the name implies, amacrine cells do not have clearly distinct axons and dendrites, in either morphological or in functional terms. All processes receive synaptic input and synapse onto other neurones. Amongst the cells in the ganglion cell layer, neurons are found which lack an axon, and which have their processes localized in the IPL. Hence their name “displaced” amacrine cells. Although the prefix “displaced” could suggest that they are due to a developmental error, this has never been proven, and their abundance and regularity in some retinas, suggests otherwise.

The amacrine cells form the most heterogeneous group of neurons in the retina. Ramon y Cajal (1892) described 15 different types on the basis of light microscopic features after Golgi stain, in chicken retina. Intracellular recordings have shown that amacrine cells can respond in a sustained or a transient fashion, and spiking amacrine cells have also been recorded (see Dowling and Dubin, 1984). There seems a good correlation of the morphology of different types of
amacrine cells with their biochemistry, as indicated by the substance they are likely to use as neurotransmitter, and this may correlate with their physiology as well. Many compounds which are good candidates as transmitters in neurons in other parts of the nervous system, are found in amacrine cells as well (TABLE 1.3) (Morgan, 1983). For most of them, there is not yet sufficient evidence as to whether they are indeed used as neurotransmitters by the different amacrine cells in the retina.

Interplexiform cells are in many ways indistinguishable from amacrine cells, amongst which their cell bodies are localized, but send an additional process to the OPL. The presence of unipolar cells, whose processes interconnect both plexiform layers, has already been described by Ramon y Cajal (1892), in dog and teleost retina using Golgi stain. Boycott et al. (1975), also using a metal impregnation stain, described comparable cells in cat, rhesus monkey, and squirrel retina. Because it is not readily distinguishable with general histological methods, this cell type attracted little attention. The discovery of dopamine-containing interplexiform cells in teleost retina and New World monkey, which could be readily visualized in retinal sections by means of the monoamine histofluorescence method (see Ehinger, 1976), increased the interest in interplexiform cells. In the OPL, interplexiform cell processes have been found to be presynaptic only, thus they mediate a centrifugal flow of information, which differentiates them from bipolar cells.

The synaptic basis of the enormous processing capacity of the retina is poorly understood. Insight into the synaptic connections and chemistry of the different classes of amacrine cells, may lead to understanding of their function in the IPL, and will help to explain the processing capacities of the retina in synaptic detail. In addition,
<table>
<thead>
<tr>
<th>Photoreceptor</th>
<th>aspartate, glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horizontal cells</td>
<td>GABA*</td>
</tr>
<tr>
<td>Bipolar cells</td>
<td>aspartate, glutamate*, serotonin, GABA, glycine</td>
</tr>
<tr>
<td>Amacrine cells</td>
<td>dopamine, glycine, GABA, serotonin, acetylcholine</td>
</tr>
<tr>
<td>Peptides</td>
<td>vasoactive intestinal peptide (VIP), somatostatin, enkephalin, substance P, glucagon, neurotensin, bombesin, cholecystokinin, luteinizing hormone releasing hormone (LHRH), neuropeptide Y</td>
</tr>
<tr>
<td>Interplexiform cells</td>
<td>dopamine in teleost fish and New World monkeys, GABA in cats, glycine in goldfish</td>
</tr>
<tr>
<td>Ganglion cells</td>
<td>glutamate, neuropeptides</td>
</tr>
</tbody>
</table>

*not in mammals
findings concerning mechanisms of synaptic transmission in the retina, may be extrapolated with caution to other parts of the nervous system, or should at least stimulate the search for similar mechanisms.

This section will review the data concerning dopamine's role in the vertebrate retina. First, the question of whether dopamine is a transmitter will be addressed. Experimental tests carried out on this question will be discussed at the tissue level first, then at the cellular level. Then, the extremely limited data dealing with dopamine's physiological role will be discussed. Table 7.2 at the end of this section summarizes the data on the different aspects where observations have been made. A brief part of this section will be devoted to discussing a possible transmitter role in the retina for the other catecholamines: noradrenaline, and adrenaline.

Dopamine's presence and synthesis in the retina.

In all species investigated dopamine is present in the retina. As can be seen in table 1.4 there is generally good agreement between the different studies on the levels found. In early studies fluorimetric- and radioenzymatic methods have been used to measure catecholamine levels. In most of the more recent studies, retinal catecholamine composition has been quantified by HPLC-ED. GC-MS has been used for catecholamine detection in the retina of rat, where noradrenaline could not be detected (Faven et al., 1978a).

The dopamine level of 1.0-4 pmol/g wet weight (about 1-5 ng/mg protein) ranks the retina of rat about midway in the rank order of 100 brain areas for dopamine content (see Falkovitz, 1979). This does not suggest that there is a massive dopaminergic system in the retina. With the possible exception of the cow, in all species investigated dopamine has been found to be the predominant catecholamine present in the
GENERAL INTRODUCTION. 1.4 Dopamine in the retina.

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Table 1.4: Catecholamine levels in vertebrate retina.

<table>
<thead>
<tr>
<th></th>
<th>Dopamine light</th>
<th>Noradrenaline light</th>
<th>Noradrenaline dark</th>
<th>Adrenaline light</th>
<th>Adrenaline dark</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>goldfish</td>
<td>4.2</td>
<td>trace</td>
<td>trace</td>
<td></td>
<td></td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>carp</td>
<td>1.1</td>
<td>trace</td>
<td>trace</td>
<td></td>
<td></td>
<td>Watling et al., 1982a</td>
</tr>
<tr>
<td>frog</td>
<td>5.6</td>
<td>8.9</td>
<td>trace</td>
<td>1.1</td>
<td></td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>toad</td>
<td>1.1</td>
<td>2.0</td>
<td>trace</td>
<td>trace</td>
<td></td>
<td>Makino-Tatsako et al., 1985</td>
</tr>
<tr>
<td>pigeon</td>
<td>0.16/0.97</td>
<td>0.59/0.51</td>
<td>trace</td>
<td>trace</td>
<td>0.38</td>
<td>Schwarcz and Coyle, 1976</td>
</tr>
<tr>
<td>chicken</td>
<td>0.84</td>
<td>0.1</td>
<td>trace</td>
<td>trace</td>
<td>0.38</td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>mouse</td>
<td>2.8</td>
<td>2.2</td>
<td>trace</td>
<td>trace</td>
<td>0.38</td>
<td>Parkinson and Rando, 1983b</td>
</tr>
<tr>
<td>rat</td>
<td>3.4</td>
<td>1.9</td>
<td>trace</td>
<td>trace</td>
<td>0.38</td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>RSC rat</td>
<td>2.0</td>
<td>1.9</td>
<td>trace</td>
<td>trace</td>
<td>0.38</td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>guinea pig</td>
<td>1.6</td>
<td>1.1</td>
<td>trace</td>
<td>trace</td>
<td>0.38</td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>hamster</td>
<td>1.9</td>
<td>1.0</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>rabbit</td>
<td>1.1</td>
<td>0.027</td>
<td>0.069</td>
<td></td>
<td></td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>cat</td>
<td>0.76</td>
<td>trace</td>
<td>trace</td>
<td></td>
<td></td>
<td>Da Prada, 1977</td>
</tr>
<tr>
<td>cow</td>
<td>5.9</td>
<td>0.41</td>
<td>0.37</td>
<td></td>
<td>0.22</td>
<td>Osborne and Nesselhut, 1983</td>
</tr>
<tr>
<td>human</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frederick et al., 1982</td>
</tr>
</tbody>
</table>

The following assumptions have been made to convert some of the data (Ehinger, 1983a):
- rabbit - assuming a wet weight of 60mg
- guinea pig - assuming a wet weight of 20mg
- rat - assuming a wet weight of 10mg

lg wet weight = 0.12g protein.
retina. At the very least, on the basis of catecholamine levels dopaminergic neurons are quantitatively more important than (nor)adrenergic neurons in the retinas of most vertebrates.

There are no reports on phenylalanine hydroxylase activity in vertebrate retina. In diabetic rats, high serum levels of neutral amino acids have been detected (Fernstrom et al., 1984). These amino acids compete with tyrosine for the transport carrier across the blood-brain barrier, resulting in low levels of tyrosine in brain (see Fernstrom, 1983). Diabetic rats also show low tyrosine levels in retina, which can be explained by a comparable competition for the blood-retina carrier. Compared to normal rats, diabetic rats show a lower rate of DOPA accumulation after inhibition of DDC in vivo, but normal TH capacity in vitro, measured radio-enzymatically. This diminished retinal catecholamine biosynthesis can be increased by intraperitoneal administration of L-tyrosine, which also results in increased levels of tyrosine in the retina (Fernstrom et al., 1984). In normal rats, the light-induced increase in dopamine turnover in retina, as indicated by the increase in DOPAC levels, could be further enhanced by administration of L-tyrosine (Gibson et al., 1983). These observations suggest that the retina is not self-sufficient for tyrosine, and support the idea that tyrosine is the first substrate for retinal catecholamine synthesis.

TH activity has been demonstrated radioenzymatically in retinal homogenates from goldfish, turtle, bullfrog (Lam, 1975), chick (Schwarcz and Coyle, 1977), rat (Iuvone et al., 1978a; Cohen and Neff, 1981) and rabbit (Lam et al., 1981). In Xenopus, the conversion of intravitreal radiolabelled tyrosine into dopamine has been observed (Sarthy et al., 1981). In rat (Morgan and Kamp, 1982), chicken and rabbit (Parkinson et
the formation of DOPA has been studied after inhibition of DDC activity in vivo. With respect to the cofactor requirements, substrate affinities, and pH optima, the properties of retinal TH of rat in vitro (Iuvone et al., 1978b; Iuvone et al., 1982) are similar to those described for other parts of the nervous system (see Lazar et al., 1982). The consistent finding is that TH is the rate-limiting enzyme and the site of regulation of catecholamine synthesis (see Iuvone and Neff, 1981).

DDC activities have been measured in retinal homogenates from rabbit (Parkinson and Rando, 1983a) and chicken (Parkinson and Rando, 1983b). Although DDC activity exceeds TH activity 1000 times in vitro, levels of the intermediate metabolite DOPA have been found to be relatively high. Whereas in the striatum of rat, the DOPA level is 0.1% of the dopamine level (Westerink, 1985), in the retina of rat (Da Prada, 1977), chick (Parkinson and Rando, 1983b), and rabbit (Parkinson and Rando, 1983a), DOPA levels are 33%, 17%, and 2% respectively of dopamine levels. Retinal DDC is effectively inhibited after intraperitoneal administration of NSD1015 (n-hydroxylbenzyl hydrazine) (Da Prada, 1977; Proll et al., 1982), or by intravitreal administration of alpha-fluoromethyl-DOPA (Parkinson et al., 1981). The subsequent accumulation of DOPA has been used as a marker for the rate of dopamine synthesis in vivo.

Activation of the dopaminergic system by light and drugs.

In frogs, a decrease in retinal dopamine levels upon exposure to light has been observed, whilst in other species either an increase or no effect at all has been reported (TABLE 1.4). A change in dopamine levels indicates a new equilibrium of dopamine synthesis and breakdown,
and thus a change, but not the direction of change, of dopamine turnover. A change in turnover is not necessarily accompanied by changes in steady-state levels of metabolites, so that the absence of significant effects of light on dopamine levels does not necessarily indicate an absence of effects of light on dopamine turnover.

In rat retina, the diurnal variations in dopamine levels have been reported to be independent of light cues, and were claimed to be generated by a circadian rhythm (Wirz-Justice et al., 1984). In contrast, Melamed et al. (1984) reported that the changes in dopamine- and metabolites levels were driven by light, and no significant component could be due to an internal circadian rhythm. Rats with inherited retinal dystrophy, which causes progressive photoreceptor degeneration, do not show a diurnal variation in dopamine levels, also indicating that the effects of light on the dopaminergic system are mediated by photoreceptors (Frucht et al., 1982; Frucht and Melamed, 1984). Since various authors in addition have demonstrated considerable effects of light on dopamine turnover (see below), a circadian rhythm is likely to be a minor contributor to the observed variation in dopamine during the dark-light cycle.

The rate of decrease in dopamine pools after inhibition of dopamine synthesis, with alpha-methyl-p-tyrosine, alpha-fluoromethyl-DOPA, or NSD1015 has been used to examine dopamine turnover in vivo. In rats maintained at a 12h dark-light cycle, the half-life of retinal dopamine was 18min after exposure to light for 2h, compared to 60min during the dark phase. Retinal dopamine turnover was increased 4-fold after exposure to light (Iuvone et al., 1978a; Iuvone and Neff, 1981). A comparable difference in turnover has been observed in animals exposed to light or dark for 96h (Iuvone et al., 1978b). In rabbits exposed to
light for 48h, the half-life of retinal dopamine was 40min, and in animals kept in the dark for the same period, the half-life was 72min. Dopamine turnover was double the dark value in the light-exposed animals (Parkinson and Rando, 1983a). In chicken the half-life times of dopamine were 28min and 85min in retinas of animals kept in the light or the dark respectively for 48h, and a 3-4 fold difference in turnover was observed (Parkinson and Rando, 1983b). Due to the efficient regulation of dopamine synthesis, the blocking of synthesis can itself be expected to change turnover rates. Hence, turnover data obtained by enzyme blockade do not give the real values in vivo. These results nevertheless demonstrate a considerable effect of environmental light on the turnover of dopamine in the retina. This might indicate, but does not prove of course, that the release of dopamine is increased during the light.

Inhibition of DDC with NSD1015 or alpha-fluoromethyl-DOPA in the retina conversely increases DOPA levels. Light has been found to stimulate DOPA accumulation in the retinas of rat (Da Prada, 1977), chicken (Parkinson and Rando, 1983b) and rabbit (Parkinson and Rando, 1983a). As little as 5 minutes exposure to light intensities as low as five lux, evoked this stimulation of DOPA accumulation in rat retina (Proll et al., 1982). After exposure to light for 2h, the accumulation of DOPA returned to dark-adapted rates within 10min of putting the rats back in the dark (Proll and Morgan, 1982). In all three species, the rate of DOPA accumulation was 3 to 4 times greater in the light than in the dark, which is consistent with the previously described effects of ambient light on the decrease in dopamine pools after inhibition of dopamine synthesis. These results show that dopamine synthesis is rapidly regulated in the retina in vivo, and indicate an activation of
Again, these effects are not simply due to light activation of dopamine synthesis, but are caused by a combination of activation by light and adaptive responses of the dopaminergic system itself, since as DOPA levels increase, there will be feed back inhibition of dopamine synthesis.

Iuvone et al. (1978a) have demonstrated that exposure of rats to light actually increases retinal TH activity in vitro, and they have further studied the possible mechanism underlying the light activation of TH (see Iuvone and Neff, 1981; Iuvone, 1984a). Shortly after exposing animals to light (15min) in vivo, the affinity of TH in vitro for the pterin cofactor increased, while the V_max and affinity for tyrosine were unchanged. After prolonged light exposure (96h), the affinity of TH for the pterin cofactor returned to the dark value, while the V_max was doubled compared to dark values (Iuvone et al., 1978b). These results suggest that there is a dual mechanism for the light activation of TH, with activity increased in the short term by modulating the affinity of the enzyme for the pterin cofactor, and a separate mechanism for long-term changes. Whether the effects of prolonged light exposure have any physiological relevance is debatable.

The kinetic changes after short term light activation can be mimicked in vitro by cAMP, in combination with phosphorylation favouring conditions (ATP, Mg^{++}). These effects are not additive to light activation, suggesting a shared molecular mechanism. The spontaneous deactivation in time after light activation in vivo, or after phosphorylation in vitro, are both inhibited by protein phosphatase inhibitors (Iuvone et al., 1982). In retinal cell suspensions, TH can be activated by 8-bromo-cAMP, showing that the dopamine containing cells have sufficient cAMP-dependent protein kinase in the proper subcellular
location to activate TH (Iuvone and Marshburn, 1982). As in other parts of the nervous system and in adrenal medulla (see Chapter 1.2), it is very likely that phosphorylation of TH is the molecular basis for short-term regulation of its activity in the retina.

Recently it has been reported that intravitreal administration of tetrahydrobiopterin to dark-adapted animals increased TH activity in vivo, as measured by DOPA accumulation after DDC block. In contrast, tetrahydrobiopterin had no effect on TH activity in light-activated retinas. Endogenous biopterin levels were unaffected in either of these lighting conditions (Iuvone, 1984a). Because of the apparent limiting biopterin levels, these observations suggest that the increased affinity of TH for its cofactor indeed could play a role in the rapid increase in dopamine biosynthesis upon exposure to light.

Prolonged exposure to light increased the apparent $V_{\text{max}}$ of TH in retinal homogenates from rat. No increase in TH measured by radio-immuno assay could be detected, suggesting that the increase in $V_{\text{max}}$ is not due to more enzyme, but rather to an increased specific activity of the enzyme (Iuvone et al., 1979). Effects of prolonged light exposure on tetrahydrobiopterin levels have not been described.

In the rat, the increase in TH capacity ($V_{\text{max}}$) was only detected after at least 3 days exposure to light. In retina of rabbit (Parkinson and Rando, 1983a) and chicken (Parkinson and Rando, 1983b), TH activities in vitro, assayed by DOPA accumulation after DDC blockade, were higher in retinas from animals light-adapted for 48h, than from dark-adapted animals. TH assay conditions were saturating for pterin cofactor, thus the increased activities represent an increase in $V_{\text{max}}$; under those conditions changed affinities would pass unnoticed. Thus in contrast to the rat, in chicken and rabbit TH capacities may be
increased after 48h light. This could be due to differences in experimental conditions (in particular, light intensities have not been specified by the authors), but might also represent species variability. At this stage it would not be safe to generalize to all species the dual mechanisms of TH activation by light found in the rat.

The light activation of dopamine synthesis has been used as probe to search for which transmitter systems could be in contact with the dopaminergic system. In cell suspensions of rat retina, GABA and GABA agonists inhibit TH, suggesting but not proving, a direct inhibitory GABAergic input to dopaminergic neurons. In vivo, low doses of GABA were found to increase TH activities, while higher doses decreased TH activities measured in vitro, suggesting that a GABA-driven interneuron might be involved as well (Marshburn and Iuvone, 1981). Kamp and Morgan (1981) have shown in vivo that GABA antagonists increase dopamine turnover, measured as the rate of dopamine decrease after synthesis inhibition, in dark-adapted rat retina, suggesting that endogenous GABA tonically inhibits the dopaminergic system in the dark. This idea was further strengthened by the observation that inhibition of GABA-transaminase by amino-oxyacetic acid or gabaculine, or GABA uptake by nipectic acid inhibited the light evoked increase in DOPA accumulation after DDC blockade (Proll and Morgan, 1983). Subsequent studies have shown that the light activation of the dopaminergic system can be inhibited by benzodiazepines (Kamp and Morgan, 1982) and barbiturates (Kamp and Morgan, 1980), further indicating the involvement of a GABA receptor in the regulation of dopaminergic activity. Thus in the retina of the rat, GABAergic neurons seem to inhibit dopaminergic neurons, and the light activation of the dopaminergic system seems to involve a decrease in this GABAergic inhibition. This observation also may not be
generalized across species, since Parkinson (personal communications) has found that GABA agonists do not affect dopamine turn-over in chicken retinas.

In contrast, in similar experiments it has been shown that acetylcholine and glycine agonists and antagonists have no effects on light-activation of the dopaminergic system in rat retina (Morgan and Kamp, 1983a; Iuvone, 1984b). This suggests that cholinergic and glycinergic neurons do not influence the pathway from light to the dopaminergic system, or the dopaminergic system itself.

The excitatory amino acid-analogue quisqualate, increases the rate of accumulation of DOPA in vivo after inhibition of DDC in dark-adapted retinas of rat. Neither kainate nor N-methyl-D-aspartate (NMDA) exerted this effect (Kamp and Morgan, 1983). This could suggest that dopaminergic neurons themselves, or neurons presynaptic to them possess quisqualate-preferring receptors.

Dopamine antagonists, such as haloperidol, sulpiride, and chlorpromazine, activate the dopaminergic neurons in rat retina, as judged by the increased levels of dopamine metabolites, together with constant or decreased levels of dopamine (Da Prada, 1977; Cohen et al., 1983). An increased affinity of TH for the pterin cofactor has been observed after treatment with dopamine antagonists (Cohen et al., 1981), favouring a mechanism involving kinetic changes of TH in these effects. Similar changes in TH affinity have been observed after high-potassium-evoked depolarization of cell suspensions of rat retina (Iuvone and Marshburn, 1982). These data seem to indicate the presence of feedback regulation, mediated by a presynaptic autoreceptor, as described for striatum (Zivkovic and Guidotti, 1974). Cohen and Neff (1982a) reported the development of tolerance in rat retina after
prolonged treatment with dopamine antagonists. These results could not be reproduced in other laboratories (Scatton et al., 1977; Barbaccia et al., 1982; Melamed et al., 1983). If there is genuine tolerance, its development is probably mediated by neuronal feedback loops, as in the striatum (Zivkovic et al., 1975).

**Dopamine release in the retina.**

Light flashes have been reported to release in a frequency-dependent manner, exogenous $^3$H-dopamine in perfused eye cups maintained in living, but heavily anaesthetized cats (Kramer, 1971). Stimulation with flashing lights slightly increased the efflux of $^3$H-dopamine from isolated, perfused rabbit eyecups (Bauer et al., 1980), but, the minor increase in efflux seems of doubtful experimental use. In perfused isolated pieces of bovine retina, a more substantial $^3$H-dopamine efflux has been observed upon stimulation with flashing lights (Reading, 1983). The onset of increase in efflux was however, 15 min after the termination of the stimulus, which does not seem to make it very relevant physiologically, even though the light-evoked increase in efflux was calcium dependent.

Recently, it has been reported that the efflux of $^3$H-dopamine from isolated retinas of cat (Hamasaki et al., 1986) and pigeon (Krempels et al., 1986) is higher during the dark than during constant light. In both species a strong efflux was observed at light off, which could account for the enhanced efflux with flashing lights, reported previously. These results indicate that dopamine release is inhibited by constant light, an effect opposite of that anticipated from the turnover experiments.
In isolated carp retina, light flashes were found to increase levels of cAMP (Dowling and Watling, 1981), the likely mediator of dopamine's effects in the retina (Watling et al., 1979; see *Dopamine receptors and response in the retina*). In contrast, constant light decreased levels of cAMP (Dowling and Watling, 1981). Recently it has been shown that a physiological condition evoked by dopamine application, reduced responsiveness and receptive field size of cone horizontal cells (see *Dopaminergic interplexiform cells*), could also be elicited by prolonged dark adaptation *in vivo* in the carp retina (Mangel and Dowling, 1985). So, the results obtained by using physiological effects of dopamine as a marker for released dopamine, are consistent with the direct observations that (constant) light inhibits the release of dopamine.

Only in human retina has high-potassium-evoked efflux of endogenous dopamine been demonstrated (Frederick et al., 1982). High-potassium-evoked increases in efflux of exogenous $^3$H-dopamine has been described in the retinas of goldfish (Sarthy and Lam, 1979), carp (Kato et al., 1982), *Xenopus* (Sarthy et al., 1981), chicken (Tapia and Arias, 1982), rat (Pycock and Smith, 1983), rabbit (Bauer et al., 1980, Dubocovick and Weiner, 1981; Lam et al., 1981), cat (Hamasaki et al., 1986), cow (Reading, 1983) and human (Frederick et al., 1982). In all preparations, the increase in efflux appeared to be calcium-dependent, and in some preparations, sensitivity to cobalt and high concentrations (20 mM) of magnesium were tested, and found to block the efflux of dopamine. These divalent cations block calcium channels and by preventing calcium influx, block vesicle-mediated release. In goldfish retina, it has also been demonstrated that under conditions where an increase in $^3$H-dopamine efflux was observed, no change in the efflux of
exogenous $^3$H-tyrosine or $^{14}$C-glucose was seen (Sarthy and Lam, 1979). So, all these data point to the presence of a classical, vesicular release mechanism for dopamine in vertebrate retina.

An increase in efflux of $^3$H-dopamine has also been elicited by electrical stimulation of pieces of rabbit retina (Dubocovich and Weiner, 1981) and chicken retina (Dubocovich, 1984b). In this experimental set-up, pieces of retina are spread between two electrodes, and exposed to electrical field potentials in the horizontal direction, changing in polarity with low frequency (3Hz). The rationale of this method and its advantages over other methods are not quite clear, and this type of stimulation is certainly not natural.

The release of dopamine has been used as a probe to detect neuronal pathways in the retina. GABA and GABA agonists had no detectable effects on the efflux of exogenous dopamine in isolated rabbit retina (Bauer et al., 1980). However, the delayed response to high-potassium complicates all results obtained in this study. In isolated pigeon retina, GABA antagonists blocked the efflux of dopamine during dark and light, indicating that GABAergic neurons were interposed between the photoreceptors and dopaminergic neurons (Krempels et al., 1986).

Serotonin, at a concentration of 0.5 uM, has been demonstrated to increase the efflux of exogenous dopamine in isolated carp and frog retina, but not in rat retina (Kato et al., 1982). The latter observation is consistent with the observation that the rat retina does not contain indoleamine-accumulating neurons (see Ehinger and Floren, 1980). In isolated rabbit retina, slightly higher doses (> 1 uM) of serotonin affected the efflux of exogenous dopamine, but the stimulated release was not calcium-dependent (Dubocovich, 1983). The observed efflux of radioactivity may be due to exchange rather than release.
Rabbit retina does possess indoleamine-accumulating neurons (Ehinger and Floren, 1980), thus it seems that indoleaminergic control of dopaminergic activity is not automatically a feature of a retina, even if it contains indoleamine-accumulating neurons.

Alpha-melanophore-stimulating-hormone (alpha-MSH) (or a metabolite), but not glutamate, kainate, and glycine, increased the efflux of exogenous dopamine in isolated rabbit retina (Bauer et al., 1980). Enkephalin (in rabbit), and melatonin (in rabbit and chicken), have been found to inhibit the electrically-evoked release of exogenous dopamine in isolated retinas (Dubocovich, 1983; Dubocovich and Weiner, 1983; Dubocovich, 1984b). These observations await confirmation and further examination before any of these putative neurotransmitters can be considered to be actually involved in regulating the release of dopamine in vivo.

In isolated rabbit retina, the electrically-evoked increase in the efflux of dopamine was inhibited by dopamine agonists, whilst dopamine antagonists increased the release. These results have been interpreted as indicating that the release of dopamine is regulated via D_2-type autoreceptors (Dubocovich and Weiner, 1981). Based on results obtained by a similar approach, the involvement of alpha_2-adrenergic receptors in the regulation of dopamine release has also been suggested (Dubocovich, 1984a). However, because of the unusual binding characteristics of the dopamine autoreceptors, these results should be cautiously interpreted.

Dopamine receptors and response in the retina.

Dopamine receptors were initially detected in retinal homogenates of the cow and rat by measuring dopamine-stimulated adenylate cyclase (Brown and Makman, 1972). Dopamine was found to be the most potent
catecholamine in stimulating adenylate cyclase activity. In addition, the stimulation of adenylate cyclase by dopamine agonists could not be blocked by isoproterenol, propranolol, or other adrenergic antagonists, but was potently blocked by dopamine antagonists (Brown and Makman, 1973). Hence, the properties of dopamine-sensitive adenylate cyclase have been interpreted as indicating a specific dopamine receptor in the retina.

Since then, dopamine-sensitive adenylate cyclase activity has been demonstrated in the retinas of several species, including goldfish, carp, chicken, rat, mouse, guinea pig, rabbit, cat, cow, Cebus monkey, rhesus monkey, *Macaca Mulatta* monkey, and ground squirrel (Makman et al., 1975; Schwarcz and Coyle, 1976; DeMello, 1978; Watling et al., 1979; Redburn et al., 1980a; Ferrendelli et al., 1980; Schorderet and Magistretti, 1980; Joo and Wolleman, 1980; Watling and Dowling, 1981).

Under comparable assay conditions, the EC50 of dopamine for retinal adenylate cyclase is the same as that found in corpus striatum. In addition, the Ki values for inhibition by dopamine antagonists are similar for striatal and retinal dopamine-sensitive adenylate cyclase (Watling et al., 1979; Makman et al., 1980; Watling and Iversen, 1981; Watling and Dowling, 1981). Although the absolute increase in cAMP levels evoked by stimulation of adenylate cyclase in the retina is comparable to the increase in striatum, basal levels of cAMP in retina are lower than levels found in other tissues. Especially in teleost fish (Watling et al., 1979; Redburn et al., 1980a), and Cebus- and rhesus monkeys (Makman et al., 1975) the relative increases in cAMP measured in the retina are therefore markedly higher than found in other tissues, a property which makes the retina an extremely valuable model system for studying various aspects of adenylate cyclase stimulation by...
Dopamine receptors have also been demonstrated in retina using ligand binding techniques. Since carp and guinea pig retina lacked significant binding of $^3$H-domperidone, a specific ligand for $D_2$-receptors, and is rich in dopamine-stimulated adenylate cyclase activity, it was suggested that the retina only contains $D_1$-receptors (Watling et al., 1979). However, domperidone shows high non-specific binding in retinal homogenates, which could easily mask a low number of $D_2$-binding sites (Creese et al., 1983). The pharmacological profile of $^3$H-spiroperidol (= spiperone) binding suggests the presence of $D_2$-receptors in retina of carp, goldfish, chicken, rat, guinea pig, rabbit, cat, cow, Cebus monkey and Macaca Mulatta (Magistretti and Schorderet, 1979; Redburn et al., 1980a; Schaeffer, 1980; Makman et al., 1980; Watling and Iversen, 1981; Makman et al., 1982; Ventura et al., 1984). Retinas of these species show high-affinity (nM) binding sites for spiroperidol, and reveal low $K_i$-values (nM–μM) for various other ligands in replacement studies. Yet, the $K_i$-values for inhibition of dopamine-stimulated adenylate cyclase are in the μM–mM range. Thus, the high-affinity spiroperidol binding sites probably represent $D_2$-receptors (Iversen, 1975; Creese et al., 1983). However, the number ($B_{max}$) of these binding sites in retina is very low compared to striatum, and the retina has the highest reported ratio of $D_1$- to $D_2$-receptors. Based on the pharmacological properties of drug-induced modulation of dopamine release in rabbit retina, Dobocovich and Weiner (1985) suggested that the $D_2$-binding sites may represent autoreceptors on the dopaminergic neurons.
The postsynaptic dopamine receptor, as characterized by dopamine-sensitive adenylate cyclase, shows light regulated sensitivity in the retina. On first exposure to light, dopamine-sensitive adenylate cyclase switches from a supersensitive to a less sensitive state in the retina of chicken (DeMello et al., 1982; Ventura et al., 1984) and rabbit (Parkinson and Rando, 1984). In mature rats a supersensitive state can be reached by light deprivation for 65h (Spano et al., 1977). Increased binding of $^3$H-spiroperidol in rabbit retina after one week of light deprivation suggests that $D_2$-receptor sensitivity is also affected by light (Dubocovich et al., 1985).

Retinal cAMP levels were increased after stimulation by flashing light (carp; Dowling and Watling, 1981), and depolarization evoked by high-potassium (carp, rat, rabbit, and cow; Brown and Makman, 1972; Ferrendelli et al., 1980; Dowling and Watling, 1981); stimuli which have been reported to release dopamine. Although these effects in carp retina were shown to be blocked completely by dopamine antagonists, other endogenous ligands might also contribute to the light-regulated activation of adenylate cyclase. In rat retina, carbachol potentiates dopamine's stimulatory action on adenylate cyclase, suggesting that there is cholinergic modulation of the dopamine response (Brown and Rietow, 1981). Glucagon activates adenylate cyclase in chicken retina (Kuwayama et al., 1982). In carp retina, neither carbachol or glucagon had effects on cAMP levels (Dowling and Watling, 1981; Watling and Dowling, 1983). Vasoactive-intestinal-protein (VIP) has been found to activate adenylate cyclase in the retinas of chicken (Koh et al., 1984; Koh and Chader, 1984) as well as carp (Watling and Dowling, 1983). So, the modulation of dopamine-sensitive adenylate cyclase or the regulation of adenylate cyclase by other compounds may vary with species. At this
stage it cannot be concluded whether the various effects on adenylate cyclase are species-specific, or correlate with the presence of dopaminergic interplexiform cells (e.g. carp) or dopaminergic amacrine cells (e.g. chicken and rat) in the retina. Calcium/calmodulin-sensitive adenylate cyclase has been characterized in bovine retina (Gnegy et al., 1984a) and has also been demonstrated in goldfish, rat and rabbit (Gnegy et al., 1984b). This intracellular regulating system of adenylate cyclase might be a common feature of the retinas of many species.

**Inactivation of dopamine in the retina.**

Vertebrate retinas have dopamine uptake systems with characteristics similar to those found in other areas of the nervous system rich in dopaminergic terminals. In the retina of *Xenopus*, chick, cat and human, uptake has been used as the essential preliminary for subsequent release experiments (see *Dopamine release in the retina*), but the uptake systems have not been characterised further. The affinity for dopamine of the retinal uptake systems in goldfish (2.6 x 10^{-7} M, Sarthy and Lam, 1979), carp (3.5 x 10^{-7} M, Kato et al., 1981a), rabbit (5.6 x 10^{-7} M, Ehinger and Floren, 1978) and cow (2.9 x 10^{-7} M, Reading, 1983) are essentially the same as described for the striatum of rat (3-4 x 10^{-7} M, see Ehinger and Floren, 1978). The affinity of retinal dopamine uptake in rat retina (1.9 x 10^{-6} M) reported by Pycock and Smith (1983) is significantly lower than for the other species.

In all species investigated, dopamine uptake appears to be highly temperature sensitive. In addition, in rabbit retina uptake is blocked by ouabain (Ehinger and Floren, 1978) and in goldfish retina is sodium-dependent (Sarthy and Lam, 1979). These results suggest that retinal
dopamine uptake is energy-dependent, like dopamine carrier systems found in other parts of the nervous system. The observation that a specific dopamine carrier blocker, benztrapine, inhibits dopamine uptake in goldfish retina (Sarthy and Lam, 1979), further strengthens the idea that retinal dopamine uptake is transported by the same carrier as that found in other dopaminergic tissues.

Removal of dopamine from the extracellular space has been shown to represent a physiologically important mode of transmitter inactivation in dopaminergic tissues (see chapter 1.2). The presence of highly efficient dopamine uptake systems in the retina suggests a similar significance for dopamine inactivation in the retina. In perfused preparations of rabbit retina, it has been shown that the uptake blocker nomifensine increased the amount of dopamine detected in the perfusate after stimuli which evoke release (Dubocovich, 1984a). Thus it is very likely that dopamine uptake is an important mode of dopamine inactivation in the retina.

Light has not been shown to affect the affinity of the carrier, although after prolonged darkness, a change in the apparent $V_{\text{max}}$ of retinal dopamine uptake has been observed (Kato et al., 1981a). The directions of change were opposite in rat and carp retina (see Ehinger, 1983a). Since light activates dopamine turnover, and the concomitant changes in endogenous dopamine pools might cause a variation in the dilution of the radiolabelled dopamine used in uptake studies, the presence of a light-regulated change in capacity is not yet proven.

The dopamine metabolites DOPAC and HVA have been detected in chicken (Parkinson and Rando, 1983b), rat (Cohen et al., 1983; Melamed et al., 1983) and rabbit (Parkinson and Rando, 1983a) retina, thus indicating the presence of the enzymes MAO and COMT. MAO activities
have been directly demonstrated in retinal homogenates of chicken, rabbit and cow. In rabbit retina (Lam et al., 1981) MAO-A is present whilst in the retinas of chicken (Suzuki et al., 1977) and cow (Sparks et al., 1981) the B-type predominates. The increase in levels of dopamine metabolites after light shows that both MAO and COMT are actively involved in deactivation of dopamine in the retina.

Dopaminergic neurons in the retina.

The observation of greenish fluorescent neurons in rat retina after formaldehyde treatment, as described by Malmfors (1963), does not indicate which catecholamine is present in those neurons. Both dopamine and noradrenaline produce greenish fluorophores with Falck's monoamine histofluorescence method (Falck et al., 1962). However, soon after this initial observation, it was shown biochemically that dopamine is the dominant catecholamine in the retina of rabbit (Haggendal and Malmfors, 1963). It was therefore concluded that the fluorescent neurons were dopamine- rather than noradrenaline containing (Haggendal and Malmfors, 1965).

With microspectrofluorometry it has been shown that the formaldehyde-induced fluorescence is due to dopamine and not noradrenaline in teleost fish (Ehinger et al., 1969; Hayashi, 1980) and rat (Kato et al., 1980) retina. Following reserpine depletion of all monoamines from carp retina, uptake of catecholamine, as monitored by reappearance of histofluorescence, could be inhibited by benztropine but not by desmethylimipramine, suggesting that the carrier involved is dopaminergic rather than noradrenergic (Hayashi, 1980). It is now generally accepted that the endogenous fluorescent product is derived from dopamine.
In the retinas of rabbit and guinea pig, the intensity of dopamine-related histofluorescence appeared to increase after light adaptation (Nichols et al., 1967), consistent with the reported effects of ambient light on retinal dopamine levels (see Dopamine's presence and synthesis in the retina). In rat, lower intensities of fluorescence could only be observed when animals were kept in constant dark from birth (Kato et al., 1980). The lack of effects of environmental light on the fluorescence intensity of dopaminergic neurons of mice (Kato et al., 1981b) and rats (Wyse and Lorscheider, 1981) with inherited photoreceptor degeneration, shows that photoreceptors may be involved. These results support the assumption that the intraneuronal dopamine, as visualized by histofluorescence, indeed represents the retinal dopamine for which a transmitter role is suspected.

Ehinger and coworkers have carried out systematic studies in the retinas of several species, using the Falck histofluorescence technique. The general appearance of dopamine-containing cell bodies did not differ significantly from amacrine cells in most species. They described five principal types of dopamine containing neurons, based on the location of the cell body (FIGURE 1.10) (see Ehinger, 1976; Ehinger, 1983a). Most often the dopamine-containing neuron was found in the innermost layer of the INL, adjacent to the IPL. This type has been named a "junctional" cell. In some species there were additional dopamine cells found amongst the ganglion cells or in the middle of the IPL. These have been named "alloganglion" cells and "eremite" cells respectively. In Cebus monkey, cells in the middle of the INL have been described and named "pleomorph" cells. In all species investigated a dense plexus of varicose processes could be found in the IPL at the border with the INL. Often there were additional, more or less specific, layers where processes could be seen.
Figure 1.9: Schema of the distribution of catecholamine-containing neurons in the retina of different species (from Ehinger, 1983).

The figure is based on observations after monoamine histofluorescence staining of the retina. All species have been reported to contain a few fluorescent cells in the ganglion cell layer, except for birds, where none were detected.

Ph, photoreceptor layer
ONL, outer nuclear layer
OPL, outer plexiform layer
INL, inner nuclear layer
IPL, inner plexiform layer
G, ganglion cell layer.
Because the cell bodies were located amongst the amacrine cells, and the arborizations of processes were found in the IPL only, the dopamine-containing neurons are amacrine cells in most species examined. The frequency of occurrence of alloganglion-, eremite-, and pleomorph cells is very low. Since their synaptic organization is similar to that of the junctional cell, they are often regarded as displaced junctional cells (Holmgren-Taylor, 1982b). In avian retina only one type of dopamine-containing neuron has been found, the junctional type (Kato et al., 1980; Araki et al., 1983; Kato et al., 1984). Unless specified otherwise, dopaminergic amacrine cell refers to the "junctional" type of dopamine-containing neuron.

Antibodies against dopamine have so far only been used in flat mounted preparations of rat retina, which visualized the same population of neurons as seen by histofluorescence (Botteri et al., 1985). Immunohistochemical detection of TH has been done in retinas from several species (Nguyen-Legros et al., 1981; Nguyen-Legros et al., 1984; Brecha et al., 1984; Ballesta et al., 1984; Oyster et al., 1984; Zucker and Yazulla, 1984; Versaux-Botteri et al., 1984; Osborne et al., 1984b; Witkovsky et al., 1984). The TH-positive cells show essentially similar morphological characteristics to the neurons visualized by the original histofluorescence method. DBH-immunoreactivity has been demonstrated in retina of Macaca monkey and cow, where it was associated with neurons in the ganglion cell layer, possibly ganglion cells (Osborne and Patel, 1985). DBH-immunoreactivity could not be detected in retinas of frog, rat, rabbit, or pigeon (Osborne and Patel, 1985), nor in chick, and rat, and other rodent retina (Ballesta et al., 1984) (see Other putative catecholamine transmitters in the retina). This suggests that TH-immunoreactive neurons found in the amacrine cell layer, are indeed
dopaminergic. Distribution of MAO, as revealed by histochemistry, does not parallel that of dopamine (Shantaveerappa and Bourne, 1964). This can be expected considering the universal role of this monoamine-catabolizing enzyme.

In teleost fish, New World monkeys, and human, dopamine-containing processes are also found in the vicinity of horizontal cells. This network in the OPL is connected with the junctional type of dopamine-containing cells, and these neurons represent the dopaminergic interplexiform cells (Ehinger et al., 1969; Dowling et al., 1975; Frederick et al., 1982; see Chapter 1.3).

Surprisingly, TH-immunoreactive interplexiform cells have been detected in the retina of cat (Oyster et al., 1984) and rat (Nguyen-Legros et al., 1981; Nguyen-Legoros et al., 1982; Ballesta et al., 1984). In the retinas of several rodents (Ballesta et al., 1984), Old World monkey, and human (Nguyen-Legros et al., 1984), ascending TH-immunoreactive processes have been detected in the INL, suggesting that dopaminergic interplexiform cells exist in these species as well. Neither of these species show dopamine-related histofluorescence in the OPL. Unlike teleost and New World monkeys, where all dopaminergic neurons seem to be of the interplexiform type (Dowling and Ehinger, 1978b; Dowling et al., 1980), some other species may have a minor population of dopaminergic interplexiform cells in addition to their dopaminergic amacrine cell population.

As in other nervous tissues, the dopaminergic neurons in the retina show efficient catecholamine-accumulating capacity. This uptake mechanism forms the basis for autoradiographic localization and cytotoxic destruction. Several catecholamine congeners have been used to preload the neurons in order to enhance fluorescence intensity after
formaldehyde treatment. Although this is convenient for quantitative
description of cell distribution, or for visualizing fine arborizations
of processes, preloading can diminish the selectivity of the method.

Preloading rabbit retina with a range of compounds, including DOPA,
dopamine, noradrenaline and alpha-methylnoradrenaline, can increase the
intensity of fluorescence. 5,6-Dihydroxytryptamine (5,6-DHT) was the
only monoamine tested which increased the number of fluorescence cells
at low doses (Dowling and Ehinger, 1978a). In carp however, all these
compounds increased the number of fluorescent neurons (Negishi et al.,
1979). Fukuda et al. (1982) detected 3 populations of monoamine-
accumulating neurons in rat retina, on the basis of results obtained by
simultaneous injection of 5,6-DHT, noradrenaline and dopamine (or
DOPA). The existence of a third population of monoamine-accumulating
neurons has also been reported in chicken retina, where the results
obtained with very high doses of monoamines even led to claims of 5
populations of fluorescent neurons (Kato et al., 1984).

In most cases, the additional monoamine-accumulating neurons are,
because of their different morphology and localization in the INL,
easily distinguishable from the classical dopaminergic cell bodies.
Moderate doses of dopamine or noradrenaline will, in most cases,
specifically enhance fluorescence of dopaminergic neurons in the
retina. However, the significant species differences with respect to
the overlap of substrate selectivities of the different uptake systems,
makes a careful consideration of the specificity of a method based upon
accumulation of monoamines essential.

For initial electron microscopic analysis, dopaminergic neurons
were stained with 5,6-DHT, which apart from reacting with formaldehyde
for histofluorescence also causes typical, electron microscopically
detectable cell degeneration (Dowling and Ehinger, 1975; Adolph et al., 1980). 5,6-DHT is also uptaken by indoleamine-accumulating neurons, so this problem was overcome by preliminary destruction of the indoleamine-accumulating neurons with 5,7-dihydroxytryptamine (5,7-DHT), a cytotoxin which under the right conditions, was claimed to be selective for indoleamine-accumulating cells, although selectivity was assessed by histological examination only (Dowling and Ehinger, 1978a; 1978b; Dowling et al., 1980). 6-OHDA has also been used for electron microscopic staining, since short term toxic degeneration can be detected under the electron microscope. 6-OHDA, at appropriate doses, does not affect indoleamine-accumulating neurons, and thus is a more selective stain than 5,6-DHT (Dowling and Ehinger, 1978a; 1978b). However, 6-OHDA only weakly forms fluorophores with formaldehyde (Dowling and Ehinger, 1978), and therefore cannot be used for simultaneous light microscopy.

The monoamine carrier systems have also been used for autoradiographic examination of the dopaminergic neurons, and in cat and rabbit accumulated radioactivity is found in the same areas in which dopamine-originating fluorescence is found (Kramer et al., 1971; Ehinger, 1981). In retinas of goldfish, frog, turtle, pigeon, rabbit, cow, and Old World monkeys, the distribution of $^3$H-dopamine uptake sites is similar to the distribution of TH-immunoreactivity, although anti-TH stained the whole neuron and $^3$H-dopamine was generally accumulated more in the processes (Osborne et al., 1984b; Witkovsky et al., 1984). In cell cultures of rabbit retina, only TH-immunoreactive neurons accumulated $^3$H-dopamine (Osborne et al., 1984a). Autoradiographic localization has been carried on to the electron microscopic level, in the retina of rabbit, cat, and human (Holmgren-Taylor, 1982b; Pourcho,
1982; Frederick et al., 1982). In contrast with the 5,6-DHT and 6-OHDA techniques, which are based upon toxic degeneration, autoradiography preserves the fine structure of processes and synapses.

In summary, two major classes of dopamine-containing neurons have been observed. Dopaminergic amacrine cells are localized in the INL. Their processes ramify in the IPL. Dopaminergic interplexiform cells in addition have processes in the OPL. Only teleost fish and New World monkeys possess a large number of dopaminergic interplexiform cells. Other species were initially thought to have a pure population of the amacrine type of dopaminergic neurons, but in an increasing number of species, TH-immunoreactive interplexiform cells have been detected.

Dopaminergic interplexiform cells.

The synaptic connectivity of dopaminergic interplexiform cells has been studied in goldfish and Cebus monkey using cytotoxic staining of the cells with 6-OHDA and 5,6-DHT, in combination with preliminary destruction of the indoleamine-accumulating neurons with 5,7-DHT (Dowling and Ehinger, 1975; Dowling and Ehinger, 1978b; Dowling et al., 1980). Recently TH-immunoreactivity has been employed for electronmicroscopic studies of dopaminergic neurons in goldfish retina (Zucker and Yazulla, 1984). $^3$H-dopamine accumulation and subsequent autoradiography at the electronmicroscopic level has been performed on human retina, which apart from dopaminergic amacrine cells also contains a small population of dopaminergic interplexiform cells (Frederick et al., 1982).

Dopaminergic interplexiform cells participate in conventional synapses only (Dowling and Ehinger, 1975; see Holmgren-Taylor, 1982b; Ehinger, 1983a; Nguyen-legros, 1984). In early studies dopaminergic
Interplexiform cells were found to be presynaptic to amacrine cells only, in goldfish and *Cebus* monkey (Dowling and Ehinger, 1978b; Dowling *et al.*, 1980). However, in goldfish retina about 12% of the output from TH-immunoreactive processes appears to be onto bipolar cell terminals (Zucker and Yazulla, 1984). In the same quantitative study, no input from bipolar cells was observed, consistent with the earlier observations in goldfish and *Cebus* monkey. In human retina, there may be bipolar cell terminals presynaptic to $^3$H-dopamine-accumulating neurons, not necessarily interplexiform neurons (Frederick *et al.*, 1982). Output from dopaminergic interplexiform cells in IPL exceeds the input they receive from other neurons in all species investigated. Some laminar variation of the output-to-input ratio has been described in goldfish retina (Zucker and Yazulla, 1984).

In the OPL dopaminergic interplexiform cells were found to be presynaptic only (Dowling and Ehinger, 1975; see Ehinger, 1983a; Nguyen-Legros 1984). They synapsed predominantly onto the horizontal cell bodies, but also onto bipolar cell dendrites in goldfish and *Cebus* monkey (Dowling and Ehinger, 1978b; Dowling *et al.*, 1980). The latter make up for only 4% of interplexiform contacts in the OPL in goldfish (Zucker and Yazulla, 1984). In human retina no contacts with bipolar cells have been observed (Frederick *et al.*, 1982). In neither species have contacts between dopaminergic interplexiform cells and photoreceptors been observed.

Since no input synapses onto the dopaminergic interplexiform cells have been observed in the OPL, they seem not to receive input in the OPL, and must be driven from the IPL only. So, it is very likely that dopaminergic interplexiform cells channel a centrifugal stream of information from the IPL to the OPL. The observation of output synapses in the IPL, however, suggest a function within the IPL as well.
Double staining at the light microscopic level has revealed that in carp retina the dopaminergic interplexiform cell bodies are encircled by acetylcholine-esterase (AChE)-positive processes (Hayashi, 1980). This does not prove the existence of functional synapses, and acetylcholine esterase may be a poor marker for cholinergic neurons. However, cholinergic drugs have been shown to affect the intensity of monoamine histofluorescence in teleost retina (Negishi et al., 1980a). In addition, the electrophysiological effects of dopamine on horizontal cells can be mimicked by acetylcholine (Drujan et al., 1980). Together these results point towards an intimate contact between cholinergic neurons and dopaminergic interplexiform cells in the teleost retina. In goldfish retina synapses from TH-immunoreactive to $^3$H-GABA accumulating processes have been observed in both the OPL and, in both directions, in the IPL (Zucker and Yazulla, 1984). These results suggest direct communication between dopaminergic and GABAergic neurons, although $^3$H-GABA uptake does not necessarily indicate that the cell is GABAergic (Zucker et al., 1984).

In isolated goldfish retina, dopamine was shown to depolarize the membrane potential of L-type cone-driven horizontal cells (Dowling et al., 1976; Hedden and Dowling, 1978). In addition, responses of these cells to wide-field light stimulation were attenuated. Since the balance of centre-surround antagonism at the level of bipolar cells was shifted in favour of the centre, it was suggested that the dopaminergic interplexiform innervation of the OPL, is involved in regulation of centre-surround antagonistic properties of bipolar receptive fields by suppressing lateral inhibitory effects of horizontal cells.

Negishi and his colleagues described the uncoupling effects of dopamine on all types of horizontal cells in the superfused retina of
the fish *Eugenes plumieri* (Negishi and Drujan, 1978; Negishi and Drujan 1979; Negishi *et al*., 1982). The selectivity of the effect was less compared with that observed in goldfish retinal preparations, since noradrenaline, serotonin, and other monoamines, were also found to be effective in reducing lateral propagation of horizontal cell signals.

Cohen and Dowling (1983) found that in carp retina deprived of dopaminergic neurons by 6-OHDA treatment, cone horizontal cells had very large receptive fields. They speculated that dopamine, via D1 receptors shown to be present on isolated horizontal cells in carp (Van Buskirk and Dowling, 1981), increased intracellular cAMP levels. This idea was based on the report that cAMP regulated permeability of gap junctions and thereby, the electrical coupling between cells in culture (Flagg-Newton *et al*., 1981). However, in that paper, cAMP was reported to increase permeability. In the model proposed by Cohen and Dowling, cAMP would have to decrease the electrical coupling of horizontal cells. It was two years later that Lasater and Dowling (1985) reported that with cultured horizontal cells of perch, dopamine and dibutyryl-cAMP decreased the conductance between horizontal cells.

Meanwhile, Piccolino *et al*. (1982) showed that in the turtle retina, GABA antagonists narrowed the receptive fields of large field horizontal cells, just as did dopamine in teleost retina. Concomitantly, the electrical resistance between horizontal cells was increased and diffusion of Lucifer Yellow was restricted. This was the first report to demonstrate that receptive field size could be regulated by manipulating electrical coupling of horizontal cells via gap junctions, and was an attractive model to explain the effects of dopamine in the OPL of teleost.
Teranishi et al. (1983; 1984a) found that dopamine (1) increased the amplitude of horizontal cell responses to central receptive field stimulation, (2) decreased receptive field diameter, and (3) prevented spread of Lucifer Yellow between horizontal cells. These effects could be blocked by the dopamine antagonist haloperidol, and mimicked by dibutyryl-cAMP. Dopamine-deprived retinas, obtained by 6-OHDA treatment, showed properties consistent with massive horizontal cell coupling. The horizontal cell responsiveness to dopamine was, however, not affected by the 6-OHDA lesion.

This mechanism of dopamine regulation of horizontal cell coupling has also been demonstrated in catfish retina (Hida et al., 1984). In carp the GABA antagonist bicuculline also uncouples horizontal cells, as described for turtle retina. These effects of bicuculline were not observed in the presence of a dopamine blocker, nor when the retina was deprived of dopamine by 6-OHDA. This suggests that there is GABAergic innervation of dopaminergic interplexiform cells in carp retina (Negishi et al., 1983a).

Piccolino et al. (1984) reported that the effects of GABAergic drugs on the coupling of horizontal cells, were blocked by dopamine antagonists. In addition, dopamine itself was found to affect the coupling of horizontal cells. These results suggest that dopamine receptors are involved in the coupling of horizontal cells, and that the previously reported effects of GABA (Piccolino et al., 1982) seem to be mediated by dopamine. Intriguingly, there is no indication of an endogenous dopaminergic ligand in the OPL of turtle retina. Monoamine histofluorescence, histofluorescence after catecholamine accumulation, immunohistochemistry against TH, and autoradiography after 3H-dopamine accumulation, did not show dopaminergic innervation in the OPL of turtle.
retina (Witkovsky et al., 1984). A possible explanation for this discrepancy is that the coupling of the horizontal cells in the turtle retina is affected by dopamine, which is released from the IPL and diffuses to the OPL (Gerschenfeld, personal communications). This could be a significant indication of action at a distance for a neurotransmitter.

Thus the effects of dopamine in the OPL have been well documented. A key question remaining, is how the second messenger cAMP affects gap junction permeability. cAMP-regulated protein phosphorylation is a likely candidate (Wiener and Loewenstein, 1983; see Goodall, 1985), but its role is, as yet, not demonstrated.

Based on these effects it has been hypothesized that the role of dopamine may be in the regulation of the antagonistic centre-surround receptive field organization of bipolar cells. The reduced receptive field sizes observed after application of dopamine, are usually interpreted as relating to a light-adapted retina. However, recently it was demonstrated that the physiological condition evoked by dopamine application, reduced responsiveness and receptive field size of cone horizontal cells, could also be brought on by prolonged dark adaptation in vivo in the carp retina (Mangel and Dowling, 1985). In addition, the few studies on light-affected dopamine release, favour a diminished release rather than increased release during the light (see Dopamine release in the retina). Thus, the exact role of dopamine in the OPL is not completely understood yet.

Dopaminergic amacrine cells.

The ultrastructure of dopaminergic amacrine cells has been studied in rabbit retina by cytotoxic staining with 6-OHDA and 5,6-DHT, in
combination with preliminary destruction of the indoleamine-accumulating neurons by 5,7-DHT (Dowling and Ehinger, 1978a). In mudpuppy retina only 5,6-DHT was used (Adolph et al., 1980), consequently, dopaminergic amacrine cells and indoleamine-accumulating cells were not distinguished. The retina of *Cynomolgus* monkey does not contain indoleamine-accumulating cells, so that 5,6-DHT specifically stains dopaminergic neurons (Holmgren, 1982). $^3$H-dopamine accumulation and subsequent autoradiography has been used for electronmicroscopic observation of synaptic connectivity in the retinas of rabbit (Holmgren-Taylor, 1982a), cat (Pourcho, 1982) and human (Frederick et al., 1982).

Synaptic connectivity of the dopaminergic amacrine cells is similar to that of dopaminergic interplexiform cells in the IPL. In all species investigated, labelled processes participate in conventional synapses only (Holmgren-Taylor, 1982b; see Ehinger, 1983a; Nguyen-Legros, 1984). Dopaminergic presynaptic elements were localized at the varicosities, and made contacts onto processes as well as cell bodies. Input from other cells was predominantly received at intervaricose elements. However, $^3$H-dopamine autoradiography revealed some input contacts at the varicosities in rabbit retina (Holmgren-Taylor, 1982a). In cat (Pourcho, 1982) and rabbit (Holmgren-Taylor, 1982b) retina, the output synapses from dopaminergic neurons made up 95% of the total number of synapses in which the dopaminergic neurons participated. Because the input from other cells was mainly at intervaricose elements, input contacts were hard to detect, so the actual proportion could be larger than 5%.

In the retinas of rabbit, cat and *Cynomolgus* monkey, dopaminergic amacrine cells only contacted other amacrine cells, including other dopaminergic neurons (Dowling and Ehinger, 1978a; Holmgren, 1982;
Holmgren-Taylor, 1982a; Pourcho, 1982). No contacts with bipolar or ganglion cells have been observed. In human retina, a contact onto a presumed bipolar cell terminal has been observed, however, the frequency of this phenomenon has not been determined (Frederick et al., 1982). In mudpuppy retina, some contacts between 5,6-DHT-stained processes and bipolar cells have been observed (Adolph et al., 1980), but these might represent contacts of indoleamine-accumulating neurons, which in other species appear to feed-back onto bipolar cell terminals. The idea emerges that the majority, if not all, contacts of the dopaminergic amacrine cells are with amacrine cells only (see Ehinger, 1983a; Nguyen-Legros, 1984).

In cat retina, the dopaminergic amacrine cell has been identified as the Golgi A18-type amacrine cell (Pourcho, 1982). Processes of these amacrine cells form distinctive rings, about 10 um in diameter, which encircle the AII-type amacrine cells (Tork and Stone, 1979), and it has been suggested that these cells may be involved in switching the retina between scotopic and photopic conditions (see Stone, 1982; Sterling, 1983). This could be the morphological substrate for dopamine's hypothetical role in regulating the light sensitivity of the cat retina (see Sterling, 1983; Ehinger, 1983a; Nguyen-Legros, 1984).

Ligand binding to dopamine receptors has been found to be enriched in bovine retinal subcellular fractions containing fragments from the IPL (Redburn et al., 1980), and the same fractions from rabbit retina contained dopamine-sensitive adenylate cyclase activity (Thomas et al., 1978). In a microdissection study, most of the retinal dopamine-sensitive adenylate cyclase was found in the IPL of rabbit and ground-squirrel (Ferrendelli et al., 1980; De Vries et al., 1982). The observations that the retinas of rodless mice contained dopamine-
sensitive adenylate cyclase, and that the activity of this enzyme decreased after cytotoxic destruction of the INL and the IPL by glutamate, are further indications of a localization in the IPL of dopamine-sensitive adenylate cyclase (Makman et al., 1975). In rat retina, protein-1 (synapsin-1), a substrate for cAMP-dependent protein kinase (Nestler et al., 1984), has been demonstrated in the INL immunohistochemically (De Camilli et al., 1979b). However, none of these entities has been demonstrated to be present in neurons actually receiving dopaminergic input. These results indicate that the molecular machinery which is thought to mediate dopamine's physiological response via cAMP, is present in the IPL as well.

Dopaminergic agonists and antagonists have been found to affect electroretinograms (ERGs) in cat (Gutierrez and Spiguel, 1973), rabbit (Jagadeesh and Sanchez, 1981) and human (Fornaro et al., 1984), but the effects are different from species to species. Dopamine depletion by treatment with 6-OHDA did not affect the ERG in rabbit, goldfish (Ehinger and Nordenfelt, 1977) and cat (Maguire and Smith, 1985). However, in a recent study it was reported that oscillatory potentials, a component of the b-wave thought to be generated in the inner retina, were increased in 6-OHDA-treated retinas of frogs (Citron et al., 1985). As noted by Ehinger (1983), the essentially tangentially orientated dopaminergic neurons cannot be expected to have much influence on the ERG, which essentially represents radially directed potential differences. Although the ERG could be useful for assessing the physiological conditions of an experimental preparation (see e.g. Ames and Nesbett, 1981), the complexity of the ERG gives it limited use for establishing the function of a particular class of neurons, specifically the dopaminergic neurons in the retina.
Single unit recordings from the optic tract of cat showed that iontophoretically applied dopamine inhibited ganglion cells (Straschill and Perwein, 1969). Both spontaneous activity as well as the excitatory component of the light-evoked response were reduced by dopamine, noradrenaline, and the dopamine agonist amphetamine. The inhibitory phase after light stimulation was augmented by these compounds. In isolated rabbit retina, dopamine was found to show variable effects on the different types of ganglion cells (Ames and Pollen, 1969). Superfusion with dopamine, noradrenaline or adrenergic agonists decreased spontaneous and light-evoked responses of ON-centre and ON-OFF ganglion cells, in agreement with results found in cat. In contrast to the cat, spontaneous activity of OFF-centre cells increased during dopamine superfusion. Light-evoked responses of the latter cells were not affected. In rabbit retina, the adrenergic blockers phentolamine and propranolol were found to affect ganglion cell spontaneous and light-evoked activities (Ames and Pollen, 1969). Thus, dopamine seems to be involved in the generation of ganglion cell responses. However, the method is too gross, and the data too limited, to specify the effects of dopamine in the IPL.

**Dopaminergic transmission in the vertebrate retina.**

Table 1.5 summarizes the results of the tests done on the various species, to examine a transmitter role for dopamine in the vertebrate retina. In a variable degree of detail, dopamine has been shown to be present in the retina, and the synthetic mechanism for dopamine synthesis has been demonstrated in retinas of many species. Both these entities have been localized in two major subsets of retinal neurons: dopaminergic interplexiform cells, and dopaminergic amacrine cells. In
Table 1.5: Overview of tests done regarding neurotransmitter status for dopamine in various vertebrate species.

| Species       | DOPAMINE PRESENT | affected by light | TYROSINE HYDROXYLASE | affected by light | TURNOVER | affected by light | DOPAC | 3-MT | HVA | DDC | MAO | COMT | RELEASE | by light | by high-potassium | RECEPTOR BINDING | ADENYLATE CYCLASE | UPTAKE | HISTOFLOURESCENCE | dopamin | TYROSINE HYDROXYLASE | UPTAKE | TH ELECTRON MICROSCOPY | UPTAKE | ELECTRON MICROSCOPY |
|---------------|------------------|-------------------|-----------------------|-------------------|----------|-------------------|-------|-----|-----|-----|-----|------|----------|----------|----------------------|----------------|-------------------|--------|---------------------|---------|---------------------|--------|---------------------|
| Teleost Fish  |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Amphibia/Reptiles |                |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Bird          |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Rat           |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Other Rodents |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Cat           |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Rabbit        |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Con           |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| New World     |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Old World     |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |
| Human         |                  |                    |                       |                   |          |                    |       |     |     |     |     |      |         |          |                      |               |                   |        |                     |         |                    |        |                     |

In various species, the results indicate the presence of a molecular dopamine release mechanism. Both receptor binding and adrenergic transmission seem to be a common feature of the vertebrate retina; the considerable variability of dopamine’s presence and operational effects of dopamine have been demonstrated in most representative species. The studies have illustrated the presence of dopamine in a variety of functional roles across species. It is important to realize that in no species has a complete set of dopamine’s receptors been identified, and a careful conservation of what functions the particular dopaminergic system partakes in. By type rather than species, hence, the idea emerges that dopamine is probably involved in the control of retinal cell activity under various conditions. The dopamine levels are approximated by the catecholamine measurements of catecholamines in plasma, which are relatively constant, and by the catecholamine measurements of dopamine, which are variable.
various species, the results indicate the presence of a vesicular dopamine-release mechanism. Both receptor binding and cell physiological effects of dopamine have been demonstrated in most representative species, and these studies demonstrated the presence of \( D_1 \)-type rather than \( D_2 \)-receptors. Taken together, the idea emerges that dopamine is probably a neurotransmitter in vertebrate retina. However, it is important to realize that in no species has a complete set of available tests been done (see TABLE 1.5).

So, although the presence of dopaminergic transmission seems to be a general feature of the vertebrate retina, the considerable variability of the basic pattern between species, observed in detailed histology and physiology, requires a careful description of the dopaminergic system in the retina for each species under investigation, and a careful consideration of what functions that particular dopaminergic system performs.

Other putative monoaminergic transmitter in the retina.

Both noradrenaline and adrenaline have been detected only in trace amounts in retinas of most species (see *Dopamine's presence and synthesis in the retina*, TABLE 1.3), except for bovine retina where levels similar to dopamine levels have been reported (Nesselhut and Osborne, 1982; Osborne and Nesselhut, 1983). Accurate measurements of retinal noradrenaline levels are hampered by the noradrenergic innervation of the choroid, which is a source of contamination (Haggendal and Malmfors, 1965), and further the determination of catecholamine levels by HPLC can be cumbersome, as illustrated by the large variation in retinal dopamine levels reported by the group of Osborne (compare Osborne, 1981 with Nesselhut and Osborne, 1982; see
TABLE 1.3). Following superior cervical ganglionectomy, most of the low levels of noradrenaline disappeared from rat retinal homogenates (Hadjiconstantinou et al., 1983). The remaining minute amount of noradrenaline, however, cannot be entirely ignored. Levels of adrenaline were not affected by ganglionectomy and seem to be of retinal origin in the rat (Hadjiconstantinou et al., 1983).

Bovine retinas have been shown to possess the capacity to convert $^{14}$C-dopamine to $^{14}$C-noradrenaline (Osborne, 1981). Recently DBH activity has been demonstrated radioenzymatically in retinal homogenates from cow (0.77 U/min.g wet weight), monkey (0.58), rabbit (0.47), rat (0.38), and lizard (0.29). No DBH activity (the detection limit was 0.2 U/min.g wet weight) could be detected in frog retina. The activity per g tissue in cow retina was higher than in the vitreous or choroid/pigment epithelium. Thus, DBH activity seems of retinal origin in the cow (Osborne and Patel, 1985).

PNMT activity has so far been demonstrated only in the retinas of rat (Hadjiconstantinou et al., 1983) and cow (Osborne and Nesselhut, 1983), and in the rat, was not affected by cervical ganglionectomy. Both species showed similar PNMT activities (18 fmol/min.g wet weight).

A high-affinity ($K_m$ 0.5 x $10^{-9}$ M) uptake of $^{14}$C-noradrenaline has been detected in isolated pieces of bovine retina. This uptake was temperature sensitive and sodium dependent, and could be partially blocked by desipramine, whilst the effects of the dopamine uptake blocker benztropine were less pronounced (Osborne, 1981). These data point to the presence in bovine retina, of a noradrenaline carrier, distinct from the dopamine uptake systems. Exogenous noradrenaline has been released from isolated cow retina by high-potassium. This release was calcium dependent and could be blocked by cobalt (Osborne, 1981).
The increase in noradrenaline efflux was, however, still detected 10 min after the 5 min pulse of high-potassium (50 mM), which is too long to readily accept the increase in efflux as demonstrating release.

Alpha2-adrenergic receptor binding has been reported in membrane fractions from bovine retina, enriched in particles from IPL (Osborne, 1982). In rat retina, in vivo alpha2-adrenergic drugs have been found to affect TH activity, measured in vitro; antagonists increased TH in dark-adapted retinas, and this effect could be blocked by the agonist clonidine (Iuvone and Rauch, 1983). Retinal dopamine turnover in vivo was demonstrated to be affected similarly, by alpha2-adrenergic drugs in the rat (Hadjiconstantinou et al., 1984a). The pharmacological characterization of this type of receptor does not indicate what the endogenous ligand is, since both adrenaline and noradrenaline activate alpha2-adrenergic receptors. In addition, the effects of alpha2-adrenergic drugs might be mediated by the dopaminergic autoreceptors (Dubocovich, 1984a).

To look for noradrenergic or adrenergic neurons at the cell level, the monoamine histofluorescence technique is not readily suitable, because it does not discriminate between dopamine and noradrenaline, while adrenaline forms only weak fluorophores with formaldehyde (Falck et al., 1962). Further, since the levels of noradrenaline and adrenaline are low, high fluorescence due to endogenous contents of these catecholamines cannot be expected. Simultaneous intravitreal administration of 5,6-DHT, noradrenaline and dopamine led to the visualization of a population of neurons which preferentially accumulated noradrenaline in rat retina (Fukuda et al., 1982). These noradrenaline-accumulating neurons do not seem to convert DOPA into noradrenaline, since high doses of DOPA, which is expected to accumulate in all
monoaminergic neurons, did not lead to their visualization. Also in chicken retina, catecholamine-accumulating neurons, as distinct from indoleamine-accumulating neurons and dopamine-containing neurons, have been described (Kato et al., 1984).

DBH-immunoreactive neurons have been detected in the retinas of cow and monkey in the ganglion cell layer. These neurons could well be ganglion cells, since the optic nerve in the cow has the same activity of DBH per wet weight as the retina (Osborne and Patel, 1985). Autoradiographic examination of the 3H-noradrenaline accumulation in cow retina, revealed the presence of radioactivity in the ganglion cell layer and in the IPL at the junction with the ganglion cell layer (Osborne, 1981; Osborne and Patel, 1985). No 3H-noradrenaline-accumulating neurons, nor DBH-immunoreactivity could be detected in retinas of frog, pigeon, rat, and rabbit, even though the latter two species have been demonstrated to have retinal DBH enzyme activity (Osborne and Patel, 1985). The antiserum used was raised against bovine DBH, so the lack of immunoreactivity could be due to species differences in antigenetic properties, and the negative results do not prove that there are no DBH-containing neurons. In rat retina PNMT-immunoreactive neurons have been demonstrated (Hadjiconstantinou, 1984a). These cells were found amongst the amacrine cells and send processes into the IPL, but were clearly distinct from the TH-immunoreactive cells described by Nguyen-Legros et al (1981). Very recently it was shown that these PNMT-positive neurons in retina, like some in the posterior hypothalamus, could not be stained with antibodies against DBH nor TH (Foster et al., 1985). Possible explanations for these findings have been discussed elsewhere (see Chapter 1.2). An interesting possibility is that these cells could be the same as the noradrenaline-accumulating neurons
described by Fukuda et al. (1984), which seem to lack the capacity to convert DOPA into noradrenaline (see above).

The frequency of the putative noradrenergic and adrenergic neurons is similar to the frequency of dopaminergic neurons in the respective species. Thus, considering the low levels of noradrenaline and adrenaline in rat retina (Hadjiconstantinou et al., 1983), this indicates that there would be an extremely low steady-state pool of these catecholamines in those neurons. In bovine retina, where relatively high levels of noradrenaline and adrenaline have been reported (Nesselhut and Osborne, 1982; Osborne and Nesselhut, 1983), intraneuronal levels comparable to dopamine can be expected, which is not in agreement with the absence of noradrenaline-related histofluorescence in bovine retina.

In bovine retina, the data obtained seem to favour a transmitter role for noradrenaline (Osborne, 1981). Since it has been demonstrated in the rat that some of these entities relate to the sympathetic innervation of the choroid, special care must be taken to ensure that the results obtained in the cow, truly reflect retinal capacities. In rat retina, some of the observations done at the tissue level seem to disagree with the data from the cellular studies. So, for both noradrenaline and adrenaline a transmitter role cannot be accepted. Equally, it cannot be decisively rejected as yet. If future research favours a transmitter role for adrenaline in rat retina, the neurons involved would be interesting in the sense that they may have an alternative mechanism to obtain their transmitter, which avoids the need for TH and DBH.

The tests for neurotransmitter roles for noradrenaline and adrenaline have so far predominantly been done in retina of the cow and
the rat. So, especially considering the exceptionally high levels of noradrenaline and adrenaline claimed for cow retina, the findings in these species alone cannot be generalized to the vertebrate retina. In some species, however, there is an indication of an actual lack of noradrenaline or adrenaline related entities. Thus it seems that, of the catecholamine transmitter candidates, dopamine is the major one present in all species, except bovine retina, and is the only one present throughout all vertebrate species.
Chapter 2.

TYROSINE HYDROXYLASE AS A BIOCHEMICAL MARKER FOR DOPAMINERGIC NEURONS IN CHICKEN RETINA.

Introduction.

Dopamine is the most obvious biochemical marker for dopaminergic neurons in nervous tissue. Although it, as well as being a putative transmitter in its own right, is also a precursor for the other catecholamine transmitters, the presence of significant amounts of dopamine is a good indication of the presence of dopaminergic neurons. The levels of dopamine result from dopamine turnover in individual neurons, and therefore only provide limited information about the number of dopaminergic neurons in a given tissue. Although the activity of the synthetic enzyme TH is highly regulated (see Chapter 1.4), the activity measured under saturating conditions in vitro might be expected to show less rapid adaptive changes than the amount of transmitter itself, and could be useful as a quantitative marker for dopaminergic neurons, especially when combined with information about the levels of dopamine and dopamine metabolites.

The biosynthesis of dopamine from the dietary amino acid tyrosine occurs in two steps and is mediated by the enzymes TH and DDC. DDC activity is indistinguishable from more general AADC activity and is therefore not a selective marker for dopamine synthesis (see Chapter 1.2). Due to the overlap in substrate specificities of TH and phenylalanine hydroxylase, tyrosine hydroxylating activity could originate from phenylalanine hydroxylase, however, this enzyme is unlikely to be found in the retina (see Chapter 1.4), or in nervous tissue in general (see Chapter 1.2). This leaves TH activity as the best marker for sites of dopamine synthesis.
TH is a mixed function oxygenase and requires molecular oxygen and a reduced pterin cofactor for catalytic activity. TH hydroxylates tyrosine to DOPA, while the pterin cofactor is oxidized and water is released (FIGURE 2.1). With the availability of high specific activity radiolabelled tyrosine, several radioenzymatic assays have been described for measurement of TH activity in vitro. Nagatsu et al. (1964) developed an assay in which the formation of DOPA from radiolabelled tyrosine is measured. To prevent the decarboxylation of DOPA, DDC has to be inhibited during incubation. Since TH is inhibited by catechols, including DOPA (see Chapter 1.2), the accumulation of DOPA could affect TH activity in vitro. Another radioenzymatic assay is based upon the combined activities of TH and DDC, and measures $^{14}$CO$_2$ formation from [1-$^{14}$C]-L-tyrosine (Waymire et al., 1971). This coupled assay only reflects TH activities if the DDC activity exceeds the TH activity. Therefore, additional DDC, which has to be isolated first, is included in the incubation medium. These problems are avoided by measuring the formation of $[^{3}$H]-H$_2$O from [3,5-ring-$^{3}$H]-L-tyrosine, as described by Karobath (1971). In addition, the separation of radiolabelled DOPA from the pool of substrate by alumina chromatography, is less reliable than the separation of $[^{3}$H]-H$_2$O from all catechol compounds in the tritium-water-release method. Thus the method measuring the formation of the side-product, H$_2$O, of the hydroxylation of tyrosine, is potentially the least complicated, yet most reliable method available of determining TH activities in vitro, and has been used as the basis for the assay used in this thesis.

A major problem in assaying TH activities in vitro is to control the redox conditions of the assay, since both molecular oxygen and cofactor in the reduced state are necessary for TH activity. Non-
Figure 2.1: Hydroxylation of tyrosine by tyrosine hydroxylase (TH).

BH$_2^*$, pterin cofactor
BH$_2^*$-H$_2^*$, reduced pterin cofactor
DOPA, "dihydroxyphenylalanine.

Note that one hydrogen atom in the resulting water, originates from the phenyl ring of tyrosine.
The hydroxylation of tyrosine by the catechol  

dopamine hydroxylase gives the distal side product 

dopa.
enzymatic oxidation of the substrate can cause high blank values, and
premature oxidation of the cofactor can reduce the sensitivity of the
assay. A variety of reducing systems and antioxidants have been
described by several laboratories (see e.g., Nagatsu et al., 1964;

The hydroxylation of tyrosine is the rate-limiting step in
catecholamine biosynthesis, and is the site for regulation of
catecholamine biosynthesis (see Chapter 1.2). Thus, the activity of TH
in vivo reflects the activity of the neuron. It is very likely that
short term modulation of TH activity is based on allosteric regulation
of kinetic properties of the enzyme (see Chapter 1.2). It has been
shown that the different allosteric states of TH can be preserved under
assay conditions in vivo (Zivkovic et al., 1974; Lovenberg et al., 1975;
Morgenroth et al., 1975; Lazar et al., 1982; Mestikawy et al., 1983;
Vrana and Roskoski, 1983; Iuvone, 1984b). This means that TH activities
measured with sub-saturating substrate concentrations in vitro reflect
the allosteric state of the enzyme, and thus indicate the activity of
the catecholaminergic neurons in vivo. As mentioned above, TH
activities measured with saturating substrate concentrations should be
more closely related to the number of dopaminergic neurons.

This chapter describes the optimization of a radioenzymatic assay
for TH in chicken retina, based on the tritium-water release method of
Karobath (1971). Due to the low number of dopaminergic neurons in
vertebrate retina, low TH activities were expected. A TH assay with
high sensitivity was therefore required, and special consideration has
to be given to defining conditions for obtaining realistic blank
values. To be able to use TH activities in vitro as marker for the
number of dopaminergic neurons, there should be a large range of
linearity in the sample size - activity relationship. To give information about the kinetic states of TH in vivo, the assay should be sensitive and reproducible under sub-saturating cofactor conditions.

Methods.

Chickens.

One day-old male chickens (White Leghorn X Black Australorp, *Gallus domesticus*) were obtained, and raised under a 12-12h light-dark cycle with ample access to water and food. Chickens at least one week old were used for the experiments. Chickens were killed with ether, the eyes were removed, and cut open around the iris. The iris, lens, and vitreous were removed, and the retinas, often together with the pigment epithelium and choroid, were isolated, weighed, and either stored frozen in 1 ml H$_2$O at -20°C, or directly homogenized. Unless specified otherwise, for all experiments described in this thesis, chickens were obtained and reared, and retinas were isolated, as described in this section.

Tyrosine hydroxylase assay.

For all experiments in this thesis, TH activity was determined in retinal homogenates using a modification of the tritium-water release method, first described by Karobath (1971). 30 µl of retinal homogenate (about 200 µg protein) was added to 70 µl incubation medium resulting in the following final concentrations: 400 mM Tris-acetate pH 6.0, 10 mM sodium-ascorbate, 2.0 mM 6,7-dimethyl-5,6,7,8-tetrahydropterine (DMPH$_4$ in 25 mg lots from Calbiochem), 20 mM beta-mercaptoethanol, 2000 U catalase (65000 U/mg, Boehringer), and 200 µM [3,5-ring-$^3$H]-L-tyrosine (about 0.8 µCi, from New England Nuclear, 54.6 Ci/mmol), as substrate.
Blank incubations additionally contained 500 μM 3-iodotyrosine, which was added before the incubation was started. The incubation was started by adding the homogenate, immediately followed by the substrate. After 20 min incubation in a metabolic shaker bath at 37°C in open test tubes, the reaction was stopped by adding 100 μl 10 % (w/v) tri-chloroacetic acid (TCA) solution. Samples were centrifuged for 10 min at 1000 xg, and 100 μl of the supernatant was run over columns, which consisted of 10 mm (6 mm diameter) Dowex 50W-X4 (H⁺-form, Biorad) layered over 7 mm (6 mm diameter) Dowex 1-X8 (OH⁻-form, Biorad). After 15-30 min the loaded columns were eluted with 2 times 500 μl H₂O. Combined eluates were counted for radioactivity in 8 ml scintillation mixture, consisting of 5 g 2,5-diphenyloxazole (PPO) per litre toluene/triton-X-100 (2/1 by volume).

Protein determination.

For all experiments in this thesis, protein was measured by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Results.

Figure 2.2 demonstrates the absolute requirement for catalase in the incubation mixture. Without catalase there was clearly more non-enzymatic oxidation, especially in incubation mixtures where heat-deactivated retinal homogenate was used. Inactivating the samples by boiling has been reported to favour peroxide formation which may cause non-enzymatic oxidation of the substrate (Coyle, 1972). Catalase is commonly used as a peroxide scavanger (Coyle, 1972; Lerner et al., 1977), and in the presence of catalase, both the heat-deactivated sample
Figure 2.2: The effect of catalase on the amount of non-enzymatic $[^3\text{H}]$-$\text{H}_2\text{O}$ release.

The TH assay was performed under standard conditions, except for the following modifications:

- A, without catalase
- B, with 2000 U catalase
- $\sigma$, no inhibitor added
- $\Delta$, heat-inactivated samples (5min 100°C)
- $\times$, TH was inhibited with 3-I-tyrosine (500uM final)
- $\bullet$, TH was blocked by the addition of TCA, prior to the incubation

A major cause for $[^3\text{H}]$-$\text{H}_2\text{O}$ release from $[3,5$-ring-$^3\text{H}]$-L-tyrosine, in addition to TH activity, is non-enzymatic oxidation. Heat-inactivated samples show a large rate of non-enzymatic oxidation, which can be reduced by the addition of catalase in the incubating medium. The non-enzymatic oxidation in 3-iodotyrosine-inhibited samples is less than in heat-deactivated samples, but can be slightly reduced by the addition of catalase.
and the addition of the irreversible TH blocker 3-iodotyrosine, gave about the same blank values. The addition of TCA prior to incubation, resulted in lowest blank values, however this does not represent blank activities realistically. Using 3-iodotyrosine to obtain blank values, the experimental/blank value ratios ranged from 0.20 to 2.5, depending upon the TH activity of the sample. In an attempt to lower the blank values, $^3$H-tyrosine was purified prior to use by alumina-, followed by cation-exchange chromatography (Nagatsu et al., 1964; Coyle, 1972). Using purified $^3$H-tyrosine, slightly lower blank values were obtained, and the experimental/blank value ratio improved slightly. Approximately 70% of the radioactivity was lost during the purification, which is excessive waste considering the slight improvement in experimental/blank value ratio obtained. Even without purification, in absolute terms, less than 0.1% of the radioactivity ran through the column after incubation in the presence of 3-iodotyrosine.

The TH assay medium was also optimized. Figure 2.3 shows the sharp pH dependency of TH activity. Best TH activity was obtained by using a rather high buffer concentration of 400 mM Tris-acetate compared to 200 or 100 mM (TABLE 2.1). A 200 mM acetate-buffered incubation medium showed significantly less activity than one buffered with 200mM Tris-acetate. Both the addition of extra CaCl$_2$, and the chelation of endogenous Ca$^{++}$ with ethyleneglycol-bis-(beta-aminoethylether)N,N'-tetra-acetic acid (EGTA), decreased TH activity in the assay (TABLE 2.1). Increasing the ionic strength by addition of NaCl also decreased measured TH activity (TABLE 2.1).

The availability of reduced cofactor seems to be crucial for TH activity. Therefore, DMPH$_4$ was obtained in small lots (25 µg) and dissolved in 1 M beta-mercaptoethanol. Although TH activities were not
The pH dependency of chick retinal TH activity *in vitro*.

The TH assay was performed under standard conditions, except for the variation in pH. Incubation time was 45 min. The pH was adjusted with 2M acetic acid in the incubation medium, prior to the addition of the sample. The pH under standard conditions is 6.0. Bars represent SEM (n=3-4).
TH activity (pmol/mg protein.min)
Table 2.1: Ionic requirements of chick retinal TH \textit{in vitro}.

\begin{center}
\begin{tabular}{|l|c|}
\hline
\textbf{standard buffer (n=3, SEM < 8\%)} & TH activity (n=3) \\
200mM Tris-acetate & 100\% \\
100mM Tris-acetate & 79\% \\
200mM acetate & 71\% \\
\hline
\textbf{calcium (n=3, SEM < 13\%)} & \\
1mM EGTA & 72\% \\
1mM EGTA & 41\% \\
+ 1mM CaCl\textsubscript{2} & \\
1mM EGTA & 76\% \\
+ 10mM CaCl\textsubscript{2} & \\
\hline
\textbf{ionic strength (n=3, SEM < 4\%)} & \\
150mM NaCl & 87\% \\
300mM NaCl & 51\% \\
\hline
\end{tabular}
\end{center}

incubation time was 45 minutes.
Table 2.2: The effects of reducing agents on chick retinal TH activity in vitro.

<table>
<thead>
<tr>
<th>TH activity</th>
<th>45min</th>
<th>10min</th>
</tr>
</thead>
<tbody>
<tr>
<td>standard*</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>without ascorbate</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>without betamercaptoethanol</td>
<td>81%</td>
<td></td>
</tr>
<tr>
<td>40mM betamercaptoethanol</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>20mM dithiothreitol</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>10mM NADPH</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>0.5mM FeSO₄</td>
<td>57%</td>
<td>200%</td>
</tr>
</tbody>
</table>

(n=3, SEM < 12%)

* under standard conditions DMPH₄ is dissolved in betamercaptoethanol, resulting in 20mM betamercaptoethanol in the incubation.
significantly lower when using a three week-old DMPH₄ solution, solutions more than one week-old were routinely discarded. In experiments where the concentrations of DMPH₄ were crucial, the cofactor was made up fresh each day. Table 2.2 shows the effects of a variety of reducing agents on TH activity. Increasing the concentration of beta-mercaptoethanol apparently decreased TH activity, although it appears essential that the cofactor is dissolved in beta-mercaptoethanol, or that a low concentration of beta-mercaptoethanol is present. Interestingly, ferrous ions, often used to increase TH activity (Nagatsu et al., 1964; Coyle, 1972; Lerner et al., 1977) increased TH activity during the first 10min of incubation, but decreased the activity over a 45min incubation period.

The separation of [³H]-H₂O from other radioactivity is an essential step in the assay. Table 2.3 shows that the use of more cationic or anionic retaining resin, did not improve the retaining capacity of the columns. Pretreatment of the column with tyrosine or 3-iodotyrosine did not affect the retaining capacity either (data not shown). Thus, the standard columns have sufficient capacity. Increasing the standard elution volume of 1000 µl, did not elute more radioactivity (TABLE 2.3). Elution with 750 µl was, however, not sufficient to elute all the radioactivity. Additional experiments (data not shown) indicated that charcoal, which is used in a comparable assay for tryptophan hydroxylase (Beever et al., 1983), is not as efficient as the ion retaining resins. The use of a batch method, where the resin is mixed with the sample, did not sufficiently separate [³H]-H₂O from other radioactivity.

TH activities in retinal homogenates in water were the same as in homogenates in a physiological buffer (TABLE 2.4). Triton-X-100 (0.2 % w/v), which has been used to increase the yield of soluble, more active
Table 2.3: The retention capacity and elutions of the columns.

<table>
<thead>
<tr>
<th>Column Composition</th>
<th>Radioactivity in Eluate (in %)</th>
<th>Elution Volume</th>
<th>Radioactivity in Eluate (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOWEX-1 OH^-</td>
<td>DOWEX-50 H+</td>
<td>0.307</td>
<td>750µl 0.084</td>
</tr>
<tr>
<td>200µl</td>
<td>0µl</td>
<td>0.099</td>
<td>1000µl 0.096</td>
</tr>
<tr>
<td>200µl</td>
<td>150µl</td>
<td>0.096</td>
<td>1250µl 0.098</td>
</tr>
<tr>
<td>200µl</td>
<td>300µl</td>
<td>0.087</td>
<td>1500µl 0.098</td>
</tr>
<tr>
<td>200µl</td>
<td>600µl</td>
<td>0.177</td>
<td></td>
</tr>
<tr>
<td>0µl</td>
<td>300µl</td>
<td>0.128</td>
<td></td>
</tr>
<tr>
<td>100µl</td>
<td>300µl</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>200µl</td>
<td>300µl</td>
<td>0.104</td>
<td></td>
</tr>
</tbody>
</table>

TH assays on chick retinal homogenates were performed under standard conditions with the presence of 500mM 3-I-tyrosine (standard blanks), (n=3, SEM < 5%).

* 200µl Dowex-1 resin slurry results in 7mm (x 6mm diameter) columns
500µl Dowex-50 resin slurry results in 10mm (x 6mm diameter) columns
Dowex-50 was layered on top of the Dowex-1.
Table 2.4: The effects of homogenization conditions on chick retinal TH activity *in vitro*.

<table>
<thead>
<tr>
<th>homogenized in</th>
<th>incubated with</th>
<th>TH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>water, pH 6.0</td>
<td>standard conditions</td>
<td>100%</td>
</tr>
<tr>
<td>water, pH 6.0</td>
<td>0.06% Triton-X100</td>
<td>84%</td>
</tr>
<tr>
<td>water, pH 6.0</td>
<td>physiological buffer*</td>
<td>95%</td>
</tr>
<tr>
<td>0.2% Triton-X100</td>
<td>physiological buffer* (30 µl/100 µl incubation mix)</td>
<td>91%</td>
</tr>
<tr>
<td>physiological buffer*</td>
<td>standard conditions **</td>
<td>97%</td>
</tr>
</tbody>
</table>

* physiological buffer: 118mM NaCl, 4.7mM KCl, 1mM CaCl₂, 1.2mM MgCl₂, 24.9mM NaHCO₃, 10mM glucose, carbogen bubbled, pH 7.3

** the triton and physiological buffer present in the sample, will also be present during the incubation.

incubation time was 20 minutes (n = 3, SEM < 7%).
Table 2.5: The stability of chick retinal TH activity.

<table>
<thead>
<tr>
<th>Condition</th>
<th>TH activity in vitro (pmol/mg protein.min + SEM (n))</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated and homogenized</td>
<td>6.2 ± 0.4 (3)</td>
<td></td>
</tr>
<tr>
<td>Homogenized and stored</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week 4°C</td>
<td>4.0 ± 0.3 (4)</td>
<td>2P &lt; 0.5%</td>
</tr>
<tr>
<td>1 week -20°C</td>
<td>2.6 ± 0.2 (4)</td>
<td>2P &lt;&lt; 0.1%</td>
</tr>
<tr>
<td>Whole retina stored</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 month -20°C</td>
<td>5.4 ± 0.3 (5)</td>
<td>2P &lt; 20%</td>
</tr>
</tbody>
</table>

*compared to freshly isolated and homogenised retina (two-tailed Student's t-test)
Figure 2.4: The linearity of the TH assay with the protein concentration.

The TH assay was performed under standard conditions. The line is fitted by the method of least squares, assuming linearity (n=4, SEM<5%, r=0.996).
Sample (mg protein/incubation)

TH activity (pmol/20min)
Figure 2.5: Chick retinal TH activity with sub-saturating concentrations of cofactor in vitro.

The TH assay was performed under standard conditions.
- •••, with 2000 μM DMPH₄
- ••, with 200 μM DMPH₄.

The apparent $K_m$ of chick retinal TH for the pterin cofactor is 200 μM (see Chapter 3).
Bars represent SEM (n=6).
TH (Coyle, 1972) in the homogenization medium, did not stimulate TH activity in the sample. If added to the incubation medium, Triton-X-100 appeared to inhibit TH activity (TABLE 2.4). So when using retinal homogenates, the addition of Triton-X-100 is potentially deleterious, rather than beneficial. Table 2.5 shows the effects of various methods of storage on TH activity. Once homogenized, TH appeared to be very sensitive to freezing/thawing. The intact retina could be stored frozen for at least one month without much loss in TH activity, but retinas assayed immediately after homogenization showed the highest TH activities in my experiments.

Figure 2.4 demonstrates that TH activity measured in the assay is linearly related to the sample size over a 10-fold range, for up to 45 min of incubation. Figure 2.5 demonstrates that the assay for TH activity has sufficient sensitivity to reveal TH activity under sub-saturating conditions for its cofactor (the apparent $K_m$ of TH for DMPH$_4$ in this assay is $200 \pm 20 \mu$M, see Chapter 3).

Discussion.

High blank values in the TH assays may be due to contamination of the substrate, non-enzymatic oxidation of the substrate, and imperfect retention by the columns, or other, unknown sources. In absolute terms, the blank values I have obtained are low compared to those obtained in comparable assays, since less than 0.1% of radioactivity runs through the column after incubation in the presence of 3-iodotyrosine. For example, Leighton and Waggoner (1981) reported 0.14, 0.21, and 2.6% following the incubation of hippocampal, hypothalamic, and striatal synaptosomes.
Incubations without sample have been used to obtain blank values for TH assays (Karobath, 1971; Boarder and Fillenz, 1978). Such blanks give low values, but several constituents present in the sample probably contribute to the real blank values. Thus, incubations without sample indicate blank values inherent to the assay method, but underestimate the real blank values. Alternatively, heat-inactivated samples have been used to obtain blank values (Nagatsu et al., 1964; Coyle, 1972; Waggoner et al., 1980), but heating seems to increase peroxide formation, leading to some increase of the blanks (Coyle, 1972). While this problem can be controlled to some extent by adding catalase, it is inconvenient for routine assays, to divide each sample into two portions and heat-inactivate one of them. 3-Iodotyrosine is an irreversible competitive inhibitor of TH (see Roth, 1979). TH activity has been reported to be totally abolished in the presence of equimolar concentrations of tyrosine and 3-iodotyrosine (Waymire et al., 1971; Karobath, 1971; Coyle, 1972), thus providing a blank which covers both sample and assay contributions. To be on the safe side, I have used a 3-iodotyrosine to tyrosine ratio of 2.5. Although it is a very potent inhibitor, 3-iodotyrosine probably does not totally block TH. Nevertheless, 3-iodotyrosine-inhibited release of $[^3\text{H}]\text{H}_2\text{O}$ seems to be the best available assay of the enzymatic hydroxylation of tyrosine, and is probably slightly conservative in practice.

In contrast to other reports (Nagatsu et al., 1964; Coyle, 1972; Lerner et al., 1977), the addition of ferrous ions did not increase the activity of chick retinal TH in vitro. Based on the apparent dependence of TH on ferrous ions in vitro, it has been suggested that TH requires ferrous ion as a cofactor (Nagatsu et al., 1964). However, these observations were made on crude tissue extracts, and do not demonstrate
that ferrous ion is a true cofactor. It is known, for example, that catalase utilizes a iron-containing porphyrin as cofactor, and that ferrous salts themselves are able to neutralize peroxide (Lehninger, 1975, p. 185). Thus, it is likely that the particular conditions of the assay in vitro, the source and degree of purification of the TH-containing sample, and the particular catalase used in the assay, may determine whether a stimulatory effect of ferrous ions will be detected.

The aim of optimizing the radioenzymatic assay for TH was, to provide a biochemical marker for the number and activity of dopaminergic neurons in chicken retina. It appears that under conditions described here, measured TH activity is highly proportional to the amount of retinal homogenate. In addition, the assay appears sensitive enough to yield reproducible results, even under sub-saturating conditions with respect to the cofactor. These properties make the assay suitable for measuring TH capacity and affinity in chicken retina in vitro.
DOPAMINE AS A NEUROTRANSMITTER IN CHICKEN RETINA.

Introduction.

Although the observations from various species strongly support the hypothesis that dopamine is a neurotransmitter in vertebrate retina, in no single species has a complete set of available experimental tests been performed (see chapter 1.4).

Dopamine has been shown to be present in chicken retina (Schwarcz and Coyle, 1976; Da Prada, 1977). With the radioenzymatic assay used, only trace amounts of the other catecholamines have been detected. The highly sensitive HPLC-ED method has been used to quantify dopamine (Parkinson and Rando, 1983b), but has not been used yet to examine the other catecholamines in chicken retina.

TH activity has been demonstrated by radioenzymatic assay in homogenates of chicken retina. The assay used was sub-saturated for tyrosine (Schwarcz and Coyle, 1976; Schwarcz and Coyle, 1977), so that the kinetic properties of TH in chicken retina could not be studied. The activation of TH in chicken retina by light has been studied using an indirect assay, in which DDC was blocked alpha-fluoromethyl-DOPA and DOPA accumulation was measured (Parkinson and Rando, 1983b). A change in the kinetic properties of TH after light exposure, as described for TH in rat retina (see Iuvone, 1984b), has not been reported for chicken retina.

Exogenous dopamine can be released from chicken retina by electrical stimulation in vitro (Dubocovich, 1984b). High-potassium-evoked release of dopamine, has been demonstrated in chicken retina (Tapia and Arias, 1982), but light-stimulated dopamine release has not.
The aim of this chapter is to strengthen the arguments that dopamine acts as a neurotransmitter in the chicken retina. The catecholamine composition of chicken retina, studied by means of both cation-exchange and ion-pair reverse phase HPLC-ED, will be described. The sensitive modifications of a radioenzymatic assay for TH, described in chapter 2, will be employed to demonstrate TH activity and to study the allosteric regulation of kinetic properties of TH by light. Further, high-potassium-evoked release of exogenous dopamine, and uptake mechanisms for dopamine will be characterized in chicken retina.

**Methods.**

Chickens were reared and the retinas were removed as described in chapter 2. Retinal protein and TH activities were determined as described in chapter 2.

HPLC-ED.

The chromatographic system consisted of a Gilson 302-pump, Gilson 802-manometric module, Rheodyne 7125-injection valve with Alltech 100 ul sample loop, and a Whatman Partisil\5-ODS-3 reverse phase column (250 x 4.6 mm), protected with a Brownlee MPLC NewGuard\ RP18 cartridge column. The electrochemical detector was a BAS glassy-carbon TL5A electrode cell, and a BAS LC-4B-amperometric controller, coupled to a Gilson NI-chart recorder, and a Gilson 620-datamaster controlled by an Apple IIe+ computer, which stored the data on disk.

Levels of monoamines were quantified using an external standard method. Peak heights were measured from the chromatograms obtained with the chart recorder. The chromatograms shown are computer plots of the data stored on disk, and give slightly less detail than the
chromatograms from the chart recorder, which were less suitable for reproduction.

Ion-pair reverse phase HPLC-ED was performed according to the method of Duda and Moore (1985). Neural retinas were isolated and homogenized in 500-750 μl mobile phase. Retinal tissues including pigment epithelium and choroid were homogenized in 750-1000 μl mobile phase. Homogenates were centrifuged for 3min at 12000 xg, and supernatants were centrifuged through 0.47 μm Nylon-66® membranes at 15min 2000 xg. The filtered samples were either stored at -20°C, or analyzed on the same day. No deterioration was observed for up to 2 months at -20°C.

The mobile phase consisted of 0.1 M citric acid sodium-phosphate buffer pH 2.3, 0.1 mM ethylenediamine-tetraacetate (EDTA), 1.4 mM sodium-octylsulphate, and 10 % (v/v) methanol. The flow rate was 1-1.5 ml/min, and the applied oxidative potential was +700 mV.

With ion-pair reverse phase HPLC, it was possible to separate dopamine, noradrenaline, adrenaline, serotonin, and many of their metabolites and precursors within a chromatographic run of 20 min (FIGURE 3.1). The retention times of NA and MOPEG, and DOPA and DOPAC were too close, however, to distinguish them. MTA was slightly slower than 5-HTP in this chromatographic system, which caused a partial overlap of those peaks, but the MTA and 5-HTP peaks were distinguishable. DOPEG, adrenaline, dopamine, 5-HIAA, HVA and serotonin were clearly separated from each other.

At an oxidation potential of 700mV the oxidation of dopamine was near saturation, whereas oxidation of adrenaline and noradrenaline was not (FIGURE 3.2). However, the response of the detector for adrenaline and noradrenaline was sufficient, at the set potential of 700mV (TABLE
Figure 3.1: Ion-pair reverse phase HPLC of monoamines.

100 ul standard solutions containing 50 pmol DOPA, dopamine (DA), DOPAC, noradrenaline (NA), adrenaline (A), DOPEG, 5-HTP, serotonin (5-HT), or 5-HIAA, or containing 800 pmol HVA, 2200 pmol MTA, or 650 pmol MOPEG were injected. The applied oxidative potential for ED was 700mV.

The chromatograms at the bottom of the figure show the separation of dopamine from its precursor DOPA, and its metabolites DOPAC, HVA, and MTA. DOPA and DOPAC co-migrate, but the other compounds are well separated from each other. The chromatograms in the middle show that adrenaline and the metabolite DOPEG are well separated, but noradrenaline comigrates with the metabolite MOPEG. Serotonin, its precursor 5-HTP, and its metabolite 5-HIAA separate clearly, as shown by the chromatograms at the top of the figure. Some standards were dissolved in a solution of ascorbic acid and pargyline, but these were not retained on the column.
Figure 3.2: Oxidative currents of noradrenaline, adrenaline and dopamine.

100 ul (30 pmol) standard solutions were injected in ion-pair reverse phase HPLC-ED system.

♦—♦, noradrenaline
■—■, dopamine
▲—▲, adrenaline.

At the routine applied potential of 700 mV, only the response to dopamine is near saturation.
Table 3.1: Electrochemical response of some monoamines at 700mV (in nA/pmol).

<table>
<thead>
<tr>
<th></th>
<th>Response (nA/pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dopamine</td>
<td>0.14</td>
</tr>
<tr>
<td>noradrenaline</td>
<td>0.12</td>
</tr>
<tr>
<td>serotonin</td>
<td>0.15</td>
</tr>
<tr>
<td>DOPA</td>
<td>0.13</td>
</tr>
<tr>
<td>adrenaline</td>
<td>0.17</td>
</tr>
<tr>
<td>5-HTP</td>
<td>0.12</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.19</td>
</tr>
<tr>
<td>DOPEG</td>
<td>0.18</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0.18</td>
</tr>
<tr>
<td>MTA(^1)</td>
<td>0.0055</td>
</tr>
<tr>
<td>MOPEG(^3)</td>
<td>0.011</td>
</tr>
<tr>
<td>HVA(^2)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

100µl (30-80pmol) standard solutions were injected in the ion-pair reverse phase HPLC-ED system

\(^1\) 2200pmol

\(^2\) 800pmol

\(^3\) 650pmol.
3.1). At 700mV the response for MOPEG, HVA and MTA was very low, less than 0.02 nA/pmol. This means that a peak which by its retention time could be noradrenaline or MOPEG, in practical terms may be interpreted as originating either from noradrenaline or from massive amounts of MOPEG. Similarly, since DOPAC levels in chicken retina are at least 50% higher than DOPA levels (Parkinson and Rando, 1983b), and since the response of the ED to DOPAC is 50% higher than the response to DOPA, a DOPA/DOPAC peak is likely to be predominantly derived from DOPAC. This peak will be referred to as "DOPAC/dopa". Several authors have determined retinal levels of DOPAC and/or DOPA, using ion-pair reverse phase HPLC-ED (Parkinson et al., 1981; Barbaccia et al., 1982; Frucht et al., 1982; Cohen et al., 1983). However, only Proll et al. (1982) demonstrated the actual separation of these two compounds, using a mobile phase with 0.15% methanol and of slightly higher pH than I used (pH 2.75). So it is not clear whether this problem of co-migration of DOPAC and DOPA was encountered by others.

For cation-exchange HPLC-ED, a Biorad cation-exchange column (195-6002, Sydney) was used. The mobile phase consisted of 0.06 M sodium-phosphate buffer pH 5.2, and 5% (v/v) methanol. It was found that in retinas homogenized in 5% (v/v) perchloric acid, no dopamine could be detected, which was surprising, since various laboratories homogenize tissue in perchloric acid, for catecholamine analysis with HPLC-ED (see e.g. Parkinson et al., 1981; Nesselhut and Osborne, 1982; Hadjiconstantinou et al., 1983). Therefore retinas were homogenized in the mobile phase of the ion-pair reverse phase chromatographic system, as described above. After each injection of a retinal sample, the column was stripped with 15 ml mobile phase containing an additional 0.5 M NaCl. The flow rate was 1 ml/min, and the oxidative potential was 600 mV.
Standards (0.5-5.5 mM) were dissolved in 1 mM HCl (DOPEG, MOPEG, DOPAC, dopamine, 5-HIAA, and HVA) or in a solution of ascorbic acid (5 mM) and pargyline (25 mM) (noradrenaline, adrenaline, DOPA, 5-HTP, and MTA), and stored frozen at -20°C. A mixture of standards (diluted 10^-3) was prepared in mobile phase each day.

Ultra-pure H₂O (resistance > 18 Mohm) was obtained with a Whatman Milli-Q water purification system.

Exposure to light and dark.

After at least 3h exposure to the light during the normal day-night cycle, chickens were placed in the dark, or exposed to two 150 watt globes at 50 cm distance (100 cd/m²). Both groups had access to water, but were not supplied with food. Retinas of the light-exposed animals were removed in ambient light, and the retinas of dark-exposed animals were removed using a deep-red safety light. Since the emphasis was on a quick isolation, retinas were not routinely separated from pigment epithelium and choroid. However all TH activity, and most of the monoamines (76% of dopamine) were found to be associated with the neuronal retina (TABLE 3.2).

Dopamine uptake.

Retinas were isolated at room temperature, and immersed in physiological buffer, consisting of 143 mM Na⁺, 4.7 mM K⁺, 1 mM Ca²⁺, 1.2 mM Mg²⁺, 126 mM Cl⁻, 24.9 mM HCO₃⁻, and 10 mM glucose. The buffer was bubbled with carbogen (95% O₂, and 5% CO₂) for at least 1h prior to use. The pH was adjusted to 7.30-7.35 with 1 M HCL at 37°C. Unless specified otherwise, retinas were cut into 200 um squares, using a McIlwain tissue chopper, and washed 3 times with buffer. Uptake in such retinal pieces was similar to that measured in whole retinas.
Table 3.2: Distribution of some monoaminergic markers in chicken retina and supportive tissue.

<table>
<thead>
<tr>
<th></th>
<th>monoamine content (pmol/retina) + SEM (n)</th>
<th>TH activity pmol/min.retina + SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dopamin + SEM (n)</td>
<td>DOPAC/ dopa</td>
</tr>
<tr>
<td>retina</td>
<td>32 + 1 (5)</td>
<td>30 + 3 (5)</td>
</tr>
<tr>
<td>retina including</td>
<td>42 + 3 (3)</td>
<td>34 + 8 (3)</td>
</tr>
<tr>
<td>pigment epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and choroid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Whole retinas (about 6 mg protein), or retinal pieces (about 0.4 mg protein) were preincubated in 900 μl physiological buffer for 5 min at either 37°C in a metabolic shaker water bath, or at 0°C in ice. The incubation was started by adding 100 μl [8-^3^H]-dopamine (0.3 μCi, from New England Nuclear, 24.7 Ci/mmol) together with ascorbic acid, EDTA, and pargyline, resulting in final concentrations of 100 nM dopamine, 500 μM ascorbic acid, 50 μM EDTA, and 25 μM pargyline. Incubations were stopped by adding 4 ml of ice-cold buffer, and placing the test tubes in ice. The tissue samples were washed three times over Whatman GF-A, or Schleicher-Schull GF-101 filters, with 4 ml volumes of ice-cold buffer. The filters were transferred to scintillation vials, and the tissue was osmotically shocked with 1 ml H₂O. 8 ml of the scintillation fluid (see chapter 2) was added, and after at least 24 h, the samples were counted for radioactivity.

Radioactivity retained on the filters was at least 4 times greater at 37°C then at 0°C. This amount at 0°C, representing nonspecific dopamine-accumulation, was always subtracted. The accumulation of ^3H^-dopamine was not saturated after 120 min incubation, and was linear with time up to 20 min incubation, for 0.1-0.5 mg protein per sample.

Dopamine release.

Retinas or retinal pieces were loaded with ^3H^-dopamine as described in "uptake", with the exception that 0.5 μCi of dopamine per incubation was used. The uptake was stopped after 30 min incubation at 37°C by diluting the sample with buffer. Tissues were washed three times immediately with 4 ml buffer over Whatman GF-A filters at 37°C. The filters were transferred to perfusion chambers. The total volume of the chambers and the tubing was 2.3 ml. Retinal tissues were perfused with
buffer at a flow rate of 2.3 ml/min at 37°C. 2.3 ml samples were collected, mixed with 12 ml scintillation fluid, and counted for radioactivity.

Release was evoked by perfusion with buffer containing 60 mM K⁺, substituted for an equivalent amount of Na⁺. For calcium-free conditions, the 1 mM Ca²⁺ was replaced by 1 mM EGTA.

Release was quantified by comparing radioactivity released during perfusion with high-potassium buffer, with radioactivity released during similar time slots before and after high-potassium buffer.

Results.

Figure 3.3 shows a chromatogram obtained by ion-pair reverse phase HPLC-ED of chicken retinal homogenate. By means of a comparison of retention times, and addition of the range of standards to homogenates, peaks corresponding to dopamine, 5-HIAA, and serotonin were clearly identifiable. A minor peak could often be detected, and was identified as originating from HVA.

A peak originating from DOPAC/dopa (see Methods) was detected as a shoulder on a large peak of unknown origin, which makes quantification of retinal DOPAC/dopa levels less reliable than quantification of the other compounds. There was no evidence for the presence of endogenous noradrenaline, or of adrenaline in chicken retinal homogenate. However, as these catecholamines appeared in a fairly crowded region of the chromatogram, this analysis system is not decisive with respect to the catecholamine composition of chicken retina.

To further investigate the levels of noradrenaline and adrenaline from chicken retina, cation-exchange HPLC was used to separate dopamine, noradrenaline, and adrenaline from homogenates of chicken retina.
Figure 3.3: Ion-pair reverse phase HPLC of chicken retina.

100 ul of a retinal homogenate (1 retina/1000 ul) was injected. Lower tracing 100 ul (15 pmol) dopamine solution.
NA, noradrenaline
A, adrenaline
DOPAC, 3,4-dihydroxyphenylacetic acid
DA, dopamine
5HIAA, 5-hydroxyindoleacetic acid
5HT, serotonin

DOPAC/dopa (see Methods), dopamine, 5-HIAA, and serotonin could always be detected in retinal homogenates. Noradrenaline and adrenaline peaks would run in a crowded region of the chromatogram.
Figure 3.4: The catecholamine composition of chicken retina.

Retina, 100 ul retinal (1 retina/500 ul) sample
Standards, 100 ul standard mix (20 pmol adrenaline, 20 pmol noradrenaline and 15 pmol dopamine)
Spiked retina, 100ul retinal sample together with the standard mix injected.

The lower chromatogram shows that with cation-exchange HPLC-ED adrenaline, noradrenaline and dopamine are well separated from each other. In the retinal sample (chromatogram in the middle) a peak with the same retention time as dopamine is seen. No peaks with retention times similar to adrenaline and noradrenaline can be seen. The region of the chromatogram where these catecholamines would appear, is clear from peaks of unknown origin. There is no indication for the presence of peaks from catecholamine metabolites.
spiked retina

retina

standards

\( \text{spiked retina} \)

\( \text{retina} \)

\( \text{standards} \)

\( A \quad NA \quad DA \)

\( 0 \quad 10 \quad R_t (\text{min}) \)
Figure 3.4 shows that, by cation-exchange HPLC, adrenaline, noradrenaline, and dopamine were clearly separated from each other, and from the void volume. Analysis of chicken retinal homogenates in this chromatographic system did not reveal noradrenaline, or adrenaline. Assuming linearity of the ED response for the various catecholamines, and with a detection limit of 0.05nA, levels of noradrenaline and adrenaline would be less than 0.02 nmol/g wet weight in retina of chicken, compared to 0.48 nmol/g wet weight for dopamine.

After 24h of exposure to light, retinal dopamine levels were 80% higher than the levels in animals, kept in the dark for the same period (TABLE 3.3). In addition, DOPAC/dopa levels were elevated 5 times, and MTA levels became detectable, whereas the retinal MTA levels in dark-exposed animals always were below detection limit. These data point towards a higher turnover of dopamine in retinas of animals exposed to light for 24h.

The onset of this apparent light activation of the dopamine turnover was studied by exposing animals to light for 15min, after they had been kept in the dark for 24h (TABLE 3.3). 15min exposure to light was sufficient to increase retinal dopamine and DOPAC/dopa levels to the same extent as observed in animals which were kept in the light for 24h. MTA levels, however, appeared only in some retinal homogenates to have been increased beyond the detection limit.

TH activity was found in homogenates of chicken retina and could be inhibited by 3-iodotyrosine (Chapter 2). The apparent kinetic properties of TH of chicken retina in vitro were $K_{m, tyr}$ 57 ± 1 μM (n=6), $K_{m, DMPH_4}$ 200 ± 20 μM (n=10), and $V_{max}$ 6 ± 1 pmol/min·mg protein (n=16).

Exposure to light for 24h had no effect on the TH capacity of chicken retina, as measured with saturating cofactor conditions (TABLE
Table 3.3: Levels of dopamine and metabolites in chick retina after exposure to light.

catecholamine levels pmol/retina + SEM (n)

<table>
<thead>
<tr>
<th></th>
<th>24hrs dark</th>
<th>24hrs light</th>
<th>24hrs dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15min light</td>
<td></td>
</tr>
<tr>
<td>dopamine</td>
<td>16 ± 2 (7)</td>
<td>30 ± 3 (7)**</td>
<td>30 ± 3 (7)*</td>
</tr>
<tr>
<td>DOPAC/dopa</td>
<td>23 ± 2 (5)</td>
<td>120 ± 20 (3)**</td>
<td>118 ± 5 (3)**</td>
</tr>
<tr>
<td>MTA*</td>
<td>n.d. (&lt;240)</td>
<td>present (≥600)</td>
<td>sometimes detected</td>
</tr>
<tr>
<td>HVA</td>
<td>200 ± 40 (3)</td>
<td>210 ± 30 (3)</td>
<td>200 ± 40 (3)</td>
</tr>
</tbody>
</table>

statistical significance, compared to dark values (two-tailed Student's t-test)

* 2p < 1%
** 2p < 0.5%
*** 2p << 0.1%

+ in our system a just detectable peak corresponds with about 600pmol/retina; detection limit 240pmol/retina.
Table 3.4: Activation of retinal TH by ambient light.

<table>
<thead>
<tr>
<th></th>
<th>TH activity in vitro (pmol/min.mg protein) ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at saturating (DMPH₄)</td>
</tr>
<tr>
<td>24 hours dark</td>
<td>3.51 ± 0.08 (5)</td>
</tr>
<tr>
<td>24 hours light</td>
<td>3.6 ± 0.1 (7)</td>
</tr>
<tr>
<td>24 hours dark, 15 min. light</td>
<td></td>
</tr>
</tbody>
</table>

* saturating (DMPH₄): 2000µM; subsaturating (DMPH₄): 200µM

Km, DMPH₄, 200µM (during the day)

Statistical significance, compared to dark values (two-tailed Student’s t-test): * 2p < 0.5%
** 2p << 0.1%.
Figure 3.5: Hight-potassium-evoked release of exogenous dopamine from chicken retina.

Isolated retinas were preloaded with $^3$H-dopamine for 30 min. and perfused with physiological buffer (open circles). Release was evoked by perfusion with buffer containing 50mM K$^+$ during the time, indicated with a bar (triangles). For Ca$^{++}$-free conditions (right hand graph) the 1mM CaCl$_2$ was replaced by 1mM EGTA. Bars represent SEM (n=4).

The spontaneous efflux of $^3$H-dopamine reached a plateau after 16min superfusion with physiological buffer, but continued longer under calcium-free conditions. Superfusion with high-potassium increased the efflux only when calcium was present in the physiological buffer.
Figure 3.6: Dopamine uptake of chicken retina *in vitro*.

Isolated retinas were incubated in the presence of $^3$H-dopamine for 30 min as described in the *Methods*. Bars represent SEM (n=3).
3H-dopamine retention (cpm/mg protein. 30min)

- Na⁺
  37°C

+ Na⁺
  0°C

+ Na⁺
  37°C
3.4), neither was the activity, measured with sub-saturating tyrosine concentrations affected by these exposures to light (data not shown). Retinal TH activity measured in vitro with sub-saturating concentrations of the pterin cofactor was increased by almost 40% compared to activities measured in vitro in animals kept in the dark for the same period. This demonstrates that the affinity of retinal TH for the cofactor was increased after exposure to light. This apparent increase in affinity could be brought about by exposure to light for 15min after animals had been dark-exposed for 24h.

High-potassium evoked release of exogenous $^3$H-dopamine from isolated chicken retina (FIGURE 3.5). During perfusion with high-potassium buffer, the efflux of radioactivity was elevated by more than 70%, compared to the spontaneous efflux. In the absence of calcium, there was only a 10% increase, so that the major portion of high-potassium-evoked dopamine release appeared to be calcium-dependent.

The histogram in figure 3.6 demonstrates the temperature and sodium dependency of the dopamine-accumulating system found in isolated retina of chicken. These results suggest that accumulation of dopamine is active and energy-dependent, and not just due to diffusion. The dopamine accumulation showed a high affinity for dopamine $K_m$ 590 ± 80 nM (n=4), and a capacity of $V_{max}$ 140 ± 20 fmol/min.mg protein (n=4).

**Discussion.**

The only catecholamine detected in chicken retina by the highly sensitive HPLC-ED method was dopamine. If noradrenaline or adrenaline are present in homogenates of chicken retina, their levels must be less than 4% of dopamine levels. These biochemical studies substantiate indications, based on observations made with spectrofluorimetric and
radio-enzymatic techniques, that dopamine is the major catecholamine present in retina of chicken (Schwarcz and Coyle, 1976; Da Prada, 1977). The detection limit for the radio-enzymatic assay used by Schwarcz and Coyle was 0.10 nmol/g wet weight. The lack of demonstrable DBH activity in homogenates of chicken retina (Schwarcz and Coyle, 1976) is a further indication that dopamine is the end product in catecholamine biosynthesis. Morgan and Chubb (personal communications) have not been able to detect DBH-like or PNMT-like immunoreactivity in chicken retina.

The dopamine levels found in chicken retina, 0.28-0.51 nmol/g wet weight, are slightly lower than those found radioenzymatically, 0.62-0.84 nmol/g wet weight (Schwarcz and Coyle, 1976; Da Prada, 1977), but very much in agreement with results from Parkinson and Rando (1983b who found 0.38-0.46 nmole/g wet weight using HPLC-ED. Dopamine levels in chicken retina are in the range as described in other species (see Chapter 1.4, TABLE 1.4).

TH activity has been demonstrated indirectly in chicken retina in vivo, by blocking DDC activity and determining accumulated DOPA (Parkinson et al., 1981). The same method has been employed for an estimation of TH activity in vitro (Parkinson and Rando, 1983b). The resulting retinal TH activity is considerably lower than I found using a direct assay, (0.70-0.99 pmol DOPA/min.mg protein vs. $V_{max}$ 6 pmol $[^3H]$-$\text{H}_2\text{O}$/min.mg protein). It is likely that the accumulation of DOPA inhibits TH (Nagatsu et al., 1964; Karobath, 1971; Waggoner et al., 1980) and that the indirect assay therefore does not reflect full TH capacity. My results are of the same order of magnitude as those of Schwarcz and Coyle (1976), who found a TH activity of 2.3 pmol/min.mg protein in chick retina in vitro, using a radioenzymatic assay. The fact that they assayed TH under sub-saturating conditions, probably explains the lower activities that they found.
Light-evoked dopamine release has proven to be difficult to demonstrate reliably in any species (see Chapter 1.4). These results confirm the observation by Tapia and Arias (1982) that exogenous dopamine can be released from chicken retina by high-potassium, in a calcium-dependent fashion. This points to the existence of a vesicular release mechanism for dopamine, as has been described for other systems, where dopamine has been accepted as neurotransmitter (see Cooper and Meyer, 1984). Further support for vesicular release mechanisms, comes from the demonstration of calcium-dependent release of exogenous dopamine evoked by an electrical stimulation (Dubocovich, 1984b).

The reuptake of dopamine seems to be an important mechanism for inactivating the compound in various nervous tissues (see Horn, 1979). Although the existence of such a mechanism is not crucial, the observation of a dopamine carrier in the chicken retina with characteristics similar to the carrier found in other parts of the nervous system, supports the assumption that dopamine is a neurotransmitter in chicken retina. The $K_m$ of 590 ± 80 nM, is similar to those as reported for the retinas of goldfish: 260 nM (Sarthy and Lam, 1979), carp: 370 nM (Kato et al., 1981a), cow: 290 nM (Reading, 1983), and rabbit: 560 nM (Ehinger and Floren, 1978), but like all other observations, lower than that reported for rat retina: 1900 nM (Pycock and Smith, 1983). In addition to inactivation by uptake, MAO has been demonstrated in chicken retina in vitro (Suzuki et al., 1977). The presence of the dopamine metabolites DOPAC, HVA, and, under certain conditions, MTA, as described here and previously by Parkinson and Rando (1983b), further indicates that dopamine is metabolized by MAO and COMT as in other nervous tissues (see Westerink, 1979).
With respect to the criterion that a neurotransmitter should have a physiological action, dopamine receptors, pharmacologically characterized as the D₁-type mediate such an effect, have been described in chicken retina by ligand binding techniques (Ventura et al., 1984; De Carvalho and De Mello, 1985). In addition, dopamine-sensitive adenylate cyclase, thought to represent the cellular response in dopamine transmission, has been demonstrated in retina of chicken in vitro (Schwarcz and Coyle, 1976; De Mello, 1978; De Mello and De Mello, 1983). There is at present no indication as to whether and how, the increased cAMP levels elicited by dopamine change the properties of the post-synaptic neurons, and in particular, changes their electrophysiological responses. The demonstrated effects of dopamine on second messenger levels in the chicken retina do indicate, however, that dopamine has a potential function of this kind, which now needs to be defined.

The different levels of dopamine in retinas from animals which had been exposed to different light levels for 24h, indicates a change in dopamine turnover depending upon environmental light. In parallel with the variation in dopamine levels, a variation in levels of catabolites of dopamine was observed, pointing towards an increased turnover of retinal dopamine in chickens exposed to light as compared to those kept in the dark. Similar differences in retinal dopamine and metabolite levels have been described for chickens kept in the light for 48h, compared to animals kept in the dark for the same period (Parkinson and Rando, 1983b). In that study dopamine turnover was found to be more than 3 times faster in the light than in the dark, with half-lives for dopamine of 28min and 72min respectively. The observed increases in MTA levels in these studies and in those of Parkinson and Rando (1983b) are
of special significance, because they suggest that increased dopamine turnover results in an increased extracellular O-methylation of dopamine, which may indicate an increased release of dopamine during exposure to light (Westerink, 1985).

The total capacity of retinal TH did not appear to be affected by exposure to light for up to 24h. However, activities measured under sub-saturating conditions for the pterin substrate, did show considerable differences of TH activity in vitro, for the different experimental groups. The results show that retinal TH from chickens exposed to light for 24h, has an increased affinity for the cofactor compared to TH from chickens kept in the dark for the same period. This kinetic change appeared to be accomplished within 15min of exposure to light.

Parkinson and Rando (1983b) have concluded that the capacity, rather than the affinity of retinal TH was increased after chickens had been exposed to light for 48 hrs. This conclusion was based on measurements of the rate of DOPA accumulation after inhibition of DDC in vitro. The apparent activity of TH is likely to be affected by the accumulation of DOPA itself (Nagatsu et al. 1964, Karobath, 1971 Waggoner et al., 1981), and thus, this accumulation is a poor marker for the capacity of TH. In the rat, in contrast to the results of Parkinson and Rando but in agreement with my results on 24h light exposures, an increase of TH's affinity for the cofactor, as assessed by a radioenzymatic assay for TH under sub-saturating conditions, has been observed up to 96h after exposure to light, but was maximal at 15min after exposure to light (Iuvone et al., 1978b). An increase of the capacity of retinal TH could only be observed after the rats had been exposed to light for at least 96h.
Of course, the increase of retinal TH capacity could indeed be accomplished faster in the chicken than in the rat. However, there is no direct evidence to substantiate such a species difference. It seems, likely that in chicken retina, like rat retina (see Iuvone, 1984b), and the dopaminergic system of other parts of the nervous system (see Almgren et al., eds., 1975), short-term activation of dopamine turnover is based on allosteric regulation of the kinetic properties of TH.

Dopamine has been shown to be present in the chicken retina, together with the enzyme specific for its synthesis. The existence of a vesicular release mechanism for dopamine is supported by the demonstrated high-potassium-evoked calcium-dependent release of exogenous dopamine. Chicken retina possess a high-affinity dopamine uptake system and has the capacity to metabolize dopamine, as indicated by the presence of metabolites. Dopamine receptors and dopamine-sensitive adenylate cyclase have been characterized by others in chicken retina. The characteristics of these biochemical aspects of dopamine in chicken retina parallel the characteristics as found in other parts of the nervous system.

Together with observations made at the cell level (see chapter 4), these results strongly indicate that dopamine is a neurotransmitter in chicken retina. Although decisive evidence is lacking of a demonstrated physiological role for dopamine in chicken retina, light has been found to affect dopamine levels, dopamine turnover, and the kinetic state of TH, which strongly supports an involvement of dopamine in the functioning of the chicken retina. Chicken is now along with the rat, the only vertebrate species where most of the available biochemical tests examining a transmitter role for dopamine in the retina, have been performed.
DOPAMINERGIC NEURONS IN CHICKEN RETINA.

Introduction.

Dopamine-containing neurons have been described in the retinas of several species using Falck's histofluorescence technique, sometimes in combination with microspectrofluorometry. On the basis of the localization of the cell bodies, several types of dopaminergic neurons have been described (see Ehinger, 1983a, Chapter 1.4). Most often, the dopaminergic neurons are located amongst the amacrine cells on the border of the IPL. This so-called junctional type of dopaminergic amacrine cell sends processes into the IPL. In all species investigated, a dense plexus of processes is found in sublamina 1 in the IPL bordering the INL. Depending upon species, there are often additional layers where processes are found. Avian retina has been reported to contain only junctional types of dopaminergic amacrine cells, with a major plexus of processes in sublamina 1, and much weaker processes in sublaminas 3 and 5 of the IPL (Ehinger, 1976; Araki et al., 1983; Kato et al., 1984).

In this chapter catecholamine-accumulating neurons in chicken retina will be compared with dopamine-containing and TH-immunoreactive neurons. The morphology and histology of these dopaminergic neurons will be described and discussed relative to previous reports from other laboratories. Data dealing with several aspects of the distribution of dopaminergic neurons in chicken retina, i.e., density, horizontal variation in density, and regularity, will be presented and discussed.
Methods.

The chickens were reared as described in Chapter 2.

Histofluorescence.

Chickens under ether anaesthesia were injected intravitreally using a hand-held Hamilton 50 μl syringe with 250 pmol noradrenaline (in 10 μl 0.9 % saline with 1 mg/ml ascorbate), 4h prior to enucleation. After removal of the iris and lens, the dissected eyes were immersed in FaGlu (Nakamura, 1979), consisting of 4 % (w/v) paraformaldehyde, 0.5 % (v/v) glutaraldehyde, 35 % (w/v) glucose, in 0.1 M sodium-phosphate buffer pH 7.4, for at least 24h before sectioning or the preparation of whole mounts. For whole mount preparations the sclera was cut away and the choroid was removed. Retinas including pigment epithelium were spread on microscope slide with the vitreous-side (ganglion cell layer) facing up. For transverse sections, a longitudinal strip retina-sclera was glued onto the cutting table of a Lancer series-1000 vibratome with Supaglue. The tissue was immersed in FaGlu, and 10-20 μm transverse sections were cut. Sections and whole mount preparations were dried at ambient temperature, sealed with Depex (Histo-Labs), and examined under a fluorescence microscope (Leitz, fitted with Plomopak D-filter system). Photomicrographs were taken on Kodak Tri-X Pan film. In some cases, dried whole mount preparations were dehydrated in 100 % ethanol (lmin), and 2 times lmin xylene, prior to sealing. This treatment preserved the initial fluorescence intensity for several days.

Immunohistochemistry.

TH-immunohistochemistry was done by Dr. Tom Millar, who provided the photomicrograph presented in figure 4.1, using an indirect
immunofluorescence method (see Millar et al., in preparation). Rabbit 
anti-TH, diluted 1:2000, was supplied by Drs. Powell and Smith, Oxford 
University, U.K. Its specificity had been reported previously (Van den 
Pol et al., 1984).

Nearest neighbour analysis.

A measure of regularity of a distribution is the width of the 
frequency distribution of the nearest neighbour distances. Wassle and 
Riemann (1978) found that the frequency distribution of nearest 
neighbour distances of several retinal cell types could be described by 
a normal distribution (equation 4.1), and they proposed to express 
regularity of neuron distribution in the retina, by means of the ratio 
of the mean nearest neighbour distances to the standard deviation (which 
has been named the regularity index). The distribution of retinal cell 
types involved in transducing the mosaic of the visual world, such as 
cones in cat and monkey retina, were found to be highly regular, with 
regularity indices of 6 and 11 respectively (Wassle and Riemann, 1978).

Often the frequency distribution of nearest neighbour distances 
cannot be described by a normal distribution (Brecha et al., 1984; 
Mariani et al., 1984). Alternative statistical functions have been 
developed to describe the mosaic in exact mathematical terms (Mariani et 
al., 1982). This surpasses, however, the practical purpose of nearest 
neighbour analysis. If the frequency distribution is symmetrical around 
a mean, the width of the distribution will reflect the regularity of the 
mosaic.

Montage pictures, covering an area of around 1.5 mm², were analyzed 
for regularity of dopaminergic cell distribution. The locations of the 
dopaminergic cell bodies were entered into an Apple IIe+ computer, using
a graphics-pad. Data from several montages were pooled. The computer was programmed to calculate the cell density, the mean nearest neighbour distance, and the appropriate probability functions for a normal, and a random distribution (equation 4.2). The fit of the actual data with a normal distribution was tested for statistical significance (chi-squared test).

\[ p(r) = k e^{-\frac{1}{2} \left( \frac{r-u}{s} \right)^2} \quad \text{(equation 4.1)} \]

\[ r, \quad \text{nearest neighbour distance,} \]
\[ u, \quad \text{mean nearest neighbour distance,} \]
\[ s, \quad \text{standard deviation.} \]

\[ p(r) = 2\pi d e^{-\pi d r^2} \quad \text{(equation 4.2)} \]

\[ r, \quad \text{nearest neighbour distance,} \]
\[ d, \quad \text{density in cells/area unit.} \]

**Results.**

Figure 4.1 shows micrographs obtained by the three methods employed to visualize dopaminergic neurons in chicken retina. Since noradrenaline could not be detected in homogenates of chicken retina by HPLC-ED (see chapter 3), it is assumed that the greenish fluorescence originates from dopamine rather than from noradrenaline. Although the endogenous dopamine content is sufficient to produce detectable fluorescence in Falck's formaldehyde treatment, the intensity of fluorescence is not high enough for good micrographs (FIGURE 4.1a). In addition, the fluorescence intensity is not sufficient to reveal details of the processes of the dopaminergic cells. Only some processes in
Figure 4.1: Dopaminergic neurons in chicken retina as seen in transverse section.

The borders of the inner nuclear layer (INL) and inner plexiform layer (IPL) are outlined in photo B.

A, Histofluorescence for endogenous monoamines. A large, greenish fluorescent (originating from catecholamines) cell body is seen in the INL at the border with the IPL.

B, Histofluorescence for accumulated noradrenaline. A very brightly fluorescent cell body is seen in the INL at the border with the IPL. In addition varicose processes are seen in the IPL, especially in sublamina 1, bordering the INL. Small noradrenaline-accumulating neurons (arrow) are seen in the middle of the INL.

C, TH-immunohistochemistry. An anti-TH-positive neuron is seen in the INL at the border with the IPL. Processes are seen in the IPL only.

Bar, in photo C, represents 10 μm.

All three methods seem to stain the same population of neurons in the chicken retina.
sublamina 1 of the IPL were visible after monoamine histofluorescence staining of the untreated retina. To enhance fluorescence intensity, neurons were preloaded with noradrenaline by intravitreal administration of this catecholamine prior to enucleation. Noradrenaline has been reported to form the most intense fluorescence of a range of catecholamines (Negishi et al., 1982e). After preloading with noradrenaline, the same cell types seen in figure 4.1a were brightly fluorescent (FIGURE 4.1b), and details of processes became visible in sublaminas 1, 3 and 5 of the IPL. In addition, a second population of neurons became detectable, with the cell bodies located deeper in the INL. Since the cell bodies of these neurons were clearly smaller than the dopaminergic neurons, this additional cell type did not interfere with the specificity of the detection method. Figure 4.1c shows a transverse section of the retina after the immunohistochemical localization of TH. It is clear that histofluorescence of endogenous dopamine, histofluorescence after catecholamine accumulation, and immunohistochemistry against TH, stain neurons which according to their morphology are similar. It is therefore concluded that all three methods visualize dopaminergic amacrine cells in chicken retina.

Histofluorescence after noradrenaline accumulation has been employed to obtain all subsequent results. The micrographs in figure 4.2 show various features of the dopaminergic amacrine cells in transverse section. Enough background fluorescence is present to distinguish the retinal layers. Cell bodies were found in the INL at the border with the IPL. The cell bodies appeared relatively large and slightly oval-shaped, with a diameter of around 10 \( \mu \text{m} \) which is of comparable size as observed in other species (see Chapter 1.4). One very bright process left the cell body, and connected the soma with the
Figure 4.2: Fluorescence micrographs of catecholamine-accumulating neurons in transverse sections of chicken retina.

ONL, outer nuclear layer
OPL, outer plexiform layer
INL, inner nuclear layer
IPL, inner plexiform layer
GL, ganglion cell layer.

The relatively large cell bodies of the dopaminergic amacrine cells are seen in the INL, at the border with the INL (Photos B and C). A dense network of varicose processes is seen in sublamina 1, but some processes can be seen in the middle (sublamina 3), and bordering the ganglion cell layer (sublamina 5) in the IPL (arrows, photo C). Dopaminergic cell bodies are not typically found so near to each other as suggested by photo A. The bars represent 50 μm in photo A, and 10 μm in photos B and C.
Figure 4.3: Fluorescence micrographs of catecholamine-accumulating neurons in flat-mounted chicken retina.

The large cell bodies of dopaminergic amacrine cells (open arrows) are easily distinguished from the smaller cell bodies of an additional population of catecholamine-accumulating neurons (arrow) (photo A). In the far peripheral retina a dense network of varicose processes is seen (photo A). Photos B and C illustrate the difference in density of dopaminergic cell bodies in the peripheral and central retina respectively. The bars represent 50 μm.
plexus of processes in sublamina 1 of the IPL. More than one process coming out of the cell body was never observed. Often fine, non-varicose processes crossed the whole IPL, and connected the plexuses of processes in sublaminas 1, 3 and 5. In sublamina 1, a very dense plexus of processes was found, whereas the plexuses in sublaminas 3 and 5 of the INL were less dense. It was not possible in these experiments to trace the processes of an individual cell horizontally within the IPL. The processes consisted of very brightly fluorescent varicosities, with estimated diameters of up to 1μm, joined by much finer diameter intervaricose segments.

In retinal flat mounts, both cell bodies and processes were visible (FIGURE 4.3). After preloading with noradrenaline, an additional population of neurons became detectable, which did not contain sufficient endogenous catecholamines for histofluorescence, and which was not TH-immunoreactive, as mentioned before. Because of their smaller size, this additional population of neurons could be easily distinguished from the dopaminergic neurons (FIGURE 4.3a). The density of dopaminergic cell bodies was about twice as high in the central part of the retina as in peripheral areas (compare FIGURES 4.3b and 4.3c). Dopaminergic processes were more easily visible in peripheral areas of the retina. Since in transverse sections no significant centro-peripheral variation in the densities of processes was observed, this is probably due to differences in thickness of the retina. The central retina is more than twice as thick as the periphery, and processes in the central part may be more difficult to see with the the higher intensity of background fluorescence.

Figure 4.4 summarizes dopaminergic cell density results obtained from 30 pictures from 6 different retinas. The highest density
Figure 4.4: The density of dopaminergic amacrine cells in chicken retina.

The figure is a schematic representation of a flat-mounted retina, the position of the pecten is indicated. The density of dopaminergic amacrine cells does not vary very much throughout the retina, peripheral densities are slightly lower than those in the central area, and the dorso-temporal region has the lowest density of dopaminergic neurons. The enlarged area shows the exact location of the area with highest density.
CELLS/MM² (±S.E.M.)

NASAL

cells /1/4 mm²

22 ± 2

26 ± 2

30 ± 1

25 ± 1

21 ± 1
encountered in a frame measuring 0.25 mm$^2$, was observed in the central retina and amounted to 48 cells/mm$^2$. Based on almost 2000 cells counted, the average density of dopaminergic cells was 30 ± 1 cells/mm$^2$ in the central retina. The area with the highest density of dopaminergic neurons was slightly nasal to the optic disc, as can be seen in the inset matrix shown in figure 4.4. This region matches the highest densities of cones (Mayer and May, 1973) and ganglion cells (Ehrlich, 1981), and corresponds to a lateral position in the visual field. The lowest density of dopaminergic neurons was found in the temporo-dorsal region of the peripheral retina, corresponding to a ventral-frontal position in the visual field.

The frequency distributions of the nearest neighbour distances of dopaminergic neurons in different regions of the retina are shown in figure 4.5. The actual frequency distributions can clearly not be described by the function describing the frequency distribution of nearest neighbour distances of a random arrangement of the cells. Although the histograms are bell-shaped, they are significantly different from a normal distribution (TABLE 4.1). The histograms are not multimodal, which supports the assumption that there is only one population of dopaminergic neurons in the chicken retina. The widths of the three histograms shown do not differ, as expressed by the regularity indices (TABLE 4.1). These results indicate a similar degree of regularity of the distribution of dopaminergic neurons throughout the chicken retina. The mean nearest neighbour distances are, as expected, inversely related to the observed densities of dopaminergic neurons in different areas of the retina.
Figure 4.5: The frequency distribution of nearest neighbour distances of dopaminergic amacrine cells in chicken retina.

Histograms, actual distribution
Left-hand curves, probability function for random distribution
Right-hand curves, probability function for normal distribution
(See also Table 4.1).

None of the actual distributions can be described by a probability function for a random distribution.
Table 4.1: Nearest neighbour analysis of the dopaminergic amacrine cells in chicken retina.

<table>
<thead>
<tr>
<th></th>
<th>nasal</th>
<th>central</th>
<th>temporal</th>
</tr>
</thead>
<tbody>
<tr>
<td>density, cells/mm² + SEM</td>
<td>26 ± 2</td>
<td>30 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>mean nearest neighbour distance, µm + SEM</td>
<td>134 ± 3</td>
<td>119 ± 1</td>
<td>142 ± 3</td>
</tr>
<tr>
<td>regularity index</td>
<td>2.7</td>
<td>2.8</td>
<td>2.9</td>
</tr>
<tr>
<td>statistical significance for non-fit with a normal distribution</td>
<td>2p &lt; 0.1%</td>
<td>2p &lt; 0.1%</td>
<td>2p &lt; 5%</td>
</tr>
<tr>
<td>total number of cells counted*</td>
<td>1539</td>
<td>1954</td>
<td>1371</td>
</tr>
</tbody>
</table>

* in 12 photomontages of each area.
**Discussion.**

Fluorescence based on endogenous dopamine content is sufficient to study dopaminergic neurons under the microscope, but for ease of analysis of micrographs it is more convenient to work with preparations where the neurons have been preloaded with noradrenaline. After preloading, an additional population of neurons, deeper in the INL, was seen. According to their soma size and shape, and their position in the INL, this additional population of cells is probably composed of the indoleamine-accumulating neurons. Indoleamine-accumulating neurons have been described in the retinas of several species (see Ehinger and Floren, 1980), including chicken retina (Hauschild and Laties, 1973; Osborne, 1982; Kato et al., 1984). They preferentially accumulate indoleamines, but they will also accumulate catecholamines. After intravitreal coinjection of catecholamine and indoleamine in chicken, Kato et al. (1984) recently described, apart from dopaminergic amacrine cells and indoleamine-accumulating neurons, a third population of neurons, which specifically accumulated catecholamines. These neurons had small soma sizes, and were located between dopaminergic and indoleamine-accumulating neurons. I saw no indication of populations of additional labelled neurons, after noradrenaline preloading.

In chicken retina I only detected one type of dopaminergic neuron, with its cell body located in the INL at the border with the IPL. This is consistent with previous histological studies of avian retina which show only the junctional type of dopaminergic neurons (Ehinger, 1976; Araki et al., 1984; Kato et al., 1984). I paid special attention to looking for indications of dopaminergic processes in OPL, but none were detected. Nor was any seen after TH-immunohistochemistry (Millar and Morgan, personal communication). On the basis of the localization of preparations, which had a dense retic field in areas of high dopaminergic...
cell bodies amongst the amacrine cells, processes in IPL, and lack of processes in OPL, these results confirm the classification of the dopaminergic neuron in chicken retina as a dopaminergic amacrine cell (Ehinger, 1976).

Dopaminergic amacrine cells in chicken retina appear to have an overall density of 25 cells/mm². Table 4.2 summarizes the data obtained in other species. Using double staining techniques on flat mounts, it has been calculated that only 0.1-1 % of total amacrine cells are dopaminergic, in fish (Negishi et al., 1981; Negishi et al., 1981; Hayashi, 1980), rabbit (Negishi et al., 1984), and pigeon (Karten, personal communication). In chicken retina, the density of cholinergic amacrine cells is 2100 - 7200 cells/mm² of cholinergic displaced amacrine cells, 1700 - 7400 cells/mm² (Ishimoto et al., submitted for publication), of somatostatin-immunoreactive cells, 300 - 1650 cells/mm² (Ishimoto et al., 1986), of neurotensin-immunoreactive cells, 200 - 1650 cells/mm², and of leu-enkephalin-immunoreactive cells, 350 - 2000 cells/mm² (Ishimoto and Morgan, personal communication). Although the relative density of the dopaminergic amacrine cells in chicken was not determined, these data show a low proportion of dopaminergic amacrine cells compared to several classes of other amacrine cells.

Despite the low frequency of the neurons, the dopaminergic neurons support a very dense network of processes in chicken retina, especially in sublamina 1 of the IPL. This aspect has been quantified in only a few species. A conservative estimate is that any region in rabbit retina is covered by the processes of at least 3 dopaminergic neurons (Brecha et al., 1984). In cat retina, dopaminergic amacrine cells have been identified as the A18 amacrine cell detected in Golgi stained preparations, which has a dendritic field in excess of 500 μm diameter.
Table 4.2: The densities of dopaminergic neurons in vertebrate retina in cell bodies/mm².

<table>
<thead>
<tr>
<th>species</th>
<th>density (range)</th>
<th>method**</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>lamprey</td>
<td>60 (15 - 131)</td>
<td>+</td>
<td>Negishi et al., 1982d</td>
</tr>
<tr>
<td>goldfish</td>
<td>344 (295 - 440)</td>
<td>+</td>
<td>Negishi, 1981</td>
</tr>
<tr>
<td>carp</td>
<td>131 (99 - 198)</td>
<td>+</td>
<td>Negishi et al., 1980b</td>
</tr>
<tr>
<td></td>
<td>54 (43 - 69)</td>
<td>0</td>
<td>Negishi et al., 1981c</td>
</tr>
<tr>
<td></td>
<td>34 (20 - 86)</td>
<td>0</td>
<td>Negishi et al., 1983c</td>
</tr>
<tr>
<td></td>
<td>106 (75 - 182)</td>
<td>+</td>
<td>Negishi et al., 1981e</td>
</tr>
<tr>
<td></td>
<td>51 (29 - 92)</td>
<td>+</td>
<td>Negishi et al., 1980b</td>
</tr>
<tr>
<td>toad</td>
<td>76 (72 - 79)</td>
<td>0</td>
<td>Makino-Tasaka et al., 1985</td>
</tr>
<tr>
<td></td>
<td>45 (24 - 99)</td>
<td>0</td>
<td>Karten (pers. comm.)</td>
</tr>
<tr>
<td>pigeon</td>
<td>50 (43 - 69)</td>
<td>0</td>
<td>Versaux-Boiteri, 1984</td>
</tr>
<tr>
<td>mouse</td>
<td>31 (24 - 69)</td>
<td>anti-TH</td>
<td>Ehinger and Aberg, 1981</td>
</tr>
<tr>
<td>guinea-pig</td>
<td>36 (24 - 69)</td>
<td>+</td>
<td>Ehinger and Aberg, 1981</td>
</tr>
<tr>
<td>rabbit</td>
<td>37 (24 - 69)</td>
<td>+</td>
<td>Brecha et al., 1984</td>
</tr>
<tr>
<td>cat</td>
<td>19 (13 - 23)</td>
<td>anti-TH</td>
<td>Negishi et al., 1984</td>
</tr>
<tr>
<td></td>
<td>54 (31 - 96)</td>
<td>+</td>
<td>Tork and Stone, 1979</td>
</tr>
<tr>
<td></td>
<td>7 (peak)</td>
<td>+</td>
<td>Oyster et al., 1984</td>
</tr>
<tr>
<td>rhesus monkey</td>
<td>26 (10 - 40)</td>
<td>0</td>
<td>Nelson et al., 1984</td>
</tr>
<tr>
<td>chimpanzee</td>
<td>22 (22 - 22)</td>
<td>anti-TH</td>
<td>Nguyen-Legros et al., 1984</td>
</tr>
<tr>
<td>human</td>
<td>21 (21 - 21)</td>
<td>anti-TH</td>
<td>Nguyen-Legros et al., 1984</td>
</tr>
</tbody>
</table>

\* not corrected for tissue shrinkage, the actual densities using monoamine histofluorescence could be up to 10% too high (Negishi et al., 1981).

\*\* 0 monoamine histofluorescence, endogenous catecholamines

+ monoamine histofluorescence after preloading with catecholamine

Negishi and co-workers have published densities of dopaminergic neurons in several other species of teleost fish (Negishi et al., 1980b, 1981b, 1981c, 1983c).
(Pourcho, 1982). At an estimated density of 40-50 cells/mm² this would result in an almost 35-fold coverage (dendritic area x density). In goldfish, the average arborization area of a dopaminergic interplexiform cell is 37000 μm², which at the density of 131 cells/mm² results in a 5-fold coverage (Negishi, 1981). Although no exact data is available, the density of the plexus observed in chicken, suggests that there is a high degree of overlap.

The regularity in horizontal distribution of the dopaminergic amacrine cells, as expressed by the regularity index of 2.9-3.4, is amongst the lowest observed in chicken retina. The regularity index of cholinergic amacrine cells is 3.8-5.4, of cholinergic displaced amacrine cells 3.4-5.6 (Ishimoto et al., submitted for publication), of somatostatin-immunoreactive cells 3.3-3.4 (Ishimoto et al., 1986), of leu-enkephalin-immunoreactive cells 3.8-4.0, and of neurotensin-immunoreactive cells 2.9-3.6 (Ishimoto and Morgan, personal communications). Also in other species, a similarly low regularity in the horizontal distribution of dopaminergic amacrine cells has been reported. The regularity index in rabbit was 3.5 (Brecha et al., 1984), and in monkey 2.4 (Mariani et al., 1984). High regularity has been associated with high spatial acuity (Wassle and Rieman, 1978; see Methods). Thus, the low regularity of dopaminergic amacrine cells in chicken and in other species, could indicate that the neuron as-a-whole, is not involved in the preservation of high spatial acuity.

There was no marked horizontal specialization of the dopaminergic system in chicken retina, as judged by the limited variation in cell densities. The highest density observed in central areas, is only twice the lowest density. In contrast, densities of cones (Meyer and May, 1973), ganglion cells (Ehrlich, 1981), and cholinergic amacrine cells
(Ishimoto et al., in preparation) show a 5-10 fold difference between highest and lowest densities. These cells are concentrated in the area of most acute vision in the chicken retina, even though it does not have a well-defined fovea. The lack of a similar concentration of dopaminergic neurons in this area suggests that dopaminergic transmission may not be involved in this functional specialization.

Recently it has been reported that in the retinas of rhesus monkey, cat and opossum, the dopaminergic amacrine cells parallel rods in spatial distribution, but do not parallel that of cones, and ganglion cells (Mariani et al., 1984; Kolb and Wang, 1985). These results have been interpreted as indicating that dopaminergic amacrine cells might be related to rod-driven pathways. A similar situation may exist in chicken retina, since in chicken the distribution of rods shows little horizontal specialization (Meyer and May, 1973), as described for the distribution of the dopaminergic amacrine cells.

Thus, in all species investigated, dopaminergic networks seem to be similarly organized, characterized by a low frequency of cell bodies, yet a dense network of processes. It has been suggested several times that such an organisation does not provide a suitable morphological substrate for a role of dopaminergic transmission in functions requiring high spatial acuity (see e.g., Ehinger, 1983; Nguyen-Legros, 1984). An organization where a few cells innervate a large number of neurons is compatible with a basal regulatory function for the dopaminergic neurons in the retina. The observed low regularity of the distribution of the cell bodies, and the absence of high densities in areas with most acute vision, further support this notion.

In this argument the dopaminergic amacrine cell is regarded as one single physiological unit. However, observations on cholinergic
amacrine cells in rabbit retina indicate that local regions of the amacrine cell dendritic tree may be physiologically autonomous (for review, see Masland and Tauchi, 1986). Cholinergic amacrine cells have been demonstrated in many species, including chicken. In rabbit, they have wide dendritic fields, as large as 800 μm in diameter, and have high densities of 350-1600 cells/mm², resulting in a 30-70 fold coverage. Cholinergic amacrine cells synapse directly onto ganglion cells, despite the broad receptive field of the amacrine cell driving it, the ganglion cells can detect fine spatial details of the visual world. As an explanation for this apparent paradox, it has been proposed that cholinergic dendrites release neurotransmitter at the local sites of activation, and thus preserve fine spatial details of the input signal. Although the morphology of the cholinergic neuron as a whole indicates poor spatial acuity, the neuron may not act as one unit, so, the observed wide-field morphology may still sustain tasks involving high acuity.

In summary, chicken retina has been shown to contain a population of neurons, which based upon their catecholamine content, TH-immunoreactivity, and catecholamine-accumulating capacity, is likely to use dopamine as a neurotransmitter. The localization of these neurons in the INL at the junction with the IPL, together with the absence of detectable processes in the OPL, confirm previous reports that these neurons are dopaminergic amacrine cells. The sparse distribution of cell bodies, could provide a starting point in looking for the function of dopaminergic transmission in general regulatory processes (see Chapter 7), although the possibility of local functional units in the processes cannot be discounted.
Chapter 5.

DEVELOPMENT OF THE DOPAMINERGIC SYSTEM IN CHICKEN RETINA.

Introduction.

The general embryogenesis of chicken retina has been comprehensively reviewed recently by Grun (1982). All cells enter their last mitosis within the first week of development. The first cell type to be recognized in histological studies is the ganglion cell at ED3. Photoreceptors are first reported at ED6, but they remain immature until just before hatch (ED21). On ED8 horizontal cells and amacrine cells appear. By ED 10, all retinal layers are well identifiable, but it is not until ED 14, that bipolar cells can be recognized.

An IPL appears on ED6-7, and is then mainly composed of transverse processes. On ED8, horizontal processes of ganglion cells and amacrine cells appear, which were identified by their cytoplasmic density under the electron microscope. Contacts between these two types of neurons are seen from ED10 on, and conventional synapses with synaptic vesicles appear on ED13. One day later, synapses with ribbons are found, indicating bipolar cell contacts.

From ED7, a small positive potential, presumably from the pigment epithelium, was found to be evoked by stimulation with light. The first appearance of the photoreceptor component of the ERG was found at ED13, coinciding with the first appearance of photoreceptors outer segments (Hanawana et al., 1976). But it is not until ED17 (Hanawana et al., 1976) to ED20 (Witkovsky, 1963) that complex ERGs, consisting of contributions of other cell types than photoreceptors, are observed. During that developmental stage a sharp increase in glutamine synthetase (GS) activity (Moscona, 1983) and choline uptake (Bader et al., 1978) is
observed. These features indicate that it is not until ED17-20 that the photoreceptors are able to drive the retina.

The neurochemical differentiation of the different transmitter systems has been poorly investigated (see Grun, 1982). The appearance of biochemical correlates of cholinergic transmission has been described in some detail (Shen et al., 1956; Baughman and Bader, 1977; Bader et al., 1978; Crisanti-Combes et al., 1978; Woolston, 1980; Thompson, 1982). Morphological aspects of the development of dopaminergic neurons have been studied using monoamine histofluorescence techniques (Araki et al., 1983; Kato et al., 1984). Only the appearance of dopamine-stimulated adenylate cyclase, as marker for the formation of dopaminergic synapses, has been studied extensively by De Mello and coworkers (De Mello, 1978; Ventura, 1984; De Mello and De Mello, 1985). However, little is known about the ontogeny of other biochemical correlates of dopaminergic amacrine cells in chicken retina.

It has been reported that the first detectable appearances of dopaminergic entities coincide with the first exposure to light, in rat retina (DaPrada, 1977; Cohen and Neff, 1982; Kamp and Morgan, 1982; Morgan and Kamp, 1984) and in rabbit retina (Lam et al., 1981, Fung et al., 1982; Parkinson and Rando, 1984). In addition, dopaminergic transmission seems to reach maturity well beyond the time when other transmission systems have reached maturity, in those species.

To relate in chicken, the activity of dopaminergic transmission with the developmental stage of the dopaminergic neurones, and with the development of the retina in general, I have measured retinal TH activities and dopamine levels, and characterized dopamine uptake during and beyond embryogenesis.
Methods.

Protein and TH activity was determined as described in Chapter 2. Dopamine levels, determined by ion-pair reverse phase HPLC-ED and by cation-exchange HPLC-ED, and dopamine uptake, were measured as described in Chapter 3.

Eggs.

Fertilized eggs were obtained from a local dealer, and hatched in a chicken-incubator, where the chicks hatched at ED21 (PHO).

Glutamine synthetase (GS) assay.

For the determination of retinal GS activity, 25 μl retinal homogenate (about 150 μg protein) was preincubated for 10min in a metabolic shaker water bath at 37°C, with 825 μl substrate solution, resulting in the following final concentrations: 58.8 mM sodium-acetate buffer pH 5.4, 141 mM L-glutamine, 5.88 mM NaH₂PO₄, and 58.8 μM ATP. After this preincubation, 150 μl reagents, consisting of 100 mM sodium-acetate buffer pH 5.4, 200 mM NH₂OH.HCl, and 40 mM MnCl₂, was added. For blanks, the MnCl₂ was omitted. After 30min incubation in a metabolic shaker water bath at 37°C, the reaction was stopped by adding 750 μl stop-solution, consisting of equal parts of 2.5 N HCl, 15 % (w/v) TCA, and 5 % (w/v in 0.1 N HCl) FeCl₃. Samples were centrifuged for 10min at 1000 xg, and optical densities (ODs) of the supernatants were measured at 500 nm. GS activity is expressed as OD units, minus blank values.
Results.

The growth of the retina, as expressed by its protein content, remained constant after ED12, but increased again after hatch (FIGURE 5.1a), which is in agreement with earlier observations (Woolston, 1980; see Grun, 1983). The increase in protein content of the retina plus supportive tissues (pigment epithelium and choroid) was less steep than the increase of protein content of the retina alone. In the mature situation, the neural retina was the major source of protein in the often used preparation consisting of retina, pigment epithelium and choroid.

TH activity became first detectable at ED12, although activity at that stage is just above the detection limit of the assay (FIGURE 5.1b). From that day on TH capacity increased steadily until hatch. During that period protein content remained constant, so that specific enzyme activity increased during late embryogenesis (TABLE 5.1). Immediately after hatch TH capacity increased at least 4-fold within 4 days, which was more than the increase in protein, so that specific activity increased during the first 4 days after hatch (TABLE 5.1). After PH4, TH capacity still increased during maturation. The increase in specific activity was however less substantial than during the first 4 days after hatch. Comparing the increase of specific TH activity with the increase of specific GS activity, an enzymatic marker for Muller glial cells (Moscona, 1983), the first 4 days after hatch, suggests the dopaminergic system is selectively activated during those first 4 days. But after PH4-8, the increase in specific TH activity was comparable with the observed increase in specific GS activity during that period.
Figure 5.1: Biochemical parameters of the development of the dopaminergic amacrine cells in chicken retina.

Top,
- o---o, retinal protein content per retina
- +---+, protein content of the retina including pigment epithelium and choroid.

Bottom,
- o---o, TH activity *in vitro*
- +---+, dopamine levels
- Δ---Δ, dopamine uptake *in vitro.*
Table 5.1: Change of specific activities of some cellular markers during maturation of chicken retina.

<table>
<thead>
<tr>
<th>Age</th>
<th>Specific activities + SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days after hatch</td>
<td>TH pmol/min.mg</td>
</tr>
<tr>
<td>PH1</td>
<td>2.0 ± 0.2(5)</td>
</tr>
<tr>
<td>PH4-8</td>
<td>3.9 ± 0.3(4)</td>
</tr>
<tr>
<td>PH43</td>
<td>4.3 ± 0.2(5)</td>
</tr>
</tbody>
</table>
At ED7 and ED12, chromatograms obtained by ion-pair reverse phase HPLC-ED of retinal homogenates, showed a large peak, comigrating with dopamine standards (FIGURE 5.2). With cation-exchange HPLC-ED, no dopamine could be detected in those homogenates, which demonstrates that the peak shown in ion-pair reverse phase HPLC-ED did not represent dopamine (FIGURE 5.3). From ED15 on, chromatograms obtained by ion-pair reverse phase HPLC-ED showed a peak, slightly slower than the peak from unknown origin as seen at ED7 and ED12. By adding dopamine to the homogenates, the peak seen from ED15 on, was identified as dopamine. Thus at ED15, retinal dopamine reached detectable levels. Retinal dopamine levels increased during embryogenesis (FIGURE 5.1b). A slight drop in dopamine levels was observed immediately after hatch, though is not significant (2p < 10 %, two-tailed Student's t-test). Apart from that, dopamine levels remained essentially constant, thus it seemed that mature dopamine levels are reached before hatch.

The chromatograms showed a qualitative difference in catecholamine composition during pre- and post-hatch periods (TABLE 5.2). At ED15 and PH1, DOPAC/dopa (see Chapter 3, Methods) levels were relatively low compared to dopamine levels, the DOPAC/dopa to dopamine ratio increased substantially between PH1 and PH14 (0.43 up to 0.97). HVA levels became just detectable at ED15, and increased after PH1 but remained around the limit of detection (50-100 pmol/retina). At PH14, HVA peaks were always present in the chromatograms. These results may indicate a change in dopamine turnover after hatch.

At ED5, the retina could not be reliably isolated, and whole eyes were used to examine dopamine uptake. This preparation showed dopamine uptake activity of 390 fmol/min/mg protein, more than 10 times larger than seen in retinas isolated from chicks at ED10, which showed an
Figure 5.2: Monoamine analysis by ion-pair reverse phase HPLC-ED of chicken retina during development.

DOPAC, 3,4-dihydroxyphenyl acetic acid
DA, dopamine
5HIAA, 5-hydroxyindoleacetic acid
5HT, serotonin.
Retinas were isolated as described in Methods, and homogenized in mobile phase. ED7-15, 4 retinas/500 ul, ED18 and PN1, 2 retinas/500 ul, PN14, 1 retina/500 ul.
The Y-scale is corrected for the different tissue/mobile phase ratios, so all chromatograms can be compared quantitatively.

During development, the chromatograms show increasing complexity, some peaks have been identified, on the basis of their retention times. At ED7 and ED12 a peak "X" comigrating with dopamine is seen (next page), however see FIGURE 5.3.
Figure 5.3: Monoamine analysis by cation-exchange HPLC-ED of chicken retinas during early development.

ED7, ED12, 4 retinas/500 ul
spiked ED12, 100 ul retinal sample together with 15pmol dopamine was injected.

The peak comigrating with dopamine in ion-pair reverse phase HPLC (see FIGURE 5.2), could be interpreted as indicating massive amounts of dopamine present in the retina at ED7 and ED12. In cation-exchange HPLC no peak with a retention time similar to dopamine was seen in retinal homogenates at ED7 and ED12.
<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Catecholamine Content pmol/retina + SEM (n)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dopamine</td>
<td>DOPAC/dopa</td>
</tr>
<tr>
<td>ED15</td>
<td>$10.8 \pm 0.4$ (6)</td>
<td>$4.0 \pm 0.4$ (6)</td>
</tr>
<tr>
<td>PHI</td>
<td>$29 \pm 2$ (5)</td>
<td>$11.3 \pm 0.6$ (4)</td>
</tr>
<tr>
<td>PHI4</td>
<td>$34 \pm 2$ (15)</td>
<td>$30 \pm 3$ (4)</td>
</tr>
</tbody>
</table>

*in some retinal homogenates a peak corresponding with HVA was just detectable; detection limit of our method was 50-100pmol HVA/retina.
Table 5.3: Kinetic properties of $^3$H-dopamine uptake during development of chicken retina.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>$K_m$ nM ± SEM (n)</th>
<th>$V_{max}$ fmol/min·mg protein ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED5</td>
<td>7500 ± 200 (2)</td>
<td>5400 ± 300 (2)</td>
</tr>
<tr>
<td>ED12</td>
<td>340 ± 20 (2)</td>
<td>160 ± 20 (2)</td>
</tr>
<tr>
<td>ED15</td>
<td>700 ± 30 (2)</td>
<td>180 ± 30 (2)</td>
</tr>
<tr>
<td>PH12</td>
<td>320 ± 20 (2)</td>
<td>140 ± 20 (2)</td>
</tr>
<tr>
<td>PH14-28*</td>
<td>590 ± 80 (4)</td>
<td>140 ± 20 (4)</td>
</tr>
</tbody>
</table>

*see Chapter 3

Between ED10 and ED12, the activity of retinal dopamine uptake increased almost 4-fold, and remained essentially constant after ED12 until hatching (Figure 5.1a). After hatching, the retinal dopamine uptake activity increased greatly again, which was only partially explained by the generalized growth of the retina, indicated by the increasing protein levels, but also appeared due to an increase in specific activity (Table 5.1). The increase in dopamine uptake activity leveled off during the first week after hatching. Specific activity remained constant after ED12. Further studies will be essential to determine the factors affecting dopamine uptake during early development.

The first appearance of a dopamine-related entity was high-affinity dopamine uptake, which became measurable at ED10. Around the same time, aputtation/dopamine uptake activity was first detected (Morgan et al., 1983). In contrast, several markers for cholinergic activity were detected far earlier during development. Nerve fiber (Thompson, 1968; Creutz-Reedman et al., 1985; Wulster, 1986; Langston and Oster, 1977), which also the first DA-immunoreactive cells were seen (Spindel et al., in preparation). Also the onset of GABA and glutamate were first detected at ED15 (Koody and Rudy, 1977).
uptake activity of 30 fmol/min/mg protein. Dopamine accumulation at ED5, differed significantly in kinetic characteristics from retinal uptake observed later during development (TABLE 5.3). The low-affinity and high-capacity observed at ED5, suggest that the uptake at that developmental stage is mediated by a different carrier, possibly a non-specific monoamine carrier present on most cells (see Chapter 1.2). From ED12 on, the kinetic characteristics of the retinal dopamine uptake were similar to those in mature chicken, and the kinetics indicate the presence of the genuine dopamine carrier, present on dopaminergic nerve terminals.

Between ED10 and ED12, the activity of retinal dopamine uptake increased almost 4-fold, but remained virtually constant after ED12 until hatch (FIGURE 5.1b). After hatch, the retinal dopamine uptake activity increased sharply again, which is only partially explained by the general growth of the retina, indicated by the increasing protein levels, but also appeared due to an increase in specific activity (TABLE 5.1). The increase in dopamine uptake activity, leveled off during the first week after hatch. Specific activity remained constant after ED8.

Discussion.

The first appearance of a dopamine-related entity was high-affinity dopamine uptake, which became detectable at ED10. Around the same time somatostatin-immunoreactivity was first detected (Morgan et al., 1983). In contrast, several markers for cholinergic activity were detected far earlier during development, from ED7 on (Thompson, 1982; Crisanti-Combes et al., 1978; Woolston, 1980; Baughman and Bader, 1977), when also the first CAT-immunoreactive cells were seen (Spira et al., in preparation). Also the uptake of GABA and glutamate were first detected at ED6-7 (Bondy and Purdy, 1977).
The first appearance of an entity depends upon the detection limit of the analysis system, a situation which is worsened in the case of the dopamine amacrine cells, because of their low density compared to many other sorts of amacrine cells in the chicken (Chapter 4). In rat retina, TH-immunoreactive neurons (Nguyen-Legros et al., 1983) were seen only one day before TH activity could be measured in vitro (Morgan and Kamp, 1982), so both methods have essentially the same detection limit. In chicken retina, TH activity was first detected at ED12, so it is doubtful that TH-immunohistochemistry will be helpful in detecting dopaminergic neurons in an earlier stage.

The appearance of high-affinity dopamine uptake on ED10, was shortly after the first formation of horizontal arborizations of processes of the amacrine cells (Coulombre, 1955), and coincided with the first observation of synaptic contacts between amacrine- and ganglion cell processes (Sheffield and Fishman, 1970; Fisher, 1982). These observations suggest that the appearance of high-affinity dopamine carriers may be part of the development of synaptic specializations of the dopaminergic amacrine cells, which would provide an extremely useful indicator.

Although TH activity is present from ED12 on, dopamine could only be detected at around ED15. These results confirm, in a more quantitative way, the indications obtained by morphological observations. Using a highly sensitive modification of the monoamine histofluorescence method, catecholamine containing neurons were detectable from ED13-14 (Araki et al., 1983; Kato et al., 1984). From that day on, dopamine-related fluorescence increased, which is consistent with the observed levels of dopamine in the retina. Early dopamine-related histofluorescence was only seen in cell bodies, processes became detectable later.
The apparent activation of the dopaminergic amacrine cells between ED12-15, as judged by the increase in dopamine levels and TH activities, coincides with the appearance of bipolar cell (ribbon) synapses in the IPL (Hanawana et al., 1976; Fisher, 1982). However, it is not until ED17-20 that photoreceptors become active (i.e. start releasing transmitter in the dark) (see Grun, 1982). So at this stage, light cannot be the trigger for activation of the dopaminergic amacrine cells.

Pharmacological studies of the dopamine-stimulated adenylate cyclase during development, in chicken retina, suggest that the D1-type receptors switch from a supersensitive state to a less sensitive state, around ED13-14 (Ventura et al., 1984). A comparable decrease in D1-receptor sensitivity has been observed in rabbit retina, around the time when endogenous dopamine levels and TH- and DDC activities increased (Parkinson and Rando, 1984). The change of receptor sensitivity around the time when I observed detectable dopamine levels, indicates that some dopamine is released as soon as neurons start producing it. The low amounts of dopamine metabolites around ED15 compared to PH14 indicate a slow turnover, and perhaps low release of dopamine during embryological stage.

Dopamine-related activities increased steadily up to a few days before hatch. A comparable increase in activity was observed for the uptake of GABA and choline (Bondy and Purdy, 1977), for AChE and CAT activities (Crisanti-Combes, 1978; Bader et al., 1978; Woolston, 1980), and for acetylcholine receptor binding sites (Woolston, 1980). This indicates an essentially similar pattern of development for these (putative) transmitter systems, up to the stage when the photoreceptors are thought to drive the retina.
The maturation of the dopaminergic system after hatch, may be slightly prolonged compared to the maturation of the other systems. The uptake of GABA, serotonin, choline, and glutamate remained constant, or increased slightly after ED18–21 (Bondy and Purdy, 1977). Also the increases in various markers for cholinergic transmission levelled off within the first two days after hatch (Woolston, 1980). In contrast, specific activities of TH and dopamine uptake were still increasing at PH5. Also in rabbit and rat retina, an increase in dopamine related enzyme activities has been observed, which continued for days longer than the increase in markers for other transmission systems, and well beyond the time of opening of the eyes (Lam et al., 1981; Fung et al., 1982; Parkinson and Rando, 1984; Morgan and Kamp, 1982; Cohen and Neff, 1982).

As an index for physiological maturity of the dopaminergic system in the rat, the light activation of TH has been observed. Although TH activity in rat retina can be detected at birth, light activation starts on the 20th post natal day (PN20), a week after the eyes open. The development of this light activation proceeds faster when the animals are exposed to light (Cohen and Neff, 1982), thus demonstrating the involvement of light stimuli in the maturation of the dopaminergic system. It would be interesting to examine this in chicken as well.

Some generalizations with respect to the development of the dopaminergic system in the retina emerge. In chicken retina, both the establishment of synaptic contacts, as well as the first exposure to light, may trigger events in the development of the dopaminergic system, which seems to be similar to the development of other transmission systems in the retina. However, the activation of the dopaminergic system in chicken, as in other species, seems to be more prolonged than the maturation of other transmitter systems.
Chapter 6.

SELECTIVE LESION OF DOPAMINERGIC TRANSMISSION IN CHICKEN RETINA BY
6-OHDA.

Introduction.

Despite the strong evidence that dopamine is a retinal neurotransmitter (see Chapter 1.4) little is known about its function as yet. Since the initial observation by Tranzer and Thoenen (1967; Thoenen and Tranzer, 1968) that 6-OHDA destroyed noradrenergic nerve terminals, 6-OHDA has been used widely to disrupt catecholaminergic pathways in many areas of the central and peripheral nervous system (for review, and detailed references, see Kostrzewa and Jacobowitz, 1974; and Jonsson et al., eds., 1975). Since dopamine appears to be the only catecholamine neurotransmitter in chicken retina (Chapter 3), this approach is perfectly suited for examining dopaminergic amacrine cells in chicken.

Toxicity induced by 6-OHDA is not specific for catecholaminergic neurons as such. The specificity of neurotoxicity induced by 6-OHDA is believed to be determined by the fact that catecholaminergic neurons have high-affinity uptake systems for catecholamines, and hence concentrate the toxin (see Jonsson and Sachs, 1975). Once inside the neuron, the highly reactive products generated by 6-OHDA, such as peroxides, quinones and free OH radicals, probably react nonspecifically with intracellular structures and destroy the neuron. Thus, the specificity of the toxin is determined by the extracellular concentration of the compound. High doses of 6-OHDA can be taken up by non-specific low-affinity systems, and have been shown to exert non-specific effects (see e.g. Butcher et al., 1975; Javoy et al., 1975).
A considerable variability in the efficacy of 6-OHDA as catecholaminergic lesioning agent has been observed. In many cases it seems that the destructive effects of 6-OHDA are restricted to the fibre terminals, whereas the axons are swollen and show irregular beading along their course, but do not vanish, and the perikarya appear to be intact. The extent to which catecholaminergic neurons are destroyed, depends upon the part of the nervous system under study.

These variable morphological effects are accompanied by comparable variability in biochemical effects. For example, in lamb stellate ganglion an acute decrease in TH activity was observed after 6-OHDA treatment (Cheah et al., 1971), whilst in rat sympathetic ganglia TH activities did not change at all after 6-OHDA treatment (Brimijoin and Molinoff, 1971). Studies of Iversen and Uretsky (1970; 1971) recently confirmed by Fety et al. (1984) showed variable effects of 6-OHDA treatment on dopamine and noradrenaline levels and TH activities, depending upon the brain area. Throughout the literature the changes in catecholamine levels range from -95 to +85% after 6-OHDA treatment (see Kostrzewa and Jacobowith, 1974).

6-OHDA has been used to study dopaminergic neurons in the retinas of fish, chicken, rabbit, rat, and cat (Dowling and Ehinger, 1978a; 1978b; Ehinger and Nordenfelt, 1977; Negishi et al., 1982b; 1982c; Cohen and Dowling, 1983; Teranishi et al., 1983; Negishi et al., 1983a; Hida et al., 1984; Maguire et al., 1985; Shimizu and Hokano, 1985). In general, only the morphological effects of 6-OHDA on dopaminergic neurons have been described, and little attention has been paid to examining whether the lesions were both effective and selective in these studies.
This study will focus on defining the conditions for an effective and selective lesion of the dopaminergic amacrine cells in chicken retina by 6-OHDA. The efficacy of neurotoxicity induced by 6-OHDA has been determined by its effects on retinal levels of dopamine and metabolites, activity of TH, dopamine uptake and the capacity for dopamine release. The selectivity has been studied by examining the effects on biochemical markers for various transmitter systems. Finally, the morphological destruction of the dopaminergic amacrine cells has been studied with monoamine histofluorescence microscopy.

Methods.

The chickens were reared, and the retinas were removed and homogenized as described in chapter 2. Retinal protein, and TH activities were determined as described in chapter 2. Levels of serotonin, dopamine, and dopamine metabolites were measured by ion-pair reverse phase HPLC-ED, as described in chapter 3. The uptake of $^3$H-dopamine, and the high-potassium evoked release of exogenous dopamine, were both studied in vitro, as described in chapter 3. GS activities were determined as described in chapter 5. Histofluorescence light microscopy was done as described in chapter 4.

6-OHDA treatment.

One week-old chickens were intravitreally injected in the left eye with various amounts of 6-OHDA (Sigma, in 10 μl of 0.9% (w/v) saline containing 1 mg/ml ascorbate) using a hand-held Hamilton 50 ul syringe, under ether anaesthesia. This treatment was repeated up to 4 times at 24h intervals, as indicated. Fourteen days after the last injection the chickens were killed with ether. Untreated contralateral eyes were
generally used as control retinas. Unless specified otherwise, the 6-OHDA-treatment mentioned in this thesis, refers to 3 injections of 200 nmole 6-OHDA at 24h intervals.

Treatment with dopaminergic drugs.

Dopamine (100 nmol, together with 500 nmol pargyline and 60 nmol ascorbic acid in 10 μl) and haloperidol (Serenace®, Searle, Australia, 390 nmol in 30 μl, and 130, 43, and 13 nmol in 10 μl) were intravitreally injected, and the retinas were isolated 2h after the injection.

Choline acetyltransferase (CAT) assay.

Retinal CAT activities were determined according to Haywood et al. (1975). 10 μl of retinal homogenate (about 0.6 mg protein) were preincubated in a metabolic shaker water bath at 37°C for 5-10min. After the preincubation 50 μl of incubation medium, consisting of 20 mM potassium-phosphate pH 7.0, 200 mM KCl, 0.2 mM EDTA, 140 μM acetyl-CoA (trilithium salt, Boehringer), 0.2 mM eserine sulphate (Sigma), and 1 % (v/v) [14C]-acetyl-CoA (59 mCi/mmol, Amersham, U.K.), was added, and the mixture was incubated for 60min in the water bath. The incubation was stopped by adding 250 μl of 0.1 M K2HgI4 in hexylmethylketone (2-octanone). After allowing the aqueous and hexylmethylketone phases to separate for 30-60min, 50 μl of the hexylmethylketone phase (upper phase) was added to 8 ml scintillation fluid (see chapter 2), and counted. For blanks, the hexylmethylketone was added before the incubation.
Amino acid analysis.

For amino acid analysis, homogenates were mixed with an equal volume of 20% (w/v) sulphosalicylic acid. After acid digestion overnight, debris was spun down at 10000 x g for 1 min, and 50 µl supernatant was analyzed for amino acids by the Research School of Biological Sciences’ Amino Acid Analysis Service.

Light microscopy.

Eyes were removed as for retinal isolations (see Chapter 2). The eye ball was cut in half, the vitreous removed, and the posterior part of the eye was immersed in fixative consisting of 0.1 M sodium-cacodylate pH 7.2, 0.185% (w/v) CaCl₂, 0.035% (w/v) KCl, 3% (v/v) glutaraldehyde, and 1% (w/v) paraformaldehyde. After at least 4h, the tissue was washed several times in 0.1 M sodium-cacodylate pH 7.2, and left overnight in buffer. Then, the tissue was further fixed in 0.1 M cacodylate buffer pH 7.2, containing 1% (w/v) OsO₄, for 2h. The tissue was dehydrated through a series of alcohol, from 70-100% (v/v), washed twice with 100% acetone, and once with 50:50 mixture of acetone with araldite resin, the latter solution consisting of 40% resin (Araldite epoxy M, Ciba-Geigy), 40% hardener (HY 964, Ciba-Geigy), 10% dibutrylphthalate (Ciba-Geigy), and 4% DMP30 (Ciba-Geigy) (all v/v). The acetone was slowly substituted by araldite solution, after which the tissue was embedded in araldite for 48h at 60-70°C. 1-2 µm sections were cut on a Reichert OmU-2-microtome. The sections were stained with 1% (w/v) Toluidine Blue in 1% (w/v) sodium-tetraborate. Sections were examined with a Leitz microscope. Photomicrographs were taken on Kodak Pan-X film.
Results.

Based on treatment schemes reported previously, intravitreal injections of 400 nmol 6-OHDA were given, and this dose resulted in a decrease in retinal TH activities by 50% (FIGURE 6.1). Higher doses did decrease TH activities further, and the maximum observed decrease was around 70%, obtained by injections of at least 800 nmol.

A single injection of 800 nmol 6-OHDA caused a range of side-effects (TABLE 6.1). CAT activity, an enzymatic marker for cholinergic transmission, was decreased by 58%. Taurine levels, an amino acid which is abundantly present in photoreceptors (Mandel et al., 1976), were decreased by 54%, which suggests that the photoreceptors were affected. Also the levels of the (putative) neurotransmitters GABA and serotonin were decreased after a high dose of 6-OHDA. In addition, the changes of retinal levels of several amino acids suggest a general necrosis in the retina (see Perry, 1982). This was confirmed by light microscopy, in which necrosis in all retinal layers was observed (FIGURE 6.2c).

To improve the selectivity of the 6-OHDA treatment, the effects of repeated low doses were examined (FIGURE 6.1). It appeared that the maximum decrease in TH activities, seen after a single dose of at least 800 nmol, also could be obtained by a daily injection of 200–400 nmol for 3–4 subsequent days. The mildest of these treatments, 200 nmol daily for 3 days hereafter referred to as 3x200 nmol 6-OHDA, was examined for side-effects. None of the drastic side-effects as seen after a single dose of 800 nmol, were seen after 3x200 nmol 6-OHDA (TABLE 6.1). A minor, though significant, decrease in CAT activities by 13%, and a small decrease in GABA levels by 10% (2p < 10%, two-tailed Student's t-test) was observed after 3x200 nmol 6-OHDA. These effects were
Figure 6.1: Dose dependency of the effect of 6-OHDA on retinal TH activity

Retinal TH activities 14 days after intravitreal 6-OHDA injections, in % compared to untreated contralateral eyes. Bars represent SEM.

The maximum observed decrease in TH activities can be obtained by a single dose of at least 800 nmol, and by 3 daily injections of 200 nmol 6-OHDA.
Tyrosine hydroxylase activity (% of control)

Amount of 6-hydroxydopamine injected/eye (nmol)
Table 6.1: Specificity of intravitreal 6-OHDA. Effects expressed as percentage compared to untreated contralateral retinas. ± SEM (n>5), all assays have been done 14 days after the last injection.

<table>
<thead>
<tr>
<th>Dose (nmol)</th>
<th>3 x 200</th>
<th>3 x 400</th>
<th>1 x 800</th>
</tr>
</thead>
<tbody>
<tr>
<td>weight</td>
<td>101 ± 4</td>
<td>109 ± 4</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>protein</td>
<td>100 ± 4</td>
<td>101 ± 1</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>tyrosine hydroxylase</td>
<td>25 ± 4**</td>
<td>22 ± 10**</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>choline acetyltransferase</td>
<td>87 ± 2*</td>
<td>92 ± 3</td>
<td>42 ± 3*</td>
</tr>
<tr>
<td>glutamine synthetase</td>
<td>98 ± 3</td>
<td></td>
<td>94 ± 8</td>
</tr>
<tr>
<td>serotonin</td>
<td>96 ± 10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Aminoacid analysis**

<table>
<thead>
<tr>
<th>Aminoacid</th>
<th>3 x 200</th>
<th>3 x 400</th>
<th>1 x 800</th>
</tr>
</thead>
<tbody>
<tr>
<td>taurine</td>
<td>100 ± 3</td>
<td></td>
<td>46 ± 2*</td>
</tr>
<tr>
<td>GABA</td>
<td>90 ± 2</td>
<td></td>
<td>32 ± 5*</td>
</tr>
<tr>
<td>glycine</td>
<td>99 ± 2</td>
<td></td>
<td>151 ± 13*</td>
</tr>
<tr>
<td>aspartate</td>
<td>98 ± 12</td>
<td></td>
<td>148 ± 27</td>
</tr>
<tr>
<td>glutamate</td>
<td>97 ± 3</td>
<td></td>
<td>84 ± 5</td>
</tr>
<tr>
<td>tyrosine</td>
<td>104 ± 4</td>
<td></td>
<td>269 ± 21*</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>103 ± 6</td>
<td></td>
<td>234 ± 45*</td>
</tr>
</tbody>
</table>

Significance has been determined with Student's t-test (two-tailed).

compared to control: *2p < 5%

**2p << 0.1%

compared to 3 x 200nmol 6-OHDA: #2p < 5%

*2p << 0.1%
Figure 6.2: Micrographs of chicken retina (transverse sections).

A, control
B, 14 days after 3 x 200 nmol intravitreal 6-OHDA administration
C, 14 days after a single intravitreal injection of 800 nmol 6-OHDA
INL, inner nuclear layer
IPL, inner plexiform layer.
Bars represent 10 um.

No necrosis is observed after 3x200 nmol 6-OHDA. The single dose of 800 nmol, however, caused massive disruption in all retinal layers.
Figure 6.3: Time course of the effect of 6-OHDA on retinal TH activity.

Retinal TH activities after 3 x 200 nmol intravitreal 6-OHDA administration, in % compared to untreated contralateral eyes. Bars represent SEM (n>6).

At 2 weeks after the last injection with 6-OHDA, TH are still depressed.
Tyrosine hydroxylase activity (% of control) vs. Time (days)
studied in more detail (see below). Light microscopic examination of retinal sections, did not reveal any necrosis (FIGURE 6.2b). Further, immunohistochemical observations of CAT, serotonin, somatostatin, enkephalin, substance P, and GABA, did not reveal any effects of 3x200 nmol on these (putative) neurotransmitter systems (Millar, personal communications). It should be noted that GABA-immunoreactivity is abundantly present in chicken retina, so that an effect on a minor population of neurons could easily be missed.

These results demonstrate that treatment with 3x200 nmol 6-OHDA selectively and effectively affected the dopaminergic enzyme activities. The efficacy of this treatment on the dopaminergic amacrine cells was investigated further.

The time course of decrease in TH activities after 3x200 nmol 6-OHDA is shown in figure 6.3. A tendency for slight recovery in TH activities seems to be present. It is clear from the time course that the decrease in TH activity is not due to a direct suppression of activity by 6-OHDA, since one would not expect 6-OHDA to be present up to 14 days after the last injection.

Dopamine levels were decreased by 59 % after 3x200 nmol 6-OHDA (TABLE 6.2). The levels of the metabolites DOPAC and HVA were unaffected by the lesion. Interestingly, the pigment epithelium/choroid from 6-OHDA lesioned eyes contained a compound comigrating with HVA in the ion-pair reverse phase HPLC system used. This HVA-like compound was also revealed in retinal homogenates treated with 6-OHDA in vitro (data not shown), and may well be 6-hydroxy-homovanillic acid. Whatever it is, the compound seems to be accumulated and stored within the pigment epithelium/choroid for at least 14 days. Also dopamine-uptake activity in vitro, was affected by the 6-OHDA treatment, a decrease in uptake by
Table 6.2: Efficacy of 6-OHDA in lesioning dopaminergic transmission.

Retinas were lesioned by 3 intravitreal injections of 200nmol 6-OHDA, experiments were done 14 days after the last injection, untreated contralateral eyes served as controls. * SEM (n).

<table>
<thead>
<tr>
<th></th>
<th>Control retinas</th>
<th>6-OHDA-treated retinas</th>
<th>statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>tyrosine hydroxylase in pmol/min/mg protein</td>
<td>5.5 ± 0.4 (14)</td>
<td>1.4 ± 0.2 (14)</td>
<td>2p &lt; 0.1%</td>
</tr>
<tr>
<td>dopamine in pmol/mg protein</td>
<td>7.3 ± 0.3 (8)</td>
<td>3.0 ± 0.2 (9)</td>
<td>2p &lt; 0.1%</td>
</tr>
<tr>
<td>DOPAC in pmol/mg protein</td>
<td>5.2 ± 0.5 (4)</td>
<td>4.5 ± 0.8 (5)</td>
<td>ns</td>
</tr>
<tr>
<td>HVA in pmol/mg protein^2</td>
<td>-74</td>
<td>-78</td>
<td></td>
</tr>
<tr>
<td>^3H-dopamine uptake in pmol/min/mg protein</td>
<td>120 ± 10 (8)</td>
<td>75 ± 7 (8)</td>
<td>2p &lt; 0.5%</td>
</tr>
<tr>
<td>^3H-dopamine release^3</td>
<td>73 ± 12 (12)</td>
<td>39 ± 9 (11)</td>
<td></td>
</tr>
<tr>
<td>1mM Ca** present</td>
<td>12 ± 5 (7)</td>
<td>25 ± 7 (6)</td>
<td></td>
</tr>
<tr>
<td>without Ca**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 two-tailed Student's t-test
2 detection limit of our method was 50-100pmol HVA/mg protein
3 high-potassium-evoked release: % of released radioactivity during perfusion with high-potassium, compared to released radioactivity during similar time slots before and after high-potassium
4 1mM Ca** had been replaced by an equimolar amount of EGTA.
Figure 6.4: \( ^{3}H \)-dopamine release.

Retinas were preloaded with \( ^{3}H \)-dopamine in vitro and perfused with physiological buffer (circles). Release was evoked by perfusion with buffer containing 60mM K\(^+\) during the time, indicated with a bar (triangles). For Ca\(^{2+}\)-free conditions, the 1 mM CaCl\(_2\) was replaced with EGTA. Vertical bars represent SEM (n=4).

Lesioned retinas show a slightly faster decline in the spontaneous efflux of \( ^{3}H \)-dopamine, than control retinas. High-potassium elicits an clear increase in efflux in both control and lesioned retinas, however the increase in efflux is more substantial in control retinas. Under calcium-free conditions, control retinas show hardly any increase in efflux during perfusion with high-potassium, whereas in lesioned retinas the efflux is clearly increased. Table 6.2 gives the qualitative changes in \( ^{3}H \)-dopamine release under these various conditions.
\[ \text{control} \quad \text{Ca}^{2+} \text{-free} \]

\[ \text{lesioned} \quad \text{Ca}^{2+} \text{-free} \]

\text{3H-dopamine release (cpm/2.3ml)}

Perfusion time (min)
48% was observed (TABLE 6.2). After 3x200 nmol 6-OHDA, the retinas showed a diminished increase in efflux of \(^3\)H-dopamine elicited by physiological buffer containing high-potassium (60mM) (39% vs 73% in control retinas) (Figure 6.4, TABLE 6.2). In 6-OHDA-treated retinas, most of the high-potassium-stimulated increase in efflux of \(^3\)H-dopamine was not calcium-dependent, and only 16% of the increase in efflux was calcium-dependent, vs 61% in control retinas.

Histofluorescence studies were done on retinas, under the same conditions which caused the specific decrease in tyrosine hydroxylase and dopamine release, after preloading the tissue with noradrenaline, as described in chapter 4. The morphology of the dopaminergic neurons in histological preparations of control retinas, did not differ from previously described (Chapter 4).

In lesioned retinas, the density of the small fluorescent neurons, which are likely to represent the indoleamine-accumulating neurons (see Chapter 4), was not different from control preparations.

In lesioned retinas the number of cell bodies was decreased by 46% of that in controls (TABLE 6.3). This decrease in number of cell bodies was highest in peripheral, and lowest in central parts of the retina (-61% vs -31%). The cell bodies in 6-OHDA-treated retinas looked different than those from in control retinas, as was seen in transverse sections of the retinas (FIGURE 6.5b). Most cell bodies were not homogenously fluorescent and looked collapsed. The effects of 3x200 nmol 6-OHDA on cellular processes was striking in both flat mounted preparations and transverse sections (FIGURE 6.5b). The network of fluorescent processes was less dense, but of increased fluorescence intensity in lesioned retinas. The varicosities were swollen to such an extent that they sometimes were hard to distinguish from smaller cell
Figure 6.5: Fluorescence micrographs of the chicken retina.

Transverse sections, bar represents 10 um.
Inset: flat-mounted preparations, bar represents 20 um.
A, control
B, 14 days after 3 x 200 nmol intravitreal 6-OHDA administration.

In both transverse sections as well as flat-mounted retinas, swollen processes and a disrupted plexus of processes was seen in lesioned retinas.
bodies. In flat-mounted 6-OHDA-treated retinas, few normal-looking processes could be detected. In transverse sections, the processes also looked to be disrupted. In particular the plexus of processes in sublamina 1 appeared broader, less dense and brightly fluorescent. However, the processes in sublaminas 3 and 5 were also clearly affected by the treatment.

Negishi et al. (1982b) reported that 6-OHDA totally eliminated dopaminergic cell processes and cell bodies in carp retina. In their experiments pargyline and ascorbate seemed to be essential for the total destruction of the dopaminergic neurons. In chicken retina, the addition of pargyline to the injection-mixture did not increase the morphological destructive action of 6-OHDA (TABLE 6.3). However, coinjection of ascorbic acid was, as judged by the effects on TH activities, essential for maximum disruption of the dopaminergic system.

Disruption of the dopaminergic transmission could affect the activity of neurons innervated by the dopaminergic neurons. Thus, the earlier described effects of 3x200 nmol 6-OHDA on CAT activities and GABA levels (see TABLE 6.1), may be due to either non-selectivity of the 6-OHDA treatment, or they may be caused by the interference with dopaminergic input of those transmitter systems. This was investigated by examining the acute effects of dopamine and the dopamine antagonist, haloperidol.

The intravitreal injection of dopamine and haloperidol did not affect biochemical markers for gross retinal condition, GS activities, taurine levels, and retinal protein contents (TABLE 6.4). Of the biochemical markers for a variety of (putative) neurotransmitter systems tested, only GABA ergic neurons seemed affected by dopaminergic drugs. Haloperidol decreased retinal GABA content, which is consistent with the
Table 6.3: The effects of pargyline and ascorbic acid on the efficacy of the 6-OHDA lesion.

Cell counts and tyrosine hydroxylase activities were done 14 days after the last injection. 3 x 200 nmol 6-OHDA, 60 nmol ascorbic acid, 500 nmol pargyline. * SEM (n).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tyrosine hydroxylase pmol/min.mg protein</th>
<th>Cells per mm$^2$*</th>
</tr>
</thead>
<tbody>
<tr>
<td>control retinas</td>
<td>5.3 ± 0.2 (6)</td>
<td>25.1 ± 0.7 (5)</td>
</tr>
<tr>
<td>6-OHDA in saline</td>
<td>2.6 ± 0.3 (6)</td>
<td></td>
</tr>
<tr>
<td>6-OHDA in saline with ascorbic acid</td>
<td>1.6 ± 0.2 (9)</td>
<td>13.6 ± 0.9 (4)</td>
</tr>
<tr>
<td>6-OHDA in saline with ascorbic acid and pargyline</td>
<td>1.7 ± 0.2 (10)</td>
<td>14 ± 1 (3)</td>
</tr>
</tbody>
</table>

*a total of at least 500 cells have been counted; n refers to the number of retinas.
Table 6.4: The effects of dopaminergic drugs on various cells in chicken retina

<table>
<thead>
<tr>
<th></th>
<th>control activity per retina</th>
<th>haloperidol 150nmol n=5</th>
<th>dopamine 100nmol n=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein</td>
<td>8.7 ± 0.2mg</td>
<td>-2</td>
<td>-1</td>
</tr>
<tr>
<td>glutamine synthetase</td>
<td>48 ± 10D/h</td>
<td>-5</td>
<td>+2</td>
</tr>
<tr>
<td>choline acetyltransferase</td>
<td>5.3 ± 0.1nmol/min</td>
<td>-7</td>
<td>-1</td>
</tr>
<tr>
<td>amino acid analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>taurine</td>
<td>3160 ± 70nmol</td>
<td>-7</td>
<td>+1</td>
</tr>
<tr>
<td>glutamate</td>
<td>319 ± 7nmol</td>
<td>+2</td>
<td>+3</td>
</tr>
<tr>
<td>glycine</td>
<td>123 ± 10nmol</td>
<td>+11⁺</td>
<td>+6</td>
</tr>
<tr>
<td>GABA</td>
<td>990 ± 10nmol</td>
<td>-24⁺</td>
<td>+18⁺</td>
</tr>
</tbody>
</table>

Significancy was determined with Student's t-test (two-tailed):

⁺2P < 10%

*2P << 0.1%
Figure 6.6: The effects of intravitreal haloperidol on retinal GABA levels.

Chickens were intravitreally injected with various amounts of haloperidol. 2hr after the injections the retinas were isolated and analyzed for GABA, as described in Methods.

Statistical significance (two-tailed Student's t-test):
* \(2p < 5\%\)
** \(2p < 1\%\)

Bars represent SEM (n=4).
observed decrease in GABA levels after dopaminergic depletion by 6-OHDA (Table 6.1). Although the increase in GABA levels after injection of dopamine was not significant (p < 0.05, two-tailed Student's t-test), the direction of change is opposite the direction observed for the administration of dopamine antagonist, suggesting a genuine effect, rather than random variation. Figure 6.3 shows the dose-response relationship of the effects of dopamine-activated GABA levels.

While no clear proof of presynaptic specificity of the treatment was obtained when the effects on all transmitter systems have been considered, the data has been suggestive of dopamine effects on GABA. A variety of mechanisms have been considered to explain the role of dopamine in the regulation of GABA release and synthesis. The results of the present study are consistent with the hypothesis that the dopamine-dopamine interaction affects GABA release and synthesis. In addition to these results, another major finding of the study was the high dose of 6-OHDA (1.0 nmol) did cause clear presynaptic effects. This positively reinforces the data that the dose-effect curve for dopaminergic activity is shifted towards higher dose of 6-OHDA. The data of these observations suggested that the dopamine-activated GABA levels, after the administration of dopamine antagonist haloperidol, indicate a minor neuroleptic effect on the dopaminergic neurons cells as well.
observed decrease in GABA levels after dopamine deprivation by 3x200 nmol 6-OHDA (TABLE 6.1). Although the increase in GABA levels after injection of dopamine was not significant (2p < 10 %, two-tailed Student's t-test), the direction of change is opposite the direction observed after the administration of a dopamine antagonist, suggesting a genuine effect, rather than a random variation. Figure 6.6 shows the dose-response relationship of the effects of haloperidol on retinal GABA levels.

Discussion.

While absolute proof concerning specificity of the 6-OHDA treatment is only obtained when the effects on all transmitter systems have been tested, this has not been attempted, given the large diversity of putative transmitters in chicken retina (see Chapter 1.3, TABLE 1.3). Two principal observations indicate the selectivity of the treatment with 3x200 nmol 6-OHDA. Firstly, this treatment did not affect biochemical markers for various (putative) neurotransmitter systems, nor were there any morphological indications for any non-specific effect. In addition to these negative results, secondly, the higher doses of 6-OHDA (> 400 nmol) did cause clear non-specific effects. This positively demonstrates that the dose-effect curves for non-dopaminergic entities is shifted towards higher doses of 6-OHDA. On the basis of these observations it is concluded, that the treatment with 200-300 nmol 6-OHDA daily, for 3-4 subsequent days, selectively affects the dopaminergic amacrine cells in chicken retina, although the small decrease in CAT activity, which could not be obtained with the dopamine antagonist haloperidol, indicates a minor neurotoxic effect on the cholinergic amacrine cells as well.
Various aspects of dopaminergic transmission were examined to determine the efficacy of the 6-OHDA treatment. If decrease in number of cell bodies by 46% reflects the loss of dopaminergic neuronal material, this would imply that 6-OHDA selectively removed dopaminergic neurons which contained almost twice the amount of TH as the remaining neurons, since retinal TH capacities decreased by 75%. In the several histological studies done in various species (for detailed references see Chapter 1.4), including chicken (Millar, personal communications), there was no indication of such a variability in TH-immunoreactivity of retinal dopaminergic neurons. On the other hand, cell bodies may survive without their processes, so the observed decrease of 46% in number of cell bodies may underestimate the actual loss of dopaminergic neuronal material. Since no unaffected processes were detected after the 6-OHDA treatment, it is likely that the loss of dopaminergic tissue exceeds 46%, and may be reflected in the observed decrease of 75% of retinal TH activities. If TH activities are used to reflect the mass of dopaminergic neuronal material, this would imply that the neurons surviving the 6-OHDA treatment are enriched in dopamine-content, since retinal dopamine levels dropped by 59% only. This dopamine accumulation, is consistent with the observed decrease in dopamine-releasing capacity of the lesioned retina. In addition, an increase in fluorescence has been observed in some lesioned retinas, which were not preloaded with noradrenaline.

TH is the site for regulation of dopamine synthesis (see Chapters 1.2 and 1.4), and it is likely that also in the dopaminergic amacrine cells of chicken retina, the capacity of TH can be regulated (Chapter 3). This imposes some caution in interpreting the decrease in TH activities in vitro as representing the decrease in dopaminergic
neuronal material. Taking this consideration into account, it seems reasonable to conclude that 3x200 nmol 6-OHDA decreases the amount of dopaminergic neuronal material by a figure between 25 and 46 %. The possibility remains however, that the other dopaminergic markers, i.e. dopamine uptake or dopamine levels, reflect the loss of dopaminergic neuronal material.

In addition to this loss of dopaminergic tissue, the function of dopaminergic transmission seems significantly impaired, as indicated by the lack of calcium-dependent release of dopamine elicited by high-potassium. Although the physiological relevance of the efflux of dopamine stimulated by high-potassium which was not dependent upon calcium, has to be examined, it is clear that the 6-OHDA-treatment as described, considerably disrupts the dopaminergic transmission in the chicken retina.

There was a small dose-window in which 6-OHDA was both selective and effective in the retina. It has been reported in studies in other parts of the nervous system, that dopaminergic neurons in general are less susceptible to 6-OHDA than noradrenergic neurons (Iversen and Uretsky, 1971). Therefore, higher doses of 6-OHDA seem necessary to affect dopaminergic neurons, which results in smaller values of selectivity. The greater effects of repeated low doses of 6-OHDA have also been found in other parts of the nervous system (Iversen and Uretsky, 1970; 1971), and this is in accord with the presumed mechanism for 6-OHDA induced neurotoxicity. 6-OHDA has to be accumulated by the cells to exert its toxic effects (Ljungdahl et al., 1971; Jonsson and Sachs, 1975), and at low extracellular concentrations the compound will predominantly be accumulated by the catecholaminergic neurons, which possess high-affinity catecholamine uptake systems.
The partial morphological destruction of the dopaminergic amacrine cells in chicken retina, is in agreement with findings in other parts of the nervous system, where 6-OHDA generally eliminates terminals, affects axons, and spares the cell bodies (Thoenen and Tranzer, 1968; Malmfors and Sachs, 1968; Ungerstedt, 1968; for review see Kostrzewa and Jacobowith, 1974). Since the processes are the major site for synaptic transmission, such a partial destruction is still quite useful as a tool in studying the function of a catecholaminergic system. However, it can only be claimed that transmission has been severely impaired, rather than eliminated.

A similar partial destruction has been described in the retinas of rabbits after doses of 40 - 80 nmol 6-OHDA on two successive days (Ehinger and Nordenfelt, 1977). The same treatment was reported to totally eliminate the dopaminergic neurons in fish retina (Negishi et al., 1982b; 1982c). However, in all those reports, effects of the lesion were assessed by histological examination only, and the possibility that these extensive morphological destructions are accompanied by nonspecific side-effects, are very likely, considering the low dose range for selectivity.

Recently, Kato et al. (1984) reported that 60 nmol 6-OHDA on two successive days totally destroyed the dopaminergic cell bodies in developing chicken retina. Their photomicrographs demonstrate the presence of processes, which were able to accumulate noradrenaline, and thus a total elimination of the dopaminergic amacrine cells cannot be claimed. If true, this is rather peculiar, since it would be the first observation of processes being sustained without soma. The densities of dopamine amacrine cells are low in chicken retina (Chapter 4), and are even lower after 6-OHDA treatment. The most likely explanation is that
they failed to detect the (few) dopaminergic cell bodies, thus, their claim of total destruction by 6-OHDA is not founded. In addition, the selectivity of the 6-OHDA-treatment was not examined. It would be interesting, however, to examine whether in the retina, like in other parts of the nervous system (see e.g. Angeletti, 1971; Lytle et al., 1971; and for reviews, Kostrzewa and Jacobowith, 1974; Jaim-Etcheverry and Zieher, 1983), the dopaminergic neurons have an increased susceptibility to 6-OHDA early during development. If this is the case, then the treatment of chicken eyes with 6-OHDA early during development, could be employed to obtain a retina which is truly devoid of the dopaminergic amacrine cells.

An interesting observation in these studies regarding the selectivity of 6-OHDA, was that GABA levels were affected by dopaminergic drugs. Assuming a volume of 1 ml for chicken eyes, haloperidol significantly affected GABA levels at a concentration of more than 130 μM. This is in the presence of endogenous dopamine at the receptor site, so it is hard to give a verdict over the affinity of the receptor involved in these effects. In carp retina, Dowling and Watling (1981) needed 100 μM haloperidol to inhibit the increase in adenylate cyclase activity upon high-potassium evoked release of endogenous dopamine, which is a dose similarly high as I needed to affect retinal levels of GABA. Since adenylate cyclase in retina of carp and chicken exhibits the same affinity for dopamine, 10 and 7 μM respectively (Dowling and Watling, 1981; Schwarcz and Coyle, 1976), this may indicate that the effects of 100-500 μM haloperidol on GABA levels in chicken retina are mediated by D₁-receptors, however the specificity and nature of receptors involved have to be tested yet.
The effects of dopaminergic drugs on the GABAergic system in chicken retina, are not in accord with the findings from Nistico et al. (1983), who reported a lack of effects of 1330 nmol intraperitoneally administered haloperidol. This route of administration may be less favourable pharmacokinetically, for manipulation of transmission systems in the retina. A close relation between dopaminergic and GABAergic transmission has been demonstrated in retina of rat, where it has been suggested that GABAergic neurons tonically inhibit dopaminergic neurons (Marshburn and Iuvone, 1981; Proll and Morgan, 1983; Kamp and Morgan, 1984). In the rat, no reciprocal effects, where dopamine affects GABAergic transmission have yet been demonstrated. In retina of carp and goldfish, dopamine has been reported to modulate the release of exogenous GABA (Kato et al., 1985; O'Brien and Dowling, 1985). Synapses between the two transmitter systems have also been observed (see Chapter 1.4). Fish retina, however, posses the interplexiform type of dopaminergic neurons, and it seems unwise to generalize between the two principal types of dopaminergic neurons. Electron microscopy, coupled with immunohistochemistry and autoradiography is now needed, to substantiate the possible dopaminergic contacts with the GABAergic system.

In conclusion, conditions have been established, for obtaining effective decreases in dopamine-related activities by 6-OHDA. These effects were selective, as judged by the lack of effects on biochemical measures of other cell types. Although morphological destruction of the dopaminergic amacrine cells was not complete, dopaminergic processes, which are probably the site for dopaminergic transmission, are clearly affected. In addition, calcium-dependent release of $^{3}$H-dopamine, a biochemical parameter closely related to dopaminergic transmission, is
significantly decreased after 6-OHDA lesioning. Given the selectivity and effectivity of this impairment of dopaminergic transmission, the 6-OHDA lesion should be useful in studies concerning dopamine's function in the retina.

In vertebrate retina dopaminergic input to cells from dopaminergic amacrine cells occurs in the IPL. In some species there is also dopaminergic input in the GCL from dopaminergic interplexiform cells. The effects of the interplexiform cells have been well documented (see Chapter 1.4). In fish and certain retina dopamine decreases the electrical coupling of horizontal cells by decreasing the permeability of gap junctions via the activation of dopamine-sensitive adenylate cyclase and the intracellular accumulation of cAMP. The subsequent action of cAMP results in a narrowing of the horizontal cell diameters, and an increase in horizontal cell conductance (Minnett et al., 1984; Lassiter and Belding, 1984). Based on these observations, it has been hypothesized that dopamine is involved in the segregation of centre-surround receptive field organization of bipolar cells (Sawtell et al., 1976; Magnoni and Drulon, 1978).

The effects of dopamine in the IPL are much less well understood (see also Chapter 1.4). In cat retina, dopamine inhibited spontaneous and light-driven activity of the ganglion cells (Braschell and Freund, 1980; Shier and Alden, 1986). In rabbit retina the effects of dopamine depended upon the ganglion cell type. Anik and Poling (1989) reported that ON-centre and ON-OFF cells were inhibited by dopamine, whereas OFF-centre cells were excited. These results were confirmed and extended by Jensen and Haw (1981; 1984), who concluded that dopamine may play an important role in the formation of the centre-surround organization of ganglion cells, and in adjusting the state of adaptation in the IPL.
Chapter 7.

PHYSIOLOGICAL ROLE OF DOPAMINE IN CHICKEN RETINA.

Introduction.

In vertebrate retina dopaminergic input to cells from dopaminergic amacrine cells occurs in the IPL. In some species there is also dopaminergic input in the OPL from dopaminergic interplexiform cells. The effects of the interplexiform cells have been well documented (see Chapter 1.4). In fish and turtle retina dopamine decreases the electrical coupling of horizontal cells by decreasing the permeability of gap junctions via the activation of dopamine-sensitive adenylate cyclase and the intracellular accumulation of cAMP. The uncoupling action of dopamine results in a narrowing of the receptive field diameter, and an increase in horizontal cell sensitivity to localized might stimuli (Teranishi et al., 1983; Piccolino et al., 1984; Hida et al., 1984; Lasater and Dowling, 1985). Based on these effects, it has been hypothesized that dopamine is involved in the antagonistic centre-surround receptive field organization of bipolar cells (Dowling et al., 1976; Negishi and Drujan, 1978).

The effects of dopamine in the IPL are much less well understood (see also Chapter 1.4). In cat retina, dopamine inhibited spontaneous and light driven activity of the ganglion cells (Straschill and Perwein, 1969; Thier and Alder, 1984). In rabbit retina the effects of dopamine depended upon the ganglion cell type. Ames and Pollen (1969) reported that ON-centre and ON-OFF cells were inhibited by dopamine, whereas OFF-centre cells were excited. These results were confirmed and extended by Jensen and Daw (1983; 1984), who concluded that dopamine may play an important role in the formation of the centre-surround organization of ganglion cells, and in adjusting the state of adaptation in the IPL.
I used this idea as starting point in the studies described in this chapter, in which the possible involvement of dopaminergic transmission in retinal sensitivity has been investigated. Experiments were carried out in which the dopaminergic system was disrupted by 6-OHDA, as described previously (see Chapter 6). Light sensitivity of lesioned retinas was compared with sham-treated retinas. Sensitivity was assessed by (1) measuring the pupillary diameter at different light levels, and at the level of the ganglion cells, by analysis of the (2) intensity-response (I/R) functions, (3) increment threshold functions, and (4) dark adaptation curves.

**Methods.**

**6-OHDA-lesion.**

4-6 week-old chickens (see Chapter 2) were injected intravitreally with 200 nmol 6-OHDA, daily for 3 subsequent days, as described in chapter 6. Eyes injected with the vehicle were used as controls. Experiments were done 2-4 weeks after the last injection. To check the efficacy of the lesion, the chickens were killed with ether when the experiments were finished, the retinas were isolated, and TH activity in retinal homogenates was determined as described in Chapter 2.

**Visual evoked potentials (VEPs).**

The VEP is the summation of responses of a group of ganglion cells, and can be recorded from the retinal surface, the optic tract, or the primary retino-recipient nucleus, which in the avian visual system is the optic tectum (see Duff and Cohen, 1975; Holden, 1977). The experimental procedure suited for VEP recording in chicken, had been developed by Dr. David Dvorak (see Dvorak and Morgan, 1983).
Chickens were anaesthetized by intraperitoneal injection of 6-10 ml 15% (w/v) urethane. The animal was wrapped in cotton wool and aluminium foil, and the head was secured by means of ear bars and a beak support. The skin and bone overlying the contralateral tectum was removed, exposing the medio-lateral surface of the optic tectum. To stop bleeding, the optic tectum was treated with Topostasin®. A silicon oil drop prevented the dura from drying. Animals were dark-adapted for 30 min prior to experimentation.

A 150 Watt quartz halogen lamp was used as light source. The output of the light source was focussed onto the entrance of a 7 mm diameter glass-fibre light conductor. Neutral density filters were used to attenuate the light intensity, and a computer controlled electronic shutter was used to set stimulus duration. The exit of the glass-fibre light conductor was positionned 5 mm from the eye, providing whole field stimulation. Maximum stimulus intensity was 55 cd/m², and ambient light levels in the darkened laboratory were more than 7 log units below this. All light intensities are expressed relative to the maximum intensity.

Tungsten in glass micro electrodes, having an exposed tip length of 3-15 μm, and an impedance of 0.4-2 Mohm (at 100 Hz) were mounted on a Narashigi oil-driven microdrive system. Recordings were made in the superficial layer (100-500 μm) of the optic tectum to ensure that responses were from ganglion cell axons and terminals only (see Holden, 1977). Moderate light stimuli (~5.0 log units) were given to measure VEPs. VEPs were amplified and visualized on a Tektronix 5113-oscilloscope. Responses were also digitized, and stored and displayed by an Apple IIe+ computer.
A typical recording of VEP from optic tectum is shown in figure 7.1. The ON-response was more prominent than the OFF-response. I have focussed on the amplitude of the ON-response of the VEP. Response amplitude was defined as the peak-to-peak amplitude of the first positive and the first negative waves of the on-response. The detection limit was 10 uV.

For I/R functions, test flashes 500msec in duration were presented at 20sec intervals.

For increment threshold measurements, a second light source was positioned. The images from both sources were projected onto the entrance of the glass-fibre conductor. Each light source could be independently controlled by means of neutral density filters, and a computer driven shutter. The maximum light intensity of the second light source was adjusted to 55 cd/m². One light source provided the background illumination, and the other light source provided test flashes. After 30sec adaptation to a given background luminance, 3 test flashes of 150msec duration were given at 10sec intervals. The magnitude of the responses to these test flashes, were compared with the I/R curve, which had been obtained previously, to see how far above threshold the intensities of the test flashes were. This figure was subtracted from the value for the intensity of the test flash, which gave the increment threshold.

Pupillary diameter.

The chicken was anaesthetized, restrained, and light was projected onto the eye as described above. After 15min dark adaptation, the eye was exposed to the specified light intensity for 30sec, after which the eye and a calibration bar were photographed, using a 35 mm camera with
Figure 7.1: Visual evoked potential (VEP) of chicken retina.

The VEP was recorded from the superficial layer (100-500 \text{um}) of the contralateral optic tectum. The light intensity of the whole-field stimulus (lower tracing) was $-5.0 \text{ log units}$ (see Methods).
results.
Under fully dark-adapted conditions, pupillary diameters of 6-OHDA-treated eyes did not differ significantly from control eyes (8.56 vs 8.60 mm, see FIGURE 7.1), suggesting that 6-OHDA had no effect on innervation of the dilatory vessels of the iris. Exposed to light, an initial constriction of the iris. In control eyes, the pupils were more constricted than in control eyes over the entire range of light intensities tested, and in treated eyes, a constriction of 12% was reached at light intensities of 1 unit less than in control eyes. These results may indicate a decrease in light sensitivity of the neuronal system that regulates the pupillary light reflex following dopamine depletions in the retina.
In control retinae the threshold light intensity for the detection of red's NER was 8.3 log units (FIGURE 7.3). The I/E function rose steeply with increasing light intensities, and reached saturation at -4.8 log units, resulting in a dynamic range of 4.5 log units. Beyond saturation, a more complex VEP was recorded (data not shown), and the I/E function increased with increasing light intensities.
Retinas treated with 6-OHDA showed a higher absolute sensitivity to light, as judged by the observed threshold difference of 0.7 log units (-7.0 vs -6.3, see FIGURE 7.1). In 6-OHDA-treated retinae, the steep rise in the I/E function at higher light intensities had the same
flash. The camera angle and distance to the eye were held constant in all experiments. The pupillary diameter was measured from photographic negatives, with the aid of a photographic enlarger.

Results.

Under fully dark-adapted conditions, pupillary diameters of 6-OHDA-treated eyes did not differ significantly from control eyes (3.56 vs 3.60 mm, see FIGURE 7.2), suggesting that 6-OHDA had no effect on innervation of the dilatory muscles of the iris. Exposure to light caused only a slight constriction of the iris. In control eyes, the observed diameter at highest light intensities available, was 16% decreased compared to the diameter of the dark-adapted eye. In 6-OHDA-treated eyes, the pupils were more constricted than in control eyes over the entire range of light intensities tested. And in treated eyes a constriction of 15% was reached at light intensities 3 log units less than in control eyes. These results may indicate a higher light sensitivity of the neuronal system which drives the pupillary light reflex, following dopamine deprivation of the retina.

In control retinas the threshold light intensity for the detection of a VEP was -6.5 log units (FIGURE 7.3). The I/R function rose steeply with increasing light intensities, and reached saturation at -4.0 log units, resulting in a dynamic range of 2.5 log units. Beyond saturation, a more complex VEP was recorded (data not shown), and the I/R function decreased with increasing light intensities.

Retinas treated with 6-OHDA, showed a higher absolute sensitivity to light, as judged by the observed threshold difference of 0.5 log units (-7.0 vs -6.5, see FIGURE 7.3). In 6-OHDA-treated retinas, the steep rise in the I/R function at higher light intensities had the same
Figure 7.2: The effects of treatment with 6-OHDA on the pupillary reflex in chicken.

Chickens were intravitreally injected with 200 nmol 6-OHDA daily for 3 subsequent days. Sham injected animals served as controls (see Methods). The pupillary reflex was measured 2 weeks after the last injection as described in Methods.

•-•, control eyes
○-○, 6-OHDA-treated eyes.

* Statistical significance (two-tailed Student's t-test)
\[ 2p < 5\% \]

Bars indicate SEM (n=3).
Figure 7.3: Intensity-response (I/R) functions for visual evoked potentials (VEPs) in control and 6-OHDA-treated retinas.

Chickens were intravitreally injected with 200 nmol 6-OHDA daily for 3 subsequent days. Sham injected animals served as controls (see Methods). VEPs were measured 2 weeks after the last injection as described in Methods.

- - - , control retinas (n=5)
○ - ○ , 6-OHDA-treated retinas (n=4).
Bars represent SEM.
As observed in control retinae, the response in 9-OH-pilocarpine-treated retinae was slightly larger than in control retinae, and the function of log VD was observed to be a 1.5 log units, resulting in a dynamic range of 2.5 log units.

Figure 7.4 shows the log threshold functions for control and 9-OH-pilocarpine-treated retinae. The threshold was observed in the presence of background illumination with a maximum retinal threshold (see below, 1972). As an aid in the analysis, the data was plotted in figure 7.4a, in log-log format, above the threshold of background illumination of -5.5 log units. The data of these flash thresholds (corrected for the flash and had to be an accurate measurement of the flash threshold increases intensity and the 90% of threshold of this 1.2 log units.

In control retinae, the Weber fraction was 0.08 log units. Beyond this, the Weber fraction tended to 1.0. This quite modest change in Weber fraction has been shown for physiological reasons and can be explained by a possible change of neural pathways with the retina, where the retina changes with a change in 9-OH-pilocarpine. The functions and the data of these flash threshold was slightly shifted over the dynamic range, and the change in the Weber function occurred at a background intensity of -5.2 log units, which was at a lower background intensity than were in control retinae. These results illustrate that the flash threshold changes in the retina in response to a physiological change (Horien, 1972).
slope as observed in control retinas. The maximum response in 6-OHDA-treated retinas, was slightly larger than in control retinas, and saturation of the VEP was observed at -3.5 log units, resulting in a dynamic range of 3.5 log units.

Figure 7.4 shows increment threshold functions for control and 6-OHDA-treated retinas. The threshold stimulus, measured in the presence of background illumination has been named the increment threshold (see Barlow, 1972). As an aid in explaining what it means, the first data-point in figure 7.4a for example, shows that with a background illumination of -5.5 log units, the light intensity of the test flash (superimposed on the background) had to be at least -5.2 log units, to evoke a VEP. With increasing background intensities, the increment threshold increases linearly, and the slope of this function represents the Weber fraction ($\Delta I/I$).

In control retinas the Weber fraction was 0.59 up to a background intensity of -3.0 log units. Beyond this, the Weber fraction jumped to 1.75. This quite sudden change in Weber fraction has been known from psychophysical studies, and is explained by a possible change of neuronal pathways within the retina, when the retina changes from a scotopic to a photopic conditions (Barlow, 1972).

In 6-OHDA-treated retinas, the Weber fraction at lower background intensities, was slightly larger than in control retinas (0.72 vs 0.59, see FIGURE 7.4b) The increment threshold was slightly elevated over this entire initial range, and the change in the Weber fraction occurred at a background intensity of -3.2 log units, which was at a lower background intensity than seen in control retinas. These results indicate that the 6-OHDA-treated retina is more sensitive to background illumination than control retinas. The change in the Weber fraction was
Figure 7.4: Increment threshold functions for visual evoked potentials (VEPs) in control and 6-OHDA-treated retinas.

Chickens were intravitreally injected with 200 nmol 6-OHDA daily for 3 subsequent days. Sham injected animals served as controls (see Methods). Experiments were done 2 weeks after the last injection, and increment thresholds were determined as described in Methods. The lines were fitted by the method of least squares, assuming linearity between -6.0 and -2.5 log units, and between -3.5 and -0.0 log units.

A, control retinas, (r=0.936 and 0.990, n=2)
B, 6-OHDA-treated retinas, (r=0.986 and 0.996, n=2).
BACKGROUND INTENSITY (log I_{backgr.})

A  control
weber fraction 0.59

weber fraction 1.75

B  6-OHDA-treated
weber fraction 1.12
weber fraction 0.72

INCREMENT THRESHOLD (log I_{thr.})
in 6-OHDA-treated retinas, not as spectacular as seen in control retinas (0.72 up to 1.12 vs 0.59 up to 1.75). However, the value of the Weber fraction at higher background intensities, was significantly smaller than the value in control retinas (1.12 vs 1.75). This means that the treated retinas had higher increment sensitivities under photopic conditions.

After exposure to high light intensities (7 log units above threshold) for 10 min, control retinas showed a 3.6 log units elevation in visual threshold (FIGURE 7.5a). After 12 min, control retinas returned to dark-adapted sensitivities. If fitted to a double exponential function, two phases can be recognized in the recovery to the dark. Up to 2 min after the bleach, the recovery is fast, with a time-constant of 0.56 min$^{-1}$. From 2 min after the bleach, the recovery is much slower with a time-constant of 0.21 min$^{-1}$. This biphasic recovery function can be explained by possible differences in the rate of recovery for different neuronal pathways in the retina (see Barlow, 1972). The early, fast phase of dark adaptation may reflect the cone-related recovery, and the second, slower phase has been allocated to rod-related recovery.

In 6-OHDA-lesioned retinas, the same bleach resulted in a almost full log unit higher elevation of threshold than in control retinas (4.4 vs 3.6 log units). Thus, the bleach appeared more efficient in the 6-OHDA-treated retinas, again pointing towards an increased sensitivity to light after 6-OHDA treatment.

Also in the 6-OHDA-treated retinas, 2 phases in the recovery function were distinguished. The initial phase, with a time-constant of 0.44 min$^{-1}$, was slower than observed in control retinas (0.56 min$^{-1}$). The second phase in recovery, with a time-constant of 0.21 min$^{-1}$ was the same as observed in control retinas.
Figure 7.5: Dark adaptation curves for visual evoked potentials (VEPs) in control and 6-OHDA-treated retinas.

Chickens were intravitreally injected with 200 nmol 6-OHDA daily for 3 subsequent days. Sham injected animals served as controls (see Methods). Experiments were done 2 weeks after the last injection as described in Methods. The retinas were exposed to light intensity of -0.0 log units (6.5-7 log units above threshold) for 10 min. The curves were fitted by the method of least squares, assuming a double-exponential relationship:

\[
\log I(t) = \log I_t=0 \exp(-at),
\]

with time-constant:

\[
T_{1/2} = \frac{0.693}{a} \text{ (in min}^{-1}).
\]

A, control retinas, (n=5) \hspace{2cm} T_{1/2}: 0.56 (r=-0.978) \hspace{1cm} 0.21 (r=-0.965)

B, 6-OHDA-treated retinas, (n=5) \hspace{2cm} T_{1/2}: 0.44 (r=-0.979) \hspace{1cm} 0.21 (r=-0.9681).
The effects of 6-OHDA treatment on retinal sensitivity to light have been studied. The results of the tests for retinal sensitivity to light are shown in the graphs. The sensitivity of the retina to light is plotted against time after bleaching. The sensitivity is expressed as the change in threshold for light detection. The sensitivity decreases with time after bleaching, and the decrease is more pronounced in the 6-OHDA-treated group compared to the control group.
Discussion.

It has been shown previously that the 6-OHDA-treatment employed for these experiments, decreased dopaminergic activity and almost completely blocked calcium-dependent dopamine release evoked by high-potassium. Moreover, the treatment is specific in these effects, as judged by the lack of effects on biochemical and morphological parameters of a variety of other neurons in the retina (Chapter 6). The efficacies of the lesions in each retina of the chickens used for these experiments, have been determined by measuring TH activities in retinal homogenates. TH activities in 6-OHDA treated retinas were never greater than 30% of activities found in control retinas. It is therefore concluded that every 6-OHDA-treated retina in these experiments represents a retina with disrupted dopaminergic transmission.

The results of the tests for retinal sensitivity all show a common feature: after dopamine-deprivation the retinas show an increased sensitivity to light. Lesioned eyes need less intensities of light to contract the pupillary diameter. The absolute sensitivity for VEP of the dark-adapted retina is 0.5 log units higher after dopamine deprivation. In lesioned retinas, lower intensities of background illumination are sufficient to elevate the VEP threshold (increment threshold). Finally, lesioned retinas are more effectively bleached by high-intensity light, and take considerably longer to dark adapt.

These results are consistent with reports in cat retina (Straschill and Perwein, 1969), where intravenous application of DOPA decreased the amplitudes of VEPs. At the single unit level, dopamine agonists were shown to decrease the activity of all types of ganglion cells. The observation that dopamine agonists have such effects, does not prove that an endogenous ligand is present. An isolated report, describing
very limited effects of 6-OHDA-treatment on ganglion cell responses in
cat, suggested a minor involvement of dopaminergic transmission in
ganglion cell responses (Maguire and Smith, 1985). However, the two eyes
checked for lesion efficacy showed a marked variation in the decrease of
dopamine, so that study cannot be regarded as decisive. The results
presented here, are based on the demonstrated selective and effective
deprivation of dopamine, and substantiate the assumed involvement of
dopaminergic transmission in the ganglion cell responses.

In rabbit retina, ON-centre and ON-OFF ganglion cells were
inhibited, but OFF-centre cells were excited by dopamine (Ames and
Pollen, 1969; Jensen and Daw, 1983). Since these cell types are present
in about equal numbers (Ames and Pollen, 1969), the overall effect of
dopamine on the VEP would probably be inhibition, rather than
excitation, consistent with the results obtained in retinas of cat and
chicken. I did not examine individual ganglion cell responses, and my
results do not exclude variable effects on individual ganglion cells.

A major part of the enormous capacity of the eye to adapt its
operating characteristics to the prevailing light conditions, is thought
to be established by neuronal compensation within the retina itself (see
Werblin, 1971; Barlow, 1972). The bend in the increment threshold
curve, is explained by a transition from rod- to cone-driven networks
(Werblin, 1972). The increment threshold function for lesioned retinas
was clearly different at higher light intensities, when the cone-driven
pathway is thought to operate. Similarly, dark-adaptation curves also
show multiple phases, an early, cone-related, recovery can be
distinguished from a slower phase in recovery, allocated to rod-related
recovery (Werblin, 1971; Barlow, 1972). In my experimental set-up, the
bleach was not sufficient to cause a clear biphasic recovery curve, but
fitting exponential functions, revealed two phases in dark adaptation, characterized by different time-constants. In lesioned retinas, the time-constant describing the early rate of recovery (cone-related), was different in lesioned retinas, whilst the second phase did not appear to be affected. Since Barlow (1972) reported that higher bleach does not affect the time-constants, the difference I observed in lesioned versus control retinas is probably due to the disruption of dopaminergic transmission, rather than due to the higher efficacy of the bleach in lesioned retinas.

Thus, both the increment threshold functions and the dark-adaptation curves, indicate that the cone-driven pathway is affected by the lesion. This leaves open the question whether the dopaminergic amacrine cells are an integral part of cone-pathways, or whether they are involved in the regulation of the cone-driven circuits. Since dopamine seems to be involved in the sensitivity of the dark-adapted retina (see above, I/R-functions), when the retina is driven by the rods only (Werblin, 1971) dopaminergic amacrine cells may have a role in the rod-driven pathway as well.

Recently Jensen and Daw (1984) reported that dopamine antagonists, administered by injection in the external carotid artery, reduced the antagonistic surround responses of ganglion cells. In cat, Thier and Alder (1984) described that iontophoretic application of dopamine caused a shift in favour of the centre in the centre-surround receptive field organization of the ganglion cells. Of course, dopamine antagonists do not necessarily have to have the opposite effects as dopamine agonists. However, the fact that dopamine agonists and antagonists have the same effect, may also be explained by the different method of drug application. Iontophoretically applied dopamine, as employed by Thier
and Alder, affects neurons within a localized patch of the retina, and results obtained this way this may be misleading, because neighbouring areas may be essential for the specialization of ganglion cell receptive fields (Werblin and Copenhagen, 1974; see Sterling, 1983). Using a method for the uniform manipulation of the dopaminergic system, the conclusion of Jensen and Daw, that dopamine may be involved in the formation of the antagonistic centre-surround organization, seems more favourable.

So, the results obtained in vertebrate retina show that dopamine has effects on the sensitivity of the dark-adapted, rod-driven retina, the cone-driven pathways at higher light levels, and the receptive field organization of the ganglion cells, which are known to change with increasing ambient light (Enroth-Cugell and Robson, 1966). The common denominator in this diverse list of retinal properties, is that all these aspects change during exposure to increasing illumination. So it seems possible to hypothesize that dopaminergic transmission is involved in the regulation of the changes in visual performance, which accompany the transition from the scotopic to the photopic adaptive state, rather than that it is a discrete part in a multitude of different functional pathways.

The observation of D_1-type receptors, (Redburn et al., 1980b), dopamine-sensitive adenylate cyclase activity (Makman et al., 1975; De Vries et al. 1982), dopamine elicited cAMP accumulation (Ferrendelli et al., 1980), and gap-junctions between several types of neurons (Teranishi et al., 1984b), all in the inner retina, suggests that dopamine's effects in the inner retina may be comparable to the effects described in the outer retina. This may indicate that dopamine in the IPL exerts its function via mechanisms similar to the OPL, involving the
regulation of the degree of lateral coupling of amacrine cells.

The results presented here, are in line with the general tenet in the literature, that dopamine-deprived retinas are more sensitive to light, and the idea emerges that dopaminergic transmission is involved in the regulation of the changes in visual performance of the retina during different states of adaptation. However, as pointed out by Werblin and Dowling (1969), electrical recording at the level of the optic nerve may specify many of the functions performed by the retina, but this approach can only indicate, not demonstrate, how the retina organizes the visual messages. The techniques so far employed, are not suitable for investigating the position of the dopaminergic amacrine cell within the wiring diagram of the retina, but form a useful basis for further research.
GENERAL DISCUSSION:

8.1 Dopaminergic amacrine cells in the chicken retina.

A central question in this thesis is the neurotransmitter status of dopamine in the retina. In discussing neurotransmission (Chapter 1.1), the release of a neurotransmitter, its recognition by a receptor, and the physiological effects mediated by the receptor, are pivotal criteria for assessing the neurotransmitter role of a compound. In addition, evidence for the presence of the compound and its synthetic mechanism give important indications for a transmitter role, although it is possible to imagine a transmitter system operating by uptake rather than synthesis.

The results presented in Chapters 3 and 4 strongly favour a neurotransmitter role for dopamine in chicken retina. Dopamine was shown to be present by HPLC-ED, and localized in neurons by histofluorescence. The synthetic enzyme TH was well characterized, and was localized by immunohistochemistry in the same morphological class of amacrine cells as were detected by histofluorescence of endogenous dopamine, or after preloading with noradrenaline. Dopamine was demonstrated to be released in a calcium-dependent manner. It has been demonstrated previously that dopamine elevates levels of cAMP via D1-type receptors in chicken retina (Schwarcz and Coyle, 1976; De Mello, 1978; De Mello and De Mello, 1985), and ligand binding has been described previously as well (Ventura et al., 1984; De Carvalho and De Mello, 1985). Further, a high-affinity dopamine uptake system was characterized, and the presence of the dopamine metabolites, DOPAC, MTA, and HVA, indicated a dopamine inactivation via MAO and COMT as well. Finally, dopamine turnover was increased by light, which in the case of
the retina, is an important additional indication for neurotransmitter status. Similar findings have been reported in retinas of other vertebrate species (see Chapter 1.4). On the basis of all these data, dopamine without doubt can be considered as a compound with the potential for being a neurotransmitter in the chicken retina, and fulfills all the biochemical criteria for transmitter status, including recognized post-synaptic effects in the form of changes in cAMP levels.

In chicken retina, all dopaminergic neurons were found to be amacrine cells, as judged by their morphological features, which were visualized with monoamine histofluorescence and immunohistochemistry at the light microscope level (Chapter 4). The cell bodies were found in the INL at the border with the IPL. A dense plexus of processes was found in sublamina 1 of the IPL, and in addition, processes were seen in sublaminas 3 and 5 of the IPL. The dopaminergic amacrine cells were present at a characteristically low density of cell bodies, which nevertheless support a major network of processes. Similar findings have been reported in other species (see Chapter 1.4), and it has been suggested (Ehinger, 1983a), that such an organization would be well suited to neurons supporting a general function, rather than a function involving high acuity. However, the possibility of local functional units in the processes, as described for the cholinergic system (Masland and Tauchi, 1986), cannot be discounted.

The development of the dopaminergic system was studied by following the activities of various biochemical markers for dopaminergic activity. Although there was some indication that the duration of maturation of the dopaminergic system after hatch, exceeded that of other amacrine cell systems, the development of dopaminergic amacrine cells was similar to that of other amacrine cells.
Manipulation of the dopaminergic system with dopaminergic drugs affected retinal levels of GABA, indicating a dopaminergic output towards GABAergic neurons, possibly involving D1-receptors. Levels of glycine and glutamate, and cholinergic activity were not affected, suggesting that there is no dopaminergic input to the amacrine cells involved in those transmitter systems. These results await further conformation, by e.g. immunohistochemistry or autoradiography at the electronmicroscope level.

In conclusion, dopamine has been demonstrated to be a likely neurotransmitter in chicken retina, and considerable progress has been made in the morphological description of the dopaminergic amacrine cells. The dopaminergic amacrine cells could be distinguished from other amacrine cells by their content of dopamine and TH, by their ability to accumulate dopamine and noradrenaline, by the characteristic large size, junctional location and low density of their cell bodies, and by the laminar distribution of their processes. They are activated by exposure to light and are able to release dopamine, possibly at synapses onto GABAergic neurons. The postsynaptic effects and the function of dopaminergic transmission in the chicken retina need now to be established.
GENERAL DISCUSSION:

8.2 Physiological effects of dopamine in the chicken retina.

Some of the post-synaptic effects of dopamine in the chicken retina have been documented. Dopamine stimulates a dopamine-sensitive adenylate cyclase via D₁-receptors, leading to increased levels of cAMP (Schwarzc and Coyle, 1976; De Mello, 1978; Ventura et al., 1984; De Carvalho and De Mello, 1985; De Mello and De Mello, 1985). However, what effects the changes in second messenger levels have on neuronal properties, is yet to be determined.

The problem of relating elevations of cAMP levels to electrophysiological or other changes in neurons post-synaptic to the dopaminergic cells is a more general problem. Reviewing work done on other dopaminergic systems (Chapter 1.2), it appeared that most of those networks are not sufficiently understood for detailed studies of the cellular response to dopamine. The major problems are, that the circuitry is often not known in sufficient detail, that the inputs to and outputs from the circuit under study are often complex and difficult to control, and that the neurons are often inaccessible and too small for intracellular recording techniques. In addition, in many of those systems, dopamine both increases as well as decreases intracellular levels of cAMP via D₁- and D₂-type receptors respectively. These problems are largely overcome in the SCG, which is a confined, simple circuit, consisting of input, an interneuron, and output. In the SCG, dopamine's effects on the sEPSP, were convincingly shown to be mimicked by butyryl-cAMP (see Libet, 1979; Chapter 1.2), thus causally relating the activation of a dopamine receptor to a final cellular response.
The biochemical features of dopaminergic transmission described in chicken retina, are similar in detail to those found in other parts of the nervous system, and all findings seem to be generalizable to the whole nervous system. So, insight into the cells' physiological response to dopamine in the retina may be generalizable, and would significantly contribute to the understanding of dopaminergic transmission in general.

The vertebrate retina has certain features which makes it advantageous over other neuronal tissues to use as model system to study dopamine's physiological response.

Its relatively pure population of \( D_1 \)-receptors (Watling and Iversen, 1981; Redburn et al., 1980a; see Chapter 1.4), and the relatively large activation of adenylate cyclase by dopamine (Schorderet and Magistretti, 1980; Iuvone and Neff, 1981; Van Buskirk and Watling, 1984), make the retina the ideal place to study the effects of raised cAMP levels in cells receiving dopaminergic input. Another major advantage of the retina as model system, is its natural stimulation by light, providing a high degree of control over the input of the neuronal circuit. In addition there are various practical advantages, such as easy accessability, relatively easy isolation and maintenance in vitro, and the histology of the retina is, up to the level of the major cell types, easy to comprehend. Of course, there are some difficulties remaining with the retina as a model, the most important being the complexity of connections in both plexiform layers. However, given the advanced state of understanding of the ultrastructural features of retinal cells, coupled with developments in immunohistochemistry, tracing of processes at the electron microscope level is now quite feasible.
A major break through would be the identification of the cells which are post-synaptic to the dopaminergic amacrine cells. Antibodies have been used to localize cAMP immunohistochemically (Ariano and Matus, 1981). DARPP-32, is claimed to be a phosphoprotein selectively present in neurons receiving dopaminergic input in the rat CNS (Ouimet et al., 1984). These might be useful for labelling post-synaptic neurons to the dopaminergic cells. The post-synaptic neuron, once located, could be examined for neurotransmitter content, which will open up biochemical approaches to studying its response to dopamine. Intracellular recording, followed by iontophoretic filling with a dye, could be employed to trace the electrophysiological characteristics of that post-synaptic neuron. However, this latter technique might be more feasible in retinas of lower vertebrates, which generally have larger, easier to penetrate cell bodies (see Dowling and Dubin, 1984).

In conclusion, with currently available techniques it should be possible to make considerable progress in understanding the cellular physiological effects provoked by dopamine released from the dopaminergic amacrine cells in chicken retina. The advantages of the retina as a model system in general, together with the body of knowledge of the dopaminergic amacrine system in the chicken in particular, make the chicken retina an ideal model system in which to investigate the dopaminergic response. Even if the results are not completely generalizable to other dopaminergic systems, they will at least give guidance for research in these areas.
GENERAL DISCUSSION:

8.3 The role of dopaminergic amacrine cells in the chicken retina.

For studies into the function of a transmitter system, the retina has some major advantages over other systems. What most strikingly distinguishes the vertebrate retina from other model neural tissues is that the function of the retina is well-defined (see Chapter 1.3). The retina transduces photons from the visual world into membrane potentials (photoreceptors), processes the visual information, converts it into action potential codes (ganglion cells), and relays it to other parts of the nervous system (ganglion cell axons). The role of dopaminergic transmission in the retina is to contribute in the process of transforming the simple photoreceptor responses into complex ganglion cell responses. This is obviously an enormous advantage over studies in other tissues, where research into the role of dopaminergic transmission is often only guided by an vague knowledge of the overall function of the whole structure.

Various practical advantages of the retina have been discussed previously (see Chapter 8.2 Physiological effects of dopamine in the chicken retina), but with regard to studies into a function, the retina has some additional advantages. In the retina, all output is channeled through the ganglion cells and optic nerve, and the function of each interneuron must be reflected in the output of the retina. Together with the natural activation by light, this provides a high degree of input/output control for experimental manipulation of the retina. In addition, since most neurotoxins show a rather narrow window for selectivity, there are major problems with lesioning studies in other parts of the nervous system. Firstly, the application of a neurotoxin
often causes physical damage, and secondly, near the site of application a concentric concentration gradient is formed, resulting in effects varying from non-selective destruction to ineffective destruction. The neural retina is a hemisphere, and a needle driven through the sclera, just outside the iris does not damage it. If the injection is aimed at the centre of the vitreous, the whole retina may be exposed to much the same dose of toxin. Further, since each retina is pharmacokinetically isolated, drugs administered to the retina do not usually affect whole body physiology or the other eye. This makes it possible to use contralateral eyes as controls, thus minimizing interanimal differences.

In order to study the function of the dopaminergic amacrine cells in chicken retina, conditions were established in which 6-OHDA selectively disrupts dopaminergic transmission (Chapter 6). In chicken retina, a maximum efficacy of the 6-OHDA treatment was obtained by a prolonged exposure to low concentrations (200-300 nmol daily for 3 consecutive days). Injections of higher doses (> 400 nmol) resulted in considerable side-effects. The prolonged exposure to low concentrations resulted in a long-lasting (up to 31 days) decrease in TH activities by more than 75%. Also dopamine levels and dopamine uptake were diminished, but most importantly, little calcium-dependent release of exogenous dopamine could be elicited by high-potassium. The morphological destruction of the dopaminergic amacrine cells, however, was limited. All processes seemed to be affected, but the number of cell bodies was decreased by only 40%. Similar results have been obtained in other parts of the nervous system, where in general 6-OHDA has been found to eliminate nerve terminals, and spare cell bodies (see Kostrzewa and Jacobowith, 1974). The reports claiming total elimination of the dopaminergic neurons in retinas of various species by 6-OHDA, are
of dubious relevance for studying the function of dopaminergic transmission, because the selectivity of the treatment has rarely been tested (see Chapter 6). With the 6-OHDA lesion described in Chapter 6, functional deficits can, with reasonable certainty, be related to a selectively disturbed dopaminergic transmission. The limitation is that the lesion is not complete, and therefore may not be suitable for studying subtle roles of dopaminergic transmission.

Since the observation that reserpine-treated rats demonstrate photophobia (Malmfors, 1963), and strengthened by the notion (Ehinger, 1983) that the morphology of the dopaminergic neurons is consistent with a generalized regulatory function, dopamine has been thought to be involved in the regulation of overall light sensitivity of the retina. Based on this assumption, I investigated the function of these neurons in the regulation of retinal sensitivity (Chapter 7).

If dopaminergic amacrine cells have a prominent function in the retina, the disruption of dopamine transmission by 6-OHDA is likely to be sufficient to disorganize this function. However, no major changes in retinal sensitivity after 6-OHDA treatment were revealed by these studies, suggesting that the dopaminergic amacrine cells are not crucial for overall retinal sensitivity. The observed minor increases in sensitivity are, however, consistent with reports in retinas of cat (Straschill and Perwein, 1969; Thier and Alder, 1984) and rabbit (Ames and Pollen, 1969; Jensen and Daw, 1983; 1984), where a slight increase in ganglion cell responses were observed after treatment with dopamine antagonists, and a decrease after dopamine agonist.

Pooling the available data on various species, dopamine seems to be involved in a large variety of qualitative properties of the retina. The following aspects were found to be affected by manipulation with the
dopaminergic system: the sensitivity of the rod-driven (dark-adapted) retina, the saturation of VEPs with increasing light intensities, the sensitivity to background illumination in rod-drive retina, the increment threshold in cone-driven retina, the rate of cone-related (but not rod-related) recovery to dark adaptation (chicken, Chapter 7). In addition the following aspects have been reported to be affected by manipulations with the dopaminergic system: the ganglion cell responses accordingly their receptive field organization (rabbit, Ames and Polen, 1969; Jensen and Daw, 1983; 1984), and the centre-surround organization of ganglion cell receptive fields (rabbit and cat, Thier and Alder, 1984; Jensen and Daw, 1984). All these qualitative performances of the retina change with ambient light (Enroth-Cugell and Robson, 1966). These effects of dopamine could be linked together by suggesting that dopamine has a role in regulating the change from scotopic to photopic perceptive states, which would represent a single, though far-reaching, function. But, it is equally possible that the dopaminergic amacrine cells are involved in several independent processes.

Interestingly, in turtle retina, which lacks dopaminergic innervation of the OPL, dopamine has been shown to affect horizontal cell coupling, similar as described in species possessing dopaminergic interplexiform cells (see Chapter 1.4). If one accepts that dopamine released in the IPL can affect the properties of the OPL, this implicates that the approach used so far, involving monitoring of VEPs or single units at ganglion cell level, will not just reveal dopamine's effects in the IPL. To measure dopamine's effects in the IPL, an alternative approach is demanded, e.g. ganglion cell- and amacrine cell responses would have to be compared with bipolar cell responses simultaneously. This is, given current technical limitations, only
possible in lower vertebrates, and then only with great difficulty.

Results concerning dopamine's function in the retina cannot be
generalized to other dopaminergic systems, because whatever dopamine's
function in the retina may be, it must be related in some way to visual
processing.

The data obtained so far indicate that the dopaminergic amacrine
cells may have a regulatory role in the changing of the performance of
the retina, during the transition from scotopic to photopic light
conditions.
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