

The development of tetrahydrobiopterin and guanosine-5'-triphosphate cyclohydrolase: differential patterns in rat brain and pineal gland

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The developmental patterns of appearance of 5,6,7,8-tetrahydrobiopterin (BH₄) and the first enzyme in BH₄ biosynthesis, guanosine-5'-triphosphate cyclohydrolase (GTPcyc), were examined in rat brain and pineal gland. A parallel relationship between BH₄ content and GTPcyc activity was evident in both tissues during development. In brain, the maximal content of BH₄ and activity of GTPcyc was observed 2 days prior to, and 10 days after, birth. In contrast, both pineal BH₄ content and GTPcyc activity became maximal postnatally. The influence of neural input on the developmental appearance of pineal BH₄ was examined in rats that had been superior cervical ganglionectomized shortly after birth. It was found that this procedure did not alter the developmental appearance of BH₄.

There is an absolute requirement for 5,6,7,8-tetrahydrobiopterin (BH₄) by the enzymes which hydroxylate tyrosine and tryptophan, tyrosine-3-monooxygenase and tryptophan-5-monooxygenase⁸. Accordingly, BH₄ is essential for the synthesis of the widely distributed biogenic monoamines derived from these amino acids, including norepinephrine, epinephrine, dopamine, and serotonin. Similarly, in the pineal gland, BH₄ plays an essential role in the synthesis of melatonin from tryptophan via serotonin.

The development of enzymes involved with the metabolism of various biogenic amines has been examined in rodent brain^{1,5} and pineal gland⁴. We have extended this line of research in the present study by examining the developmental appearance of BH₄ and guanosine-5'-triphosphate cyclohydrolase (GTPcyc). This enzyme is of special interest because it is the first¹⁴ and, as was recently shown for rat brain⁷, probably the rate-limiting enzyme in the BH₄ biosynthetic pathway.

Pregnant Sprague-Dawley female rats timed to within 12 h of fertilization were obtained from

Zivic Miller Inc, Allison Park, PA. Animals were not segregated according to sex until 22 days after birth; after this time only male animals were used. The bilateral removal of the superior cervical ganglion was performed by the animal supplier at birth. Biopterin content of whole brains and pineal glands was determined as previously described^{3,6}. This analysis is based upon the iodine oxidation of BH₄ to biopterin, and the subsequent quantitation of biopterin by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection. Briefly, animals were killed during the light period and tissues were immediately frozen on dry ice and stored at -70 °C until assayed. Individual brains or 2–10 pineal glands were later sonicated in 0.1 M HCl containing D-erythro-neopterin as an internal standard. BH₄ in the resulting homogenate was oxidized to biopterin by the addition of a solution of 1% iodine/2% potassium iodide; the mixture was incubated at 24 °C for 1 h. Excess iodine was reduced by the addition of a solution of 1% ascorbic acid. The samples were then centrifuged and the resulting supernatants passed

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through a 0.7×5 cm column of Dowex 50W-X8 (hydrogen form). The column was washed with water and the pterins eluted with 1 M ammonium hydroxide directly onto a 0.7×1 cm column of Dowex AG1-X8 (acetate form). After washing with water, pterins were eluted from this column with 1 M acetic acid. The acetic acid eluate was lyophilized, the residue resuspended in 0.005 M HCl, filtered through a $0.45 \mu\text{m}$ Millipore filter and the resulting sample chromatographed.

The assay of GTPcyc is based upon the conversion of guanosine-5'-triphosphate (GTP) to 7,8-dihydro-*D*-erythro-neopterin-5'-triphosphate (the first pterin intermediate in BH_4 synthesis) and its subsequent oxidation and dephosphorylation to produce *D*-erythro-neopterin². This pterin is then isolated and quantitated by reverse-phase HPLC with fluorescence detection³. Individual brains were weighed and sonicated (1:3.5; w:v) in 0.1 M Tris, pH 8.0 containing 1 mM EDTA and 0.3 M KCl. Samples were centrifuged at 49,000 g for 1 h and 300 μl of the resulting supernatants were desalted by centrifugation through a 1.5 ml column of Sephadex G25 medium equilibrated with homogenizing buffer¹¹. Groups of 2-10 pineal glands were sonicated in 250 μl of homogenizing buffer and the homogenized centrifuged at 49,000 g for 1 h. Preliminary analysis showed that Sephadex G25 treatment of the pineal extract was not required for optimal GTPcyc activity. A 200 μl sample of either the desalted brain or pineal extract was incubated with 50 μl of homogenizing buffer containing 0.5 μmol GTP (final concentration 2 mM) for 1.5-3 h at 37°C. GTPcyc activity in the supernatant fractions prepared from either the adult brain or pineal gland was found to be linear with respect to the incubation times and amounts of protein assayed. The GTPcyc reaction was terminated by the addition of 75 μl of 1 M HCl. 7,8-Dihydroneopterin-5'-triphosphate generated during the reaction was oxidized to neopterin-5'-triphosphate by the addition of 25 μl of a 1% iodine/2% potassium iodide solution and incubation for 1 h at 24°C. The mixture was adjusted to pH 8.0 with 75 μl of 2 M Tris base, and the oxidized phosphorylated pterin was

dephosphorylated to produce neopterin by the addition of 0.5 mg of alkaline phosphatase (from calf intestine, type I) in 50 μl of homogenizing buffer and incubation for 1 h at 37°C. After this incubation, 4 ml of 0.1 M HCl, 250 μl of 100% trichloroacetic acid, and 0.5 μCi of [¹⁴C]guanosine (specific activity 450 mCi/mmol) were added to the samples which were then centrifuged at 3000 g for 15 min. Supernatants were treated as for the analysis of biopterin. [¹⁴C]Guanosine was added to each sample to correct for recovery of pterins through the Dowex 50 and Dowex 1 procedure. Protein content was determined by the method of Peterson¹² with the use of bovine serum albumin as standard.

HPLC analysis of biopterin and neopterin was performed with an ODS-2 reverse-phase C₁₈ column (Whatman, Inc.). The solvent system was methanol:water (1:19), and the flow rate 1 ml/min; chromatography was performed at room temperature. The eluate was monitored

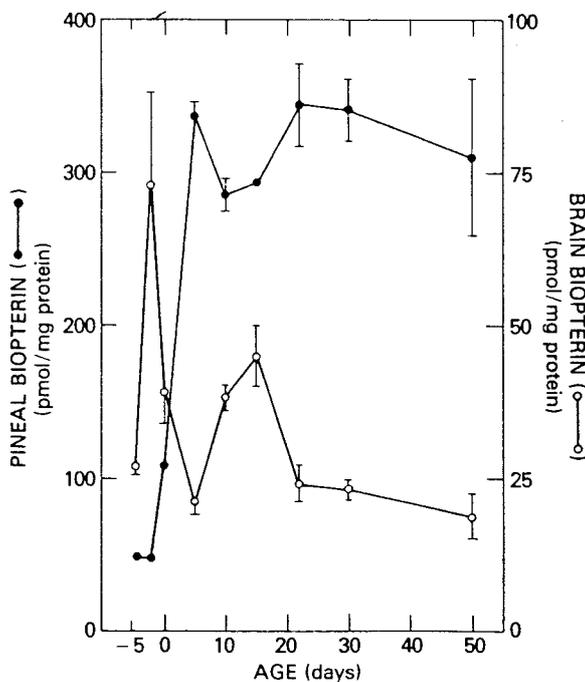


Fig. 1. Pregnant Sprague-Dawley rats timed to within 12 h of fertilization were obtained from the supplier. Animals were not segregated according to sex until 22 days after birth; after this time only males were used. Animals were killed during the light period and BH_4 and protein content of individual brains or 2-10 pineal glands were determined (see text). Each point is the mean \pm S.D. of 3-4 determinations.

for fluorescence (excitation at 330–380 nm; emission at 460–600 nm). Sample bipterin and neopterin contents were determined by peak area analysis.

We observed a marked developmental increase in rat pineal BH₄ content during the 7-day period starting 2 days prior to birth (Fig. 1). During this time the bipterin/mg protein ratio increased about 7-fold from a stable value of 50 pmol/mg protein at 4 and 2 days before birth, to an adult level of 350 pmol/mg protein by postnatal day 5. Pineal bipterin levels then declined significantly ($P < 0.05$; Student's *t*-test) to 280 pmol/mg protein by days 10 and 15. A second peak of 350 pmol/mg protein was observed on days 22 and 30 which was significantly greater ($P < 0.05$) than the previous two values.

In contrast with the pineal gland, where the highest bipterin content was observed after birth, in the brain the highest bipterin content occurred prior to birth. We found that two days before birth the bipterin/mg protein ratio in the whole brain was 75 pmol/mg protein, which is 3-fold higher than that of 50-day-old animals ($P < 0.05$) (Fig. 1). This prenatal period was the only time when BH₄ levels in brain were greater than those of pineal gland. By the fifth day, BH₄ levels decreased 3-fold to 24 pmol/mg protein ($P < 0.05$) and then, between days 10 and 15, levels increased to 40 pmol/mg protein ($P < 0.05$), a value approximately 2-fold higher than that of adult brain. After this time, BH₄ content declined somewhat; by day 22 it stabilized at the adult level of 25 pmol/mg protein. On a protein basis, adult brain contained less than 10% of the BH₄ content of the adult pineal gland.

GTPcyc activity of the developing pineal gland, expressed as pmol neopterin formed/mg protein/min, was found to be correlated with the developmental pattern of pineal BH₄ content ($r = 0.74$) (Fig. 2). The major exception to this was that enzyme activity was undetectable 4 and 2 days prior to birth, a time when BH₄ was clearly present (50 pmol/mg protein). We do not believe that enzyme activity was undetectable due to a lack of assay sensitivity, inasmuch as it was possible with the assay used to reliably detect as little as 3% of the activity detectable at birth.

Rather, we believe that prior to birth the pineal gland may not be capable of synthesizing BH₄ and that BH₄ present in the gland at these times may be derived from extra-pineal sources. The large postnatal increase in BH₄ seen in the pineal gland, as noted above, was accompanied by a large increase in GTPcyc activity. The activity of this enzyme increased almost 20-fold by the fifth day after birth.

GTPcyc activity of the developing brain, expressed as pmol neopterin formed/mg protein/h, was also found to correlate with developmental changes in BH₄ content as observed above ($r = 0.86$) (Fig. 2). In contrast with the pineal gland, however, enzyme activity was detectable in the rat brain prior to birth. Two significant peaks of GTPcyc became evident during maturation of the brain. The first, and larger, peak was observed at 2 days before birth while the second was seen at postnatal days 10 and 15. The specific activity of GTPcyc in the adult brain was approximately 1% of that of the adult pineal gland.

Many, if not all, aspects of pineal function are regulated by the sympathetic adrenergic innervation of the gland by the superior cervical ganglia⁹. In organ culture, the biosynthesis of BH₄ by the pineal gland has been shown to be inhibited by an adrenergic-cyclic adenosine monophosphate-dependent mechanism which leads to a decrease in the level of BH₄⁶. In view of this relationship, we sought to determine whether the sympathetic innervation of the pineal, which reaches adult levels by 2 weeks after birth⁴, is in some way involved in the developmental increase or the adult maintenance of pineal BH₄ levels. However, removal of the superior cervical ganglia at birth did not alter pineal BH₄ levels of 20-day-old animals killed in the light when compared to levels found in sham-operated control animals (47.9 ± 7.1 vs 44.3 ± 3.3 pmol/mg protein). An intact adrenergic input to the gland, therefore, does not appear to be required for the development of normal pineal bipterin content.

The development patterns of appearance of BH₄ and GTPcyc in the brain and pineal gland revealed by this study are of interest for several

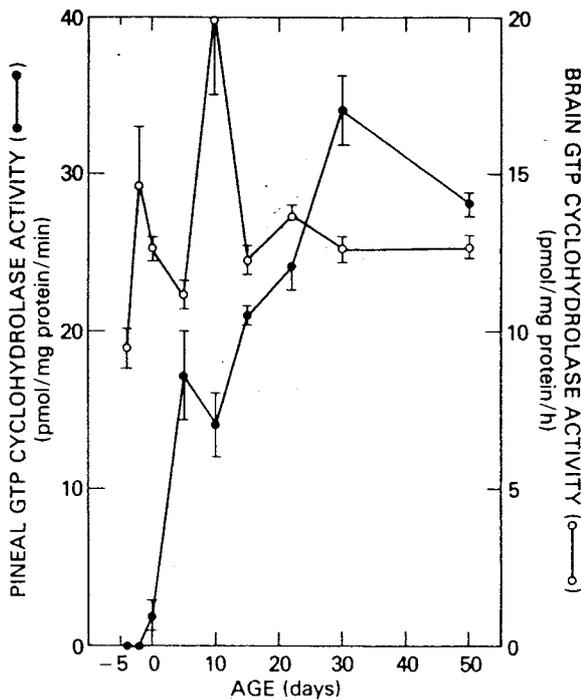


Fig. 2. Pregnant Sprague-Dawley rats timed to within 12 h of fertilization were obtained from the supplier. Animals were not segregated according to sex until 22 days after birth; after this time only males were used. Animals were killed during the light period and GTPcyc activity and protein content of individual brains or 2-10 pineal gland were determined (see text). Each point is the mean \pm S.D. of 3-4 determinations.

reasons. First, there is a clear relationship between BH_4 and GTPcyc activity during development, as indicated by the similar timing of significant changes in both parameters. Thus it would appear that a major factor regulating the amount of BH_4 in these tissues during development is the activity of GTPcyc, an observation which supports the hypothesis that GTPcyc is the rate-limiting enzyme in BH_4 synthesis. These findings also suggest that BH_4 in these tissues is primarily derived from de novo synthesis. The second point of interest is that major developmental increases in BH_4 and GTPcyc activity occur at about the same time that large changes in both tyrosine-3-monooxygenase activity in the brain¹, and tryptophan-5-monooxygenase activity of both brain⁵ and pineal gland⁴, occur. This observation would indicate that a common factor may control the schedule of developmental appearance of these components of the biogenic

amine synthesizing systems. The identity of such a factor is unknown; our studies with superior cervical ganglionectomized animals would indicate that in the case of the pineal gland, a critical factor is not elaborated by the nerves which innervate the gland. A third point of interest is the levels of GTPcyc. Our present results indicate that the activity of this enzyme is about 100-fold greater in the pineal gland than in the brain. This is consistent with previous observations that the activity of tryptophan-5-monooxygenase and the concentrations of BH_4 in the pineal gland are higher than in any other tissue. The reason for this, in part, appears to be related to the high capacity of the pineal gland to make serotonin, which is required to support the high level of melatonin production during the night.

An impressive feature of the developmental appearance of BH_4 and GTPcyc in the brain is the sharp peak at the end of the second week of life. Although it is reasonable to assume that the increase in biopterin is caused by the increase in GTPcyc activity, the significance of the increase in this enzyme activity is obscure. One possibility is that this increase reflects the transient expression of the GTPcyc gene in a differentiating cell type¹³. One might expect that this would be associated with a transient increase in the activity of either of the BH_4 -dependent aromatic amino acid hydroxylases. However, similar peaks in the developmental pattern of these enzymes have not been observed. As has been noted by others¹⁰, the concentration of biopterin in certain neuroendocrine tissues, such as the pineal gland, hypothalamus, and pituitary, exceeds the amount that would appear to be necessary to support tyrosine and tryptophan hydroxylation. Because of this apparent excess of BH_4 , there has been speculation that biopterin is involved in other types of cellular processes beyond those related to its established role as a cofactor for the aromatic amino acid hydroxylases. Perhaps the prenatal increase in brain GTPcyc, and the associated increase in BH_4 , reflect a high level of activity of a biochemical process which is required during this period of brain development, but which is not directly related to the hydroxylation of aromatic amino acids.

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