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Synaptogenesis in the Fetal Corpus Striatum, Globus Pallidus, and Substantia Nigra: Correlations With Striosomes of Graybiel and Dyskinesias in Premature Infants

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Abstract

Synaptogenesis can be detected in tissue sections by immunoreactivity for synaptophysin, a synaptic vesicle glycoprotein that serves as a marker of synaptic maturation. Reactivity was prospectively studied postmortem in sections of the striatum, globus pallidus, and substantia nigra in 172 normal human fetuses and neonates of 6 to 41 weeks' gestation. Caudate nucleus and putamen show patchy reactivity beginning at 13 weeks' gestation around some intracapsular neurons; the pattern is well developed in all regions before midgestation. Near-uniform reactivity throughout the striatum is achieved by 34 weeks, but subtle patchiness is still perceived at term. The globus pallidus shows uniform reactivity without stria from 13 weeks and the substantia nigra from 9 weeks. Synaptic patchiness in the fetal corpus striatum appears to correspond to the "striosomes of Graybiel" that define adjacent neurotransmitter-rich and neurotransmitter-poor zones. Clinical correlation is proposed with dystonic postures and athetoid movements observed in normal preterm neonates of 26 to 32 weeks.

Keywords

corpus striatum, dyskinesias, globus pallidus, striosomes of Graybiel, substantia nigra, synaptogenesis, synaptophysin, caudate, putamen

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The temporal and spatial sequence of synaptogenesis can be demonstrated reliably in formalin-fixed, paraffin-embedded sections of human fetal brain using synaptophysin immunocytochemistry.[1-5] As one of the principal structural glycoproteins of synaptic vesicular walls, synaptophysin is nonspecific in respect of the location of the synapse (axodendritic or axosomatic), function (inhibitory or excitatory), or the nature of the chemical transmitter molecule enclosed within the vesicle. First described in 1985,[6,7] synaptophysin subsequently became widely used in neuropathology, mainly with application to neoplasms and neurodegenerative diseases of adults, but rarely previously applied to fetal brain except for our preliminary study, which also established technical modification for use in fetal brain tissue.[4] Synaptophysin is a robust protein not rapidly degraded by postmortem autolysis and can be reliably demonstrated even after 5 days' delay between fetal death and tissue fixation.[5]

Synaptophysin immunoreactivity in human adult brain is confined to gray matter, but within the white matter reactivity is not only around heterotopic individual neurons but also within coarse axons.[5] This latter reactivity represents recognition by the antibody of peptides of partially constructed synaptophysin molecules that are in the process of axoplasmic transport to the axonal terminal where they will contribute to the formation of synaptic vesicles. Diffuse background synaptophysin activity is seen in fetal brain in many areas of white matter for this same reason, but not as easily resolved as punctate reactivity within axons. In an early study of mature brain, seeking contrasts with ganglion cells in cerebral neoplasms, Miller et al stated, "Normal gray matter structures all showed a diffuse punctate granular pattern of

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neuropil staining without staining of neuronal cell bodies."[8] This observation is confirmed in numerous subsequent studies, including ours, neuronal somata not exhibiting reactivity because the synapses are at the surface plasma membrane, though we have observed cytoplasmic cross-reactivity in the large sensory neurons of the dorsal root ganglia despite the total absence of synapses in dorsal root ganglia.[5] A possible explanation is that some membranous organelles within these cells have a molecular structure similar enough to synaptic vesicle membranes that the synaptophysin antibody recognizes them.

We recently demonstrated synaptogenesis in the fetal hippocampus and cerebral neocortex using synaptophysin immunoreactivity as a marker of synaptic maturation.[5] The present study was designed similarly to study the corpus striatum and globus pallidus. A parallel pattern to the "striosomes" in the developing corpus striatum demonstrated by Graybiel 3 decades ago soon became evident. Clinical correlates with the neurologic maturation of normal premature infants are proposed. These normative data provide an essential background to enable an interpretation of abnormal synaptogenesis in fetal and neonatal encephalopathies.

Materials and Methods

Materials

The 162 normal fetal brains studied prospectively for the study of hippocampus and cerebral neocortex, of both sexes and ranging in age from 6 to 41 weeks' gestation,[5] also had the basal ganglia prepared for this study and were re-examined in this context and further details of these cases are available in a previous publication of these cases.[5] An additional 10 fetal brains were examined by the same methods previously described after that initial study was completed.

Ages of the 172 fetuses and neonates with normal brains in this study were 6 to 8 weeks (n = 4); 9 to 15 weeks (n = 11); 16 to 20 weeks (n = 56); 21 to 26 weeks (n = 59); 27 to 32 weeks (n = 9); 33 to 36 weeks (n = 9); and 37 to 41 weeks (n = 24). Gestational (ie., postconceptional) ages were determined by obstetric data and also were based on fetal and brain weights and standard fetal anthropometric measurements, including crown-rump length, foot size, and other external morphologic features. The sexual differential of the fetuses was 82 male and 86 female, with 4 very young fetuses of indeterminate sex. The male-female ratio was equal in terms of statistical significance.

Fetuses of less than 22 weeks' gestational age were a mixture of induced terminations of pregnancy for nonneurologic disorders that were incompatible with extrauterine life and elective terminations for nonmedical indications. After 25 weeks, fetuses were stillborn or late gestational premature infants who died soon after birth; most of the respiratory insufficiency was inadequately compensated by mechanical ventilation despite intensive neonatal care. Consent for pathologic examination of the products of conception was obtained in accordance with provincial and hospital standards.

Identification of fetal cerebral structures was based on the authors' experience[9-13] and by standard atlases of developmental neuroanatomy.[14-18] Sections of the corpus striatum were taken at the level of the anterior limb of the internal capsule, flanked by the head of the caudate nucleus and the rostral end of the putamen. More caudal coronal sections demonstrated more posterior parts of the putamen, the tail of the caudate nucleus, and the globus pallidus. The substantia nigra in the rostral midbrain also was examined. The subthalamic nucleus and nucleus accumbens provided data only from a few brains because these structures were not included in the original prospective protocol; hence sections at the level of the thalamus did not always pass through it.

Exclusion and inclusion criteria for brains selected for this series are described in the paper focused on the hippocampus and neocortex,[5] and essentially rejected major cerebral malformations, fetal hydrocephalus, chromosomopathies, metabolic diseases, and fetal exposure to potential neurotoxins, including maternal alcohol and drugs. These cases are part of other protocols for which the present normative data are primordial. Cases with extensive postmortem autolysis were excluded. Minor lesions, such as small germinal matrix hemorrhages and acute intrapartum hypoxia or ischemia were not excluded.

Methods

Details of the techniques were previously described.[5] All brains were sectioned transversely (coronally). Anti-synaptophysin antibody was obtained from Novocastra Laboratories (Newcastle-Upon-Tyne, United Kingdom) and distributed through Vison Biosystems (Norwell, Massachusetts). Technical issues for the demonstration of synaptophysin in human fetal brain were described in a previous report.[4] A 1:25 dilution was used with thermal intensification using an HIER steamer. Automated reactivity was performed on Ventana Nexes IHC. Appropriate, simultaneously incubated control experiments were performed with postmortem term neonatal brain tissue that did not show autolysis.

Results

Corpus Striatum

Minimal, subtle reactivity was not seen until 13 weeks (Figure 1). By 15 weeks, a well-defined pattern of reactivity was seen around the intracapsular neurons between fascicles of the anterior limb of the internal capsule and a patchy, striated, or streaked reactivity within both the head and tail of the caudate nucleus and putamen. No perceptible difference was seen in the maturation of these structures. A progressive increase was seen in the intensity of synaptophysin patches and stria, with the nonreactive zones also becoming progressively more reactive until uniformly strong reactivity after 37 weeks' gestation abolished the distinction of the patches. Subtle hints of patchiness still persisted in the term neonate, but even these disappeared postnatally in the first few months (Figures 2-4 and 5C). The axonal "pencil bundles of Wilson" continue to appear in sharp contrast because of their lack of synaptophysin reactivity. The findings in different fetuses of

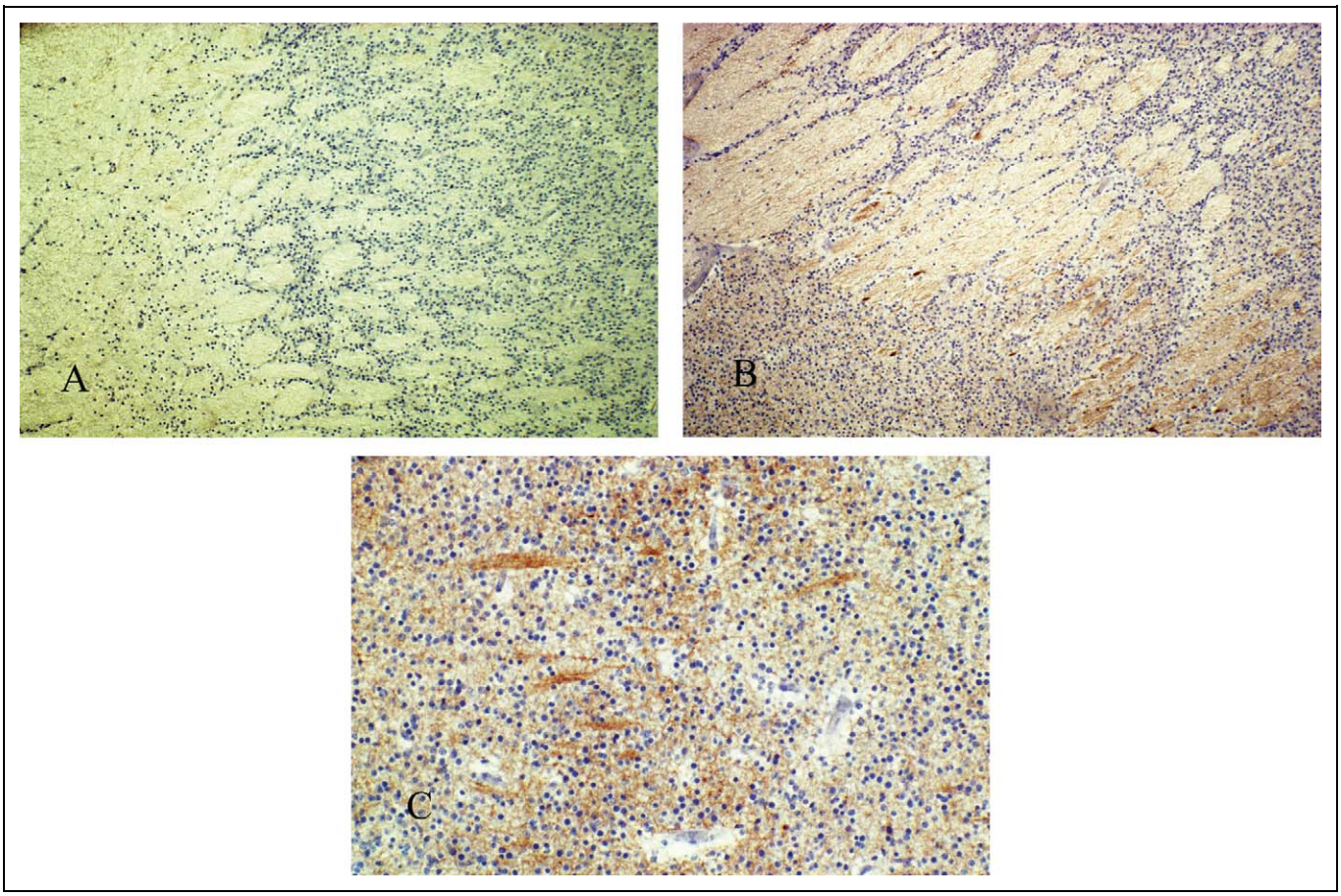

Figure 1. (A) Corpus striatum of human fetus at 9 weeks' gestation. There is no detectable synaptophysin reactivity in the caudate nucleus or putamen or in the neuronal clusters within the small anterior limb of the internal capsule. (B, C) At 13 weeks' gestation, early small linear stria (striosomes) of reactivity are seen focally around intracapsular neurons and adjacent zones, but most of the neuronal clusters are nonreactive. Synaptophysin immunoreactivity. (A and B, ×250; C, ×400).

the same gestational age were nearly identical. No sexual difference was detected.

Globus Pallidus

By contrast with the corpus striatum, the pallidum exhibited no stria or patches at any gestational age, though the multiple coarse intrinsic axonal bundles that are part of the normal architecture of this structure gave a superficial appearance of stria because they often separated the reactive gray matter into thin septa. The inner and outer segments of the globus pallidus achieved their reactivity simultaneously rather than sequentially. Weak immunoreactivity appeared at 13 weeks, simultaneous with the corpus striatum, and became strong and uniform by 15 weeks' gestation and thereafter (Figure 5). There was consistency among fetuses of the same gestational age. In the postnatal period, low magnification of the globus pallidus with synaptophysin immunocytochemistry demonstrates apparent less intense reactivity than the corpus striatum, but this deceptive appearance is due to the coarse, nonreactive intrinsic white matter fascicles dividing the gray matter into multiple thin septa (Figure 5D).

Substantia Nigra

The substantia nigra develops uniform reactivity without patches or stria, similar to the globus pallidus, initially at 9 weeks' gestation and strong by 13 to 15 weeks (Figure 6). No differences were detected in synaptophysin reactivity between pars compacta and pars reticularis, despite different neurotransmitter systems.

Subthalamic Nucleus and Nucleus Accumbens

Only preliminary data are available, but synaptophysin reactivity appears uniform in its development from about 13 weeks in both structures.

Discussion

Two important correlations can be made with the sequence of synaptic development of the corpus striatum. The first is with the pattern of "striosomes" of Graybiel, previously demonstrated by histochemical methods before the availability of antisynaptophysin antibodies. The second correlation is with

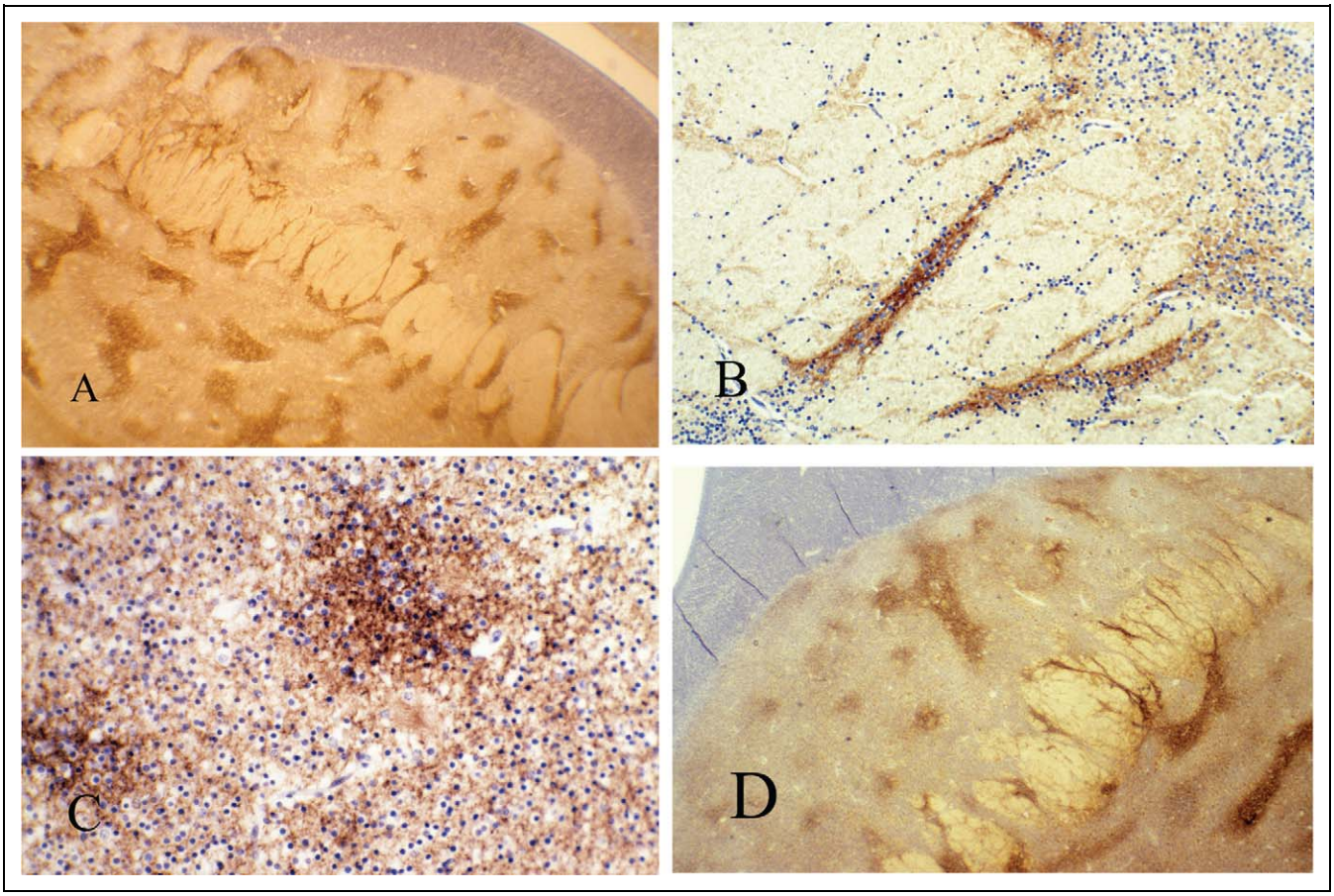

Figure 2. (A-D) Corpus striatum of 3 human fetuses, each at 19 weeks' gestation, demonstrate a high degree of consistency of normal maturation among fetuses of the same gestational age. Synaptophysin-reactive patches are seen in the caudate nucleus, putamen, and neuronal clusters between fascicles of the anterior limb of the internal capsule. (B) Higher magnification of reactive neuronal aggregates in the intracapsular neurons. Synaptophysin immunoreactivity (A,D, ×100; B, ×250; C × 400).

clinical observations of motor activity of preterm infants in the neonatal intensive care nursery.

Patchy synaptophysin reactivity in the corpus striatum, but not in the globus pallidus, was previously demonstrated by Ulfig et al with immunoreactivity similar to that used in our study, in 15 human fetuses of 15 to 22 weeks' gestational age.[19] Our results thus are confirmatory and further expand on theirs. We previously briefly noted this patchy pattern of the immature corpus striatum in preliminary form at the onset of this study,[20] but we have not identified other similar studies in our literature search. Kleinert studied synaptophysin, along with a number of other immunocytochemical markers, in 20 human embryos and fetuses of 3 to 30 weeks' gestation, attempting to compare the developmental pattern to primitive neuroectodermal tumors, but did not note the patchy reactivity in the corpus striatum.[21]

Correlations With Striosomes of Graybiel

The synaptophysin pattern in the developing fetal brain in this study showed a patchy network in the caudate nucleus and putamen that does not correspond to vascular perfusion territories or to clear anatomic boundaries but does follow the pattern of "striosomes of Graybiel" not seen before 13 weeks' gestation. It first became evident beginning in the intracapsular striatum between bundles of axons of the anterior limb of the internal capsule and adjacent zones, the pattern spreading in both directions to form patches within both the head and tail of the caudate nucleus and the putamen. These patches might be regarded as stria, as the name *corpus striatum* implies, and best correspond to the stria of the reciprocal neuropeptides substance P and somatostatin as well as acetylcholinesterase activities described in fetal rat, feline, and human brains in the late 1970s and early 1980s by Ann M. Graybiel.[22-27] In essence, there are zones or stria within the developing corpus striatum that are neuropeptide-rich and neurotransmitter-poor, and adjacent zones that are neuropeptide-poor and neurotransmitter-rich; these latter zones are the ones likely to correspond to the aggregates of strong synaptophysin reactivity at midgestation demonstrated in the present study. This hypothesis is currently being tested in our laboratory using double-labeling of synaptophysin with various neuropeptides and will be reported later as a separate study.

The projection of these striatal neurons is to the thalamus, which at midgestation already has strong synaptophysin

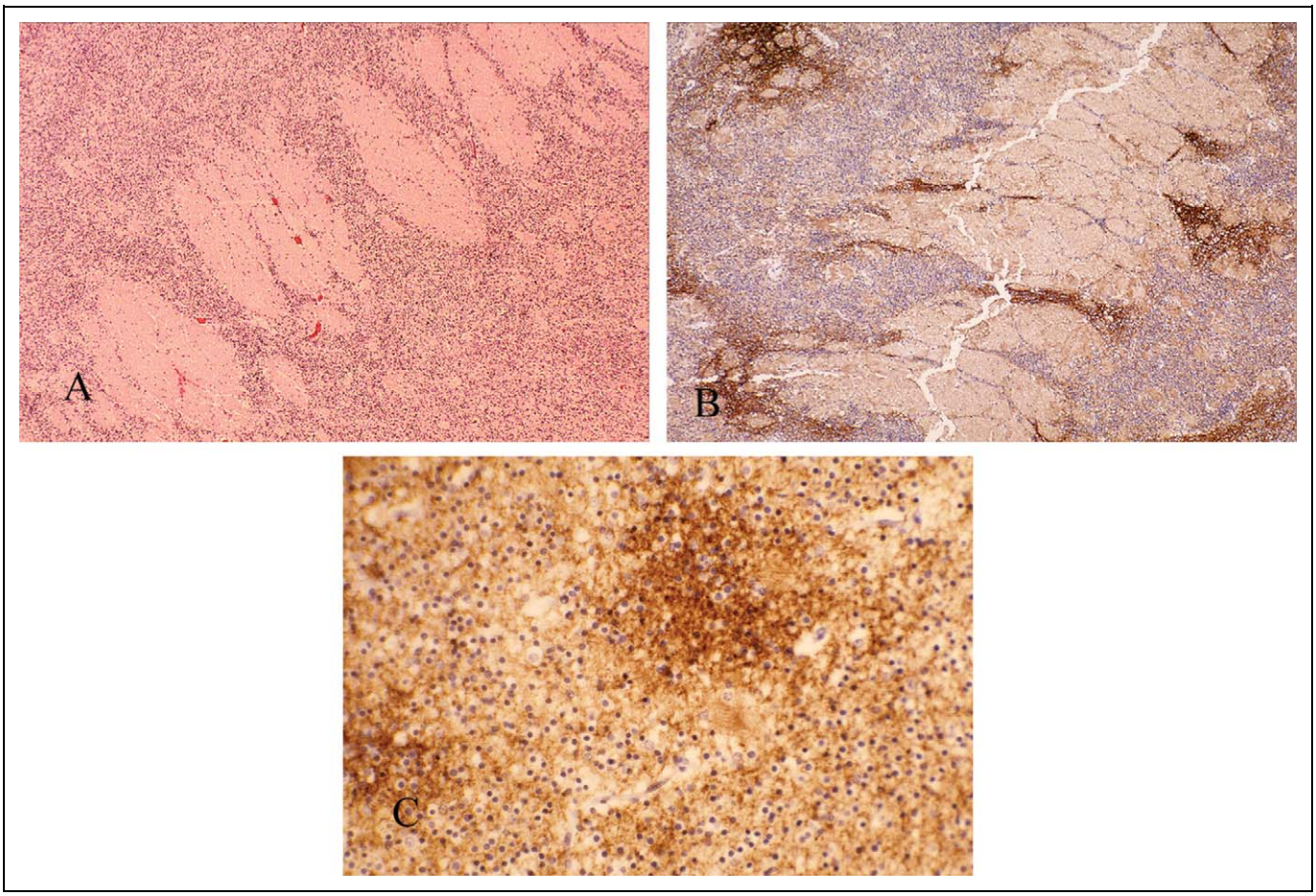

Figure 3. Corpus striatum of a 21to 22-week fetus showing (A) Histological uniformity without distinction of neuronal population. (B) High contrast between sharply demarcated patches of synaptophysin reactivity in both the head of the caudate nucleus and putamen, as well as intracapsular striatal neuronal aggregates (B). At a more caudal level, the tail of the caudate nucleus shows the same patchy expression of synaptophysin as does the head of the nucleus (C). (A, Hematoxylin-eosin; B and C, Synaptophysin immunoreactivity; A and B, ×100; C, ×250).

expression throughout. The medium-spiny neurons of the caudate nucleus and putamen receive cortical inputs from glutamatergic neurons that are excitatory and nigral inputs from dopaminergic neurons that are inhibitory. Many, but not all, GABAergic striatal interneurons coexpress calcium-binding proteins, such as parvalbumin and calretinin, and also nitric oxide synthase, the latter also expressed in other interneurons without these proteins.[28-30]

By contrast with the patchy striosomal pattern of the corpus striatum, our studies indicate that both the inner and outer segments of the globus pallidus show a uniform timing of maturation of synapses around neurons. Synaptophysin immunoreactivity initially appears in the globus pallidus at 14 weeks, simultaneous with the onset in the corpus striatum, suggesting their onset of functioning as a unit. Though the corpus striatum includes the characteristic axonal "pencil bundles of Wilson," and coarser intrinsic fascicles of the same nature occur in both segments of the globus pallidus, the synaptic maturation is quite different in these various structures often grouped together as the "basal ganglia" or "extrapyramidal system," 2 terms not entirely satisfactory.

Mitochondrial respiratory chain enzymatic activities in neurons within and outside the patches of synaptophysin immuno reactivity would be an additional important correlation, but requires frozen sections for this histochemistry; formalin-fixed, paraffin-embedded sections are not suitable. Mitochondrial activity was not studied by Graybiel in the corpus striatum. We recently demonstrated increased neuronal oxidative enzymatic activity in frozen sections of brain resected surgically for the treatment of epilepsy in children,[31] so that this type of study is indeed feasible, at least in fresh surgical tissue. Whether such examination is reliable in human postmortem tissue is uncertain because mitochondrial enzymes rapidly degrade after death. Nevertheless, we are presently pursuing this approach prospectively using freshly frozen sections of fetal brain and hope to resolve these queries and provide supplementary correlative data.

Clinical Correlates

It is difficult to isolate clinical effects of the basal ganglia on motor functions in the fetal and neonatal brain because there are 5 basal ganglionic loops, only 1 of which is motor, and

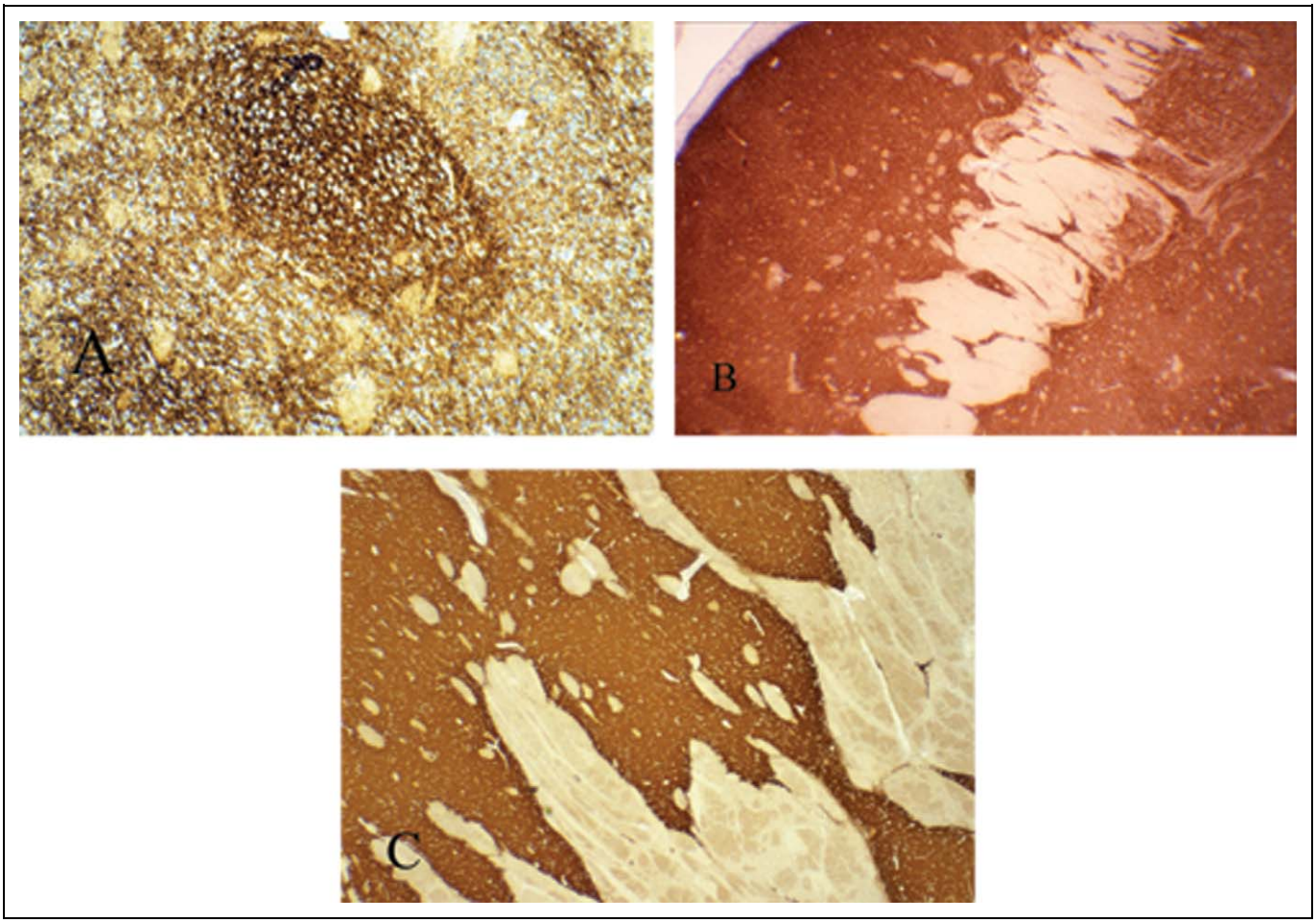

Figure 4. Corpus striatum in fetuses of (A) 26 weeks' gestation and (B) 39 weeks. (A) At 26 weeks, the patchy distribution of reactivity is still quite evident, but weak synaptophysin reactivity also is now appearing in adjacent regions that exhibited none at younger ages (Figures 2 and 3). (B) The term neonate (39 weeks' gestation) exhibits mostly uniform reactivity, but a subtle hint of the former patchy pattern still persists in some areas. The multiple small white (nonreactive) areas are the "pencil bundles of Wilson," intrinsic white matter fascicles; the internal capsule also is nonreactive. (C) This 5-month-old infant girl, born at 28 weeks' gestation, shows uniformly intense reactivity in the putamen and intracapsular nuclei of the corpus striatum. She died of nonneurologic causes and the brain was normal at autopsy. Synaptophysin immunoreactivity. (A $\times$250; B and C, $\times$100).

because all of its influence prevailing on lower motor neurons is mediated through the corticospinal and corticobulbar tracts; in the human CNS, there are no direct descending caudatospinal, putamenospinal, pallidospinal, or nigrospinal tracts, and all influences must be mediated through the corticospinal tract after intermediate synapse in the centromedian nucleus of the thalamus. The corticospinal tract is not fully mature until 2 years of age, but indeed functions prior to that time in the preterm and term neonate, influencing muscle tone and posture, reinforcement of tactile reflexes including suck and swallow, and inhibition of monosynaptic segmental spinal reflexes.[32]

In premature infants between 27 and 34 weeks' gestation, dystonic postures and shoulder movements and athetoid writhing finger and toe movements frequently are noted; these are not called *athetosis* or *dystonia* because these terms imply a pathologic condition. These unstable movements and postures were first documented in 1961 by Albrecht Peiper, a German neonatal neurologist[33] (Figure 7). More recently, dynamic real-time ultrasound studies of human fetuses have shown that such athetoid movements occur in utero before 25 weeks' gestation.[34] The clinical phenomenon may be the clinical correlate of incompletely developed patchy synaptic activity in the striosomes of Graybiel. A second phase of similar movements and postures is intensified in the first few months postnatally, until the infant begins to develop individual finger movements and pincer grasp,[33] corresponding to maturation of the corticospinal tract in terms of terminal axonal ramifications and myelination.

Some cases of attention-deficit disorder without hyperkinesis in children are attributed to mutation of the *DAT1* gene, and this gene is strongly expressed in the corpus striatum but not in the frontal neocortex, which has strong glutamatergic projections to the striatum.[35,36] Whether the aberrant development of synaptic patterns in the fetal caudate nucleus and putamen might be a basis for the later appearance of these cognitive deficits is unknown, but synaptophysin

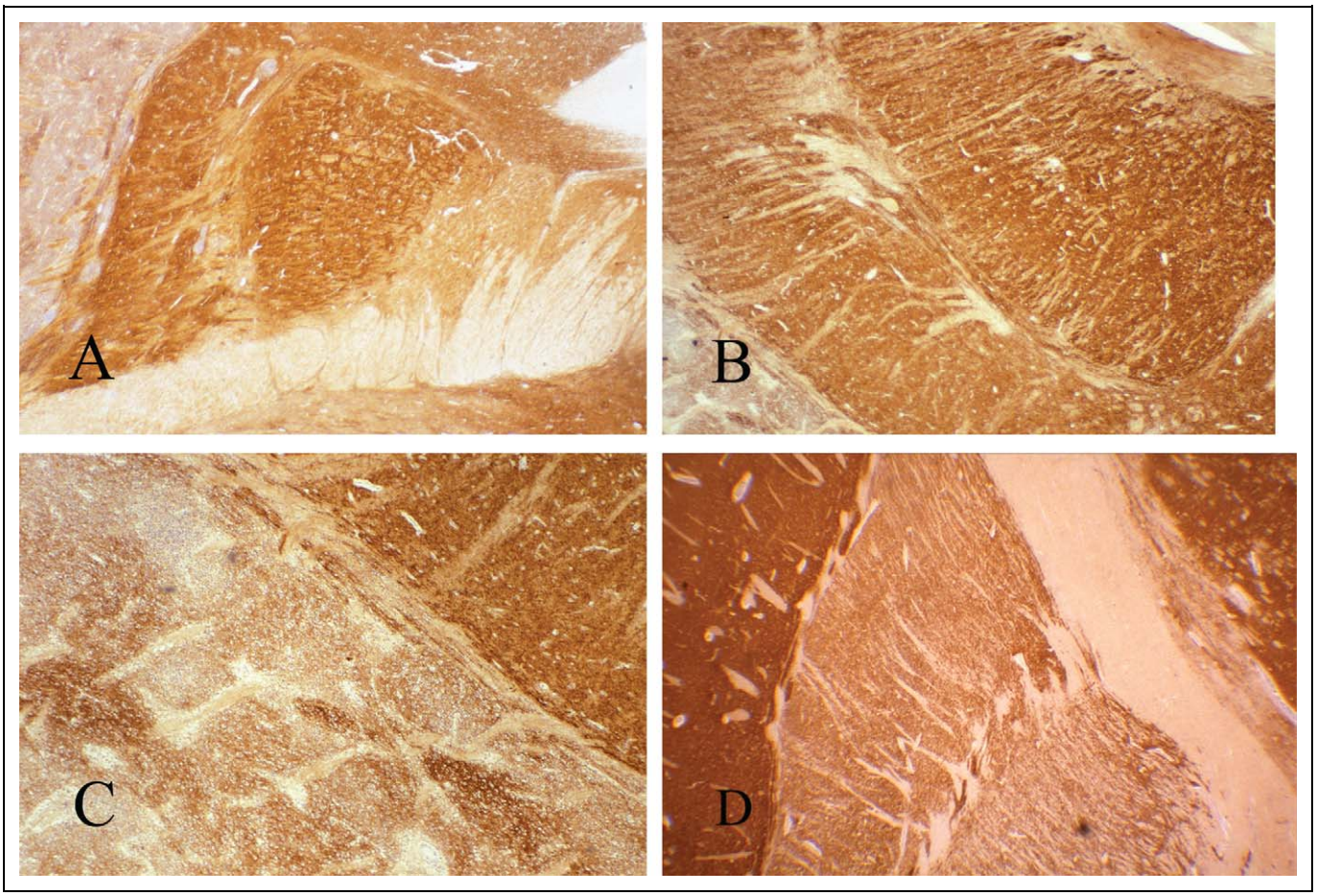

Figure 5. Globus pallidus at (A) 18 weeks and (B) 22 weeks shows strong uniform reactivity in both the inner and outer segments, with only the intrinsic coarse white matter fascicles and internal capsule being nonreactive. (C) Same section as (B) to show the expected patchy reactivity in the posterior part of the adjacent putamen. Reactivity in the globus pallidus appears at 13 weeks, similar to the corpus striatum, but never exhibits the patchy distribution seen in the caudate nucleus and putamen (compare with Figures 2-4). (D) Postnatally, as seen in this 5-month-old premature infant (same as Figure 4C), the globus pallidus appears deceptively less intensely reactive than the putamen because of the growth of the intrinsic voluminous white matter fascicles that separate the septa-like, uniformly reactive gray matter. Inner and outer segments remain equal and the external capsule separating the putamen is nonreactive. Synaptophysin immunoreactivity. (A-C, ×100; D, ×40).

immunoreactivity applied to the corpus striatum in children with such disorders who die of other causes might demonstrate alterations that can be correlated. Transient hypermetabolism is shown in the basal ganglia of neonates by PET functional imaging following perinatal hypoxic encephalopathy,[37] but the intriguing question of whether an inferred functional increase in synaptic activity is accompanied by a quantitative increase in synapses and increased synaptophysin reactivity is not yet confirmed.

Older children may occasionally develop dyskinesias after severe hypoxia. Whether the pathogenesis of these movement disorders involves a selective vulnerability of 1 of the 2 populations of neurons of the corpus striatum so easily identified in the fetus but not easily discerned in the term neonate or at all postnatally remain speculative at this time. The pathogenesis of "athetotic cerebral palsy" in children who have suffered perinatal asphyxia similarly is poorly understood, but also might be a greater selective vulnerability of 1 of the 2 populations of the fetal striosomes. Modern neuroimaging studies, including functional imaging, demonstrate changes in the basal ganglia in some neonates, particularly prematures, who have experienced an episode of severe or prolonged hypotension or intrapartum hypoxia, though the precise mechanism is incompletely understood.[38] Apart from acute hypoxia, a systematic study of the large number of genetically determined dyskinesias, inborn metabolic diseases with dyskinetic features, chromosomopathies, and acquired toxic dyskinesias, including kernicterus and maternal drug abuse, has not been performed using synaptophysin immunoreactivity. This present study of normal maturation in the human fetus provides a basis for interpreting aberrations from the expected developmental pattern in such pathologic conditions.

Neuroanatomic and Phylogenetic Correlates

The reason for the diffuse background activity in the corpus striatum of our fetal brains, but not in the internal capsule, is that the

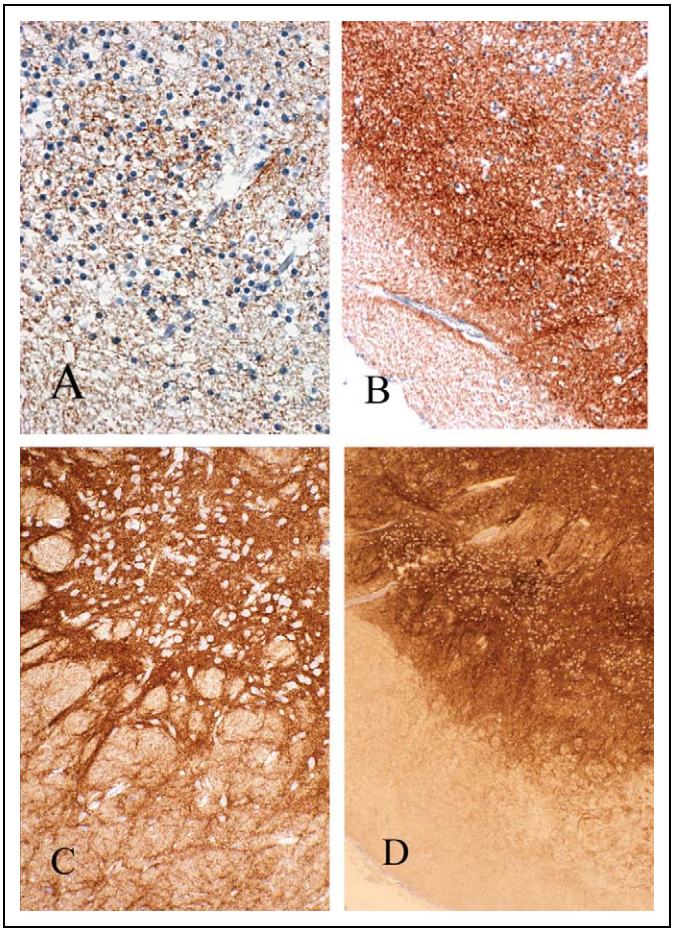

Figure 6. Substantia nigra of the rostral midbrain in fetuses of (A) 9 weeks', (B) 13 weeks', and (C) 15 weeks' gestation and (D) a 40-week full-term neonate. Weak reactivity is first detected at 9 weeks. It becomes more intense by 13 weeks, and by 15 weeks it is uniformly strong throughout, similar to the term neonate though not quite as intense qualitatively. It also is similar in distribution to the globus pallidus, without patches or distinction between pars compacta and pars reticularis. Synaptophysin immunoreactivity. (A, ×400; B and C, ×200; and D, ×100).

internal capsule was more mature because the ascending axons in the anterior limb were already well formed and with established synaptic relation with cortical target neurons, whereas the synaptic maturity in the corpus striatum is less advanced at the same gestational age, as we demonstrate in this present study. In fetal brain during the late first, second, and early third trimesters, apparently nonspecific background reactivity is seen with synaptophysin in white matter. It becomes less intense with advancing gestational age and disappears entirely from some pathways, such as the internal capsule and corpus callosum. It is still seen in some regions at term and postnatally, but in the adult white matter reactivity it is highly localized to some, but not all, axons and surrounding heterotopic white matter neurons[5] (see also H. B. Sarnat et al, unpublished data).

It is not surprising that the spatial and temporal developmental patterns of synaptophysin immunoreactivity are

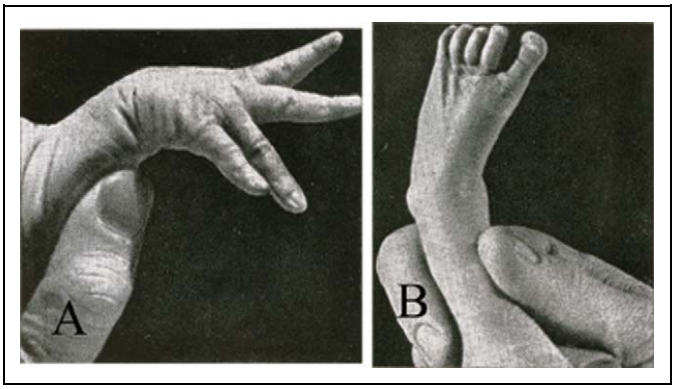

Figure 7. Reproduction of original photographs that appeared in Peiper's 1961 book,[29] published in Leipzig, East Germany, describing the physiological dystonic postures and athetoid movements of (A) the hand and (B) the foot of human preterm infants of 26 to 32 weeks' gestation. These dystonic postures and movements disappear at later gestational ages. These original astute clinical observations were lost in the literature for many years or suppressed for political reasons, but have been confirmed by many subsequent authors in Europe and North America. They correlate well with the striosomes of Graybiel and with the patchy synaptophysin reactivity we observe in the corpus striatum at corresponding fetal ages.

identical in the caudate nucleus, including both the head and tail, and in the putamen. In rodents and other small mammals in which the internal capsule is small, these structures are anatomically continuous in both the fetus[39-41] and the adult rat.[42-45] They also are in continuity in the human fetus through the intracapsular neurons interposed between the individual fascicles of the anterior limb of the developing corpus callosum, but this intracapsular component is not as evident in the adult brain because of enlargement of the internal capsule, associated with myelination, so that the fascicles become more contiguous and the neuropil spaces within it are largely obliterated. In ontogenesis, the caudate nucleus and putamen are derived from the ventromedial part of the primitive telencephalon after cleavage of the prosencephalon at 4 to 5 weeks' gestation. The globus pallidus is partly derived from this region as well, though it is partly derived from the embryonic diencephalon; the entire subthalamic nucleus is of diencephalic origin. The substantia nigra originates in the midbrain neuromere. Whereas this structure is not usually included within the structures designated "basal ganglia," its major axonal projections are to the globus pallidus and it is an integral functional part of the system of motor control, hence its inclusion in the present developmental study.

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Author Contributions

HBS was the principal investigator and is a neuropathologist. RNA is also a neuropathologist, and has experience with striosomes of Graybiel. LFS is a pediatric and neonatal neurologist and took care of the clinical correlates.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The postmortem fetal and neonatal studies described in this article complied with standards and guidelines of the University of Calgary Ethics Committee. Parental consent for autopsy was properly obtained in every case.

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