The development of rat striatal cholinergic interneurons: mechanisms important in location and maturation.

by

E.H.S. van Vulpen

1998

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate department of Anatomy and Cell Biology

University of Toronto

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The development of rat striatal cholinergic interneurons: mechanisms important in location and maturation.

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Abstract

The large aspiny cholinergic interneurons represent less than 2% of the striatal neurons in the rat, but their sensitivity to a relatively small number of afferent synaptic inputs, their tonic firing pattern, and widespread dendritic and axonal fields, place them in an excellent position to participate in the communication between the functionally distinct patch and matrix compartments of the striatum.

The overall goal of this thesis is to investigate the development of these striatal cholinergic interneurons in rats and examine possible mechanisms, such as birthdate or the exposure to the local striatal factor nerve growth factor (NGF), that may affect the location and maturation of the striatal cholinergic neurons.

In the first set of experiments, I analysed the distribution of cholinergic cells within the two compartments during development. The results demonstrate that the cholinergic interneurons become more or less homogeneously distributed between the patch and matrix compartments, although significantly more cholinergic neurons do reside in the intermediate zone (area of the matrix bordering the patch compartment), outside the patch borders in the adult. Additionally, cholinergic neurons mature differentially depending on the compartmental localization, with
cholinergic neurons in the striatal matrix compartment expressing choline acetyltransferase (CHAT) later than the cholinergic neurons in the patch compartment.

The second set of experiments examined the neurogenesis of cholinergic neurons in relation to the striatal compartments and the results suggest that birthdate can predict compartmental localization of the cholinergic neurons in the striatum, with the earliest born neurons (embryonic day 13) having a higher probability of ending up in the patch compartment and the latest born neurons having a higher chance of ending up in the matrix compartment.

The last set of experiments investigated the influence of NGF infusion on the cholinergic maturation. Cholinergic neurons in the patch and matrix compartments have distinct maturational programs, and the results suggest that NGF is able to accelerate the normally slow cholinergic maturation of the cholinergic neurons in the matrix compartment, while the cholinergic maturation of the patch cells seems to be independent of NGF signalling. I propose that the differential induction of CHAT in the striatal cholinergic interneurons might be necessary to ensure the proper connections between the striatal cholinergic neurons and the functionally distinct patch and matrix compartments, in a temporally co-ordinated way.
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ABBREVIATIONS

ABC-  avidin-biotin complex
AChE-  acetylcholinesterase
ACh-  acetylcholine
BDNF-  Brain derived neurotrophic factor
bFGF-  basic fibroblast growth factor
BrdU-  bromodeoxyuridine
CHAT-  cholineacetyltransferase
CNS-  central nervous system
CNTF-  Ciliary neurotrophic factor
DA-  dopamine
dAB-  diaminobenzidine
DNA-  deoxynucleic acid
E-  embryonic day
ENK-  enkephalin
EPN-  entopenduncular nucleus
GABA-  gamma-aminobutyric acid
Glu-  glutamate
GP-  globus pallidus
IZ-  intermediate zone
LAMP-  Limbic system associated membrane protein
mg-  milligram
ml-  milliliter
mRNA-  messenger ribonucleic acid
NGF-  nerve growth factor
NMDA-  N-methyl-D-aspartate
NOS-  nitric oxidesynthase
NPY-  neuropeptide Y
P-  postnatal day
PF- parafascicular nucleus of the thalamus
PBS- phosphate buffered saline
PV- parvalbumin
p75 low affinity neurotrophin receptor
SEM- standard error of the mean
Sub P- substance P
Snr- substantia nigra pars reticulata
TBS- tris buffered saline
TH- tyrosine hydroxylase
TrkA tyrosine receptor kinase A
ug- microgram
ul- microliter
Acknowledgements

I would like to thank everyone who contributed in any way to the realization of this thesis and who supported me throughout all my endeavors in the Toronto lab. A special thank you to all the people who stimulated my original enthusiasm for science over the years and whom without this thesis wouldn’t exist. Last but not least a special thank you to all family and friends who made it possible to continue in spite of all the “detours”.
GENERAL INTRODUCTION

The neostriatum is the largest component of the basal ganglia, receiving inputs from the cerebral cortex and thalamus and sending processed information through the other parts of the basal ganglia and thalamus to areas of frontal cortex implicated in motor planning and execution (Graybiel, 1991; Gerfen, 1992). Dysfunction of the basal ganglia leads to disturbances of movement and cognition exemplified in Parkinson’s disease and Huntington’s disease (Albin et al, 1989; Sullivan et al, 1989). Studies of the cellular organization of the neostriatum have focused mainly upon the spiny projection neurons, which represent the vast majority of neurons, but recent work has revealed the identities and some possible modes of action of the interneurons in the neostriatal circuitry (Kawaguchi et al, 1995).

This thesis deals specifically with the development of one population of these striatal neurons: the cholinergic interneurons. Although the cholinergic neurons represent only a small part of the total striatal population, the levels of acetylcholine and choline acetyltransferase in the neostriatum are one of the highest in the central nervous system (Wetherell et al, 1989). Moreover the excitatory cholinergic neuronal system plays an integrative role in the final circuitry of the striatum. As the establishment of synaptic contact and the subsequent maturation of the cholinergic processes are vital for striatal function, it is essential to gain a better understanding of the development of the striatal cholinergic neurons.

The overall goal of this thesis is to investigate the spatial and temporal development of the striatal cholinergic interneurons and investigate possible mechanisms such as birthdate or the exposure to the local striatal nerve growth factor (NGF) on the location and maturation of the striatal cholinergic neurons.

The compartmental organization of the striatum

The striatum can be divided into two areas, the caudate and the putamen. In cats, monkeys and humans these two areas are physically separated from one another by the fibers of the internal capsule. In rodents however, this subdivision is poorly defined and the two are often referred to as the caudate putamen or simply (neo)striatum (Graybiel and Ragsdale, 1979). The entire striatum can be segregated into two complementary compartments: small patches embedded in a matrix background, although it is clear from serial section reconstructions that the patches actually form an apparently continuous labyrinthian compartment through the striatum.
(Graybiel and Ragsdale, 1978; Groves et al, 1988). This intricate interdigitation of the patch and matrix compartments has only emerged with mammalian evolution, and is precisely conserved across mammals. Independent of mammalian order, the patch compartment makes up approximately 15% of the total striatal volume and the area of individual patches increases with the size of the striatum, with the total number of patches remaining the same (Johnston et al, 1990). The patch-matrix organization was first identified in the adult striatum with the report of enriched μ opiate receptors in patches (Pert et al, 1976). Later Graybiel and Ragsdale (1978) reported that acetylcholinesterase (AChE) staining was weak in zones that they termed striosomes. The acetylcholinesterase poor striosomes appeared to exactly coincide with the μ opiate receptor patches (Herkenham and Pert, 1981). These neurochemical markers, together with somatostatin fibers and calbindin-immunoreactive neurons (which are both located in the matrix) display a fairly consistent patch-matrix distribution throughout both the dorsal and ventral striatum, with the exception of the shell region of the nucleus accumbens. Other neurotransmitters, receptors, enzymes and even neuronal connections have also been found to respect the boundaries of the two compartments in the adult (Gerfen, 1984; Graybiel, 1984; van der Kooy and Fishell, 1987).

**Organization of striatal neurons**

The principal neuronal cell type in the neostriatum is the medium spiny projection neuron and as much as 95% of the total striatal population belongs to this group (Kemp and Powell, 1971; Graveland and DiFiglia, 1985). Striatal projection neurons can be divided into two general categories: the striatonigral and striatopallidal systems. The striatopallidal neurons contain gamma-aminobutyric acid (GABA) and Enkephalin (ENK) and project to the globus pallidus (GP). The striatonigral system contains GABA, substance P, and dynorphin and provides axon collaterals predominantly to the substantia nigra pars reticulata (SNr) and the entopeduncular nucleus (EPN) (Gerfen and Young, 1988). The medium spiny projection neurons projecting in either the striatonigral or the striatopallidal system are located in roughly equal percentages in both the patch and the matrix compartments. Several studies in the rat have demonstrated that the dendrites of the projection neurons are restricted to the compartment of the parent neuron (Gerfen, 1985; Penny et al, 1988; Kawaguchi et al, 1989), although some dendritic crossings from one compartment into the other have been reported in the cat, ferret and primate (Bolam et al,
This suggests that inputs that are confined to the patch compartment will mainly affect patch output neurons, whereas inputs directed to the matrix will affect matrix output neurons. Therefore, the patches and matrix are thought to comprise a system of functionally distinct parallel input-output processing channels, although information from one compartment may influence the other via indirect pathways. Communication between the two compartments is thought however to be mainly mediated by the striatal interneurons, which can extend their dendrites across compartmental boundaries (Penny et al, 1988; Kawaguchi et al, 1989). These interneurons make up approximately 5% of the total neuronal population and are characterized by cell bodies of variable size and aspiny or sparsely spiny dendrites (Dimova et al, 1980; Chang et al, 1982; Bolam et al, 1984). Four major classes of interneurons have now been identified and these classes differ in firing modes, spatial territories of dendrites and axons, and neurochemical identity (Kawaguchi, 1992, 1993; Kawaguchi et al, 1995). They are: (1) the large cholinergic neurons, which are identifiable by the presence of choline acetyltransferase (CHAT); (2) GABAergic interneurons that contain parvoalbumin, one of the calcium binding proteins; (3) GABAergic interneurons that contain calretinin; and (4) a class of interneurons that contain somatostatin, NADPH-diaphorase, and nitric oxide synthase (NOS), and that perhaps employ GABA as well. Double-labeling studies have shown these classes of cells to be non-overlapping. Smaller numbers of cholecystokinin and vasoactive intestinal peptide (VIP) positive aspiny neurons have also been described (Takagi et al, 1984; Theriault and Landis, 1987).

In the adult striatum, the GABA-ergic interneurons containing parvoalbumin, as well as the cholinergic and somatostatin population have been reported to be more or less homogeneously distributed within both compartments, with a tendency to reside on patch/matrix borders with axons and dendrites frequently crossing the boundaries between the patch (striosome) and matrix compartment (Cowan et al, 1990; Gerfen, 1984; Graybiel et al, 1986, 1990; Rushlow et al, 1996). These neurons are therefore located ideally to play a role in patch/matrix integration.

**Function of striatal compartments**

The most convincing evidence that the patch and matrix compartments are functionally specialized is that the input-output connectivity of the striatum is organized in relation to these
compartments. The patches receive afferent fibers from the deep layers of the prelimbic and cingulate cortices and send projection fibers to the substantia nigra pars compacta. The matrix on the other hand receives afferent fibers mainly from the superficial layers of the frontoparietal cortical areas and sends projection fibers to the pallidum and substantia nigra pars reticulata. Patches with their special neurochemistry and special linkages to limbic structures such as limbic cortex (Gerfen, 1984; Donoghue and Herkenham, 1986) and amygdala (Ragsdale and Graybiel, 1988) are thought to modulate motivational and emotional information, whereas the matrix receiving input from sensorimotor cortical areas (Gerfen, 1984; Donoghue and Herkenham, 1986) might be more tightly linked to specific sensory and motor systems.

Formation of the striatal compartments

The striatum originates from an enlarged area of forebrain ventricular zone called the ganglionic eminence (Smart and Sturrock, 1979). The period of neurogenesis in the rat neostriatum occurs mainly between embryonic day 12 (E12) and postnatal day 2 (P2) (Bayer, 1984; Marchand and Lajoie, 1986), with the majority of striatal neurons becoming postmitotic between E13 to E20. After striatal neurons become postmitotic, they leave the ventricular zone and migrate radially and tangentially to assume their position within the striatum (Halliday and Cepko, 1992).

Some progress has been made in unravelling the mechanisms that underlie the formation of the striatal compartments. First, of neuronal proliferation with $[^3]$H thymidine show that birthdate can predict compartmental fate. The earliest striatal neurons to leave the mitotic cycle in the rat embryo become restricted to the patch compartment (Lanca et al, 1986; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987; Fishell and van der Kooy, 1991) while neurons that become postmitotic at later embryonic times, join the matrix compartment (van der Kooy and Fishell, 1987). The patches of $[^3]$H thymidine labelled striatal cells can be also detected after specific embryonic injections in cat (Graybiel and Hickey, 1982) and monkey (Brand and Rakic, 1979). Also, cell lineage analysis studies suggest that patch/matrix lineage segregation is a critical developmental separation. Chimeric and retroviral tracing analysis of proliferating cell lineages (Fishell et al, 1990; Krushel et al, 1993a) showed that apparently there are separate precursors in the embryonic germinal zone of the forebrain that give rise to striatal patch versus matrix neurons.
Furthermore *in vitro* studies have revealed that dissociated early born patch neurons are selectively adherent to other early born patch neurons (Krushel et al, 1989). This is in contrast with the later born neurons which are non adhesive (Krushel and van der Kooy, 1993b). The selective adherence of the early postmitotic patch cells to other patch cells, after the massive migration of later born matrix cells out of the ventricular zone and their intermixing with the patch cells already out in the striatum, is hypothesized to underlie the striatal compartmentalization (Krushel and van der Kooy, 1993b; Krushel et al, 1995). This selective adhesiveness also appears to be a characteristic of the early born deep cortical neurons. The selective adhesiveness of the early born striatal and cortical neurons is not an attribute of all early postmitotic forebrain neurons, because early born neurons dissociated from the septum/basal forebrain were found dispersed within reaggregates (Krushel and van der Kooy, 1993b).

The presence of a cell surface glycoprotein on patch neurons which makes them ‘sticky’ and adhere to each other, is a likely possibility, since many glycoproteins have been recognized as important for adhesion, cell recognition and migration (Hynes and Lander, 1993). A possible candidate for this glycoprotein might be Limbic system- Associated Membrane protein or LAMP. LAMP has been found to be selectively expressed in limbic system structures and to be selectively expressed in the patch compartment of the neostriatum (Chesselet et al, 1991). Additionally, glycoconjugate boundaries have been found to surround patches in the neostriatum during the perinatal period (O’Brien et al, 1992; Steindler et al, 1988). Two other cell adhesion molecules, Thy-1 (Shults and Kimber, 1993) and KG-CAM (Kuga et al, 1995), and members of the immunoglobulin super family have been shown to be present in patches in the early postnatal rat striatum, while homogeneously distributed in the maturing striatum and might be involved in striatal compartmentalization.

While most striatal neurons follow the general rule that birthdate can predict compartmental fate, with neurons that become postmitotic early in neurogenesis contributing primarily to the patch compartment (E12-E15), and neurons that become postmitotic late in neurogenesis (E17-E20) contributing primarily to the matrix compartment, an exception to that general rule are the cholinergic interneurons. In the adult they have been reported to be more or less homogeneously distributed between both compartments (Graybiel et al, 1986; Kubota and Kawaguchi, 1993). This is surprising, since all striatal cholinergic neurons in the rat are born
before E18, with the majority becoming postmitotic between E12 and E15 (Semba et al, 1988; Phelps et al, 1989), a time that corresponds to the “patch destiny”.

**Organization of the cholinergic striatal interneurons**

These large or giant aspiny neurons (diameter range 22-30 μm in rat) were first identified as cholinergic following their staining with AChE histochemistry (Fibiger, 1982), which was later confirmed by the histochemical localization of CHAT immunoreactivity (AChE is now known to be also expressed in the somatostatin interneurons). In the rat, the cholinergic interneurons have been reported to be more or less homogeneously distributed between the striatal compartments. This is in contrast to the heterogeneous distribution in the cat, due to the higher density of CHAT cells in the vicinity of patches within the dorsolateral striatal quadrant (Martone et al, 1993, 1994). The difference in CHAT cells density may represent a species difference, as many neuroactive compounds are more pronounced in cats and primates than in rodents.

Although the cholinergic neurons are relatively sparse, representing less than 2% of the striatal population in rats, they exert a profound influence on striatal function. Striatal cholinergic interneurons have large cell bodies that extend aspiny dendrites over relatively large domains (up to 1000 μm in extent) and extend an even more widespread axon collateral (Wilson et al, 1990), which remains confined to the striatum and makes symmetrical synapses on dendritic shafts and perikarya of the major actor in striatal function, the GABAergic medium spiny projection neuron (Phelps et al, 1985; Izzo and Bolam, 1988). The medium spiny projection neurons of both major types (GABA/SubP or GABA/Enk) are relatively enriched in the m1 and m4 muscarinic ACh receptors, with most striatal projection neurons expressing the m1 receptor, and approximately half of the striatal output neurons expressing the m4 subtype (Weiner et al, 1990; Bernard et al, 1992). So far however, no synaptic potential elicited in projection neurons by cholinergic interneurons has been identified. This input could possibly act by altering the excitability of the cells via changes in voltage sensitive ion channels instead of eliciting discrete synaptic potentials and it has been proposed that acetylcholine stabilizes the state of medium spiny cells whether they are in a depolarized or a hyperpolarized state, by modulation of the A-current (Akins et al, 1990; Kitai and Surmeier, 1993).

Anatomical studies have shown that the cholinergic interneurons predominantly receive thalamic inputs from the parafascicular nucleus (PF) of the thalamus, at least on their proximal
dendrites (Lapper and Bolam, 1992). However, afferent fibers from the PF nucleus almost exclusively innervate the matrix compartment (Herkenham and Pert, 1981) and distal dendrites of CHAT cells extend also into the patch compartment (Penny et al, 1988, Kawaguchi, 1993). These data suggest that cholinergic cells may integrate input from both the thalamic axons at the matrix and the unidentified fibers in the patch, and affect projection cells in the matrix. The thalamic inputs are hypothesized to be glutamatergic (Lapper and Bolam, 1992), and glutamate has been shown to increase striatal ACh release through the activation of the NMDA receptor (Scatton and Lehmann, 1982). Therefore thalamostriatal fibers to cholinergic interneurons acting through NMDA receptors could be responsible for the stimulation of release of ACh in vivo. Cholinergic cells express mRNA that encodes the functional receptor unit (NR1) of the NMDA receptor (Landwehrmeyer et al, 1995; Chen et al, 1996). Other glutamatergic input on the cholinergic neurons may come from the cerebral cortex, as giant cholinergic neurons receive monosynaptic cortical inputs (Wilson et al, 1990) and cortical ablation reduces striatal ACh turnover (Consolo et al, 1990). Morphological evidence however shows only rare synaptic contacts of cortical terminals on distal labelled cholinergic dendrites (Dimova et al, 1993).

SubP has been shown to release endogenous ACh in vivo and in vitro from the rat striatum and SubP positive axon terminals have been observed to make synaptic contact with dendrites of cholinergic neurons (Bolam et al, 1983,1986; Martone et al, 1992). Cholinergic neurons also express the NK-1 (neurokinin) receptor (Gerfen, 1991; Aubry et al, 1993, Kaneko et al, 1993), which indicates that the cholinergic cells are likely to be an important direct target for SubP released by axon collaterals of GABA/SubP axons of spiny neurons that project to the substantia nigra. (The cholinergic and somatostatin interneurons are the only striatal neurons that express the relevant NK-1 receptor). It has been hypothesized that striatonigral neurons may affect striatal medium spiny neurons and substantia nigra neurons by a GABA receptor mediated process and simultaneously induce release of acetylcholine from striatal cholinergic neurons by a substance P receptor mediated process.

Cholinergic cells also have the ability to respond to dopaminergic agonists and they receive a very sparse input from the nigrostriatal DA pathway via tight appositions or symmetrical synapses located at the proximal level of the cell body or dendrite (Chang, 1988; Kubota et al, 1987; Dimova et al, 1993).
Table 1. Physiological and chemical properties of cholinergic striatal interneurons

<table>
<thead>
<tr>
<th>Morphology</th>
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<tr>
<td>inputs</td>
<td>- thalamus (Parafascicular nucleus)</td>
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<td>- striatal medium spiny projection neurons via axon collaterals 2,3</td>
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<td></td>
<td>- nigral 4,6</td>
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<tr>
<td></td>
<td>- cortex (rare)</td>
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<td>- interneuron (Som and GABA/PV)</td>
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<td>axon collaterals</td>
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<tr>
<td>receptors (percentages)</td>
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<tr>
<td></td>
<td>- D2 (80%) 11</td>
</tr>
<tr>
<td></td>
<td>- m1 (muscarinic ACh) (88%) 12</td>
</tr>
<tr>
<td></td>
<td>- m2 (muscarinic ACh) (80%) 12</td>
</tr>
<tr>
<td></td>
<td>- m4 (muscarinic ACh) (80%) 12</td>
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<tr>
<td></td>
<td>- NMDA receptor 13,14</td>
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<td></td>
<td>- GluR1 15</td>
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<td>- GluR4 15,16</td>
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<td></td>
<td>- neurokinin-1 17,19</td>
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<tr>
<td></td>
<td>- 5HT1 (&lt;20%) 20</td>
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<td></td>
<td>- trkA (99%) 21,22</td>
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<td></td>
<td>- Sub P (92%) 18</td>
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1- Lapper and Bolam, 1992           12- Bernard et al, 1992
4- Chang, 1988                       15- Martin et al, 1993
5- Kubota et al, 1987               16- Tallaksen-Greene and Albin, 1994
6- Dimova et al, 1993                17- Gerfen, 1991
7- Kawaguchi et al, 1995             18- Aubry et al, 1993
8- Phelps et al, 1985               19- Kaneko et al, 1993
9- Izzo and Bolam, 1988              20- Morilak and Ciaranello, 1993
11- Le Moine et al, 1990            22- Steininger et al, 1993
                                          23- Le Moine et al, 1994
The spiny projection neurons integrate the effects of thousands of inputs but are relatively insensitive to each one and they utilize a wide range of sub threshold membrane potentials for integration of synaptic inputs over a relatively long time course (Wilson, 1993). Large aspiny cholinergic neurons on the other hand only receive a relatively small number of afferent fibers but are highly sensitive to individual synaptic inputs, resulting in firing in a tonic irregular pattern, which makes them distinct from the other striatal neurons (Wilson et al, 1990). By shaping the activity of the spiny neurons, the tonically active cholinergic neurons may modulate the spiny neurons.

Possible functions of striatal cholinergic neurons

There have been a variety of roles attributed to the striatal cholinergic interneurons over the years. They have most often been featured as afferent interneurons, relaying inputs from dopaminergic afferents to the projection neurons. The majority of the striatal cholinergic neurons is known to express the D_{2} receptor, while some of the cholinergic neurons express the D_{1} receptor subtype (Le Moine et al, 1990, 1991). Stimulation of D_{2} receptors via dopamine or D_{2} agonists participate in the control of ACh release, while D_{2} antagonists decrease ACh release. (D_{1} receptors are not involved in the regulation of the release of ACh from neostriatal tissue in vitro). The finding that most dopaminergic afferents end directly on the projection neurons, rather than on cholinergic interneurons (Freund et al, 1984) has made this hypothesis less attractive, although the cholinergic neurons do receive a very sparse input from the nigrostriatal DA pathway via tight appositions or symmetrical synapses located at the proximal level of the cell body or dendrite (Chang, 1988; Kubota et al, 1987; Dimova et al, 1993).

A second possible function is that the striatal cholinergic neuron may function as an associative interneuron in the neostriatum. The neostriatum is lacking an associative fiber system comparable to that of the corticocortical system or the associative fibers of the hippocampus. Cholinergic interneurons have large somata which can be found in both the matrix and the patches, widespread dendritic trees, extending over a region much larger than the projection neurons, and often crossing the boundaries between the two compartments, which makes these cells capable of integrating synaptic inputs over relatively large regions. Their axonal fields are also very extensive compared with those of most of the other striatal neurons (Wilson et al, 1990). Axons of the cholinergic neurons have a tendency to arborize in the matrix compartment
(Graybiel et al., 1986; Kawaguchi, 1992) which results in a very pronounced AChE or CHAT fiber staining in the matrix area, which is often used as a negative patch compartment marker. The influence of the cholinergic interneurons may therefore predominate in the matrix and the direction of associational signals has therefore also to be directed principally towards the matrix. However the m1 cholinergic receptor is more concentrated in the patch/striosomal system than in the matrix, and the enhanced m1 binding in the patches could serve to offset the lower density of cholinergic neuropil in the patches (Nastuk and Graybiel, 1989). Of special interest are the tonically active neurons (TAN’s) in the primate striatum, which resemble the tonic firing and prolonged afterhyperpolarization characteristics of the cholinergic neurons in the rodent striatum (Wilson et al., 1990; Kawaguchi, 1992), and which tend to reside on patch/matrix borders. The TAN’s could possibly act as some kind of associative interneuron, as they are thought to be involved directly in collecting reinforcement related information from the patches and influence the activity of the projection neurons in the matrix (Graybiel and Kimura, 1995).

Beyond their involvement in multiple local circuit interactions through synaptic mechanisms, cholinergic interneurons can be thought to participate in non synaptic modulation of the spiny output neurons in the striatum. The cell body of a giant CHAT neuron appears to be in direct somatic apposition with one to four perikarya of medium sized spiny neurons (Pickel and Chan, 1991) which is supported by other reports describing the participation of these giant cells on cell clustering in the neostriatum (Mensah, 1977, 1980). Furthermore the presence of nematosomes or nucleolus-like bodies within the nucleus and cytoplasma of the giant aspiny neuron in the striatum, thought to constitute a storage or transit form of nuclear derived material (Dimova et al., 1993), point to a possible dynamic role of these cholinergic neurons in the control of synaptic transmission and intercellular communication or adhesion. The tonically active cholinergic neurons may modulate (or be modulated by) non-cholinergic spiny neurons through non-synaptic somatic or dendritic apposition. Also both neurons may be simultaneously inhibited by shared afferent input. Activation of this system could facilitate coordinated movements through synchronization of cholinergic neurons and spiny projection neurons (Pickel and Chan, 1991).

The sensitivity of the giant aspiny interneuron to a relatively small number of proximal afferent synaptic contacts, its tonic firing and its widespread dendritic and axonal fields place it in an excellent position to act as a modulator of the excitability of the neostriatal projection neurons in advance of the onset of striatal activity.
Factors influencing striatal cholinergic differentiation

A variety of growth factors have been identified over the last 50 years which can act as trophic factors. Neurotrophic factors, which are a subset of growth factors acting on neural tissue, can be defined as "endogenous, soluble proteins regulating survival, growth, morphological plasticity, or synthesis of proteins for differential functions of neurons" (Hefti et al, 1993). NGF (nerve growth factor) was the first discovered neurotrophic factor to play an important role in survival, maintenance, sprouting and differentiation of neurons in the developing peripheral nervous system (e.g. Levi-Montalcini, 1975; 1987). In the central nervous system (CNS), NGF’s role as a survival factor is less clear and many of NGF’s actions in the CNS are more compatible with a role as differentiation inducing factors rather than survival factors. The most investigated NGF responsive system in the CNS; the cholinergic neurons of the basal forebrain and the striatum, show increases in choline acetyltransferase activity, hypertrophy of the cell body and sprouting of the processes in culture, during development and in the adult (discussed below). Over the years a variety of other factors have been reported to be capable of influencing cholinergic cell survival, differentiation and sprouting. Other growth factors like BDNF (brain derived neurotrophic factor), bFGF (basic fibroblast growth factor), and immune cytokines like IGF-I (insulin-like growth factor), IL-3 (interleukin), IL-6, and interferon all elevate choline acetyltransferase activity in forebrain cholinergic neurons (Hama et al, 1989; Kamegai et al, 1990; Knusel et al, 1990, 1991; Miller-Jonakait et al, 1994). From the list above only interferon (Miller Jonakait et al, 1994) has been reported to have a small effect on cultured striatal cholinergic neurons.

NGF as the local factor in the environment, is probably the most likely candidate for the differentiation of cholinergic neurons within the striatum. NGF has been shown to increase CHAT activity in fetal dissociated striatal culture (Hatanaka and Tsukui, 1986) and organotypic cultures of fetal rat striatum (Martinez et al, 1985). *In vivo* experiments in the neonatal rat also show an increase in CHAT activity after NGF infusion icv (Mobley et al, 1985; Aloe, 1987). In the adult rat, exogenous NGF chronically infused into the lateral ventricle was shown to increase CHAT immunoreactivity in the cholinergic neurons and also the size of the cholinergic cell bodies in the caudate putamen of adult rats (Gage et al, 1989; Hagg et al, 1989). Moreover, when the striatum is injured neonatally or in the adult, NGF can completely block the degenerative effects of the lesion (Aloe, 1987; Gage et al, 1989). Also chronic NGF administration in aged rats has
been reported to stimulate CHAT activity and improve behavioral performances related to the striatum (Williams and Rylett, 1990; Williams et al, 1993).

NGF was the first discovered member of a family now known as the neurotrophins (other mammalian members being BDNF, neurotrophin (NT)-3 and NT-4/5). Neurotrophins bind to two kinds of transmembrane glycoproteins, p75 and members of the trk family of receptor kinases (Meakin and Shooter, 1992; Bothwell, 1995; Chao and Hempstead, 1995). The trk receptor kinases bind neurotrophins with higher affinities than p75, distinguish between neurotrophins and are essential for functional responses in vitro (Meakin and Shooter, 1992; Bothwell, 1995; Chao and Hempstead, 1995; Greene and Kaplan, 1995; Lewin and Barde, 1996). So far three members of the family have been identified in vertebrates; trk A, B and C. It seems that under physiological conditions, trkA (p140) is the preferred receptor for NGF, although there is a possibility of cross-talk between certain neurotrophins and receptors, and there is some evidence that NT-3 and NT-4 may be able to elicit responses via trkA (Chao, 1992; Belliveau et al, 1997).

p75 binds all neurotrophins with similar affinity and was until recently not thought to be a functional receptor in the absence of a trk receptor. However recent reports show some intriguing functions of the p75 receptor. It is important for the developing nervous system because mice bearing a null mutation in the p75 gene have decreased pain sensitivity and a diminished cutaneous innervation (Lee et al, 1992) and cultured sensory neurons from these mice are less sensitive to NGF than wild-type neurons (Davies et al, 1993). Furthermore it has been proposed that p75 is capable of facilitating the maturation of cholinergic cells (Verdi et al, 1994) and p75 might also be involved in mediating cell death (Rabizadeh et al, 1993; Casaccia-Bonnefil et al, 1996; Frade et al, 1996; van der Zee et al, 1996).

Several studies have shown striatal perikarya in the developing striatum expressing the low affinity p75 NGF receptor protein and mRNA, while in intact adults either no p75 cells can be detected (Yan and Johnson, 1988; Gage et al, 1989; Koh and Higgins, 1991) or a small number limited to the ventrolateral part of the striatum. Cholinergic cells that lose their p75 receptor during the postnatal development, are however able to re-express this receptor in response to NGF infusion (Gage et al, 1989; Hagg et al, 1992) (or even tissue damage (Gage et al, 1989)), or ciliary neurotrophic factor infusion (Hagg et al, 1992). The developmental pattern of the supposedly high affinity receptor trkA on the other hand is quite different. The specific activity of high affinity NGF binding increases with age and stays high (Mobley et al, 1989). This correlates
with *in situ* hybridization for trkA which shows only a few scattered, lightly labelled trkA mRNA positive cells in early postnatal striatum, after which labelling intensity rapidly increases (Ringstedt et al, 1993). High affinity NGF binding sites are uniformly distributed in the adult neostriatum (Richardson et al, 1986; Raivich and Kreutzberg, 1987) and the distribution of these binding sites correlates with the distribution of the cholinergic neurons. Moreover trkA is present in all cholinergic neurons in the adult striatum (Holtzman et al, 1992; Steiniger et al, 1993).

**Objectives of the present study**

The striatal cholinergic interneurons are born early in striatal neurogenesis and previous studies have shown that early born neurons mainly end up in the patch compartment. However in the adult rodent striatum, cholinergic neurons appear to be homogeneously distributed within the two compartments in the striatum. In order to investigate this intriguing discrepancy, the present thesis research started with the exploration of the distributions of cholinergic cells within the two striatal compartments during development (Chapter II). Factors important in pattern formation of the striatum are also likely to be important for the cholinergic interneuronal population. Events like birthdate (Chapter III), or the exposure to the local factor NGF in the environment (Chapter IV), were also investigated for their importance in determining the location and maturation of the cholinergic interneurons. Differentiation of the cholinergic neurons may also be affected by different NGF signalling mechanisms present on the cholinergic neurons, and therefore the distribution of the p75 and the trkA receptor between striatal compartments during the perinatal period was also investigated (Chapter IV).
Chapter II

Differential maturation of cholinergic interneurons in the striatal patch versus matrix compartments

Abstract
Striatal neurons are generated in two distinct phases. Neurons that become postmitotic early in embryonic development come to be located primarily in the patch compartment of the striatum, while the majority of the neurons situated in the striatal matrix compartment are generated later in embryogenesis. The cholinergic interneurons in the striatum, which have been reported to be more or less homogeneously distributed in the adult, are all generated early in development. Given that early generated neurons are expected to be situated primarily in the patch compartment, we investigated the apparently homogeneous distribution of cholinergic neurons by analysing their localizations in the patch and matrix compartments during striatal development. To selectively mark the striatal patch compartment we made injections of the retrograde fluorescent tracer True Blue in the substantia nigra on embryonic day 20 or postnatal day (P)1, and then stained for choline acetyltransferase (CHAT) at different timepoints in development. After P7, the distribution of the CHAT positive neurons changes from an earlier preference for the patch compartment to a preference for an area of the matrix just outside of the patches. Absolute counts show that this change in distribution is caused mainly by a late turn on of CHAT by the cholinergic neurons in the matrix compartment. These data suggest that there are different compartmental subpopulations of cholinergic neurons in the striatum.
INTRODUCTION

The mammalian striatum is a heterogeneous structure that can be divided into two complementary and functionally distinct compartments; the patches and the matrix. Patches, with their connections to limbic structures such as limbic cortex (Gerfen, 1984; Donoghue and Herkenham, 1986) and amygdala (Ragsdale and Graybiel, 1988), are thought to modulate motivational information, whereas the matrix, which receives input from sensorimotor cortical areas (Gerfen, 1984; Donoghue and Herkenham, 1986), may be more tightly linked to specific sensory and motor functions (White, 1989). The medium spiny projection neurons, which are located in both the patch and matrix compartments, are the principal cell type in the neostriatum, and their dendrites appear to be restricted to the compartment of the parent medium spiny neuron (Penny et al., 1988; Kawaguchi et al., 1989). This suggests that inputs that are confined to the patch compartment will affect patch output neurons, whereas inputs directed to the matrix will affect matrix output neurons. The remaining small minority of the striatal neuronal population, the cholinergic, GABAergic and somatostatin/NPY interneurons, have been reported to be more or less homogeneously distributed within the striatum (Cowan et al., 1990; Gerfen, 1984; Graybiel et al., 1986; Graybiel, 1990). Those striatal interneurons situated in the patches and in the area of the matrix just outside the patches have been reported to send dendrites into both the patch and matrix compartments (Penny et al., 1988; Kubota and Kawaguchi, 1993). The large aspiny cholinergic interneurons (20-40 μm in diameter) represent less than 2% of the striatal neurons in rats, but may exert a profound influence on striatal function. The sensitivity of the cholinergic interneuron to a relatively small number of proximal afferent synaptic inputs, its tonic firing and its widespread dendritic and axonal fields, place it in a excellent position to participate in the communication between the patch and matrix compartments. (Wilson et al., 1990; Dimova et al., 1993; Lapper and Bolam, 1992; Kawaguchi, 1992, 1993).

The organization of the striatum into two compartments can be seen early in embryogenesis. Studies of neuronal proliferation with \(^{3}H\) thymidine demonstrated that birthdate can predict compartmental fate (Graybiel and Hickey, 1982; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987; Fishell and van der Kooy, 1991). The earliest striatal neurons to leave the mitotic cycle in the rat embryo (between embryonic days (E)12-17) become restricted mainly to the patch compartment, and neurons that become postmitotic at later embryonic times (E18
until postnatal day (P)2 join primarily the matrix compartment (Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987; Fishell and van der Kooy, 1991). Birthdate studies combined with markers for cholinergic neurons revealed that cholinergic neurons are born early in striatal neurogenesis (between E12 - E17; Semba et al., 1988; Phelps et al., 1989), and therefore would be predicted to be located mainly in the patches. However, in the adult striatum cholinergic neurons have been reported to be more or less homogeneously distributed between the patch and matrix compartments (Graybiel et al., 1986; Kubota and Kawaguchi, 1993). To study this intriguing discrepancy, we analysed the distributions of cholinergic cells within the two compartments during development. The results indicate that cholinergic striatal interneurons mature differentially depending on compartmental localization, with the cholinergic neurons located in the patches turning on CHAT earlier than the cholinergic neurons in the matrix.

MATERIALS AND METHODS

Labeling of early projecting striatonigral neurons

Timed pregnant Wistar rats (Charles River) were used, and the presence of a vaginal plug was taken as embryonic day E1 (by Charles River). In order to selectively mark the patch compartment, we labeled the early projecting striatonigral neurons with microinjections of the retrograde fluorescent tracer True Blue in the substantia nigra (Fishell and van der Kooy, 1987a). For the embryonic injected group, pregnant rats were anesthesized with pentobarbital (60 mg/kg) and laparotomies were performed. Each rat fetus (E20) was oriented inside the uterus and the dorsal calvarian suture lines were used to estimate the location of the midbrain. The uterine wall was punctured with a 25 Gauge needle and a 0.2-0.4 μl injection of 5% True Blue was injected bilaterally with a 1μl Hamilton syringe, into the ventral midbrain. A cyanoacrylic glue (Crazy Glue) was used to seal the puncture in the uterine wall and after suturing, the pregnant rats were allowed to recover until the following day (E21). The pregnant mother was reanesthesized with pentobarbital and the E21 embryos were each perfused intracardially with a 1 mM NaNO₂ phosphate buffered saline (PBS) rinse, followed by 4% paraformaldehyde, 0.5% glutaraldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4). For all the other groups (ages of sacrifice: P3, P7, P17, P40, and P75; with P75 considered as the adult timepoint), unmanipulated pregnant mothers were allowed to give birth and the day of birth was taken as P0. On P1, the rat pups were anesthesized using hypothermia, and 0.5μl of a 5% solution of True
Blue was injected bilaterally with a 1μl Hamilton syringe into the ventral midbrain. After survival times of 2 to 74 days the postnatally microinjected animals were reanesthetized with sodium pentobarbital and then perfused with phosphate buffered saline, containing 1mM NaNO2 followed by 4% paraformaldehyde, 0.5% glutaraldehyde and 15% picric acid in 0.1 M phosphate buffer (PH 7.4). After perfusion, the brains were removed and postfixed for 18-24 hr in the same fixative but without the glutaraldehyde. Prior to sectioning the brains were rinsed with Tris buffered saline (TBS) and 40μm (E21 brains) and 50μm (other age groups) frontal vibratome sections were cut.

At the times of the E20 and P1 microinjections, the majority of the patch neurons will have made their striatonigral connections, whereas the majority of the matrix neurons do not form their efferent connections to the substantia nigra until later in the first postnatal week (Fishell and van der Kooy, 1987). Long term survival experiments in a report by Fishell and van der Kooy (1987) showed that an embryonic injection of True Blue in the substantia nigra results in distinct striatal patches of labeled neurons with minimal striatal matrix labeling (regardless of survival time). On the other hand, a nigral injection at the end of the first postnatal week labels striatal neurons throughout the patches and the matrix. Therefore True Blue can only be available for axonal uptake at its injection site for a few days at the most. Given that striatal matrix neurons do not send their axons to the substantia nigra until later in the first postnatal week (Fishell and van der Kooy, 1987), the majority of the neurons that will be retrogradely labeled after the E20 and P1 microinjections (regardless of the times of sacrifice) will be the early projecting medium spiny neurons in the striatal patch compartment. Neurons retrogradely labeled by True Blue transported from the perinatal substantia nigra accurately delineate patch borders, as their distribution exactly matches the tyrosine hydroxylase fiber patches seen early in development (Fishell and van der Kooy, 1987), and as their distribution marks the same patch borders labeled by an E13 pulse of a birthdate marker (Fishell and van der Kooy, 1991). Also, the distribution of neurons labeled by an injection of a birthdate marker at E13 exactly matches the adult striatal distribution of yet another widely used patch compartment marker (i.e. the high opiate receptor binding specific for the patch compartment; van der Kooy and Fishell, 1987). Taking these factors into account, we conclude that True Blue can be used to accurately mark the patch compartment, as long as it is injected before the majority of the matrix neurons make their efferent connection to the substantia nigra.
Figure II. 1
Photomicrographs of CHAT positive neurons and True Blue labeled patches in a single coronal striatal section from a P7 rat. A P1 pup was microinjected with True Blue in the substantia nigra, sacrificed on P7 and immunocytochemically stained for CHAT. (A) shows two patches which contain True Blue retrogradely labeled striatal projection neurons (white cells), (B) demonstrates examples of CHAT positive neurons in the patches (arrowheads); in the intermediate zone (arrows) and in the rest of the matrix compartment (unmarked), (C) shows a double exposure of the True Blue labeled patches and the CHAT labeled neurons. Arrowheads and arrows point to the same CHAT positive cells in both (B) and (C). The intermediate zone was defined arbitrarily as two cell diameters outside the patches (using the average diameter of CHAT positive neurons as the standard). Scale bar = 100 μm.
Figure II. 2

A representative injection site (from a P1 injection, P7 sacrifice) in the ventral midbrain of the retrograde fluorescent tracer True Blue, showing that the injections infringed upon both the pars compacta and pars reticulata of the nigra. A cross-hatched line separates the substantia nigra, pars reticulata from the substantia nigra, pars compacta. Scale bar = 500 μm.
Immunocytochemistry

The vibratome sections were collected in 0.05M TBS (pH 7.6). After rinsing them thoroughly with TBS to rinse out the picric acid, the sections were incubated free floating for 40 hr at 4°C with the primary antibody to choline acetyltransferase (CHAT, 1:500, Chemicon). After rinsing, the sections were incubated in a goat anti rabbit secondary antibody conjugated to biotin (1:200, Vector labs), followed by an avidin-biotin complex conjugated to peroxidase (Elite ABC kit, 1:700, Vector labs). All antibodies were diluted in 0.5% Triton X-100 and 2.5% normal goat serum in 0.05M TBS. The peroxidase was visualized with 0.05% diaminobenzidine containing 0.01% H₂O₂. The sections were mounted onto chrom-alum gelatin coated slides, dehydrated and coverslipped with Fluoromount (BDH). True Blue retrogradely labeled neurons and CHAT immunoreactive neurons were visualized in the same section with a combination of brightfield and epifluorescence (at 360 nm for the True Blue labeled neurons). In this way both labels (the DAB immunoreactive cholinergic neurons and the fluorescent retrogradely labeled neurons) could be observed at the same time, which was especially important in determining the locations of cholinergic neurons with respect to the retrogradely labeled patches (Fig. 1). All results presented below are obtained from injections filling and restricted to the ventral midbrain (Fig. 2). This type of injection has been previously reported to yield a reproducible distribution of retrogradely labeled striatal neurons (Fishell and van der Kooy, 1987). As the striatal compartments were most easily delineated by True Blue labeling within the head of the striatum (head of the caudate-putamen in rat), the area of striatum studied was limited to the entire striatum rostral to the globus pallidus and dorsal to the nucleus accumbens. Behind the rostral pole of the globus pallidus, some patches coalesce into elongate stripes at all of the ages studied. Camera lucida tracings were made of the retrogradely labeled patches to determine the relative proportions of the patch and matrix compartments within the head of the striatum at the different timepoints (Fig. 3). For each timepoint, the number of rats used depended on obtaining both good retrograde labeling of the patch compartment neurons and good choline acetyltransferase immunostaining in the same animal (total of n=6 for E21, n=5 for P3, n=5 for P7, n=5 for P17, n=7 for P40, and n=6 for P75). The camera lucida drawings were also used to measure cross-sectional areas, in combination with a Macintosh computer bit pad and the software package Blueprint from Graphsoft. An estimate of the total volume of the head of the striatum was obtained by multiplying cross-sectional area, section thickness and the total number of sections. The average
cholinergic neuron diameter was measured from 50 randomly chosen cholinergic neurons in the striatum at each of the age groups, and all cell counts were corrected by the Abercrombie method (1946).

**Quantification**

The distribution of CHAT immunoreactive neurons was examined in relation to the striatal compartments. Therefore, in each animal the striatal cholinergic neurons were counted at two rostrocaudal levels (e.g. at the level of the nucleus accumbens, and at the mid caudal level of the anterior commissure decussation) throughout 2-3 sections per level in the head of the striatum. Because cholinergic neurons have been reported to lay near patch borders with some of their processes crossing the borders (Penny, 1988; Kawaguchi, 1993), we investigated if there was a higher concentration of cholinergic cells in the region just surrounding the patches. This region, named the intermediate zone, is the part of the matrix surrounding the patches and was arbitrarily defined as two cell diameters (using cholinergic cell diameter at age studied as the standard) from the patch border. Thus, the cholinergic neurons were located within one of the three following areas: the patch compartment, the intermediate zone, or the matrix area (which is the matrix compartment minus the intermediate zone). We did not notice a difference in cell counts between the two rostrocaudal levels, and therefore we first averaged the mean counts from the two levels and then averaged the counts from the animals in each age group of sacrifice. The numbers of cells in the different areas (the patch compartment, the intermediate zone and the matrix compartment minus the intermediate zone) were then transformed to a density measure (by equating the areas for volume differences). If CHAT cells are homogeneously distributed, then by equating the volumes of the three areas we expected that each group would contain 33% of the CHAT positive cells. Using a $\chi^2$ test we asked if the observed distributions of CHAT cells in each area were significantly different from those expected from a homogeneous distribution of CHAT positive cells throughout the striatum. To estimate the total number of cholinergic neurons in the different areas (patch, matrix and intermediate zone) of the striatum at each age, we calculated the total number of cholinergic neurons within the entire volume that each area comprises within the head of the striatum. The effects of age on the total cell counts in the three different striatal areas were evaluated by an analysis of variance, and then by Newman-Keuls tests.
Figure II. 3
Schematic diagrams based on camera lucida drawings of the striatal patches at different timepoints. Patch neurons (stippling) are retrogradely labeled after an injection in the substantia nigra at E20 (sacrifice at E21) or P1 (sacrifice at P3, P7, P17, P40, P75). The anterior commissure is illustrated in black on the medial side of the striatum. As the medial striatum is the last striatal area in development to take on a patchy distribution of patch neurons, a homogeneous region of retrogradely labeled cells in the medial striatum can still be visualized in the medial striatum at E21. The hatched area in the E21 diagram is the remaining ventricular and subventricular zones.
RESULTS

Estimates of compartmental volumes

Microinjections of the fluorescent retrograde tracer True Blue into the substantia nigra at E20 or P1 labeled projection neurons in the patch compartment, and these dense patches of TB labeled cells were used to estimate the percentage of the volume of the head of the striatum taken up by the patch compartment (Table 1). The same animals were also used for the cell counts described below. Because the majority of the neurons in the matrix compartment are generated between E18 and P2 (van der Kooy and Fishell, 1987; Fishell and van der Kooy, 1991), one explanation for the change in the relative proportions of the striatal patch versus matrix compartments after E21 (from 19.1% at E21 down to 10.9% at P3), is the increase of matrix neurons in the striatum. The change in the relative proportions of striatal patch versus matrix compartments after P17 (from 10.3% at P17 to 17.7% at P40), might be explained by the earlier dendritic outgrowth and maturation of the neurons (projection- and interneurons) in the patch compartment compared to the matrix compartment. The percent volume of the intermediate zone, which is dependent on the increasing cholinergic cell diameter with age in our estimates, as well as on the size and the number of patches, was calculated using the values for the patch compartments.

To estimate the total cholinergic population in the head of the striatum, we calculated the volume of the head of the striatum for each age group (Table 1). These estimates for striatal volume were corrected for the volumes that the fibers of passage comprised at each age. The volume estimates for the head of the striatum vary somewhat from two previous reports (Fishell and van der Kooy, 1991; Fentress, 1981), and this may be due to the use of vibratome sections in this study, compared to the paraffin embedded or cryostat sections used previously. The paraffin embedding procedure causes considerable tissue shrinkage and may account for the lower volume measurements reported by Fentress (1981). Volume measurements by Andén (1966) from the atlas of König and Klippel (1963) give approximately the same numbers as in this study.
Table II. 1 Mean (± S.E.M) total volumes of the head of the striatum (mm³) at different ages and the percentages of these volumes occupied by the patch compartment, intermediate zone, and the rest of the matrix compartment.

<table>
<thead>
<tr>
<th>Volume</th>
<th>E21(n=6)</th>
<th>P3(n=5)</th>
<th>P7(n=5)</th>
<th>P17(n=5)</th>
<th>P40(n=7)</th>
<th>P75(n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>3.1(±0.4)</td>
<td>7.5(±0.5)</td>
<td>12.4(±0.6)</td>
<td>16.5(±0.4)</td>
<td>20.2(±1.1)</td>
<td>27.3(±1.4)</td>
</tr>
<tr>
<td>%Patch</td>
<td>19.1</td>
<td>10.9</td>
<td>12.0</td>
<td>10.3</td>
<td>17.7</td>
<td>17.4</td>
</tr>
<tr>
<td>%IZ</td>
<td>4.6</td>
<td>3.0</td>
<td>2.9</td>
<td>2.8</td>
<td>6.0</td>
<td>7.8</td>
</tr>
<tr>
<td>%Matrix</td>
<td>76.3</td>
<td>86.1</td>
<td>85.1</td>
<td>86.9</td>
<td>76.3</td>
<td>74.8</td>
</tr>
</tbody>
</table>
Figure II. 4

The densities of CHAT neurons are expressed as the percentages of the total number of CHAT neurons in striatal areas when the areas are equated for volume differences. A $\chi^2$ statistical test was used to ask if the observed relative distributions of CHAT neurons in each striatal area (the patches, the intermediate zone and the matrix minus the intermediate zone, were significantly different from an expected homogenous distribution of CHAT neurons throughout the striatum. * indicates a significant difference ($P<0.05$) from the expected homogenous 33% value. The horizontal solid line is the 33% value predicted if CHAT neurons were homogeneously distributed. IZ = intermediate zone. Data represent means $\pm$ S.E.M.
Distributions of cholinergic neurons in the striatum

The relative distributions of the cholinergic neurons in each area changes during development (Fig. 4). From E21–P3 cholinergic neurons are located preferentially in the patches (E21, $\chi^2_{0.05} = 20.5$, df = 5; P3, $\chi^2_{0.05} = 14.7$, df = 4), when compared to the 33% value predicted from a homogeneous distribution. However on P7 and thereafter, the cholinergic distribution changes to a preferential location within the intermediate zone, when compared to the 33% value predicted from a homogeneous distribution (P7, $\chi^2_{0.05} = 46.1$, df = 4; P17, $\chi^2_{0.05} = 56$, df = 4; P40, $\chi^2_{0.05} = 17.5$, df = 6), and this preference remains in the adult (P75, $\chi^2_{0.05} = 17.5$, df=5).

From E21–P17 there was a significant lower density of cholinergic neurons in the rest of the matrix compartment, however an increase in the relative density of cholinergic neurons in the matrix compartment was observed over postnatal development such that in the adult the density of cholinergic neurons in the matrix was not significantly different from what would be expected from a homogeneous distribution of cholinergic neurons in the striatum (E21, $\chi^2_{0.05} = 18.9$, df = 5; P3, $\chi^2_{0.05} = 25.5$, df = 4; P7, $\chi^2_{0.05} = 23.8$, df = 4; P17, $\chi^2_{0.05} = 20.5$, df = 4).

These changes in the relative distributions of cholinergic neurons could be explained by the following possible mechanisms: 1- migration of the cholinergic neurons from the patches into the intermediate zone and matrix area; 2- cholinergic cell death or turn off of CHAT by some neurons in the patches; 3- late expression of CHAT by the cholinergic neurons in the matrix. To discriminate among these mechanisms, we calculated the absolute numbers of CHAT positive cells in each striatal area (the patch compartment, the intermediate zone and the matrix compartment minus the intermediate zone for each age group) (Fig. 5). An analysis of variance on the absolute CHAT cell counts within the three striatal areas across age revealed a significant interaction between age and striatal area ($F(10,84) = 12.0, p < 0.05$). Significant main effects of age were seen for each area; $F(5,28)= 4.3$, $p < 0.05$ for the patch compartment; $F(5,28)= 11.9$, $p < 0.05$ for the intermediate zone and $F(5,28)= 16.3$, $p < 0.05$ for the matrix area. In the patches, the total number of CHAT neurons reached its maximum at P3, and Newman-Keuls tests showed a significant decrease of the number of CHAT positive neurons in the patch compartment between P3 and P17 ($p < 0.05$). On the other hand, the total number of CHAT neurons in the intermediate zone increases to adult levels by P7 (with a significant increase in the number of CHAT positive neurons between P3 and P7; $p < 0.05$). The total number of CHAT positive neurons in the matrix area increases to adult levels by P17 with a significant increase
Figure II. 5

The total number of cholinergic neurons throughout the head of the striatum that are located in the different areas at different ages. IZ = intermediate zone. Data represent means ± S.E.M.
between P3 and P17; p < 0.05). The total number of all CHAT positive neurons in the striatum (the combined number of CHAT positive neurons in patches, intermediate zone and matrix area) continues to increase after P3. Approximately half of the total number of CHAT positive neurons seen in the adult (P75) are seen at P3 (t = 4.71, df = 9, p < 0.05).

DISCUSSION

Differential maturation within striatal compartments

We conclude that the change in the distribution of the cholinergic neurons from an early preference for the patches to a preference for the intermediate zone on P7 and thereafter can be explained by cholinergic neurons in the different areas turning on CHAT at different timepoints during development. We suggest that there is gradient of CHAT maturation, with the cholinergic neurons in the patches (which contain the earliest born neurons in striatal neurogenesis) turning on CHAT first, followed by the cholinergic neurons in the intermediate zone and finally by those neurons in the rest of the matrix compartment. Another marker for cholinergic neurons, acetylcholinesterase (AChE), shows a similar temporal pattern of expression as CHAT and AChE positive cell bodies have been observed associated with AChE positive neuropil patches in the developing striatum of the embryonic rat (Butcher and Hodge, 1976), cat (Graybiel et al, 1981, 1984) and human (Kordower and Mufson, 1993) Moreover the early expression of the CHAT phenotype in the cholinergic neurons in the patches is also accompanied by the high expression of choline uptake sites and AChE fiber staining in the patches, while the increase in CHAT expressing neurons in the intermediate zone and the rest of the matrix compartment happens concurrently with an increase in AChE staining and choline uptake sites in the matrix. (Butcher and Hodge, 1976; Lowenstein et al, 1989).

Because the total number of CHAT positive neurons in the striatum (the combined number in the patches, intermediate zone and matrix area) approximately doubles after P3, the increase in the number of CHAT positive neurons in the intermediate zone and the rest of the matrix compartment cannot solely be due to CHAT positive neurons migrating from the patches into the intermediate zone and matrix area. Thus, there must be a late turn on of CHAT in the cholinergic neurons in the intermediate zone and the rest of the matrix compartment. Even the decrease in the number of cholinergic neurons in the patch compartment between P3 and P17 is
more likely due to cell death or CHAT turn off by the cholinergic neurons in the patch compartment, than to migration of the differentiated cholinergic neurons out of the patches into the intermediate zone and matrix area, because the absolute decrease of cholinergic neurons in the patch compartment is much lower than the absolute increase of cholinergic neurons in the intermediate zone and matrix area over the same timepoints. However a combination of both processes, cell death and migration, cannot be excluded. Although the results indicate that there is a late turn on of CHAT by the cholinergic neurons in the matrix compartment, we cannot simply conclude that the cholinergic neurons are already homogeneously distributed among the patch and matrix compartments from embryonic times, and that the turn on of CHAT is differentially regulated in striatal compartments. Even though this seems to be the most likely explanation, another possibility is that almost all cholinergic fated neurons are in the patch compartment initially, but only a select group expresses CHAT. In this scenario, some cholinergic cells do not express their CHAT phenotype at early times (E21), but later migrate out (or are pushed out) of the patch compartment and then start expressing their CHAT phenotype postnatally when they settle in the intermediate zone or in the rest of the matrix compartment. One piece of evidence that speaks against this hypothesis is that some CHAT expressing cholinergic neurons are already in the intermediate zone and the matrix at E21 when the patches have just formed.

**NGF and cholinergic maturation**

NGF (nerve growth factor) is a likely candidate for the local factor inducing the differential maturation of cholinergic neurons within the striatum. NGF increases CHAT activity in cholinergic neurons (Hatanaka and Tsukui, 1986; Martinez et al., 1985; Mobley et al., 1985; Aloe, 1987; Gage et al., 1989; Hagg et al., 1989; Williams and Rylett, 1990) and NGF antibodies decrease the expression of CHAT in cholinergic neurons (Vantini et al., 1989). NGF binds to two different receptors: p75 and trkA. The function of p75 remains unclear, but trkA seems to be necessary for many of the biological effects of NGF (Meakin and Shooter, 1992). In situ hybridization for trkA mRNA shows only a few scattered and lightly labeled trkA mRNA positive cells in early postnatal striatum, after which time labeling intensity rapidly increases (Ringstedt et al., 1993). trkA expression increases over the same timepoints when we see an increase in CHAT positive cells in the matrix compartment, and in the adult striatum all cholinergic neurons express trkA (Holtzman et al., 1992, Steiniger et al., 1993). While NGF might be important in increasing
the number of CHAT molecules in striatal cholinergic neurons, this does not mean necessarily that NGF is the critical factor for committing striatal neurons to the cholinergic phenotype or for the initial survival of these NGF responsive neurons. Mice with null mutations of the NGF gene or of the trkA gene reveal that neither endogenous NGF nor the trkA receptor is required for the early survival of cholinergic striatal and basal forebrain neurons (Crowley et al., 1994; Smeyne et al., 1994). However, the cholinergic neurons in the NGF null mutant mice appeared smaller and also more lightly stained for CHAT and trkA. These results imply that the actions of other trophic factors might compensate for the actions of NGF in the striatal cholinergic system. Although NGF is not required for survival of cholinergic neurons in the striatum during early postnatal life, it may regulate the level of CHAT in these cells.

**Differential adhesion as a mechanism underlying compartmental location**

The question remains as to how the early born striatal cholinergic interneurons become more or less homogeneously distributed between the patch and matrix compartments, although significantly more cholinergic neurons do reside in the intermediate zone outside the patch borders in the adult. Previous studies have shown that early born neurons (E12-E17) mainly end up in the patches and that they are selectively adhesive to one another (Graybiel and Hickey, 1982; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987a; Krushel et al, 1989; Fishell and van der Kooy, 1991). This is in contrast with the later born striatal neurons (E18-P2) which mainly end up in the matrix compartment and which are not adhesive to each other (Krushel et al, 1989; Krushel and van der Kooy, 1993). There also appears to be some differential adhesion within the early born striatal population within the patches, with the earliest born patch neurons (E13) tending to be more centrally located within the patches and more adhesive than later born (E15) patch neurons within the early born generation gradient (E13-E17) (Johnston et al, 1990). This selective increased adhesiveness of the earliest born patch neurons may induce the early born patch neurons to coalesce into patches. The homogeneous distribution of the cholinergic neurons within the patch and matrix compartments of the striatum might therefore be explained by different levels of adhesiveness. We predict then that the earliest born cholinergic neurons within the cholinergic generation gradient are more adhesive (and end up in the patch compartment and turn on CHAT expression earlier) than the later born neurons within the cholinergic generation gradient that end up in the matrix compartment.
The intermediate zone

Differential striatal neuron adhesion by itself cannot explain the enrichment of cholinergic neurons in the intermediate zone; the area in the matrix compartment just surrounding the patches. The enrichment of cholinergic neurons around patch borders, could be related to the presence of transient tenascin and glycoconjugate boundaries that can be detected around the patches in the first postnatal week (Steindler et al., 1988; O'Brien et al., 1992). These transient borders in the intermediate zone might capture migrating cholinergic cells, given that tenascin exhibits some adhesive properties (Kruse et al., 1985). These tenascin and glycoconjugate boundaries cannot be detected in the adult striatum (Steindler et al., 1988; O'Brien et al., 1992), and indeed the presence of these boundaries corresponds to the period during the first postnatal week when the relative density of cholinergic neurons in the intermediate zone rises.

The increased density of CHAT positive neurons in the intermediate zone supports the hypothesis that striatal cholinergic neurons participate in the communication between the functionally distinct patch and matrix compartments. However it does not necessarily support the existence of a third annular compartment as proposed by Faull et al. (1989). This third annular compartment was shown in primate and human to contain an increased density of substance P fibers, Met-enkephalin fibers and neurotensin receptors (Graybiel and Ragsdale, 1983; Beach and McGeer, 1984; Faull et al., 1989), and corresponds to the AChE negative border surrounding the AChE poor patches. As this AChE negative border defines the border of the patch (or striosome) compartment (Graybiel et al., 1986), the increased density of substance P and Met-enkephalin fibers and neurotensin receptors would be within the patch compartment, while increased density of CHAT neurons in the present study was measured outside the patch compartment (marked by embryonic or early postnatal retrograde labeling from the substantia nigra). This discrepancy is not due to our retrograde compartment marker as this retrograde labeling of patches provides an accurate marker of patch borders, marking the same patch borders labeled by an E13 pulse of bromodeoxyuridine (Fishell and van der Kooy, 1991), and by CHAT positive fiber patches early in development (unpublished observations). It remains to be determined whether a third functional striatal compartment exists that integrates the information between the "limbic" patch compartment and the surrounding "sensorimotor" matrix compartment, or whether the border between the patches and matrix simply causes a build up of certain cells and fibers on either side of this border.
CONCLUSION

In conclusion, striatal cholinergic interneurons mature differentially depending on their compartmental location. We hypothesize that a greater adhesiveness of the earliest born cholinergic neurons could help localize them in the patch compartment, and a differential distribution of NGF signalling mechanisms may induce the earlier CHAT phenotypic maturation of patch cholinergic neurons. The differential turn on of CHAT in striatal cholinergic interneurons might be necessary to ensure the proper connections between striatal cholinergic neurons and the developmentally and functionally distinct patch and matrix compartments.

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Chapter III

**Striatal cholinergic interneurons: birthdates predict compartmental localization**

Abstract

The striatal patch and matrix compartment neurons are born at different times during rat development. The majority of the early born neurons preferentially end up in the patch compartment, while the majority of the later born neurons end up in the matrix compartment. Although the cholinergic interneurons are all born early in neurogenesis (between embryonic day (E)12 and E17), and we would therefore expect them to be located mainly in the patches, they are relatively homogeneously distributed in the adult, with a preference for the matrix area just outside the patches (the intermediate zone). To ask if birthdate can predict the compartmental localization of cholinergic neurons in the striatum, we marked new postmitotic neurons in the embryo with a maternal injection of bromodeoxyuridine (BrdU) on E13, E15 or E17 and labeled the patch compartment with an injection of the retrograde tracer True Blue into the substantia nigra on postnatal day (P) 1. The pups were sacrificed at P40 and the tissue was processed for BrdU, choline acetyltransferase, and True Blue triple labeling. Cholinergic neurons that became postmitotic at E13, had a higher chance of ending up in the patch compartment compared to either the intermediate zone or the rest of the matrix compartment. On the other hand cholinergic neurons that became postmitotic at E17 had a higher chance of ending up in the matrix compartment (including the intermediate zone). We conclude that birthdate can predict compartmental localization, with the cholinergic neurons in the intermediate zone following the same pattern as the cholinergic neurons in the rest of the matrix compartment. Cholinergic neurons show the same relative birthdate/compartment relationship as do other striatal neurons, although the absolute birthdates of cholinergic neurons are shifted earlier in neurogenesis.
INTRODUCTION

The mammalian striatum is a heterogeneous structure that can be divided into two complementary and functionally distinct compartments, the patches and the matrix (Goldman and Nauta, 1977; Goldman-Rakic, 1982; Graybiel and Ragsdale, 1983). Communication between the two compartments is thought to be mediated mainly by the striatal interneurons, which can extend their dendrites across compartmental boundaries (Kubota and Kawaguchi, 1993; Penny et al, 1988). One electrophysiologically identifiable class of neurons in the striatum (the tonically active neurons, or TANs) is thought to be involved directly in collecting reinforcement related information from the patches and influencing the activity of the projection neurons in the matrix (Graybiel and Kimura, 1995). These TANs show learning-dependent plasticity, are distributed over broad regions in the striatum and respond in a temporally coordinated way after behavioral conditioning. Based on their electrophysiological properties (Kawaguchi, 1992; Wilson et al, 1990) and their tendency to reside on patch/matrix borders, these cells are thought to be the cholinergic interneurons of the striatum (Aosaki et al, 1995).

The cholinergic neurons in the rat striatum are born early during neurogenesis (between embryonic (E) days 12-17) (Phelps et al, 1989; Semba et al, 1988), and are distributed homogeneously among the patch and matrix compartments in the adult rat, but with a preference for the matrix area just outside the patches, termed the intermediate zone (van Vulpen and van der Kooy, 1996). Striatal neurons as a whole show compartmental preferences, depending on the time that they leave the mitotic cycle (Brand and Rakic, 1979; Fishell and van der Kooy, 1991; Graybiel and Hickey, 1982; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987). The earliest striatal neurons to become postmitotic in the rat embryo (between E12- E16) become restricted mainly to the patch compartment, while neurons that are born later (E18-postnatal (P) day 2) end up primarily in the matrix compartment (Fishell and van der Kooy, 1991; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987). The cholinergic neurons in the striatum appear not to follow this general rule, as they are born early during striatal neurogenesis, but not all are located in the patch compartment in the adult rat. In the previous studies examining the different times of origin of choline acetyltransferase (CHAT) immunoreactive neurons in the rat (Phelps et al, 1989; Semba et al, 1988), the striatal cholinergic population was considered as a homogeneous population and not compartmentally identified. However our recent work suggests that there are different compartmental subpopulations of cholinergic neurons, based on their times
of maturation, with the cholinergic neurons located in the patches turning on CHAT earlier than the cholinergic neurons in the matrix (van Vulpen and van der Kooy, 1996). The present study asks if striatal cholinergic neurons with different compartmental locations are generated simultaneously, or are the differentially maturing cholinergic neurons (van Vulpen and van der Kooy, 1996) also generated along a temporal neurogenic gradient? We marked new postmitotic neurons on their birthdates with the DNA synthesis marker bromodeoxyuridine (BrdU) (Fishell and van der Kooy, 1991; Miller and Nowakowski, 1988) and then examined the distribution of double labeled neurons (BrdU+ and CHAT+) within the striatal patch and matrix compartments, as well as within the intermediate zone. The results indicate that cholinergic birthdate can predict compartmental localization, with early born cholinergic neurons (E13) preferentially ending up in the patch compartment, while the late born cholinergic neurons (E17) prefer the matrix compartment. Thus, cholinergic neurons show the same relative birthdate/compartment relationship as do other striatal neurons, although the absolute birthdates of cholinergic neurons are shifted earlier in neurogenesis.

MATERIALS AND METHODS

Labeling cholinergic neurons on their birthdates

Timed pregnant Wistar rats (Charles River) were used and the presence of a vaginal plug was taken as embryonic day (E)1. As striatal cholinergic neurons are born early in neurogenesis (between E12 and E17) (Phelps et al, 1989; Semba et al, 1988), timed pregnant rats received an intraperitoneal injection of BrdU (Sigma, 18 mg/100 gm b.wt; dissolved in 0.007M NaOH) on either E13, E15 or E17.

Labeling of early projecting striatonigral neurons

The pregnant mothers were allowed to give birth and the day of birth was taken as postnatal day (P) 0. In order to determine the localization of cholinergic neurons in the striatum, we selectively marked the patch compartment by labeling the early projecting striatonigral neurons with micro injections of the retrograde fluorescent tracer True Blue into the ventral midbrain (Fishell and van der Kooy, 1987; van Vulpen and van der Kooy, 1996). On P1 the rat pups were anesthesized using hypothermia (Fishell and van der Kooy, 1987), and 0.5 µl of a 5%
solution of True Blue was injected bilaterally with a 1 µl Hamilton syringe into the ventral midbrain. True Blue has been previously reported to be an accurate marker of the patch compartment (Fishell and van der Kooy, 1987; van Vulpen and van der Kooy, 1996). At postnatal day 40, the True Blue microinjected, BrdU containing animals were reanesthesized with sodium pentobarbital (60 mg/kg) and then perfused with phosphate buffered saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After perfusion, the brains were removed and postfixed for 48 hr in the same fixative. Prior to sectioning the brains were rinsed with Tris buffered saline (TBS) and 50 µm frontal vibratome sections were cut.

*Double immunofluorescent labeling*

The vibratome sections were collected and washed in 0.05M TBS (pH 7.6). After rinsing, the sections were placed in 1M HCl at 60ºC for 45 minutes to denature the double stranded DNA into single strands and thus expose the BrdU epitopes. Sections were rinsed with 0.05 M TBS incubation buffer containing 0.3% triton X-100, 1% normal horse serum and 1% normal goat serum 3 times for 10 minutes each. The sections were then processed for BrdU and choline acetyl transferase (CHAT) double labeling by incubating the free floating sections with a mixture of the anti-BrdU (mouse monoclonal 1:25, Becton Dickinson) and the anti-CHAT (rabbit polyclonal 1:500, Chemicon) for 48-70 hr at 4ºC. After rinsing with the incubation buffer, the sections were incubated in a mixture of goat anti rabbit conjugated to fluorescein isothiocyanate (1:100; Vector laboratories) and biotinylated horse anti-mouse IgG (1:100; Vector Laboratories) for 1 hr at room temperature. After reacting with the secondary antiserum mixture, the sections were washed and placed in a solution containing avidine-conjugated RITC (1:250: Vector Laboratories) for 1 hr at room temperature. After the final rinse, sections were mounted on chrome-alum coated slides, dehydrated, and cover slipped with Fluoromount (BDH).

*Data analysis*

All results presented below are obtained from injections filling and restricted to the ventral midbrain. This type of injection has been reported previously to yield a reproducible distribution of retrogradely labeled striatal neurons (Fishell and van der Kooy, 1987; van Vulpen and van der Kooy, 1996). Sections triple-labeled with retrogradely labeled True Blue neurons, fluorescein labeled CHAT neurons and rhodamine labeled BrdU cells were examined and photographed using
a Nikon fluorescent microscope with the appropriate filters. 400 ASA Kodak Gold film was used for photomicrographs. A drawing tube attached to the fluorescent microscope was used to outline the True Blue labeled patches and the location of the fluorescein labeled CHAT neurons with respect to the compartments. Switching to the rhodamine filter revealed whether the fluorescein labeled CHAT neurons also were double labeled for BrdU.

As the striatal compartments were most easily delineated by True Blue labeling within the head of the striatum (head of the caudate-putamen in rat), the area of striatum studied was limited to the entire striatum rostral to the globus pallidus and dorsal to the nucleus accumbens. Separate cell counts for the medial and lateral striatum at a rostral and a caudal level were made, to examine possible rostrocaudal or mediolateral differences in the percentages of BrdU double labeled CHAT neurons. The rostral level included the islands of Calleja within nucleus accumbens, while the caudal level was located at the decussation of the anterior commissure before the rostral globus pallidus appears in coronal sections. A total of n=11 for E13 BrdU, n=16 for E15 BrdU, and n=15 for E17 BrdU animals were analysed at P40 from 2 separate experiments.

**Quantification**

The distribution of BrdU/CHAT double labeled, CHAT single labeled and BrdU single labeled neurons was examined in relation to the striatal compartments (as determined by the dense True Blue labeling in the patches). Given that CHAT positive neurons have been reported to prefer an area of the matrix just outside the patches in the adult rat (van Vulpen and van der Kooy, 1996), we also looked at the distribution of single (BrdU+ or CHAT+) and double labeled cells (BrdU+/CHAT+) in the intermediate zone. The intermediate zone is defined arbitrarily as the area two cell diameters outside the patches. Labeled cells were counted in 380 × 275 μm areas within the medial and lateral striatum at two rostrocaudal levels (a middle versus a midcaudal level) at 20 times magnification. Each sample contained approximately 20% patch compartment and 80% matrix compartment (including the intermediate zone) matching the approximate percentages that the compartments make up within the overall striatum (FisheIn and van der Kooy, 1991; van Vulpen and van der Kooy, 1996).

We analysed the data at P40, when all cholinergic cells are mature and show their CHAT phenotype (van Vulpen and van der Kooy, 1996). At this timepoint, the cholinergic neurons are homogeneously distributed between the patch and matrix compartment, although there is a
significantly higher proportion of cholinergic neurons in the intermediate zone (IZ) of the matrix just outside the patches (van Vulpen and van der Kooy, 1996). Given that the different striatal areas comprise unequal volumes within the striatum at P40 (van Vulpen and van der Kooy, 1996), the numbers of double labeled (BrdU/CHAT) cells within an area were expressed as percentages of the total numbers of CHAT cells within that area. These comparisons of the percentages of CHAT cells that are double labeled makes it unnecessary to control for the volume differences in the striatal compartments. The effects of birthdate on the percentages of CHAT cells double labeled with BrdU in the different striatal areas were analyzed using analyses of variance and Newman-Keuls post hoc tests (p < 0.05). Furthermore, to investigate the peaks of cholinergic neuron generation in the different striatal areas, the average numbers of double labeled BrdU/CHAT cells that were labeled with BrdU on E13, E15 or E17 were expressed as percentages of the total of all of the double labeled BrdU/CHAT cells counted over the entire generation period in the different striatal areas.

RESULTS

The distributions of double labeled (BrdU/CHAT) and single labeled BrdU and cholinergic neurons were examined in the P40 (adult) rat in relation to the patch compartment, intermediate zone, or the rest of the matrix compartment. The dense True Blue labeling always specifically labeled the patches, independent of the time of BrdU injection, and was used as the compartment marker. In the E13 BrdU injected rats, the majority of the early born neurons were restricted to the patch compartment and the overall distribution of single labeled BrdU cells overlapped the distribution of the True Blue labeled patches (Fig. 1A). In the E17 BrdU injected rats on the other hand, the single labeled BrdU cells were mostly found outside the True Blue labeled patches (Fig. 1B), although some BrdU positive neurons could be found in the patch compartment.

A caudal to rostral generation gradient has been reported for striatal cholinergic neurons (Phelps et al, 1989; Semba et al, 1988). We originally performed separate cell counts for the medial and lateral striatum at both a rostral and a caudal level to examine possible mediolateral or rostrocaudal differences in the numbers of neurons double labeled with CHAT and BrdU expressed as percentages of the CHAT neurons. The earliest born (E13) double labeled neurons in the patches had a tendency to be located in the caudal compared to the rostral striatum, and
Figure III. 1 Photomicrographs of triple labeled sections with CHAT positive neurons (FITC, green), True Blue labeled patches (blue), and BrdU labeled neurons (RITC, red) in single coronal vibratome sections in the P40 rat. A. An E13 BrdU injection labeled neurons in the patch compartment, whose distribution completely overlaps with the pattern of True Blue patch neuron labeling. A double labeled BrdU/CHAT positive cell can be seen in the intermediate zone (arrowhead) extending a process into the patch compartment, while a single labeled CHAT positive cell (green) with labeled processes can be detected at the border of the patch compartment (arrow). B. An E17 BrdU injection labeled neurons in the matrix compartment, whose distribution is complementary to the pattern of True Blue patch neuron labeling. A CHAT positive neuron (negative for BrdU) can be detected in the patch compartment (arrow), while in the matrix compartment one lightly double labeled BrdU/CHAT positive cell can be seen (arrowhead) along with a few single labeled CHAT positive neurons (unmarked). Scale bar, 100 μm.
Figure III. 2 P40 neurons that were double labeled (BrdU positive and CHAT positive) expressed as percentages of all of the CHAT positive neurons in each of the compartments after the different embryonic day BrdU injections. IZ, intermediate zone. Data represents means ± S.E.M.
Double labeled neurons (CHAT and BrdU) as percentages of all of the CHAT positive neurons per compartment

BrdU injection age

- Patch
- Matrix minus IZ
- IZ
there was also a slight bias for the lateral part of the striatum, but these differences were not statistically significant (data not presented). Not even a hint of such a gradient could be detected for the double labeled cells in the matrix compartment at any of the ages investigated (E13, E15, or E17). Therefore, the data are presented as combinations of the rostrocaudal levels and mediolateral aspects of the striatum.

Analyses of variance revealed a significant main effect of birthdate on the numbers of double labeled BrdU/CHAT cells (expressed as percentages of all CHAT cells per area) for the patch compartment; F(2,38) = 31.9, p < 0.05, while no significant main effects of birthdate were seen for the intermediate zone nor the rest of the matrix compartment (p > 0.05). In the patches, the numbers of BrdU/CHAT double labeled neurons expressed as percentages of the CHAT cells in that compartment decreased significantly between E13 and E15, and also between E15 and E17 (p < 0.05, Newman-Keuls tests). The number of CHAT neurons double labeled with BrdU expressed as a percentage of all the CHAT cells in that compartment at E13 was also significantly higher (p< 0.05) in the patch compartment than in either the intermediate zone or matrix, while at E17 this percentage was significantly lower (p < 0.05) in the patch than in either intermediate zone or the matrix (Fig. 2). We conclude that the earliest born cholinergic neurons (E13) have a significantly higher chance of ending up in the patch compartment than in either the intermediate zone or the rest of the matrix compartment, while the cholinergic neurons that become postmitotic at E17 have a significantly higher chance of ending up in either the intermediate zone or the rest of the matrix compartment than in the patch compartment. Furthermore the cholinergic neurons in the intermediate zone show the same generation pattern as the cholinergic neurons in the rest of the matrix compartment, which suggests that the intermediate zone may have a closer developmental relationship with the matrix compartment than with the patch compartment.

The percentages of the CHAT positive neurons in the patch compartment that were double labeled with BrdU at each of E13, E15 and E17 add up to near 100%. (Fig.2). This summed percentage seems high as some patch cholinergic neurons must also be born on the days in between. Therefore, the estimates of percentages of CHAT labeled cells that are double labeled at any one day are likely overestimates. BrdU has a disadvantage compared to the other birthdate marker often used ([3H]-thymidine) in that it is more difficult to determine whether a cell is heavily or lightly labeled with BrdU. Thus, all of our estimates of cells born at any one day
probably include cells that will go through another round of division as well as cells that have their last division at the exact time of the BrdU injection.

The peak day for generating cholinergic neurons in the patches was E13 and almost all of the CHAT positive neurons in the patches were generated by E15. In contrast, the cholinergic neurons in the intermediate zone and the rest of the matrix compartment became postmitotic throughout the E13 to E17 period without any real generation peaks. It should be noted that approximately equal absolute numbers of cholinergic neurons are born on E13 and E17 (van Vulpen and van der Kooy, 1996), in contrast to all striatal neurons where the absolute number of all striatal neurons born on E17 is much higher than on E13 (van der Kooy and Fishell, 1987).

When striatal neurons are considered as a whole, a relationship between birthdate and compartment is evident (van der Kooy and Fishell, 1987). Striatal neurons are generated from E12-P2 in two distinct phases: the neurons that become postmitotic between E12-E16 become restricted mainly to the patch compartment, while the neurons that become postmitotic between E18-P2 are restricted mainly to the matrix compartment. The striatal cholinergic interneurons, make up only a small percentage of the total striatal population (1-2%), but follow a similar two phase generation pattern. The cholinergic neurons that become postmitotic early (E13) have a greater chance of ending up in the patch compartment, while the cholinergic neurons that become postmitotic at the end of the cholinergic generation gradient are mainly restricted to the matrix compartment. We conclude, therefore, that the cholinergic neurons show the same relative birthdate/compartment relationship as to the rest of the striatal neurons. However, plotting the ratios of patch neurons to matrix neurons born at the different embryonic ages for both populations of cells (all striatal neurons versus all cholinergic neurons) (Fig.3) revealed that the absolute birthdates of cholinergic neurons are shifted earlier in neurogenesis with respect to the birthdates of the total population of striatal neurons.
Figure III. 3 The P40 ratios of patch neurons labeled with BrdU to matrix neurons (including the intermediate zone) labeled with BrdU across embryonic BrdU injection ages. The ratios change in parallel across age for both CHAT striatal neurons and all striatal neurons, but the ratios for CHAT neurons are shifted earlier in neurogenesis. Ratios above the solid horizontal ratio line indicate a predominance of patch neuron birthdates and ratios below the line indicate a predominance of matrix neuron birthdates.
All neurons

CHAT neurons

Ratios of patch to matrix neurons labeled with BrdU

E13  E15  E17

BrdU injection age
DISCUSSION

The present results suggest that birthdate can predict striatal compartmental localization, with the majority of the early postmitotic (E13) cholinergic neurons preferring the patch compartment, while the later born (E17) cholinergic neurons preferentially end up in the matrix compartment. Previous work (van Vulpen and van der Kooy, 1996) has shown that the cholinergic neurons in the striatum also mature differentially, with the cholinergic neurons in the striatal patch compartment turning on CHAT first, followed by the cholinergic neurons in the intermediate zone and the matrix compartment. These data support the straightforward conclusion that the earliest born cholinergic neurons (which are preferentially located in the patches) mature before the later born cholinergic neurons in the matrix compartment mature. Because the earliest born cholinergic neurons are exposed to the striatal environment longer than the later born cholinergic neurons (by about four days: E13-E17), this longer or earlier exposure to striatal growth factors could explain the earlier maturation of the cholinergic neurons in the patches. Indeed, perinatal exposure to higher than normal control levels of nerve growth factor can induce a more rapid maturation of the cholinergic neurons in the matrix compartment (see Chapter IV). It remains to be determined whether the cholinergic neurons become restricted to a compartmental fate as a result of their initial interactions with the postmitotic striatal environment or whether the cholinergic neurons are committed to their compartmental location prior to becoming postmitotic. However, there is evidence from retroviral lineage tracing (Krushel et al, 1993a) and chimeric mice (Fishell et al, 1990) studies that striatal neurons as a whole may have their compartmental fates partially specified as proliferating progenitors within the striatal germinal zone.

The finding that the cholinergic neurons in the intermediate zone show the same generation pattern as the cholinergic neurons in the rest of the matrix compartment suggests that the intermediate zone shows a close developmental relationship with the rest of the matrix compartment. However, other evidence points to a separate group of specialized cholinergic neurons in the striatal intermediate zone. The density of cholinergic neurons in the intermediate zone is higher than in the rest of the matrix compartment or in the patch compartment (van Vulpen and van der Kooy, 1996), and the cholinergic neurons in the intermediate zone have been shown to extend their dendrites across compartmental boundaries (Kubota and Kawaguchi, 1993; Penny et al, 1988). Moreover, in the monkey the cholinergic
neurons residing on the patch/matrix borders (Aosaki et al, 1995) also correspond to the tonically active neurons (or TANs) which are thought to be involved directly in collecting information from the patches and influencing the activity of the neurons in the matrix (Graybiel and Kimura, 1995). However, the present results do not demonstrate a correlation between birthdate and the localization of the cholinergic neurons within this specialized intermediate zone of the matrix compartment, as the cholinergic neurons in the intermediate zone show the same developmental relationship as the cholinergic neurons in the rest of the matrix compartment. The specialized intermediate zone of the matrix compartment also does not correlate with the hypothetical third striatal subcompartment as proposed by Faull et al (1989). The increased staining of substance P fibers and substance P receptor positive neurons, which define the putative third compartment, is located in the outer rim of the patch (striosome) compartment (Faull et al, 1989; Jakab et al, 1996), whereas the intermediate zone with its increased density of cholinergic neurons is located instead in the inner rim of the matrix compartment, just outside the patches (van Vulpen and van der Kooy, 1996). Moreover, the tonically active cholinergic neurons of the monkey are primarily located in the matrix compartment as it borders the patches and are not found in the patch (striosome) compartment (Aosaki et al, 1995).

Although striatal neurons are generated from E13 to P2, the cholinergic striatal neurons become postmitotic selectively during early striatal neurogenesis, from E13-E17. The absolute birthdates of the striatal cholinergic neurons are consequently shifted earlier in neurogenesis with respect to the birthdates of the total population of striatal neurons. This early generation of the striatal cholinergic neurons, and especially the cholinergic neurons in the patches, raises the possibility that these neurons could influence the subsequent development of the structure of the striatum. Striatal cholinergic neurons may play a role during embryonic striatal compartmentation, as the "giant" cholinergic neurons in the adult neostriatum have been reported to participate in cell clustering (Mensah, 1977; 1980). Cholinergic neurons can be detected in direct somatic apposition with multiple perikarya of medium sized spiny neurons (Pickel and Chan, 1991), and they may play a dynamic role in the control of synaptic transmission and intercellular communication or adhesion (Dimova et al, 1993). Another critical time period in the development of the striatal structure occurs during the first postnatal week, when severing the connections between the striatum and the substantia nigra produces a massive shrinkage of the striatum that is not seen after similar embryonic or adult lesions (van der Kooy, 1996). This
critical first postnatal week period coincides not only with the naturally occurring cell death of striatal neurons (Fishell and van der Kooy, 1991) but also with a relative change in distribution of the striatal cholinergic neurons (van Vulpen and van der Kooy, 1996).

The earliest born striatal neurons (E12- E17) have been shown to be selectively adhesive to one another. This contrasts with the later born neurons (E18- P2) which are not adhesive to each other (Krushel et al, 1989; Krushel and van der Kooy, 1993b; Krushel et al, 1995). Because the majority of the early born striatal neurons preferentially end up in the patch compartment, the selective adhesiveness of the early born patch neurons may be a mechanism that coalesces the patch neurons into patches during embryonic development. As all cholinergic neurons are also early born (between E12 and E17), but not all of them end up in the patch compartment, it would be interesting to look specifically at the adhesive properties of the cholinergic neurons. Based on the birthdate compartment relationship of the cholinergic neurons, we predict that only the earliest born of all cholinergic neurons will show adhesive properties. Thus, we speculate that cholinergic birthdate might not only predict striatal compartmental location and subsequent cholinergic maturation, but perhaps also may predict the differential adhesiveness that results in the mechanical positioning of cholinergic neurons in the specific striatal compartments.

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Chapter IV

NGF facilitates the developmental maturation of the previously committed cholinergic interneurons in the striatal matrix

Abstract
Although all of the cholinergic interneurons of the striatum are generated early in development, the maturation of these neurons depends on striatal compartmental localization. The majority of the cholinergic neurons in the patches turn on choline acetyltransferase (CHAT) embryonically, while the majority of cholinergic neurons in the matrix turn on CHAT postnatally. To ask if CHAT expression can be induced earlier in the cholinergic neurons and if the facilitation is compartment specific, we injected nerve growth factor (NGF) into the lateral ventricle of either embryonic day 19 embryos or postnatal day 1 pups, and analyzed them at E21 and P3, respectively. We simultaneously marked the patch compartment by injecting the retrograde fluorescent tracer True Blue into the substantia nigra at the times of the NGF infusions. NGF induced a dramatic increase in the number of CHAT immunoreactive neurons in the matrix compartment (up to adult levels), while the NGF infusions did not increase the number of CHAT neurons in the patch compartment. Analyses of the compartmental distributions of the p75 and trkA-NGF receptors do not provide by themselves an explanation for the differential cholinergic maturation in the compartments of the control striatum, nor for the upregulation of CHAT in the striatal matrix after the NGF infusion. We conclude that NGF infusion is capable of facilitating the normally slow cholinergic maturation of the cholinergic neurons in the matrix, while the cholinergic maturation of the CHAT cells in the patch compartment seems to be largely independent of NGF signalling.
INTRODUCTION

Analysis of the distribution of the cholinergic neurons in the striatum during development indicates that cholinergic striatal interneurons mature differentially depending on compartmental localization, with the cholinergic neurons located in the patches turning on CHAT earlier than the cholinergic neurons in the matrix (van Vulpen and van der Kooy, 1996). Nerve growth factor (NGF) is a candidate inducer for this differential compartmental maturation of striatal cholinergic interneurons. In vitro, NGF increases choline acetyltransferase (CHAT) activity in fetal dissociated striatal culture (Hatanaka and Tsukui, 1986) and in organotypic cultures of fetal rat striatum (Martinez et al, 1985). In vivo, intraventricular injections of NGF increase CHAT activity in the striatum of the newborn rat (Mobley et al, 1985; Aloe, 1987) and in the adult rat exogenous NGF chronically infused into the lateral ventricle increases the number of CHAT molecules in the cholinergic neurons and also the size of the cholinergic cell bodies in the caudate putamen (Gage et al, 1989; Hagg et al, 1989). Moreover when the striatum is injured neonatally or in the adult, NGF can completely block the degenerative effects of the lesion on the cholinergic neurons (Aloe, 1987; Gage et al, 1989). Chronic NGF administration also has been reported to enhance the function of striatal cholinergic interneurons (Williams and Rylett, 1990). Thus, infusing NGF might be expected to facilitate the cholinergic phenotype of striatal cholinergic neurons.

NGF acts through two types of transmembrane glycoproteins: p75 and trkA, and the trkA receptor kinase seems to be essential for functional responses in vitro (Meakin and Shooter, 1992). The perinatal striatum contains numerous striatal perikarya expressing the low affinity p75 receptor protein and mRNA (Gage et al, 1989; Koh and Higgins, 1991), while in the adult striatum the p75 positive neurons are colocalized with only a small proportion of the numerous CHAT-ir neurons (Kordower et al, 1988; Kiss and Patel, 1989; Koh et al, 1989; Hagg et al, 1992). The pattern of development of the trkA receptor in the striatum is quite different. In situ hybridization for trkA shows only a few scattered and lightly labeled trkA mRNA positive cells in early postnatal striatum, after which time labeling intensity rapidly increases (Ringstedt et al, 1993), and in the adult striatum all cholinergic neurons express trkA (Holtzman et al, 1992; Steiniger et al, 1993).
However, mice with targetted mutations of the NGF ligand or the trkA receptor (Crowley et al, 1994; Smeyne et al, 1994), show surprisingly few deficits in the central nervous system. The cholinergic neurons in the basal forebrain and striatum in the trkA− mice differentiate into their cholinergic phenotypes (Fagan et al, 1997), although there appear to be fewer and smaller cholinergic neurons in both regions during late postnatal development, combined with a reduced expression of choline acetyltransferase. These results imply that instead of NGF being required for the initial differentiation or survival of the cholinergic neurons in the striatum, NGF might have more subtle functions such as in the maintenance of the phenotype or in neuronal plasticity (Thoenen, 1995).

I investigated the role of NGF in the development of the cholinergic neurons in the striatum by administering exogenous NGF during the perinatal period. To examine if CHAT expression in the late maturing cholinergic neurons in the matrix compartment of the striatum can be induced earlier in development and if this putative facilitation is compartment specific, I infused NGF in the lateral ventricle of either embryonic day (E)19 embryos or newborn postnatal day (P)1 rats and simultaneously labeled the patch compartment by injecting the retrograde fluorescent tracer True Blue into the substantia nigra at E19 or P1, respectively. Given that the presence of different NGF receptors on the different subpopulations of cholinergic neurons in the striatum could influence the time point that cholinergic neurons turn on CHAT, I also investigated the distribution of p75 and trkA positive cells in the striatum during early development. The results indicate that NGF infusion can facilitate the normally slow cholinergic maturation of the cholinergic neurons in the matrix compartment, while it has no effect on the cholinergic maturation of the CHAT cells in the patch compartment.

MATERIALS AND METHODS

Embryonic NGF infusions

19 day timed pregnant Wistar rats (presence of the vaginal plug was considered embryonic day (E)1) acquired from Charles River Labs (Wilmington, MA) were anesthetized with sodium pentobarbital (60 mg/kg) and laparotomies were performed. Each rat fetus (E19) was oriented inside the uterus and held firmly in place between thumb and index finger, and the dorsal calvarian
suture lines were used to estimate the location of the lateral ventricles. After puncturing the uterine wall with a 25 gauge needle, a Hamilton syringe was used to infuse 12.5 µg NGF (mouse 7S, Upstate Biological, Lake Placid, NY) in 5 µl phosphate buffer with 0.1% BSA unilaterally into the lateral ventricle over a 2 minute period. After sealing the first puncture in the uterine wall with the cyanoacrylic adhesive Crazy Glue (The Borden Comp., Willowdale, ON), the same fetus was reoriented inside the uterus and the dorsal calverian suture lines were used this time to estimate the location of the ventral midbrain. After puncturing the uterine wall with a 25 gauge needle, 0.2-0.4 µl of a 5% True Blue solution (Sigma, Oakville, ON) was injected bilaterally into the ventral midbrain. At this timepoint during development only the early projecting striatonigral neurons will be retrogradely labeled with microinjections of the fluorescent tracer True Blue in the ventral midbrain (Fishell and van der Kooy, 1987), and thus the distribution of the retrogradely labeled striatal neurons can be used to selectively mark the patch compartment. The additional punctures in the uterine wall were also sealed with Crazy Glue, and after suturing the pregnant rats were allowed to recover for two days. The pregnant mothers were reanesthetized with pentobarbital and the E21 embryos were perfused intracardially with a 1mM NaNO2 phosphate buffered saline (PBS) rinse, followed by 4% paraformaldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4).

**Early postnatal NGF infusions**

P1 (with P0 considered as day of birth) rat pups were anesthetized using hypothermia (Fishell and van der Kooy, 1987), and 25 µg NGF in 10 µl phosphate buffer with 0.1% BSA was infused unilaterally (with 10 µl Hamilton syringe) into the lateral ventricle over a 10 minute period. To selectively label the patch compartment, the same pups also received a 0.5 µl injection of 5% True Blue bilaterally into the ventral midbrain. The pups were allowed to survive for 48 hours, then were reanesthetized (P3) with sodium pentobarbital and then perfused with a PBS rinse, followed by 4% paraformaldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4). In both the embryonic and the postnatal experiments, vehicle controls were infused with 0.1% BSA in phosphate buffer into the lateral ventricle in the same volumes received by the NGF treated animals, and also received bilateral injections with 5% True Blue in the ventral midbrain. Other non lateral ventricle infused controls received only the bilateral True Blue injections in the ventral midbrain area.
**Immunocytochemistry and quantification**

After either E21 or P3 perfusion, the brains were removed and postfixed for 90 minutes in the same fixative. Prior to sectioning the brains were either rinsed with Tris buffered saline (TBS) and 50 μm vibratome sections were cut or the tissue was cryoprotected with 10% sucrose in buffer and 14 μm frozen sections were cut and thaw mounted onto chrom-alum gelatin coated slides. The embryonic sections were pretreated with a mixture of 4 parts of 100% methanol and 1 part 3% H₂O₂ to suppress endogenous peroxidase activity. All antibodies were diluted in 0.3% Triton X-100 and 2.5% normal serum in 0.05 M Tris buffered saline. The polyclonal CHAT antibody (Chemicon, Temecula, CA) was used at 1:500, the mouse monoclonal 192 IgG antibody recognizing the p75 receptor (gift from Dr. Côté) was diluted 1:10, and the polyclonal antibody (trkA) recognizing the intracellular part of the trkA receptor (gift from Dr. S.C. Feinstein, University of California, Santa Barbara, CA) was diluted 1:1600. The free floating vibratome sections were incubated for 30-40 hr with the primary antibody at 4°C, while the mounted cryostat sections were incubated for 18 hr at 4°C. After rinsing the sections in 0.05 M Tris saline buffer, the sections were incubated in a donkey-anti rabbit secondary antibody conjugated to biotin and used at a dilution of 1:100 (Amersham, Arlington Heights, IL) for CHAT and trkA, and a sheep anti-mouse secondary antibody conjugated to biotin at a dilution of 1:100 (Amersham, Arlington Heights, IL) for p75. After 1 hr in the secondary antiserum, the tissue sections were rinsed again in 0.05 M Tris buffer and the sections were incubated for 1 hr with either an avidin-biotin complex conjugated to peroxidase (Elite ABC kit, 1:700, Vector labs, Burlingame, CA) or avidin conjugated to the fluorescent CY3 (1:150, Sigma, Oakville, ON). The peroxidase was visualized with 0.05% diaminobenzidine containing 0.01% H₂O₂. The free floating sections were then mounted onto chrom-alum gelatin coated slides. All slides were dehydrated and cover slipped with Fluoromount (BDH, Toronto, ON). The distributions of CHAT, trkA or p75 immunoreactive neurons were examined in relation to the striatal compartments during early striatal development. In the adult, cholinergic neurons are preferentially located in the intermediate zone (IZ; the area of the matrix compartment just surrounding the patches; van Vulpen and van der Kooy, 1996). I quantified this region as a separate striatal area (arbitrarily defined as two cell diameters from the patch border). In each animal the striatal
immunoreactive neurons were counted at two rostrocaudal levels (a middle versus a mid caudal level) throughout 3 sections per level in the head of the striatum, and all cell counts were corrected by the Abercrombie method (1946). Unfortunately it was not possible to use the dissector method on these sections. In order to calculate the absolute number of CHAT cells per striatal area using the physical dissector method, it would be necessary to photograph all of the CHAT positive cells in relation to the True Blue marked compartments throughout the entire section, at the different levels of focus and at a sufficient magnification. This procedure would have resulted in too much fading of the True Blue staining to accurately delineate the compartments, and therefore I choose to count the CHAT cells directly under the microscope to limit fading. Moreover, in a separate experiment no differences were detected between absolute cholinergic counts throughout striatal sections calculated with the Abercrombie correction or absolute cholinergic counts corrected with the dissector method (unpublished results; also see Hagg et al, 1997). Absolute numbers of immunoreactive neurons in the different striatal areas: patch compartment, intermediate zone, and the rest of the matrix compartment (matrix area) were calculated within the entire volume that each area comprises within the head of the striatum. The compartmental volumes were estimated as described previously (van Vulpen and van der Kooy, 1996). In all experiments the number of rats analyzed depended on obtaining both good retrograde labeling of the patch compartment neurons and good CHAT, p75 or trkA immunostaining in the same animal (E19/21 CHAT staining: total of n=4 for NGF infused embryos, n=4 for vehicles controls, and n=6 for non lateral ventricle infused controls; P1/3 CHAT staining: total of n=4 for NGF infused pups, n=6 for vehicles controls, and n=5 for non lateral ventricle infused controls; E19/21 p75 staining: total of n=7 for NGF infused embryos, n=7 for vehicles controls, and n=4 for non lateral ventricle infused controls; P1/3 p75 staining: total of n=5 for NGF infused pups, n=6 for vehicles controls, and n=2 for non lateral ventricle infused controls; E19/21 trkA staining: n=6 for non lateral ventricle infused controls; P1/3 trkA staining: n=6 for non lateral ventricle infused controls). The effects of NGF infusion on the total CHAT and p75 cell counts, and the distributions of CHAT, trkA and p75-ir neurons (all in relation to the three different striatal areas), were evaluated by a \( \chi^2 \) test or by an analysis of variance followed by Newman-Keuls tests.
RESULTS

Effects of NGF infusion on CHAT expression

Both E19 and P1 NGF infusion in the lateral ventricle resulted in a dramatic increase in the absolute number of CHAT positive cells in the neostriatum at E21 and P3, respectively, compared to either vehicle infused or non-infused control animals (Fig. 1). The NGF induced increases in CHAT positive cells were seen in the matrix area and intermediate zone, but not in the striatal patch compartment. A $2 \times 3 \times 3$ (Age [E21 or P3] x Treatment [NGF infusion, vehicle infusion, or control] x Area [patch, IZ, or matrix]) analysis of variance on the absolute CHAT cell counts showed significant main effects of age, $F(1,23) = 8.24$, $p < 0.05$, treatment, $F(2,23) = 17.55$, $p < 0.05$, and area, $F(2,46) = 148.89$, $p < 0.05$. The lack of a significant interaction between Age x Treatment ($p > 0.05$) revealed that the increase in CHAT cell numbers after treatment was comparable between E21 and P3, and we therefore combined the two ages in the subsequent Newman-Keuls tests. NGF treatment affected the absolute CHAT cell counts differentially in the different striatal areas as evidenced by a significant interaction between Treatment x Area, $F(4,46) = 60.31$, $p < 0.05$. Within the patch compartment, Newman-Keuls tests showed that the NGF infusions did not increase the absolute number of CHAT positive neurons compared to the vehicle or control animals. Instead, the number of CHAT positive cells in the patches showed a small but statistically significant decrease after the NGF infusion when compared to the control animals ($p < 0.05$). Both the intermediate zone and the rest of the matrix compartment showed a significant increase in the number of CHAT positive neurons after NGF infusion, compared to either vehicle infused animals or control animals ($p < 0.05$).

In order to investigate if the increased number of CHAT positive cells after the NGF infusion reaches the adult cholinergic pattern, we directly compared the number of cholinergic cells after the embryonic or early postnatal NGF infusion in the different striatal areas with the number of CHAT cells in the different areas in the control adult (chosen as P40, because no significant increase in the absolute numbers of CHAT cells is seen after P40, van Vulpen and van der Kooy, 1996). A $3 \times 3$ (Age [E21, P3 or P40] x Area [patch, IZ, or matrix]) analysis of variance on the absolute CHAT cell counts showed no significant main effect of age on the absolute number of CHAT cells, $F(2,12) = 1.96$, $p > 0.05$, demonstrating that the absolute numbers of CHAT cells after either embryonic or postnatal NGF infusion have been increased to

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Figure IV. 1 The absolute numbers of CHAT positive neurons in the patch compartment, intermediate zone (IZ) and the matrix area in the head of the striatum in control non infused, vehicle infused and NGF infused animals in (A) E19 infused embryos (E21 sacrifice) and (B) P1 infused pups (P3 sacrifice). The numbers of CHAT cells in the different areas at E21 and P3 are compared to the numbers of CHAT cells in the patch compartment, intermediate zone, and matrix area of P40 (adult) control rats. Data represents means ± S.E.M.
the numbers of CHAT cells in the adult (P40) (Fig. 1). It is important to mention however, that the facilitation of the cholinergic maturation after NGF infusion only applies to the cholinergic cell bodies. During normal striatal development, the pattern of acetylcholinesterase (AChE) fiber staining changes from a negative matrix background with AChE rich patches in the developing striatum, to either a darkly stained matrix with AChE poor patches in the mature adult striatum in the cat (Graybiel, 1984) or to a homogeneously AChE staining in the adult rat striatum (Butcher and Hodge, 1976). In the NGF infusion experiments, some additional sections from NGF, vehicle infused and non-infused animals were stained for AChE (data not shown) (protocol according to modification of the direct thiocholine method of Karnovsky and Roots by Tago et al, 1986). No obvious differences in AChE fiber staining could be detected after NGF infusion compared to either the vehicle infused or non infused control, and the adult pattern of more AChE staining in the matrix was not evident after NGF infusion.

Although exogenous NGF administration either embryonically or at P1 can increase the numbers of neurons expressing CHAT (with the absolute numbers quickly increased up to the adult P40 level), the increase in immunostaining within individual neurons after NGF infusion seemed much more pronounced in the postnatal pups compared to the embryonic animals. The CHAT positive cells at P3 after the NGF infusion were very intensily stained, compared to the lightly stained CHAT immunoreactive cells seen in either the vehicle infused (see Fig. 2A-D) or non infused control animals. On the other hand the CHAT positive cells in the embryonic animals after NGF infusion, were relatively lightly stained, similar to the CHAT positive cells in the vehicle infused or control animals at that age. However, the number of lightly positive stained embryonic cells was increased significantly compared to either the vehicle infused or non infused controls. Although in both the NGF infused embryonic and postnatal animals the total numbers of CHAT cells were significantly increased after the NGF infusions, and paralled the adult P40 levels, the numbers of CHAT molecules within a positive CHAT neuron after NGF infusion seemed to be much higher within the postnatal animals compared to the embryonic animals. There are several possible explanations for this age effect. First, the amount of NGF used in the embryos was less than in the P1 animals. However when we used a similar concentration of NGF in the embryos as in the P1 pups, all of the embryos died within the uterus. Second, the chances of NGF dispersal (due to leakage) during or after NGF infusion were much higher in the embryos.
Figure IV. 2 Photomicrographs of CHAT positive neurons and True Blue labeled patches in a coronal 50 µm section. (A,B), This P1 pup was infused with NGF and microinjected with True Blue in the substantia nigra, sacrificed at P3 and immunocytochemically stained for CHAT. In A the CHAT neurons (grey/black) can be seen in relation to the True Blue labeled patches (blue). (B) The same section but photographed with brightfield optics. The same CHAT neurons (brown) can be seen in relation to the CHAT positive fiber patches, which overlap completely the True Blue labeled patches in A. Arrowheads point to some CHAT positive neurons in the intermediate zone, which was arbitrarily defined as two cell diameters from the patches. Dashed lines outline example of patch area. (C) CHAT staining in P1 NGF infused pup compared to P1 vehicle infused control (D). A few examples of CHAT neurons are arrowed in C and D. (E) Photomicrograph of p75 positive neurons and fibers (CY3-red), in relation to True Blue labeled patches (blue) in the E21 lateral striatum in a 14 µm cryostat section. A dashed line outlines the subcallosal streak (sc), and another dashed line outlines a patch area. The majority of the p75 staining can be seen in the matrix compartment. (F) Photomicrograph of trkA positive neurons in the lateral striatum at E21 in a 14 µm cryostat section. A few examples of trkA positive neurons are arrowed. c; cortex, cc; corpus callosum, and s; striatum. Scale bar = 100 µm.
than in the P1 animals. Third, an immature receptor signalling system, which cannot bind or internalize NGF effectively, could result in a lower immunostaining in the embryonic group.

**Perinatal distribution of p75, trkA and CHAT in the striatum**

Compartmental distributions of p75, trkA, and CHAT immunoreactive neurons during early development were studied in single labeled adjacent sections using either a combination of epifluorescence and brightfield (for True Blue and DAB) or conventional fluorescence (for True Blue and CY3). The dense True Blue labeling in the patches was used as the striatal compartment marker. At E21, trkA labeled cells are lightly labeled and most positive neurons are located in the lateral postmitotic striatum (Fig. 2F), with their distribution comparable to the distribution of CHAT positive neurons at that age. The total number of striatal trkA positive neurons steadily increases from 2,857 at E21 to 4,461 at P3, which parallels the increase of total CHAT neurons in the striatum: 2,311 at E21 to 3,910 at P3. At P3, trkA positive neurons are considerably more darkly stained than at earlier ages and can also be detected more medially in the striatum, comparable to the distribution of the CHAT positive neurons at P3. At this timepoint, light staining trkA positive fiber patches could also be detected in the lateral striatum, and there appeared to be a complete overlap between True Blue positive patches and trkA positive fiber patches. E21 is the earliest time point at which both CHAT and trkA can be reliably quantified. Although positive trkA and CHAT cell bodies can be detected before E21, the low intensity of immunostaining within the cell bodies, combined with the relatively high background, made it impossible to quantify these numbers. The intensity of immunostaining for both CHAT and trkA in cell bodies and terminal fields increased markedly from E21 through to the adult.

p75 staining on the other hand can only be detected transiently during early striatal development. The numbers of cells staining for p75 increased from E17 (the earliest timepoint investigated), and an analysis of variance revealed a significant effect of age on the absolute numbers of p75 positive cells in the striatum from E17 through E19, E21 and P3 (F(3,28) = 42.6, p< 0.05). The absolute number of p75 positive cells is maximal at E21, significantly decreases from E21 to P3 (2752 at E21 versus 1067 at P3, Newman-Keuls test, p<0.05) and p75 positive cells no longer can be detected in the second postnatal week, except for the few p75 positive cells in the ventrolateral aspect of the striatum that remain in the adult.
To investigate whether the p75 and trkA cells were homogeneously distributed among the three striatal areas at E21 and P3, or if there was a preference for one of the three areas, we transformed the number of p75 and trkA positive cells in the three different areas to a density value, by equating the areas for volume differences (see van Vulpen and van der Kooy, 1996). A $\chi^2$ test was used to examine if the observed distributions of p75 and trkA cells in each area were significantly different from the 33% value predicted from a homogeneous distribution of p75 and trkA cells in the three areas throughout the striatum. The greatest density of the p75 cell bodies was present in the matrix compartment, with a preference for the intermediate zone within the matrix compartment (E21, $\chi^2_{0.05} = 25.5$, df = 3; P3, $\chi^2_{0.05} = 56$, df = 7). The very prominent p75 fiber staining at E21 also was confined mainly to the matrix (Fig. 2E). The density of trkA positive neurons, on the other hand, was significantly under represented in the matrix compartment (E21, $\chi^2_{0.05} = 12.1$, df = 5; P3, $\chi^2_{0.05} = 23.7$, df = 5), similar to the CHAT staining at this age (see van Vulpen and van der Kooy, 1996).

We calculated the absolute numbers of p75, CHAT and trkA positive cells in each striatal area (the patch compartment, the intermediate zone and the matrix area) for E21 and P3 (Fig. 3). A $2 \times 3 \times 3$ (Age [E21 or P3] × Staining [CHAT, trkA, or p75] × Area [patch, IZ, or matrix]) analysis of variance on the absolute cell counts for p75, CHAT and trkA showed a significant 3 way interaction between age, staining and area, F (4,58) = 4.62, p<0.05. Analyzing the compartmental distributions of the p75, trkA and CHAT-ir populations between E21 and P3 (Fig. 3), revealed that in the patch compartment CHAT and trkA are expressed in similar numbers (not significantly different from each other with Newman-Keuls test, p > 0.05), with both increasing significantly between E21 and P3 (p<0.05). On the other hand, the p75 expressing population is significantly lower in number than the CHAT and trkA population at both E21 and P3 (p <0.05). In the matrix area, the numbers of CHAT and trkA positive neurons also increases significantly between E21 and P3 (p<0.05), but here the CHAT positive neuron numbers are significantly different from the trkA and p75 numbers at E21 and P3 (P<0.05). At E21 in the matrix, the trkA and p75 numbers are greater than the CHAT numbers, while at P3 (although trkA expression is still greater than CHAT expression), the p75 numbers are actually significantly decreased compared to either the CHAT or trkA numbers (p<0.05). In the intermediate zone no significant differences were detected between CHAT, trkA or p75 at E21 or P3.
Figure IV. 3 The absolute numbers of p75 immunoreactive, trkA immunoreactive, and CHAT immunoreactive cells at E21 and P3 in non infused control animals in the patch compartment (A), intermediate zone (B), and matrix area (C). Data represents means ± S.E.M.
To investigate if the p75 expressing population is the same as the trkA immunoreactive population, we performed double labeling for p75 and trkA with two fluorescent markers. The p75 immunoreactive cells seemed to be confined within the trkA expressing population (most p75 positive cells seemed double labeled for trkA, but the reverse was not true). Unfortunately, however, the quality of the staining did not allow us to quantify these results.

**Effects of NGF infusion on the p75 and trkA receptor expression**

Administration of exogenous NGF either embryonically (E19) or early postnatally (P1) was capable of inducing the numbers of neurons expressing CHAT up to the adult (P40) value. Neurotrophins have been reported to regulate the activity of responsive neurons, partially through increasing the level of their receptors (Holtzman et al, 1992; Li et al, 1995). Striatal cholinergic cells that lose their p75 receptor during postnatal development are able to re-express this receptor in response to NGF administration or tissue damage in the adult (Gage et al, 1989; Hagg et al, 1992) and trkA gene expression in the striatum is upregulated after NGF administration in the adult (Holtzman et al, 1992; Meakin and Shooter, 1992). We asked whether the induction of CHAT expression after the embryonic or early postnatal NGF infusion was accompanied by the upregulation of striatal trkA and p75 levels. We therefore investigated the trkA and p75 positive cells in the NGF infused animals, vehicles and the non-infused controls in both the E19/21 and the P1/3 experiments. Unfortunately, the quality of the trkA immunostaining did not allow us to quantify the numbers of trkA positive neurons in these experiments, but the numbers of trkA immunoreactive cells among the NGF, vehicle or control animals did not seem obviously different. A 2 × 3 × 3 (Age [E21 or P3] × Treatment [NGF infusion, vehicle infusion or control] × Area [patch, IZ, or matrix]) analysis of variance on the absolute numbers of p75 positive cells revealed no main effect of NGF treatment (F(2,25) = 0.21, p > 0.05), nor any interaction of NGF treatment with age (F(2,25) = 0.52, p > 0.05) or area (F(4,50) = 0.52, p > 0.05), showing that NGF treatment had no effect on the number of p75 positive cells. In addition, the very prominent p75 fiber staining in the matrix at E21 seemed to be unaffected by NGF, as the p75 staining in the fibers was similar in the non infused controls, the vehicle infused controls, and the NGF infused embryos.
DISCUSSION

NGF facilitates striatal cholinergic maturation in the matrix compartment

The main conclusion of this study is that NGF infusion has differential effects on the CHAT expression by the cholinergic neurons in the different striatal areas. While NGF facilitated CHAT expression in the intermediate zone and the rest of the matrix compartment, it did not increase CHAT expression in the patch compartment. The response to NGF is similar in the intermediate zone and the rest of the matrix compartment (the matrix area), which may not be surprising as the intermediate zone can be considered a part of the matrix compartment (van Vulpen and van der Kooy, 1996). NGF infusion accelerates the CHAT expression of the slowly maturing CHAT neurons in the matrix compartment by bringing the numbers of CHAT cells in the matrix up to, but not exceeding, the adult values. Accordingly, we conclude that NGF is capable of facilitating the CHAT expression of the already committed CHAT neurons in the matrix compartment earlier than is normal in development. We hypothesize that NGF may be the limiting factor endogenously for cholinergic matrix neuron maturation.

On the other hand, NGF does not appear vital for the maturation of the cholinergic patch neurons. During normal development, the number of CHAT positive neurons in the patches increases significantly between E21 and P3 (van Vulpen and van der Kooy, 1996). In the patch compartment of the embryonically NGF infused animal, the number of cholinergic neurons was not increased during this normally active maturation period in the patch compartment. We suggest that NGF is not essential for the maturation of the cholinergic patch neurons, although we cannot rule out the possibility that NGF infused at a timepoint other than E19 could have facilitated patch CHAT maturation.

During normal striatal development there is a gradient of CHAT maturation, with the cholinergic neurons in the patches (which contain the earliest born neurons in striatal neurogenesis E12-E17; see Fishell and van der Kooy, 1991) turning on CHAT first, followed by the cholinergic neurons in the intermediate zone and finally by those neurons in the rest of the matrix compartment (van Vulpen and van der Kooy, 1996). Given that endogenous levels of NGF are very low during early striatal development (Mobley et al, 1989), these low NGF levels may be limiting for the maturation of the cholinergic neurons of the matrix compartment. Exogenously applied NGF may supplement the low endogenous levels, thus inducing the earlier maturation (up to adult values) of CHAT in the cholinergic matrix neurons. It is important to note that the NGF
facilitation of CHAT expression in striatal matrix neurons is a modification or maturation of previously committed neurons. Mice with null mutations of NGF, trkA or p75 all still develop striatal cholinergic neurons, showing that the early commitment of a striatal neuron to the cholinergic phenotype is not dependent on NGF signalling (Crowley et al, 1994; Smeyne et al, 1994; Fagan et al, 1997; Yeo et al, 1997). However, the small and underdeveloped morphology as well as the low CHAT expression of striatal and other forebrain neurons in some of these knockout mice is consistent with our experiments showing that the exogenous NGF infusion can facilitate the maturation of the cholinergic neurons in the striatal matrix.

Although increased CHAT activity after NGF infusion has been reported to correspond to upregulated receptor levels (Holtzman et al, 1992; Li et al, 1995), the present results did not show any upregulation of either the p75 or the trkA receptors after either embryonic or early postnatal NGF infusion. These results do not necessarily imply that NGF does not upregulate receptor levels, but suggest that the increase or upregulation in p75 or trkA protein might have been too small to detect with immunocytochemical techniques. More sensitive mRNA techniques (northern analysis) can show minor increases in trkA mRNA levels, while these same increases are too small to detect with a non-quantitative immunocytochemical staining (Li et al, 1995). Prolonged exposure to NGF over several days is often required to increase trkA levels (see Li et al, 1995). Another possibility is that receptor activation by NGF is an early event in the cellular activation cascade, and by two days (in current experiments) the increased receptor levels have returned to baseline levels and cannot be detected anymore (Kromer and Kaplan, 1995).

**NGF signalling mechanisms as regulators for cholinergic maturation**

The present results show that during early striatal development, the trkA positive cells always overlap or precede the appearance of CHAT expressing neurons, independent of striatal area. This indicates that trkA is always present to mediate normal striatal cholinergic maturation. On the other hand the number of p75 neurons, which is maximal at E21, is less than half of the CHAT population number in the patch compartment at this time. At E21, the matrix compartment (the IZ and the rest of the matrix) contains twice as many p75 expressing cells as CHAT positive neurons, with the p75 immunoreactive cells located preferentially in the intermediate zone of the matrix compartment. These compartmental distribution patterns of the p75- and TrkA- NGF
receptors do not clarify directly the differential compartmental maturation of the cholinergic neurons or the upregulation of CHAT after the NGF infusion. However, trkA may be a limiting factor in cholinergic maturation in the matrix compartment, as the initially lower density of trkA positive neurons in the matrix coincides with the late maturation of the cholinergic neurons in the matrix compartment. Striatal CHAT and trkA increase simultaneously, and in the mature adult striatum NGF can influence cholinergic neurons (all expressing trkA) solely through trkA in the absence of p75 (see Holtzman et al., 1992). As p75 almost entirely disappears during the postnatal striatal development, it’s function in the striatum is most likely transient and p75 might be associated with the increased responsiveness to NGF during striatal perinatal development (Johnson et al., 1987). Although many roles for the p75 receptor have been suggested over the last few years, the exact role of p75 signalling during perinatal striatal development is still unclear. p75-NGF signalling in the patch compartment does not seem essential for the early maturation of the CHAT neurons in the patches as less than half of the cholinergic neurons in the patches express p75 at E21. The number of CHAT expressing neurons in the patch compartment also increases about 1.5 times between E21 and P3 (van Vulpen and van der Kooy, 1996), although p75 expression decreases between E21 and P3. In addition, embryonic NGF infusion did not increase CHAT expression in patch cholinergic neurons. The p75 receptor has been suggested to facilitate cell maturation (Verdi et al., 1994) and although cholinergic neurons in the patches mature before the matrix cholinergic neurons (van Vulpen and van der Kooy, 1996), the p75 receptor does not seem to play such a facilitating role in the cholinergic development of the patch compartment. Even in the matrix compartment, where p75 expressing cells outnumber the CHAT positive neurons at E21, a maturational role of p75 is not likely, as most of the cholinergic neurons in the matrix do not show their cholinergic phenotype until after P3 in control animals (van Vulpen and van der Kooy, 1996), and p75 expression is limited at P3 and thereafter. However p75 signalling (with or without trkA) could be involved in striatal CHAT development in other ways. As a positive regulator, p75 has been involved in the modulation of neurotrophin sensitivity (Davies et al., 1993), in more selective signalling (Clary and Reichardt, 1994), and in the mediation of the retrograde transport of neurotrophins (Anderson et al., 1995). Additionally, p75 has a role in neurite extension (Lee et al., 1994). Regarding this last alternative, the transient perinatal preferential location of p75 in the intermediate zone could be of special interest, as p75
might promote the axonal outgrowth of the cholinergic neurons in the intermediate zone and help establish compartmental connections.

p75 also can have a negative influence on cell function. As a negative regulator p75 appears to be involved in cell death (Rabizadeh et al, 1993; Casaccia-Bonnefil et al, 1996; van der Zee et al, 1996), and may also modulate trkA trophic signals (Wada et al, 1995; Maliartchouk and Saragovi, 1997). The latter option would delay specifically the cholinergic maturation of the matrix neurons, as the highest density of the p75 staining is located in the matrix compartment. Although p75 receptor induced, apoptotic death of the striatal cholinergic neurons has recently been reported in the postnatal mouse striatum (van der Zee et al, 1996), it is not clear if the transient p75 receptor expression in the perinatal rat striatum has a similar function. The p75 apoptotic signal in the mouse (occurring between P6 and P15) was hypothesized to be dependent on trkA for basal forebrain neurons, as apoptosis only occurred in the trkA negative cholinergic basal forebrain neurons. However, during early striatal rat development, when p75 expression is maximal, all cholinergic neurons express trkA (present study). Therefore, it does not seem likely that the early transient perinatal p75 occurrence in the rat striatum is involved with the type of induced cell death described in the p75 null mice.

CONCLUSION
Cholinergic neurons in the striatum mature differentially depending on their compartmental localization, with the cholinergic neurons located in the patches turning on CHAT earlier then the cholinergic neurons in the matrix. The present results indicate that NGF is capable of facilitating the normally slow maturation of the already committed cholinergic neurons in the matrix compartment, while the CHAT maturation in the patches seems to be independent of NGF signalling. Loss of function experiments with NGF or receptor knockouts suggests that NGF signalling is not the critical factor for either committing the striatal neurons to the cholinergic phenotype or for the initial survival of these NGF responsive neurons. Thus, developmentally NGF may regulate the time of matrix CHAT neuron maturation, while in the adult NGF might have more subtle effects in modulating neuronal function (Thoenen, 1995).
GENERAL DISCUSSION

The present thesis comprises an account of the development of striatal cholinergic neurons. Cholinergic neurons mature differentially, depending on the compartmental location, with the cholinergic neurons located in the patches expressing CHAT earlier than the cholinergic neurons in the matrix compartment. Possible mechanisms responsible for the location as well as the differential induction of the cholinergic phenotype have been investigated. Birthdate studies combined with a compartment marker show that birthdate can predict localization of the cholinergic neurons in the striatum, with the earliest born neurons having a higher probability of ending up in the patch compartment and the latest born neurons having a higher chance of ending up in the matrix compartment. I also found that patch cholinergic neurons show a differential distribution of NGF signalling mechanisms compared to the cholinergic neurons in the matrix, which as well points to different sub-populations of cholinergic neurons within the striatum. Increasing the local factors in the environment responsible for cholinergic maturation can accelerate the otherwise slow maturation of the cholinergic neurons in the matrix, while the cholinergic maturation in the patches seems unaffected. The following discussion will describe how a combination of separate developmental mechanisms could be involved in the formation of the adult pattern of cholinergic neurons within the striatum.

A) Cholinergic striatal interneurons mature differentially depending on compartmental localization

The results from chapter II show that there is an apparent heterogeneity among the striatal cholinergic interneurons, with respect to the time that they show their cholinergic phenotype. The earliest striatal cholinergic neurons to mature are located preferentially in the patch compartment during early development (E21-P3), while the majority of the cholinergic neurons in the matrix compartment gradually turn on CHAT later in development. I propose that the differential turn on of CHAT in the striatal cholinergic interneurons might be necessary to ensure proper connections between the striatal cholinergic neurons and the patch and matrix compartments, in a temporally coordinated way. Especially the cholinergic neurons in the intermediate zone are excellent candidates to participate in the communication between the developmentally and functionally distinct patch and matrix compartments, as they have been reported to extend their dendrites and axons into both patch and matrix compartments (Kubota and Kawaguchi, 1993;
Penny et al, 1988). Furthermore, the density of cholinergic neurons in the intermediate zone is increased relative to the patch and matrix compartments (chapter II). The tonically active neurons (TANs), which are thought to be the cholinergic interneurons based on their electrophysiological properties (Wilson et al, 1990; Kawaguchi, 1992; Aosaki et al, 1994a; 1994b; 1995), are of special interest with regards to a possible role in linking the two functionally distinct compartments. TANs show learning dependent plasticity, which is gated by dopaminergic nigrostriatal inputs (Aosaki et al, 1994a). Moreover, TANs recorded in widely separated sites in the striatum respond after learning in a temporally coordinated way to a conditioning stimulus, which suggest that TANs could have a special function in coordinating the striatal circuitry. The fact that roughly half of these neurons lie at the borders of the patches (Aosaki et al, 1995), (which corresponds with the increased density of CHAT neurons in the intermediate zone) supports the hypothesis that TANs are directly involved in collecting reinforcement related information from the patch compartment and influence the activity of the projection neurons in the matrix (Graybiel and Kimura, 1995). Because few TAN cells can be found in the patches (striosomes) (Kimura et al, 1993), the question comes to mind whether all striatal cholinergic neurons are TANs positive, or only those CHAT cells at the patch/matrix border and in the rest of the matrix compartment?

The striatal cholinergic maturation pattern, is not specific for the cholinergic neurons, as a variety of other substances in the striatum also have been reported to be transiently concentrated in the developing patch compartment with a later increase in the matrix compartment (see Table 2). Although the developmental expression of these substances, including CHAT, could be linked in a common differentiation pattern, the cholinergic maturation appears to be under independent inductive influences (chapter IV).
Table 1: Substances that are transiently concentrated in the developing patch compartment, with a later increase in the matrix compartment.

<table>
<thead>
<tr>
<th>Cell bodies</th>
<th>Enkaphalin ¹ (Enk positive fiber patches remain in the adult striatum, although not very distinct in dorsolateral striatum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sub P ¹⁻⁴</td>
</tr>
<tr>
<td>Output neurons</td>
<td>Retrograde tracers selectively label the patch compartment when injected in the nigral target area from E19-P2. If the injection is made later during development, both compartments are labeled resulting in a homogeneous striatal labeling ⁵</td>
</tr>
<tr>
<td></td>
<td>DARPP-32 Patchy prior to the patchy distribution of TH-ir terminals ⁶</td>
</tr>
<tr>
<td>Fibers</td>
<td>AChE ⁷,⁸</td>
</tr>
<tr>
<td></td>
<td>Choline uptake sites ⁹</td>
</tr>
<tr>
<td></td>
<td>TH or DA ¹⁰,¹¹</td>
</tr>
<tr>
<td>Receptor</td>
<td>Neurokinin-1 receptor ¹²,¹⁶ Receptor for SubP found on cholinergic and somatostatin interneurons in adult ¹³⁻¹⁵</td>
</tr>
<tr>
<td></td>
<td>D1 receptor binding ¹⁷,¹⁸</td>
</tr>
<tr>
<td></td>
<td>Mu-opiate receptor binding ¹⁹⁻²¹</td>
</tr>
<tr>
<td></td>
<td>Muscarinic receptors ²²</td>
</tr>
<tr>
<td>Other</td>
<td>Ca²⁺/Calmodulin-dependent protein kinase II ²³ Patchy prior to the patchy distribution of TH-ir terminals</td>
</tr>
</tbody>
</table>

¹- Song and Harlan, 1993, 1994
²- Ni and Jonakait, 1988
³- Boylan et al, 1990
⁴- Zahm et al, 1990
⁵- Fishel and van der Kooy, 1987
⁶- Foster et al, 1987
⁷- Butcher and Hodge, 1976
⁸- Murrin and Ferrer, 1984
⁹- Lowenstein et al, 1989
¹⁰- Olsen et al, 1972
¹¹- Voorn et al, 1988
¹²- Ardelt et al, 1996
¹³- Kaneko et al, 1993
¹⁴- Shigemoto et al, 1993
¹⁵- Gerfen, 1991
¹⁶- Quirion and Dam, 1986
¹⁷- Rao et al, 1991
¹⁸- Murrin and Zeng, 1989
¹⁹- Moon Edley and Herkenham, 1984
²⁰- Murrin and Ferrer, 1984
²¹- van der Kooy, 1984
²²- Nastuk and Graybiel, 1989
²³- Newman-Gage and Graybiel, 1988
B) Birthdate can predict localization of the cholinergic neurons in the striatum.

My analysis of cholinergic neurogenesis within the caudate putamen revealed that the cholinergic neurons that become postmitotic early in neurogenesis (E13) have a higher probability of ending up in the patch compartment compared to the matrix compartment. On the other hand, the majority of the later born cholinergic neurons (E17), has a higher chance of ending up in the matrix compartment. I propose that birthdate can therefore predict localization of the cholinergic neurons in the striatum. Cholinergic neurons also show the same relative birthdate/compartment relationship as to other striatal neurons, although the absolute birthdates are shifted earlier in neurogenesis.

With neuronal birthdate being a strong predictor of compartmental localization, neurons are either committed to their compartmental location prior to becoming postmitotic or are restricted to a compartmental fate as a result of their initial cellular interactions with the postmitotic striatal environment. The only way to test commitment is through directly challenging the predestination of a cell, although some evidence suggest that the determination of cellular characteristics is based not strictly on the exact birthdate, but rather the relative timing of the generation of cells (Snyder-Keller, 1995; Gillies and Price, 1993). Numerous studies over the last few years have investigated the commitment of striatal neurons through directly challenging the fate of striatal neural progenitors. Precursors specifically derived from the LGE (lateral ganglionic eminence) have been reported to incorporate primarily into the developing host striatum (Campbell et al, 1995) but are also capable of modifying their regional phenotype within the telencephalon in response to local position specific cues (Fishell, 1995). In both studies the transplanted cells differentiated into the appropriate phenotypes and made the appropriate connections with either the striatal or cortical targets. Because striatal neural progenitors consist of both future patch and matrix neurons, it is problematic however to directly challenge compartmental fate. Cholinergic precursor cells need to be individually labeled, transplanted and followed to their compartmental location to directly test whether cholinergic striatal neurons are committed to their compartmental location prior to becoming postmitotic or if cellular interactions with the postmitotic striatal environment determine the compartmental location.

One mechanism that could achieve the postmitotic compartmental segregation of the cholinergic striatal neurons is adhesion. In vitro reaggregation studies investigating the organization of striatal neurons into their appropriate compartments showed that the early born
patch neurons are selectively adhesive to one another (Krushel et al, 1989). This in contrast with the later born neurons (E18 -P2) which mainly end up in the matrix compartment and which are non adhesive (Krushel and van der Kooy, 1993b). Cholinergic neurons are born over the same early developmental period when patch neurons are born (Semba et al, 1988; Phelps et al, 1989) yet cholinergic perikarya are distributed fairly evenly across the adult striatal patch and matrix compartments (Graybiel et al, 1986). If cholinergic neurons are non-adhesive then how do some end up in the patches, and if cholinergic neurons are adhesive then how do some end up in the matrix? Because birthdate appears to be important in predicting the location of the cholinergic neurons, with the earliest born cholinergic neurons have a higher probability of ending up in the patch compartment, and the later born neurons (E17) preferring the matrix compartment, I predict that the earliest of the early born CHAT positive neurons will be more adhesive than the slightly later born cholinergic neurons, and will be more centrally located and more tightly clustered in the reaggregates than the slightly later born cholinergic neurons. Future reaggregate experiments will test to what extent cell adhesion turns on among the striatal cholinergic neurons and whether the selective adhesion of only the earliest born cholinergic neurons might explain the distribution of cholinergic neurons in the patch and matrix compartments in vivo.

C) Influence of striatal environment on cholinergic differentiation and maturation

i. Interaction with striatal afferents

Besides local factors produced in the striatum influencing cholinergic maturation (like NGF), the cholinergic phenotype could also depend on other interactions with the striatal environment. The striatal environment is made up by a multitude of factors, including the afferents from distal structures, and collaterals from local neurons. Primary afferents to the striatum originate from numerous sources and most show compartmental specificity. One of the major inputs to the striatum originates from the cortex, and contains most likely glutamate. However most of the cortical fibers terminate on the spiny projection neurons and only limited contacts are found on the cholinergic interneurons (Dimova et al, 1993). Also near complete perinatal decortication has no effect on striatal acetyl cholinesterase staining (Kolb et al, 1986).

Of special interest is the other major afferent striatal input, the dopaminergic fibers from the substantia nigra. Substantia nigra dopamine fibers innervate the striatum prior to compartmentalization (Murrin, 1983; Moon Edley and Herkenham, 1984; van der Kooy, 1984;
Voorn et al, 1988) and the distribution of dopamine (which originates entirely from nigrostriatal afferents) also changes from diffuse to patchy at a time coincident with the formation of the striatal compartments and the cholinergic maturation (Olsen et al, 1972; Voorn et al, 1988). AChE positive patches also correlate directly with the reported dopamine islands early in development (Graybiel et al, 1981; Graybiel, 1984). The D1 receptor which is expressed by approximately 25% of all striatal cholinergic neurons (Le Moine et al, 1991), is also transiently concentrated in the developing patch compartment, with a later increase in the matrix compartment (Rao et al, 1991; Murrin and Zeng, 1989). Dopaminergic fibers have been shown to terminate on the cholinergic neurons (Dimova et al, 1993) and the learning dependent plasticity of the TAN's neurons (which are thought to be the cholinergic neurons) is gated by dopaminergic nigrostriatal input (Aosaki et al, 1994a). CHAT activity is increased in striatal tissue cocultured with ventral mesencephalon (Kessler, 1986), which indicates that the early arriving dopamine nigrostriatal afferents might be involved in the induction of CHAT activity. On the other hand, dopamine might also have an inhibitory effect on cholinergic cell numbers as in striatal cultures (with the dopamine afferents cut off) the number of CHAT cells appears increased (Østergaard, 1993). Also in the adult weaver mouse, which develops a massive cell death of neurons in the substantia nigra, combined with the over 70% loss of dopamine in the striatum, the number of CHAT cells is increased by 34%, combined with an increased labeling intensity of CHAT (Smith et al, 1993). Other studies show no effect of dopamine on striatal AChE or CHAT mRNA levels. Destruction of dopamine containing nigrostriatal afferents to the developing striatum either in utero (E17-18) or early postnatal (P0 or P3) does not profoundly alter the AChE staining pattern in the postnatal striatum (Snyder-Keller, 1991) and also removal of nigrostriatal afferents of the young adult striatum by 6-OHDA lesions, does not change levels of striatal CHAT mRNA (Brené et al, 1990). Striatal cholinergic neurons can also be easily identified in either striatal explants (Walker et al, 1987), organotypic cultures (Østergaard, 1993), or in the D1 receptor mutant mouse (Xu et al, 1994), although counts of the cholinergic interneurons were not carried out. Therefore it seems more likely that the induction of CHAT activity is intrinsic to the striatum, although extrinsic neuronal connections with other areas may modulate the differential maturation of the cholinergic neurons.
ii) NGF as the local factor influencing cholinergic differentiation and maturation

The recent development of gene targeting techniques has generated several neurotrophin and receptor knockouts to study their role in the development and function of neurons in the PNS and CNS. The NGF null mutant mice displayed as expected severe sensory and sympathetic deficits due to neuronal loss, but surprisingly cholinergic neurons in the basal forebrain and striatum differentiated and maintained their phenotype for the life span of these mice (Crowley et al., 1994). The cholinergic neurons in these NGF null mice did however appear smaller and also more lightly stained for CHAT and trkA. On the other hand disruption of a single allele of the nerve growth factor gene did result in permanent cell loss in the septal area (Chen et al., 1997). This difference between homozygous and heterozygous NGF knockouts might conceivably be explained by the possibility that exposure to NGF is necessary to induce dependence of cholinergic neurons for survival (Chen et al., 1997). In the NGF null mice, other homologous trophic factors could conceivably compensate for the actions of NGF, as NGF signalling does not seem absolutely required for the survival or the early commitment to the cholinergic phenotype of the striatal and basal forebrain cholinergic neurons (Crowley et al., 1994; Smeyne et al., 1994). While NGF is not required for survival or maintenance of the phenotype of cholinergic neurons in the striatum during early postnatal life, this factor may have more subtle functions, such as regulating the level of CHAT in these cells and therefore the functional status of these cells, or in neuronal plasticity (Thoenen, 1995). During early postnatal striatal development, another alternate function of NGF might be the timing of cholinergic maturation, and as the results suggest in Chapter IV, specifically of the cholinergic neurons in the matrix compartment, since NGF does not seem vital for the cholinergic maturation of the cholinergic patch neurons. NGF would in this way act as an endogenously limiting factor for cholinergic matrix neuron maturation.

One of the major targets of the striatal cholinergic neurons are the striatal substance P projection neurons (Phelps et al., 1985; Izzo and Bolam, 1988), which are located in both the patch and matrix compartments. These substance P projection neurons might also be the possible source of NGF in the striatum because almost all substance P neurons contain cholinergic muscarinic receptors (Bernard et al., 1992) and NGF positive cells can be seen as many medium sized (10-18 μm) cells distributed in equal density throughout the medial and lateral extent of the striatum (Altar et al., 1991; Conner and Varon, 1992). Interestingly, the substance P neurons have been reported to be in a patchy distribution embryonically (Ni and Jonakait, 1988; Boylan et al,
1990), while in the complementary matrix compartment substance P is largely expressed postnatally. If the assumption, that the substance P neurons are the NGF ligand producing cells, is valid, then the heterogeneous pattern of substance P expression also might point to a heterogeneous distribution of NGF within the developing striatum, with NGF levels initially higher in the patch compartment.

Besides the traditional role of a neurotransmitter and modulator of neural transmission, substance P could also have a developmental role in the nervous system. Substance P and other NK-1 receptor agonists have been shown to elicit fiber sprouting in neuroblastoma cells (Narumi and Maki, 1987), cultured chick dorsal root ganglia (Narumi and Fujita, 1978) and explanted chromaffin cells (Barker et al, 1993) and substance P has been observed to modulate the release of chemoattractants from the floor plate in the embryonic spinal cord (De Felipe et al, 1995). Therefore substance P could possibly act as a local factor influencing cholinergic differentiation and maturation.

Varying levels of NGF will also indirectly influence the cholinergic maturation in the striatum, and factors regulating the NGF synthesis in the striatum need to be determined. In the developing hippocampus one of the factors controlling the levels of NGF is acetylcholine (other factors include glutamate and GABA, see da-Penha-Berzaghi et al, 1993). It would be interesting to investigate whether striatal cholinergic neurons themselves might influence the production of NGF in the striatum during postnatal development.

As proposed previously the differential turn on of CHAT in the striatal cholinergic interneurons might be necessary to ensure the proper connections between striatal cholinergic neurons and the functionally distinct patch and matrix compartments. As NGF is able to facilitate the normally slow cholinergic maturation of the cholinergic neurons in the matrix compartment (including the intermediate zone), it raises the question whether exogenous NGF, besides facilitating the cholinergic phenotype, is also capable of affecting the wiring of the cholinergic matrix neurons within the striatum. NGF has been shown to be capable of increasing the complexity of neuritic trees of the postnatal striatal cholinergic interneurons, with the axons being more susceptible than the dendrites (Studer et al, 1994), however no effect of NGF was found in the embryonic striatum (Martinez et al, 1985; Hartikka and Hefti, 1988). An analysis of dendritic and axonal arborizations of the cholinergic neurons in the different striatal areas after NGF infusion compared to controls, would be able to address this question.
The cholinergic striatal interneurons situated in the patches and in the intermediate zone have been reported to send dendrites into both patch and matrix compartments (Penny et al., 1988; Kubota and Kawaguchi, 1993). The time of development of these boundary-crossings is however unknown. Tenascin and glycoconjugate boundaries have been reported around the patches in the first postnatal week (Steindler et al., 1988; OBrien et al., 1992). These boundaries can not be detected in mature animals and their appearance seems to correspond with critical periods during the development and stabilization of distinct functional/morphological arrangements. It could be that these glial boundaries deter the late maturing cholinergic cells in the matrix from sending their dendrites over to the patch compartment. These boundaries are apparently "soft" boundaries, because they disappear when 6-OHDA is given in the substantia nigra during development (Steindler et al., 1988). It would be interesting to see if the 6-OHDA treatment, causes the reorganization of the cholinergic cell bodies and the neuritic tree. A reorganization of barrel neurons has been reported in the cortex after a 6-OHDA lesion, and barrel neurons were found to disobey the barrel boundaries. Because NGF can increase neurite outgrowth and complexity of striatal cholinergic neurons if given postnatally (Studer et al., 1994), I would expect to see a faster outgrowth of the neurites after postnatal NGF infusion and I also predict more boundary crossings from the cells in the patches and in the intermediate zone. Assuming that a restrictive local environment is the mechanism how the cholinergic cells in the matrix become confined within the matrix, I would also predict that they will send dendrites over to the patch compartment after the NGF infusion because the glial boundaries have not developed fully yet. I propose that low endogenous levels of NGF in the striatum during development could be important for properly setting up the connections of the cholinergic cells between patch and matrix compartment. Although the levels of endogenous NGF increase during striatal development, NGF is still very low in the fully developed adult striatum (Korsching, 1986; Hagg et al., 1989). The biological significance of such a less than maximal trophic support has been suggested to be crucial for an effective role of these neurons. Endogenous NGF can be functionally modulated to either stimulate or reduce the neuronal performance and a lower constant level of endogenous NGF might ensure that the neurons perform at a limited fixed level (Hagg et al., 1989).
(D) NGF signalling mechanisms and striatal cholinergic development

The expression of the p75 receptor and CHAT in neostriatal cholinergic neurons do not follow the same temporal pattern, and seems to be regulated by separate intracellular mechanisms. One example is that CNTF is capable of upregulating the p75 receptor, but not choline acetyltransferase in the normal striatum (Hagg et al, 1992). On the other hand the regulation of trkA and CHAT in the striatum, follows a very similar temporal pattern (Li et al, 1995a), which suggests that both are regulated by the same factor. This factor is NGF, as both exogenous and endogenous NGF, are capable of regulating the differentiation of the striatal cholinergic neurons (Li et al, 1995a). The role of trkA in NGF induced cholinergic differentiation is quite well established. NGF is capable of activating the striatal trkA receptors at P0, while NGF infusion also increases trkA expression (Li et al, 1995a, 1995b). Additionally overexpression of trkA accelerates NGF induced differentiation (Hempstead et al, 1992). In the adult striatum, NGF infusion is also able to upregulate the trkA receptor (Gibbs and Pfaff, 1994), and NGF is thought to mediate its effects solely through trkA in the absence of p75, as no p75 positive cells can be detected in the adult striatum. The role for the “other” NGF receptor during striatal cholinergic differentiation is not that clear cut, and the following discussion proposes some ways p75 might be involved in the striatal cholinergic maturation process.

Over the last few years, many roles for the p75 receptor have been suggested, with p75 having either a positive or a negative influence on cell function (for reviews see Bredesen and Rabizadeh, 1997; Carter and Lewin, 1997; Kaplan and Miller, 1997). As a positive regulator p75 has a role in the modulation of neurotrophin sensitivity (Davies et al, 1993), neurite extension (Lee et al, 1994), more selective signalling (Clary and Reichardt, 1994) and mediation of retrograde transport of neurotrophins (Anderson et al, 1995; Curtis et al, 1995). The p75 knockout (Lee et al, 1992) showed that p75 enhances the sensitivity of NGF dependent neurons to NGF, and p75 expressing populations might be able to survive or show their phenotype in situations where there are sub-saturated levels of NGF. The p75 receptor has also been thought to make cells mature faster (Verdi et al, 1994) or make them more sensitive to NGF (Davies et al, 1993).

In the striatum, the p75 receptor expression disappears almost entirely during the postnatal striatal development and in the adult striatum only a very small percentage of the cholinergic neurons express P75 (Kordower et al, 1988; Kiss and Patel, 1989). While other NGF
responsive areas, like the forebrain, express p75 into adulthood, p75 is only transiently expressed during the perinatal striatal development, at which time the striatum also shows an increased responsiveness to NGF (Johnston et al., 1987). In the early developing striatum, only half of the CHAT population expresses p75, while there are many more p75 positive cells than CHAT cells in the E21 matrix compartment (including the intermediate zone) (Chapter IV). While a maturational role for the p75 receptor in the patch compartment seems not very likely, p75 could be important in the cholinergic maturation of the matrix compartment. p75 may help CHAT matrix maturation, first, by making the cholinergic neurons in the matrix more sensitive to the limiting amounts of NGF present in the developing striatum. The cellular localization of NGF has only been established so far in the adult striatum (Conner and Varon, 1992), because the levels of NGF are virtually undetectable during early development (Mobley et al., 1989). Some evidence, however, points to a heterogeneous distribution of NGF within the striatum throughout development, with higher levels of NGF in the developing patches during early development. High levels of zinc, correlated with the storage of NGF propeptide, can be detected in heavily stained patches with Timm's staining during the first postnatal week (Vincent and Semba, 1989). The staining in the matrix increases after postnatal day 6, and the zinc levels are higher in the matrix compartment in the adult striatum. The greatest density of the p75 cell bodies in the matrix compartment during early development is concurrent with the low levels of NGF in the matrix, and the p75/trkA expressing (NGF responsive) cholinergic neurons in the matrix compartment might need the p75 receptor to make them more sensitive to NGF.

A second possibility is that p75 might support the growth of axons towards their targets. Evidence to support this comes from the p75 null mice displaying a reduced sympathetic innervation of the pineal and sweat glands (Lee et al., 1994) (although p75 not required for the survival of the NGF-dependent sympathetic neurons (Lee et al., 1992)). In the developing striatum, the transient perinatal preferential location of p75 in the intermediate zone could possibly function to promote axonal outgrowth of the cholinergic neurons in the intermediate zone towards the substance P projection neurons in both compartments, and help establish communication between the functionally distinct patch and matrix compartments (Wilson et al., 1990; Dimova et al., 1993; Lapper and Bolam, 1992; Kawaguchi, 1992, 1993).

A third alternative is that the presence of p75 in the early striatum results in more selective signalling. The coexpression of p75 and trkA permits NGF to signal more selectively over other
neurotrophins, while expressed alone, trkA is apparently capable of also responding to neurotrophin 3 (Clary and Reichardt, 1994). This promiscuity may account for the limited effects of the NGF knock out on striatal cholinergic differentiation (Crowley et al, 1994). Also in the adult striatum, NT-3 could potentially play a role in receptor activation and neuronal function, as trkA is expressed in striatal cholinergic neurons, without p75 expression (Holtzman et al, 1992; Steininger et al, 1993; Sobreviela et al, 1994).

Finally, p75 might be transiently expressed by the striatal cholinergic neurons during development to facilitate the retrograde transport of NGF by these neurons, as only areas in the brain that contain both the p75 and trkA receptor show prominent retrograde transport of NGF (Anderson et al, 1995; Curtis et al, 1995). The motoneurons are also known to transiently express the NGF receptor during development, when NGF is retrogradely transported by these neurons (Yan et al, 1988). Interestingly, the majority of the striatal p75 staining during perinatal development is confined to the matrix compartment (either in the cell bodies or in the fibers) and the presence of p75 might therefore simply reflect a way of getting NGF to the cell body where it could exert its trophic activity.

As a negative regulator p75 appears to be involved in cell death (Rabizadeh et al, 1993), and unbound p75 may also modulate trkA trophic signals (Wada et al, 1995; Maliartchouk and Saragovi, 1997). The latter option would specifically delay the cholinergic maturation of the matrix neurons as the majority of the p75 staining is located in the matrix compartment. Induced cell death of the cholinergic matrix neurons, caused by an unoccupied p75 receptor (Rabizadeh et al, 1993), does not seem very likely, as p75 positive neuronal number decreases, while CHAT positive neurons in the matrix compartment steadily increases during development, without showing a decline (Chapter II). Theoretically, it is possible though that the cholinergic neurons in the perinatal matrix expressing both trkA and p75 are in a state of dying because of an unoccupied p75 receptor, but are rescued when NGF increases in the striatal matrix later during development. However the NGF knockout mouse does not show massive striatal cholinergic cell death (Crowley et al, 1994).

For the cholinergic neurons in the patch compartment on the other hand, p75 induced cell death is a more likely possibility, as the number of CHAT positive neurons in the patch compartment decreases significantly after P3 (Chapter II). This decreasing number has been hypothesized to be due to either cell death or migration of the cholinergic neurons from the
patches into the matrix compartment (Chapter II). The number of CHAT patch cells decreasing after P3 parallels the number of p75 expressing cells in the patches perinatally, and therefore it is feasible that the CHAT cells in the patches that initially express the p75 receptor selectively die when the amount of NGF becomes limiting, while the patch CHAT cells expressing only trkA survive. However in two recent papers describing apoptotic cell death due to the p75 receptor, apoptosis selectively occurred in the trkA negative population (Frade et al, 1996; van der Zee et al, 1996). Additionally van der Zee et al (1996) described apoptotic cell death of trkA negative cholinergic neurons in the late postnatal mouse striatum (Although no striatal cholinergic cell death can be detected in a similar study by Yeo et al, 1997). The results from chapter IV show that all cholinergic neurons in the developing striatum express trkA and only a percentage expresses also p75. As P75 is only transiently expressed in the perinatal rat striatum, and almost entirely disappears in adulthood, p75 acting as a cell death receptor seems more likely in early striatal development. The discrepancy between the time of the described apoptosis of the cholinergic cells in the late postnatal mouse striatum, and the possible cell death due to the unbound p75 receptor in the early rat perinatal striatum as hypothesized above, could possibly be due to the different mouse versus rat models. Hopefully future research will enlighten us about p75’s function in cholinergic striatal development, and whether it acts as a positive or a negative regulator (or both) of cholinergic cell function.
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