Coverpicture: The ancient Egyptian hieroglyphics for the word “brain”, first appeared in a Seventeenth Century B.C. papyrus describing the symptoms, diagnosis, and prognosis of two patients wounded in the head. This writing is the earliest known reference to the brain anywhere in the human record.

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On Transplantation of Fetal Ventral Mesencephalon with Focus on Dopaminergic Nerve Fiber Formation

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Stockholm 2002
For Björn and Malte
“The Brain is wider than the sky
For put them side by side
The one the other will contain
With ease—and you beside”

Emily Dickinson
This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


V. **Törnqvist N**, Strömberg I. The localization of extracellular matrix molecules in intrastriatal ventral mesencephalic grafts in the rat model of Parkinson’s disease. Preliminary manuscript.
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INTRODUCTION

Parkinson’s disease

Clinical features and pathology

In 1817 the London-physician Sir James Parkinson described the disorder that bears his name in “An Essay on the Shaking Palsy”. His description of the typical clinical symptoms and appearance of patients with Parkinson’s disease (PD) is still applicable: “involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported, with a propensity to bend the trunk forwards, and to pass from a walking to a running pace, the senses and intellects being uninjured”.

PD is not only the most well known example of hypokinetic disorders, but also the first example of a brain disease resulting from deficiency of a single neurotransmitter, namely dopamine (DA). The disease is characterized by a slowly progressing degeneration of the dopaminergic neurons in substantia nigra pars compacta (SNC) (Trétiakoff 1919), which project to the caudate nucleus and the putamen. This causes the motor disturbances typical for PD. In the 1960’s it was shown from postmortem studies that the levels of DA were decreased in the basal ganglia of PD patients (Ehringer and Hornykiewicz 1960), and that the severity of degeneration in the SN paralleled the reduction of DA in the striatum (Bernheimer et al. 1973).

Another characteristic feature of PD and a prerequisite for the diagnosis, is inclusion bodies called Lewy bodies that can be found primarily in the SNC (Lewy 1912). The Lewy body is an intracellular eosinophilic hyaline inclusion, consisting of filamentous material, and staining positive for ubiquitin and α-synuclein (Forno 1996; Goedert 2001; Lewy 1912; Spillantini and Goedert 2000). Interestingly, in some genetic forms of PD, α-synuclein is mutated (Kruger et al. 1998; Polymeropoulos et al. 1997), but the function of the gene is unknown. Lewy bodies are present in other cell types and disease states of the brain, and around 9-13% of healthy persons above the age of 70 exhibit Lewy bodies in the SN (Forno and Langston 1993; Gibb and Lees 1988). Therefore, the reason to why the Lewy bodies are found and how they are related to PD is not fully understood.
Neuromelanin is a substance that is somewhat of a mystery. Its function is not known, but has been suggested to favor the formation of Lewy bodies (Duffy and Tennyson 1965), or to be a waste product of catecholamine metabolism, protecting the cell from harmful metabolites. It has also been suggested to interfere with the normal function of the nerve cell and lead to cell death, since it accumulates in excessive amounts with age (Mann and Yates 1974). The name substantia nigra (black substance), given because of the presence of neuromelanin, is unfortunate, since the pigment is found only in catecholamine-synthesizing neurons (German et al. 1983), which are quite rare among other neuronal types in the SN. Secondly, the pigment occurs only in primates, and thirdly, the pigment is not unique to the SN, but is found in some neurons of all groups utilizing bioamines. It has long been known that pigmented neurons of the ventral midbrain are preferentially damaged in PD. Moreover, the SN is preferentially destroyed, while the ventral tegmental area (VTA) is spared. It appears that there is a significant relationship between the neuromelanin content and the neuronal vulnerability (Hirsch et al. 1988). In other words, if the SN neurons degenerate preferentially it might be because practically all of them contain neuromelanin, and since the VTA neurons are resistant it might be due to the fact that they are devoid of the pigment.

Neuronal loss in SN with advancing age has been documented in normal aging in several studies, and was assessed to 36-48% between the age of 20 and 90 (Hirai 1968; Mann et al. 1984). The pattern of cell loss indicates that the dorsal tier of the SN is more vulnerable than the ventral tier (Fearnley and Lees 1991). In PD, there is a ten-fold increase in cell death rate compared to normal aging, and in contrary to aging the neurons of the ventral tier are most sensitive (Fearnley and Lees 1991).

Loss of DA in the putamen is more prominent than in the caudate of PD patients (Nyberg et al. 1983), and within the putamen the caudal portions are more severely affected (Kish et al. 1988). This can be explained by the more pronounced loss of dopaminergic neurons in the ventral tier that preferentially project to the putamen (Gibb and Lees 1991). Normal aging causes a reduction of striatal DA in the range of 50-60% between the age of 14 and 92. The loss is, however, the
same for the caudate and the putamen, thereby differing from the pattern seen in PD (Kish et al. 1992).

The cardinal symptoms of the disease occur when approximately 70-80% of the ventral mesencephalic dopaminergic cells have undergone degeneration (Bernheimer et al. 1973; Nyberg et al. 1983). Muscular rigidity, reduced movement ability (hypokinesia), difficulties to initiate movement (akinesia), slowness of movement (bradykinesia), and rhythmic resting tremor are the major kinetic losses (Gowers 1893; Selby 1984). Moreover, the typical patient suffering from PD is easy to recognize by the shuffling gait, flexed posture, and impaired balance (Gowers 1893; Parkinson 1817). The severity of the symptoms increases over time, but the progression of the disease differs considerably between patients (Schwab 1960). Likewise, the prevalence differs concerning geographic area, but the overall value has been calculated to increase from 0.1% in the population as a total to 1% in people 60 years or older, making it the second most common neurodegenerative disease (Langston 1998; Tanner 1996).

Patients suffering from PD do not only exhibit motor disturbances, but sometimes also lack motivation and have reduced spontaneity. Experiments have shown that a depletion of DA in the prefrontal cortex impairs the performance of monkeys in cognitive tasks. The deficit can be counteracted by the precursor of DA, L-DOPA, or by apomorphine (a DA agonist) (Brozoski et al. 1979). The cognitive symptoms of parkinsonian patients might therefore reflect a decrease in transmission in the mesocortical dopaminergic pathways originating in the VTA and terminating in the prefrontal cortex.

Examples of other brain nuclei besides the SN, known to be affected in PD, are the noradrenergic locus coeruleus, involved in dementia (German et al. 1992; Zweig et al. 1993), and some of the serotonergic raphe neurons, involved in depression (Halliday et al. 1990; Jellinger 1991).

**Etiology of PD**

The etiology of PD is unknown, but several theories have been matter of discussion over the years. However, since the disease has a mean onset at the age of 50-65 (Kurtzke 1992), age has been ascribed to the most prominent risk factor (Burton and Calne 1990; Kurland et
al. 1973). It has to date not been possible to prove the involvement of a genetic component, although the disease is more prevalent in some families. Approximately 15-19% of the PD patients have relatives that also suffer from the disease, suggesting involvement of genetic factors in PD (Duvoisin 1984; Gowers 1893; Hart 1904; Kurland 1958). Furthermore, a family history of PD is still the strongest risk factor apart from age (Semchuk et al. 1993). During the 90’s, a couple of genes linked to familial PD were discovered. Mutations in the α-synuclein gene were found in two families with autosomal dominant inheritance of PD (Kruger et al. 1998; Polymeropoulos et al. 1997), and the protein parkin was found to be involved in a recessive form of parkinsonism (Kitada et al. 1998). Mutation of specific genes, however, is rarely found to be the cause of PD. Furthermore, monozygotic twin studies lend little support to inheritance of the disease (Ward et al. 1983).

Environmental factors, like heavy metals (Rybicki et al. 1993) have been discussed as potential causes to the disease. It is well known that toxic chemicals in the environment can cause neurological diseases. One example is the Parkinson syndrome suspected to be induced by manganese in miners working with manganese ores (Feldman 1992). Another well known example is the parkinsonism caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a side product of synthetic heroin self-administered by drug abusers in California and Hawaii (Davis et al. 1979; Langston et al. 1983). Since this drug is not as potent in rodents as in humans (Boyce et al. 1984; Saghal et al. 1984; Walters et al. 1984), the finding of MPTP, apart from opening up a field of investigation of other potential toxic environmental factors, led to the development of a non-human primate model for PD.

MPTP reduces the levels of DA in mice, humans, and monkeys, and cellular degeneration takes place in the SN (Burns et al. 1983; Elsworth et al. 1987; Jenner et al. 1984; Langston et al. 1984; Rios et al. 1995). MPTP is transformed into the toxic metabolite MPP⁺, which is taken up by dopaminergic cells (Rios et al. 1995; Tipton and Singer 1993). The toxic effects have been ascribed to the ability of the ion to block the complex I enzyme in the mitochondrial membrane, which leads to energy depletion, production of free radicals and cell death.
(Nicklas et al. 1985). Interestingly, reduced levels of the complex I enzyme, as well as elevated levels of free iron and lipid peroxidation (which indicates the state of oxidative stress) have been found in brains of patients with PD (Dexter et al. 1989b; Dexter et al. 1989a; Jenner 1998; Mann et al. 1994; Schapira et al. 1990). Moreover, the pesticide rotenone is a complex I inhibitor inducing degeneration of striatal dopaminergic terminals and formation of Lewy bodies in rats (Betarbet et al. 2000). Free radicals are highly toxic agents normally trapped by protective agents such as catalase, peroxidase and glutathione. Indeed, these trapping agents are depleted in the SN of PD patients (Ambani et al. 1975; Perry et al. 1982). Therefore, any agent that interferes with inactivation of free radicals might contribute to neuronal loss. Manganese, for instance, has been reported to inhibit the scavenging of free radicals (Donaldson et al. 1982). Thus, there is substantial evidence that oxidative stress might be involved in the etiology of PD.

Finally, the involvement of infectious agents in the development of PD was clearly exemplified in 1916 when an epidemic outbreak of Encephalitis lethargica (of Von Economo) caused a large number of parkinsonian-like cases (Burton and Calne 1990).

The neurotransmitter DA

In 1948, a brain substance differing from adrenaline and noradrenaline was identified. This molecule was named enkephalin (Raab and Gigee 1951). Later it was suggested that a factor X found in high concentrations in the brain could be DA (Montagu 1957). In the late 50’s it was shown that DA was a distinct neurotransmitter and not

\[
\text{Tyrosine} \rightarrow \text{Dopa} \rightarrow \text{Dopamine} \rightarrow \text{Noradrenaline} \rightarrow \text{Adrenaline}
\]

TH AADC DBH PNMT

Fig. 1. Pathway for the synthesis of dopamine, noradrenaline, and adrenaline. Enzymes involved in the process are listed under the arrows. Tyrosine hydroxylase (TH) catalyzes the rate limiting reaction between tyrosine and dopa (dihydroxyphenylalanin), dopa-decarboxylase (AADC) synthesizes dopamine from dopa, dopamine-β-hydroxylase (DBH) converts dopamine to noradrenaline and phenylethanolamine-N-methyltransferase (PNMT) converts noradrenaline to adrenaline.
only an intermediate molecule in the synthesis of noradrenaline and adrenaline (fig. 1) (Bertler and Rosengren 1959; Carlsson et al. 1958).

During the following years these findings together with the discovery of L-DOPA by Carlsson and colleagues (Carlsson et al. 1958) and the development of the Falck-Hillarp fluorescence method (Falck et al. 1962) led to extensive mapping studies of the major catecholaminergic pathways in the CNS (Bertler and Rosengren 1959; Dahlström and Fuxe 1964).

The distribution of dopaminergic neurons in the brain was first mapped by Dahlström and Fuxe in 1964. They found small clusters of dopaminergic cells, and named each nuclei with an A followed by a number. A large portion of these neurons are located in the A9 (substantia nigra, SNC) and in the A10 (ventral tegmental area, VTA). The human SNC contains about 450 000 dopaminergic neurons in each hemisphere (German et al. 1983). In adult rat, the number of dopaminergic neurons has been estimated to 15-25 000 per side with an equal amount located in A9 and A10 respectively, while the number of dopaminergic neurons in A8 is approximately 1 400 per side (Björklund and Lindvall 1984; Fallon and Loughlin 1995).

The dopaminergic neurons of the brain are organized into four major subsystems; the tuberoinfundibular, nigro/mesostriatal, mesolimbic, and mesocortical systems (Björklund and Lindvall 1984; Fuxe et al. 1970). While the tuberoinfundibular, mesolimbic and mesocortical systems are known to participate in cognition and to be involved primarily in some of the symptoms of schizophrenia (Matthyse 1981; Stevens 1979), the nigrostriatal system plays an important role in the control of voluntary movement and, as previously discussed, selective destruction of this pathway results in the motor disturbances of PD.

The basal ganglia

The basal ganglia consist of four major distinct nuclei; the striatum, the globus pallidus internal and external segments (GPI, GPe), the subthalamic nucleus (STN), and the substantia nigra including pars reticulata and pars compacta (SNr, SNC). The corticothalamalic link running via the basal ganglia is highly segregated; each circuit originates in a specific area of the cortex and engages
different portions of the basal ganglia and thalamus. Therefore, the organization of the basal ganglia-thalamocortical motor circuit is somatotopic. The skeletomotor link, for instance begins in the premotor cortex, the supplementary motor area and the motor cortex, while the limbic circuit has other origins. Moreover, each cortical area projects to a discrete striatal area in a topographic manner. Hence, association areas project to the caudate, sensorimotor areas project to the putamen and limbic areas project to the ventral striatum (Björklund and Lindvall 1984).

*The striatum*

The striatum is the major input nuclei of the basal ganglia, and receives input from large parts of the motor-cortex and from thalamus. The striatal nucleus is anatomically and functionally highly heterogenous. It consists of two parts, the patch/striosome and the matrix compartments that differ from each other histochemically. Briefly, striosomes may be characterized by high levels of μ-opioid receptor density and the presence of axon collaterals from somatostatin containing striatal interneurons, while the matrix compartments express high levels of acetylcholinesterase (AChE) and calbindinD28k (Gerfen and Wilson 1996; Graybiel and Ragsdale Jr 1983). Most neurons (90-95%) in the striatum are medium spiny projection neurons utilizing γ-aminobutyric acid (GABA)-ergic neurotransmission (Gerfen and Wilson 1996). These cells receive the major cortical input and are also the main source of output. However, present in the striatum are also a few cholinergic local inhibitory interneurons. They have extensive collaterals reducing the activity of the striatal output neurons (Gerfen and Wilson 1996).

The SNr and the Gpi are the major output nuclei from the striatum. The main skeleto-motor circuit consists of corticostriatal, striatonigral, and striatopallidal connections of which the two latter ones further make a connecting loop to the motor-cortex via the thalamus. An important connection concerning PD is the dopaminergic nigrostriatal or mesostriatal connection, which projects from SNC to the striatum.
**DA receptors**

There are at least six known DA receptors; D₁, D₂a, D₂b, D₃, D₄, and D₅, which can be divided into two subgroups; D₁-like (D₁ and D₃) and D₂-like (D₂, D₃, D₄) (Missale et al. 1998). A biochemical distinction between the D₁-like and D₂-like receptors has been widely accepted; D₁ receptors are associated with an adenyl cyclase which is sensitive to stimulation by DA (Kebabin and Greengard 1971), while D₂ receptors inhibit adenyl cyclase (Kebabin and Calne 1979; Spano et al. 1978). The D₁ receptor is expressed in medium-sized neurons in the caudate-putamen (Gerfen et al. 1990), in the nucleus accumbens, and in the olfactory tubercle (Deary et al. 1990; Gerfen et al. 1990; Sunahara et al. 1990; Zhou et al. 1990), while the D₂-receptors are expressed in medium-sized enkephaline-positive and large cholinergic neurons of the caudate-putamen (Gerfen et al. 1990; Le Moine et al. 1990a; Le Moine et al. 1990b), in the nucleus accumbens, olfactory tubercle (Bunzow et al. 1988; Dal Toso et al. 1989), and the midbrain (Meador-Woodruff et al. 1989). This suggests that the D₂ receptor also has a presynaptic role. On terminals it acts as an inhibitory autoreceptor controlling both the rate of neuronal firing and the DA release at the terminal.

**SNC and VTA**

Both A9 and A10 dopaminergic neurons in the midbrain project to the caudate-putamen. A9, however, primarily projects to the dorsal striatum (the mesostriatal connection) while A10 makes mesolimbic connections to areas of the ventral striatum (nucleus accumbens and olfactory tubercle) and mesocortical connections to the limbic and frontal cortex (Björklund and Lindvall 1984; Fuxe et al. 1970). A9 makes further connections with the GP, STN, and neocortex, and A8 (the retrorubral field, RRF) projects to the ventral striatal areas (Björklund and Lindvall 1984).

A second type of subdivision of the A9 and A10 dopaminergic neurons can be made concerning which striatal compartment that is their target of projection. Dopaminergic neurons that project to the striosomes have their origin in A9 including a few neurons that originate from the SNr. These neurons are called ventral-tier neurons and do not express calbindin_D₂₅k (Gerfen et al. 1987; Gerfen et al. 1987;
Gerfen and Wilson 1996; Jimenez-Castellanos and Graybiel 1987). The neurons that project to the matrix have their origin in A10, the dorsal tier of A9, and in A8. These neurons express calbindin_{D28k} (Gerfen et al. 1987; Gerfen et al. 1987; Gerfen and Wilson 1996; Jimenez-Castellanos and Graybiel 1987). Calbindin_{D28k} has been shown to be expressed by dopaminergic cholecystokinin (CCK)-expressing neurons preferentially innervating ventral striatum, while dopaminergic neurons co-expressing TH and retinaldehyde dehydrogenase 1 (RALDH1) preferentially project to dorsal striatum (German and Liang 1993; Haque et al. 1997; Hudson et al. 1994; McCaffery and Drager 1994; Schultzberg et al. 1984; Seroogy et al. 1988). Therefore, simplified, most A9 neurons are calbindin_{D28k}-negative while RALDH1-positive, and innervate the dorsal striatum, while A10 neurons co-express calbindin_{D28k} and CCK, and innervate the ventral striatum.

**Development of the ventral mesencephalic dopaminergic system**

The presence of DA appears at E13 in rat, and at E14 the first dopaminergic fibers reach the striatum (Olson and Seiger 1972). In neonatal rodents, the dopaminergic systems are still under progress, and not until several weeks later the striatal innervation is fully developed (Gerfen et al. 1987; Seiger and Olson 1973).

Cells in the A8-A10 areas of the ventral midbrain undergo their final mitosis between E12 and E16 in rat (Lauder and Bloom 1974). TH-expression can be detected in cells after the time point for final mitosis with first expression appearing at E12 (Foster et al. 1988; Specht et al. 1981). Generation of several neuronal cell types in the CNS is known to depend on signaling of the protein sonic hedgehog (Shh) (Echelard et al. 1993; Ericson et al. 1995). Moreover, it has been demonstrated that Shh can induce neurons of a dopaminergic phenotype in explants (Hynes et al. 1995; Wang et al. 1995). Therefore, it is clear that Shh is involved in the dopaminergic developmental pathways. Moreover, development of midbrain structures requires the protein fibroblast growth factor-8 (FGF-8) (Crossley et al. 1996). However, many other factors probably are involved in the induction of the dopaminergic cell fate.

Recently, it was found that Nurr1, an orphan nuclear receptor belonging to the steroid/thyroid hormone receptor superfamily, is
involved in the development of the nigrostriatal circuit (Law et al. 1992; Zetterström et al. 1997). Nurr1 mRNA is expressed in many areas of the CNS, for example in the ventral midbrain area (Saucedo-Cardenas and Conneely 1996; Xiao et al. 1996; Zetterström et al. 1996). In the A8, A9, and A10 areas, Nurr1 is co-expressed with TH (Baffi et al. 1999; Bäckman et al. 1999). Since Nurr1 was shown to disappear from the ventral midbrain following a 6-hydroxydopamine (6-OHDA) lesion, it was concluded that a co-localization with TH indeed is present (Zetterström et al. 1996). In Nurr1 gene knockout mice, some of the markers known to be specific for midbrain dopaminergic neurons are lacking, and the animals die within a few days after birth (Zetterström et al. 1997; Zetterström et al. 1996). Moreover, heterozygous mice have reduced levels of striatal DA, indicating a role for Nurr1 also in mature DA neurons (Zetterström et al. 1997). These results identified Nurr1 as a critical constituent of DA neurons during development and at postnatal stages.

The classical basal ganglia model

The classical model of basal ganglia circuitry was developed in the late 80's, partly in order to explain the hypokinesia of PD, the hyperkinesia of Huntington's disease, and hemiballism (Albin et al. 1989; DeLong 1990). The internal connections of the basal ganglia follow two distinct pathways leading to opposite functions. The medium spiny striatal neurons express different neuropeptides depending on which target they project to; neurons utilizing the direct pathway express substance P and dynorphin, while neurons projecting via the indirect pathway express enkephalin and neurotensin (Gerfen and Wilson 1996).

The projection that runs from the cortex via thalamus to the striatum is an excitatory glutamatergic projection. Via the direct pathway, activation of medium spiny GABAergic neurons of the striatum causes an inhibition of the tonic activity in the output nuclei, which in turn causes a small GABAergic inhibitory input to the thalamus and cortex. Therefore, a high activity is achieved in the cortex and movement follows upon activation of the direct pathway. The indirect pathway does not include a direct connection between the striatum and the output nuclei. Instead, a GABAergic connection
between the striatum and the GPe is employed. The low activity in the GPe leads to a small inhibition of the STN to which GPe is connected. Therefore STN gains high activity. Via the only excitatory glutamatergic pathway of the basal ganglia, the STN increases the activity of the two output nuclei causing an inhibition of the thalamic and cortical activity. Hence, an activation of the indirect pathway causes a depression of movement.

DA, exerting its effect in the nigrostriatal pathway, however, causes a facilitation of movement by its differential action on these two pathways. This is due to the presence of different DA receptors in the direct and indirect pathway. DA acting on D₁ receptors in the direct pathway causes an increase in transmission, while DA acting on D₂ receptors in the indirect pathway causes a decrease in transmission. Hence, the overall effect of DA is a facilitation of movement via both pathways (fig. 2a).

It is believed that the main cause to the hypokinesia in PD is loss of dopaminergic input from SNC to the striatum. This leads to over-activity in the indirect pathway, decreased activity in the direct pathway, and therefore increased inhibition of thalamocortical neurons. These changes cause an overall depression of movement (fig. 2b). The disturbed activity in the basal ganglia pathways may be counteracted by surgical or electrical intervention of the nuclei involved, sometimes resulting in dramatic effects on some of the parkinsonian symptoms. Thalamotomy, thalamic stimulation, pallidotomy, pallidal stimulation, and STN stimulation are some examples of this kind of therapy (Narabayashi 1990).

The classical model of the basal ganglia functional anatomy has been very important for the understanding of underlying mechanisms in PD. However, the model best accounts for akinesia, while it is more difficult to explain rigidity and tremor. The existence of the direct/indirect pathways has been questioned since many striatal neurons project simultaneously to the GPe, Gpi, and SNr (Parent et al. 2000). Moreover, the effect of DA on striatal neurons is much more complex; i.e., DA does not simply activate the direct pathway and inhibit the indirect pathway (Calabresi et al. 2000), and D1 and D2 receptors are often co-localized in striatal neurons (Aizman et al. 2000). Furthermore, the classical model does not explain why thalamo-
Fig. 2 The direct pathway (grey) of the classical basal ganglia model projects from the caudate/putamen via an inhibitory GABAergic pathway to the output nuclei GPi/SNr (a). Inhibition of these nuclei causes a small inhibitory input to the thalamus and cortex, leading to a facilitation of movement. In the indirect pathway (black) the connection between the caudate/putamen and the GPe leads to a low activity of the GPe and a high activity of the STN. A high excitatory input from the STN to the GPi/SNr leads to a large inhibition of the thalamus and cortex causing depression of movement. However, DA acting on D1 receptors in the direct pathway, and on D2 receptors in the indirect pathway facilitates movement via both pathways. In (b) the cause to the hypokinesia of PD according to this model is shown. Loss of DA leads to over-activity in the indirect pathway and decreased transmission in the direct pathway, and therefore increased inhibition of thalamo-cortical neurons and an overall depression of movement.

tomy reduces tremor (Jankovic et al. 1995), and why pallidotomy eliminates dyskinesias (Lang et al. 1997). Instead, the model predicts that thalamotomy results in decreased thalamic activation of the cortex, and thereby worsens the symptoms of PD, and that pallidotomy at the internal segment diminishes inhibitory outflow from the basal ganglia and produces hemiballism. However, the classical model has been valuable in the field of hypo- and hyperkinetic research.

**Treatment strategies**

*Anti-cholinergic drugs, L-DOPA, and DA agonists*

The first effective anti-parkinsonian treatment was anti-cholinergic drugs. These were introduced long before there was any understanding of the neurochemical pathology of the disease. Belladonna (atropin) extract was introduced by Charcot in 1892 and used as main treatment until the first synthetic drugs came 50 years later. The nigrostriatal dopaminergic input normally has an inhibitory effect on acetylcholine-containing interneurons, and loss of inhibitory
innervation results in increased firing of cholinergic cells (Bartholini 1980). Anticholinergics continue to be prescribed, but the effects are greatest in reducing rigidity and only modest effects are achieved on tremor and akinesia.

In the 50's, the Swedish scientist Arvid Carlsson discovered the effect of the rate limiting enzyme and precursor of DA, namely L-DOPA. In 1957, Carlsson and his colleges had found that reserpine, a drug with antipsychotic effects, caused massive depletion of DA in the brain resulting in hypokinesia (Carlsson et al. 1957; Carlsson et al. 1958). When Carlsson's experimental animals were given L-DOPA, which in those days solely was believed to be a precursor of noradrenaline, the motor inhibition was counteracted (Carlsson et al. 1957). It was also shown that DA was preferentially located in the basal ganglia (80% of the total brain content), and constituted one half of the catecholamine-content of the brain. Intravenous administration of L-DOPA was performed in clinical trials by Birkmayer and Hornykiewicz and was proved to provide a dramatic but brief reversal of the symptoms (Barbeau et al. 1962; Birkmayer and Hornykiewicz 1961). However, when applied at a higher dose, the patients exhibited significant reduction of the parkinson-related symptoms (Cotzias et al. 1967).

In contrast to DA, L-DOPA has the ability to cross the BBB, and ever since the 70's, L-DOPA has been an efficient and widely used drug for treatment of PD. When administering L-DOPA, the few remaining neurons in the SN restore some of the nigrostriatal functional loss by synthesis to DA (Hefti and Melamed 1980). The DA might then be released in amounts large enough to exert effects on appropriate target cells. Another possibility is that dopa decarboxylase (AADC) might produce DA from orally administered L-DOPA in non-dopaminergic (for example serotonergic) cells. This has, however, been out-ruled due to the fact that lesioning of the raphe nuclei does not diminish the behavioral effects of L-DOPA on rats with nigrostriatal lesions (Melamed et al. 1980).

The fact that DA neurotransmission is maintained for a relatively long time in PD, despite a massive disappearance of DA nerve terminals, has early been explained on basis of the concept volume transmission (Agnati et al. 1986). It is probable that L-DOPA too propagates through the extracellular space by means of diffusion and
exerts its effect through non-synaptic transmission. This would make it possible to achieve an effect despite the low number of dopaminergic neurons (Fuxe et al. 1970; Zigmond et al. 1987).

L-DOPA is nowadays used in combination with decarboxylase inhibitors that do not pass the BBB, and therefore inhibit the peripheral metabolism of L-DOPA decreasing peripheral side effects and increasing the levels of the drug reaching the brain (Bartholini and Pletscher 1968; Dunner et al. 1971; Tissot et al. 1969). Sometimes concomitant treatment with DA agonists like bromocriptine, a D2-receptor agonist, is applied (Markstein and Herrling 1978), but these agonists are not as potent as DA, which acts on both D1 and D2 receptors. Other drugs in use are inhibitors of the enzymes monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT), preventing breakdown of released DA (Quinn 1995).

Although L-DOPA is a potent and widely used drug, it does not alter the course of the disease; it only controls some of the symptoms. Moreover, the positive effect declines as dopaminergic neurodegeneration progresses, and the patients often develop on-off symptoms due to fluctuations in dosage of the drug. When the concentration of the drug becomes too high the patients experience dyskinesia, while too low a dose leads to rigidity (Granéus 1978; Rinne et al. 1981).

_Grafting of fetal neural tissue_

The problems with dose-fluctuations that arose after years of treatment with L-DOPA also led to efforts in finding treatment strategies focused on cell replacement. Grafting of fetal dopaminergic neurons to the striatum became one of the most important methods (Björklund and Stenevi 1979; Perlow et al. 1979). Fetal neurons from a variety of brain regions can successfully be incorporated into the adult brain and then be identified by the neurotransmitters they produce. The first clinical experiments involving cell replacement in PD, however, were performed using catecholamine-producing chromaffin cells from adrenal medulla. In 1982 and 1983 PD patients received autografts to the caudate nucleus (Backlund et al. 1985), but the results were disappointing. The following two patients received grafts to the putamen instead. This resulted in better, but still insufficient
improvements (Lindvall et al. 1987). Further clinical trials involved chromaffin tissue supplemented with nerve growth factor (NGF) (Sydow et al. 1995), but at that time point results from fetal dopaminergic grafts appeared more promising.

In the late 60’s it was discovered that the neurotoxin 6-OHDA could cause a selective degeneration of catecholaminergic neurons in the CNS (Tranzer and Thoenen 1967; Ungerstedt 1968). The neurotoxin is absorbed by the terminals, accumulates in the dopaminergic cell bodies and degeneration follows due to the production of free radicals. Soon after this discovery, a rat model for PD was developed (Ungerstedt 1968; Ungerstedt and Arbuthnott 1970). A unilateral injection of 6-OHDA into the medial forebrain bundle (MFBB) causes a unilateral lesion of the nigrostriatal pathway, which in turn gives rise to a motor asymmetry between the right and left side. The extent of the lesion can be determined by studying drug-induced turning behavior; apomorphine causes contralateral rotational behavior due to hypersensitivity of the DA receptors in the lesioned striatum and amphetamine causes ipsilateral rotational behavior due to massive release of DA in the intact striatum (Hudson et al. 1993; Ungerstedt 1971; Ungerstedt and Arbuthnott 1970). Using this model, it was soon shown that fetal dopaminergic grafts could survive and counteract the effect induced by the neurotoxin (Björklund and Stenevi 1979; Perlow et al. 1979). Extensive animal experiments were introduced to further investigate the mechanisms of action of such grafts. It was found that not only do the neurons survive for a long time (Freed et al. 1980; Strömberg and Bickford 1996), but they also reinnervate the host striatum in a target-specific way (Björklund et al. 1981; Strömberg et al. 1992), form new synaptic connections (Bolam et al. 1987; Clarke et al. 1988; Freund et al. 1985; Jaeger 1985; Mahalik et al. 1985; Strömberg et al. 1988), normalize striatal neuronal firing rate (Fisher et al. 1991; Strömberg et al. 1985; Van Horne et al. 1990), and it was possible to prove release of DA from the reinnervated striatum (Rose et al. 1985; Strömberg et al. 1988; Zetterström et al. 1986). Moreover, some motor functions were restored (Björklund et al. 1981; Dunnett et al. 1988). Destruction or rejection of the graft caused a reversal of the improvements proving that the positive effects indeed were induced by the graft (Carder et al. 1988; Dunnett et al. 1988).
The first clinical trials involving fetal DA neurons were initiated in the late 80's (Lindvall et al. 1988; Madrazo et al. 1988), and recently two postmortem studies have shown survival of the grafts and reinnervation of the putamen (Kordower et al. 1998). This is further confirmed by $[^{18}F]$-DOPA positron emission tomography (PET)-scans (reflecting survival of the graft and outgrowth to the striatum) showing a return of $[^{18}F]$-DOPA uptake and DA release in transplanted patients (Piccini et al. 1999). To date, around 350 patients have received dopaminergic transplants (Freed et al. 1995; Freeman et al. 1995; Lindvall 1997; Olanow et al. 1997; Peschanski et al. 1994). Functional improvements have been documented in many patients, sometimes to the extent that pharmacological treatment with L-DOPA could be withdrawn (Lindvall 1997). However, many of the patients still show only moderate recovery (Lindvall and Hagell 2000), and whereas the effect on rigidity, bradykinesia/akinesia, and motor fluctuations is quite good, the effect on several other symptoms remain unaffected (Lindvall and Hagell 2000).

In 2001 the first double-blind placebo-controlled trial of grafting to PD patients was published (Freed et al. 2001). The results revealed a significant improvement only among younger patients (<60 years) one year post-grafting. Furthermore, development of dyskinesias was reported among 15% of the patients. However, the tissue had been cultured prior to implantation, and less amount of tissue as compared to previous studies had been grafted (Kordower et al. 1998; Kordower et al. 1996). Furthermore, the patients had not undergone immunosuppression. Accordingly, the number of surviving cells was lower than that found in previous reports, further demonstrated by two cases from the study that have come to autopsy. In the patients 7 000-40 000 dopaminergic neurons were found as compared to 80 000-140 000 cells in the previous clinical studies (Kordower et al. 1998; Kordower et al. 1996). It has been shown from rat experiments that as few as 200-500 dopaminergic neurons (1-5% of the grafted cells) can reverse the parkinsonian symptoms (Brundin et al. 1988). It is therefore not clear if the cause to the dyskinesia reported in the double-blind study is due to cell survival, as suggested by the authors (Freed et al. 2001).
As exemplified by the above discussed study, the symptomatic relief upon grafting is incomplete, and the efficacy still has to be improved. Not only is it necessary to achieve an increased cell survival above the 5-20% of the grafted neurons that survive today (Barker et al. 1996; Brundin et al. 1988), but also to improve the reinnervation of the host striatum. The latter issue is highly relevant since it has been shown in animal experiments that the host reinnervation process halts shortly after implantation (Barker et al. 1996). Moreover, the reinnervation is not further increased although years of survival time (Strömberg and Bickford 1996) and the nerve fiber density is lower than that normally seen in dorsal striatum (Vidal et al. 1998).

Previous results from our research group have shown the presence of two different growth patterns in striatal anlage (lateral ganglionic eminence, LGE) when cografting ventral mesencephalon (VM) and LGE using the intraocular transplant model (Strömberg et al. 1997; Strömberg and Humpel 1995). When VM and LGE are simultaneously implanted into the anterior chamber of the eye, a dense and patchy growth pattern can be seen in LGE after a few weeks. This pattern is similar to the one seen during normal striatal development (Olson and Seiger 1972). On the contrary, when LGE is implanted and allowed to mature before the VM is implanted, a diffuse and widespread growth pattern is induced (fig. 3). Therefore, it seems that LGE has reached maturity and lost its ability to attract nerve fibers from the DA neurons with capacity to form patchy growth. Interestingly, when the patchy growth is induced, the innervation of the immature striatal target continues until nerve terminals cover the entire volume. The diffuse growth terminates after a certain time period, when large portions of the LGE still have not been innervated (Strömberg et al. 1997). Therefore, it is clear that an immature striatal target gains a more robust innervation. In this context, it becomes obvious that it is not only necessary to strive for an enhanced graft cell survival. Even more important is it to consider the VM as a heterogenous area housing different subpopulations of dopaminergic neurons, and to increase the specific population of neurons within a graft that gives rise to the desired growth pattern in the striatal target.
Fig. 3 The intraocular model for cогrafts may be applied for both sequential (a) and simultaneous (b) grafting of VM and LGE. For sequential grafting, LGE is implanted at a time point before VM, leading to a diffuse and widespread innervation pattern in LGE (a). When VM and LGE are implanted to the anterior chamber of the eye simultaneously, a patchy and dense innervation pattern is found in the LGE (b).

Xenografting

Grafting of fetal dopaminergic neurons has been a matter of debate ever since introduced, due to ethical issues, and lately efforts have been made in the field of xenografting. Gifts from other species have been shown to survive well in animals under immunosuppression (Hufsker et al. 1989; Pakzaban and Isacson 1994), and graft survival as well as axonal extensions into the host brain have been shown when transplanting pig dopaminergic neurons to a patient suffering from PD (Deacon et al. 1997). Nevertheless, there is still a problem concerning the risk for cross-species transfer of infectious agents (Isacson and Breakefield 1997).

Stem cells

To bypass the ethical concerns, the ideal situation would be the production of large numbers of dopaminergic neurons from a small number of stem cells. However, the induction of a dopaminergic phenotype has been shown to be a promising but complex procedure. Several factors are, as previously discussed, involved in the process of determining dopaminergic cell fate; Shh, FGF-8 and the orphan nuclear receptor Nurr1 are some of those (Ye et al. 1998; Zetterström et al. 1997). In 1999 a dopaminergic phenotype was successfully induced in Nurr1-overexpressing neural stem cells (Wagner et al. 1999). Grafting experiments using neural stem cells have until recently, however,
shown only short term survival, a low number of surviving cells, and transient TH-expression (Ostenfeld et al. 2000; Wagner et al. 1999). Interestingly, axonal outgrowth was demonstrated when grafting neural precursor cells to rat striatum (Ostenfeld et al. 2000; Studer et al. 1998). Earlier this year, a study demonstrated that embryonic stem cells can develop into functional dopaminergic neurons after transplantation in the parkinsonian rat model (Björklund et al. 2002), suggesting a future for this area.

**Neurotrophic factors; NGF**

In the efforts to enhance the efficacy of transplantation of embryonic VM tissue to PD patients, extensive studies have been performed concerning the role of different growth factors in enhancing the survival and outgrowth of midbrain dopaminergic neurons.

The first identified molecule which was shown to exert effects on cell survival was NGF (Cohen 1959; Cohen 1960; Levi-Montalcini and Hamburger 1953). After the discovery of NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 and –4 (NT-3, NT-4) with close relationship to NGF were identified (Barde et al. 1982; Ernfors et al. 1990; Hallböök et al. 1991; Jones and Reichardt 1990; Leibrock et al. 1989; Maisonpierre et al. 1990; Rosenthal et al. 1990). Due to their potency to promote cell survival, they were assigned to the group of neurotrophic factors included in the family of neurotrophins. During the recent years neurotrophic factors have gained much attention due to their obvious survival promoting effect on neurons under degeneration.

**GDNF and TGFβ**

Gliarial cell line-derived neurotrophic factor (GDNF) was, as the name implies, derived from a glial cell line. This cell line has been shown to promote increase of dopaminergic uptake in cultured nigral neurons (Lin et al. 1993). Moreover, enhanced VM graft survival, outgrowth and functional effect in the parkinsonian rat has been demonstrated in several studies (Granholm et al. 1997; Johansson et al. 1995; Meyer et al. 1999; Rosenblad et al. 1996; Sautter et al. 1998; Strömberg et al. 1993; Wilby et al. 1999; Zawada et al. 1998). However, it has been suggested that the effect of GDNF requires the presence of
another growth factor, namely transforming growth factor beta (TGFβ) (Kriegstein et al. 1998). This factor has a critical role in the regulation of differentiation and proliferation of various cell types. Furthermore, it has been shown to regulate the survival of midbrain dopaminergic neurons (Kriegstein and Unsicker 1994; Poulsen et al. 1994).

After the discovery of GDNF, three more family members were identified; neurturin (NTN) (Kotzbauer et al. 1996), artemin (ART) (Balogh et al. 1998; Rosenblad et al. 2000) and persephin (PSP) (Milbrandt et al. 1998).

**Basic FGF and EGF**

Both basic FGF (bFGF or FGF2) and epidermal growth factor (EGF) can be referred to as mitogens, since they induce proliferation of neuronal precursor cells in *in vitro* embryonic mesencephalon (Gensburger et al. 1987; Murphy et al. 1990; Mytilineou et al. 1992; Ray et al. 1993; Santa-Olalla and Covarrubias 1995; Studer et al. 1998). Both factors are present in the SN (Bean et al. 1991; Cintra et al. 1991; Fallon et al. 1984; Grothe et al. 1991); bFGF has been proved to stimulate cell proliferation in cultures of mesencephalon (Knusel et al. 1990; Studer et al. 1998) and EGF receptor (EGFR) mRNA is present in midbrain dopaminergic neurons (Seroogy et al. 1994). The subventricular zone and the area lining the ventricles are known to house proliferating nestin-positive precursor cells (Barbe 1996; Lendahl et al. 1990; Morshead and van der Koy 1992). Interestingly, the bFGF and EGF receptors are expressed in this area (Seroogy et al. 1995; Wanaka et al. 1991) and it is known that bFGF increases markers for immaturity, such as nestin (Wahlberg 1997). This indicates that bFGF and EGF in some way are involved in the regulation of neuronal and glial progenitor cells in the embryonic brain. Indeed, both EGF and bFGF have been shown to stimulate proliferation, differentiation, and probably migration to surrounding striatal tissue of these cells in adult rat (Wahlberg 1997).

**Viral vectors**

Neurotrophic factors have been widely used to enhance VM graft efficacy, but one difficulty has been to deliver the neurotrophic compounds. For this purpose, viral vectors may be used. For instance,
genetic information can be inserted into neurons or glial cells in the SN or the striatum, leading to an increased production of DA (Leone et al. 2000), or GDNF (Kordower et al. 2000). The major drawback with this approach is that viral vectors can induce inflammatory responses in the recipient (Hollon 2000). Therefore, a nonviral transfection technique may also be applied, as demonstrated by transfer of the GDNF gene to fetal VM tissue (Bauer et al. 2000).
AIMS

The major aim of this thesis was to increase VM-derived striatal innervation density in the Parkinson model for rat. The specific aims of papers I-V were:

**Paper I**
- to morphologically and functionally characterize different striatal growth patterns in intraocular VM-LGE cografts

**Paper II**
- to increase dopaminergic VM-derived striatal reinnervation in the Parkinson model for rat by usage of factors known to be associated with striatal immaturity

**Paper III**
- to investigate whether all TH-positive neurons within a VM graft participate in the striatal reinnervation

**Paper IV**
- to investigate the possibility to induce TH-expression in Nurr1 knockout tissue *in vitro*

**Paper V**
- to study the presence of extracellular matrix molecules in intra-striatal VM grafts and possibly relate to the guidance of dopaminergic outgrowth into the host
MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (150-250 g) or fetuses from rats of the same strain were used throughout the experiments of papers I, II, III and V (B&K, Sollentuna, Sweden). In paper IV, Nurr1 mutant mice were used. The mice were generated from the C57/B1 strain, described elsewhere (Zetterström et al. 1997). Matings between heterozygous males and females occurred during nighttime, and the animals were separated in the morning. When a vaginal plug was detected, the first day following mating was considered as embryonic stage (E) 0.5 of the embryos.

All animals used were kept in rooms with controlled temperature and a standardized night/day schedule of 12/12 h. The animals had free access to water and food pellets. All experiments performed followed ethical guidelines approved by the local animal research committee.

Dissection of VM and LGE

VM and/or LGE was dissected from rat or mouse fetuses of different developmental stages, and the tissue was used for grafting or primary organotypic cultures (table 1).

Pregnant rats were anaesthetized and, by palpation, pregnancy was confirmed, and the age of the fetuses was estimated (Olson et al. 1983). The two uterine horns were removed from the pregnant rat or mouse after deep anaesthesia followed by dislocation of the neck. VM was dissected bilaterally using the mesencephalic flexure as landmark (Seiger 1985). The butterfly-shaped dissected piece of VM tissue, including bilateral A8-A10 dopaminergic cell groups, was cut in the midline. The LGE was dissected as the lateral part of the striatal anlage after cutting open the overlying cortex (Pakzaban et al. 1993). The most anterior portion of LGE was isolated and used as one piece of tissue. For fetal mouse tissue, the uterus was removed and put in a sterile petri dish, and dissections of VM and/or LGE were performed under sterile conditions.
All tissue pieces were placed in sterile, room tempered Dulbecco’s modified Eagle’s medium (DMEM, Gibco) for a maximum of two hours before used for transplantation or cell cultures.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Target area</th>
<th>Type of graft</th>
<th>Embryonic stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Iris</td>
<td>Unilat. VM</td>
<td>E15.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unilat. LGE</td>
<td>E15-17</td>
</tr>
<tr>
<td>II</td>
<td>Striatum</td>
<td>Bilat. VM</td>
<td>E13.5-15.5</td>
</tr>
<tr>
<td>III</td>
<td>Iris</td>
<td>Unilat. VM</td>
<td>E14-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unilat. LGE</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Lat.ventricle</td>
<td>Bilat. VM</td>
<td>E14-16</td>
</tr>
<tr>
<td>IV</td>
<td>Culture</td>
<td>Bilat. VM</td>
<td>E9.5</td>
</tr>
<tr>
<td>IV</td>
<td>Culture</td>
<td>Unilat. VM</td>
<td>E10.5</td>
</tr>
<tr>
<td>IV</td>
<td>Culture</td>
<td>1/2 Unilat. VM</td>
<td>E15.5</td>
</tr>
<tr>
<td>IV</td>
<td>Culture</td>
<td>LGE</td>
<td>E13.5-14.5</td>
</tr>
<tr>
<td>V</td>
<td>Striatum</td>
<td>Bilat. VM</td>
<td>E14.5</td>
</tr>
</tbody>
</table>

Table 1. Embryonic stages used for VM and/or LGE grafted to iris, striatum, lateral ventricle, or used for tissue cultures. VM was severed in two or four pieces or used as one tissue block, while LGE always was used as one piece of tissue.

**Sympathectomy and intraocular transplantations**

The *in oculo* model, described by Olson *et al.* in 1983, is a useful tool for studying brain tissue in rat experiments. It can be regarded as an *in vivo* alternative model to cell culture techniques and has been applied at our department since the late 60’s (Malmfors and Olson 1967; Olson and Malmfors 1970; Olson et al. 1983; Strömberg 1999; Strömberg et al. 1985). The anterior chamber of the eye has several advantages when compared with other grafting or culturing techniques; the cornea is transparent and allows the graft to be studied non-invasively. The eye provides a chamber where the grafts can expand in volume without being squeezed by or exerting pressure on host tissues, and the graft survives as long as the host does. Moreover, the graft is developed under *in vivo* conditions. When two pieces of tissue are implanted, connections can be formed between them, serving as a microcircuit of, for instance, two fetal brain regions such as VM and LGE. Since the tissue pieces can be implanted at different time points, this model also allows for studies on aging effects.

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Before receiving the intraocular transplants the rats were bilaterally sympathectomized under deep anesthesia. The superior cervical ganglia were removed to avoid interference from endogenous, noradrenergic innervation of the host irides. This allowed identification of catecholaminergic nerve fibers derived from the grafted tissue. A time lapse of at least ten days after the sympathectomy was employed to ensure that endogenous levels of neurotrophic factors, which are increased in the iris after the procedure, become normalized (Ebendal et al. 1983; Ebendal et al. 1985).

Fig. 4 Tissue pieces are introduced into the anterior chamber of the eye of the rat host through a slit in the cornea. The grafts are placed in the desired position by gentle pushing from the outside of the cornea with a pair of forceps.

Five minutes prior to transplantation, the eye received a drop of atropin to achieve dilation of the pupil and minimize the risk of iris injury. A slit was carefully and quickly made in the cornea using a razor blade, and a glass pipette, with a modified tip, holding the graft was inserted and emptied (fig. 4). For double-grafts, VM and LGE were placed adjacent to each other on the iris and allowed to fuse. In some experiments LGE solely was inserted at a certain time point prior to the grafting of VM. Fusion of the two grafts was thereby achieved when the LGE had already matured.
Production of EVAc rods

Bioactive rods, fabricated from ethylene-vinyl acetate, may be stereotaxically implanted into the brain, where they release for example growth factors over a time period of approx. two weeks. Apart from the easy implantation technique, using a specially designed implantation device, this method has the advantage of not causing inflammatory responses. This can be a problem when using cannulae or dialysis fibers connected to subcutaneously placed minipumps. In none of the implantation experiments did we find any sign of inflammation. Moreover, the brain tissue will not be damaged other than at the time for implantation, since the rod is not in any way connected to the skull, and therefore brain movements cannot cause injury. Bioactive rods for secretion of the factors bFGF, EGF, GDNF, and TGFβ1 were fabricated as follows.

Recombinant human EGF and bFGF (Life Technologies, Sweden) and recombinant rat GDNF and TGFβ1 (R&D Systems, Minneapolis, MN) were dissolved in 5% bovine serum albumin (BSA; Sigma) solution in a 1:1500 (w/w) ratio of growth factor to BSA for all growth factors except EGF (1:300). EVAc (Elvax40; Dupont Scandinavia, Sweden) was dissolved in dimethylchloride and devices containing 25% total protein were made by a melt extrusion method (Wahlberg 1997). The mean calculated release from the EGF rods was approx. 50 ng/day, and from the others, approx. 10 ng/day. Based on previous data the total duration of release was predicted to last 14-21 days.

Intracranial transplantations

6-OHDA lesion

Prior to implantation of VM grafts in the parkinsonian rat model, unilateral 6-OHDA lesions were performed. The lesions were stereotaxically conducted by injection of 6-OHDA (Sigma, Sweden; 2 μg/μl in 0.9% NaCl containing 0.2 mg/ml ascorbic acid). The rats (150g) were anaesthetized with halothane and fixed in a stereotaxic frame with the toothbar set at −2.3 mm. The scalp was cut opened with a pair of scissors, a hole was drilled in the bone and a small opening was made in the dura using a sharp needle. Injections were made into the MFBB via a 10 μl Hamilton syringe which was mounted to the frame (coordinates 4.4 mm posterior, 1.2 mm lateral to bregma and 7.8 mm
below the dural surface). To prevent 6-OHDA from diffusing along the needle track, there was a time lapse of one minute before the syringe was retracted from the brain tissue. The opening in the scalp was sutured using wound clips.

**Rotational behavior**

Apomorphine-induced rotational behavior was studied on a regular basis after 6-OHDA lesioning to estimate the success of the DA denervation (Herrera-Marschitz and Ungerstedt 1984; Ungerstedt and Arbuthnott 1970). The rats were placed in plastic rotometer bowls connected to a computer program that registered the number of turns made by the rat. Upon placing the animals in the rotometer bowls, spontaneous rotational behavior contralateral to the lesion was induced. When the rats had habituated and the spontaneous rotations had ceased, they received an injection of apomorphine (0.05 mg/kg diluted in 0.9% NaCl s.c.). The number of turns was counted over a time period of 70 minutes. Rats rotating more than 450 turns were selected for further grafting experiments (Heikkila et al. 1981; Herrera-Marschitz and Ungerstedt 1984; Hudson et al. 1993; Ungerstedt and Arbuthnott 1970). The animals were rotated again at certain time points postgrafting.

**Transplantations**

Animals selected for further experiments were anaesthetized with halothane and fixed in a stereotaxic frame. The toothbar was set at −3.3 mm. For intrastriatal or intraventricular grafting each rat received two pieces of unilateral VM ipsilateral to the DA lesion. Both tissue blocks were pulled into a 21G pull-push cannula mounted to the stereotaxic frame and then implanted (for coordinates, see table 2). Moreover, in paper II bioactive growth factor-secreting rods (EGF, bFGF, GDNF, TGFβ1) were implanted simultaneously with VM, using a specially designed implantation device. In cases where the effect of both GDNF and TGFβ1 were tested, two rods were implanted. There was a time lapse of one minute before retracting the cannula or the rod implantation device to prevent adhesion of the tissue or the rod to the instrument. The animals were sacrificed by perfusion 8 weeks postgrafting.
**Fluorogold injections**

The retrograde tracer fluorogold (Fluorochrome, Inc., 2% in 0.9% NaCl) was injected into the striatal portion of intraocular cografts or into the striatum ipsilateral to the grafts of intraventricularly transplanted animals (table 2) to investigate which neurons that were taking part in the VM-derived striatal innervation (paper III). The position of the striatal part of the cografts was determined before injections by follow-up inspections through the cornea, using a dissection microscope. The striatal tissue was darker than the VM and the grafts could therefore be easily distinguished from one another.

Glass micropipettes for intraocular injections were pulled from a capillary to a tip diameter of approx. 30 µm. After filling with fluorogold the micropipette was glued to the tip of a Hamilton syringe using beeswax. The rat was anaesthetized with halothane and fixed in a stereotaxic frame. Using a cannula, a small opening was made in the cornea above the striatal portion of the cograft. The syringe was mounted to a micromanipulator and the tip was inserted into the striatal graft. Fluorogold was injected (50-70 nl) at a speed of 1µl/min using a microinjection pump.

<table>
<thead>
<tr>
<th>Target sites</th>
<th>Treatment</th>
<th>Coordinates (mm to bregma and dura):</th>
<th>Ant.</th>
<th>Lat.</th>
<th>Below the dura</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paper II</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>striatum</td>
<td>VM</td>
<td>0.5</td>
<td>1.8-2.0</td>
<td>4.8</td>
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</tr>
<tr>
<td>Single rod</td>
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<tr>
<td>Anterior rod</td>
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<td>3.4</td>
<td>7.0</td>
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<td></td>
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<tr>
<td>Posterior rod</td>
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<td>3.4-4.2</td>
<td>7.0</td>
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<tr>
<td><strong>Paper III</strong></td>
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<td></td>
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<td></td>
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<tr>
<td>lateral ventricle</td>
<td>VM</td>
<td>0.5</td>
<td>1.2</td>
<td>4.5</td>
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<tr>
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<td>4.0</td>
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<tr>
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<tr>
<td>fluorogold</td>
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<td>1.9</td>
<td>4.0</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>striatum</td>
<td>VM</td>
<td>0.5</td>
<td>2.5</td>
<td>4.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Showing coordinates used for intrastriatal, intraventricular implantations, or fluorogold-injections in papers II, III, and V. EGF, bFGF, GDNF and TGFβ1-rods were implanted as single rods, or in cases when studying additive effects between GDNF and TGFβ, two rods were implanted using anterior and posterior positions.
Intraventricularly grafted rats with significantly reduced apomorphine-induced rotational behavior, i.e. functional reinnervation of the striatum had occurred, were anaesthetized with halothane and fixed in a stereotaxic frame. Fluorogold was injected in doses of 50 nl into six sites using three tracks and two depositions in each track (table 2). All the animals that had received fluorogold injections were sacrificed 5 to 7 days after the injections by perfusion, and the brains or intraocular grafts were processed for indirect immunohistochemistry.

**In vivo chronoamperometry**

In paper I, high-speed *in vivo* chronoamperometric measurements (IVEC-5, Medical Systems Corp.) were performed in the LGE portions of intraocular cografts. Nafion (Sigma)-coated single carbon fiber electrodes were manufactured for the recordings (Nagy et al. 1985). Calibration was employed using standardized solutions of ascorbic acid (20 mM) and DA (2 mM), and electrodes exhibiting high selectivity for DA (at least 500:1) and with a high degree of linearity (linear regression factor >0.997) with respect to DA sensitivity (Gerhardt et al. 1984; Strömberg et al. 1991) were selected. Nafion coating makes the electrode impermeable to ascorbic acid and other anions, resulting in a selectivity for cation neurotransmitters (e.g DA) (Gerhardt et al. 1984; Nagy et al. 1985). Square-wave pulses of +0.55 V were applied at a rate of 5 Hz to the electrode. The flow of electrons, resulting from the oxidation of DA at the surface of the electrode is measured as a current directly proportional to the concentration of oxidizable compounds in the extracellular fluid (Scatton et al. 1988). The electrode and a glass micropipette containing KCl (120 mM) were glued together at a distance of 300 μm using beeswax. The micropipette was connected to a micropressure system (BH-2, Medical Systems Corp.) allowing micropressure ejection of KCl.

For electrochemical detection of extracellular DA in *in oculo* grafts, the rats were anaesthetized with 25% urethane (1.25 g/kg i.p.),
Fig. 5 Experimental setup for recording of potassium-induced DA-release in grafted striatum. A slit is cut in the cornea so that a lid is made above the grafts. A carbon fiber electrode connected to an IVEC recording system is mounted with a glass pipette filled with KCl. The pipette is connected to a micropressure system. A chamber is placed over the eye so that the grafts are continuously superfused with EBSS at 37°C. The chamber is sealed to the skin with agarose. An Ag/AgCl reference electrode is placed outside the chamber in connection with the superfusion fluid. During measurement the carbon fiber electrode and the glass pipette are inserted into different sites of the LGE.

tracheotomized and allowed to breathe spontaneously. To maintain normal body temperature, the rat was placed on a heating pad. The skull was fixed in a stereotaxic frame, the skin around the eye was removed, and the eye was fixed in the desired position with a few sutures. A slit was made in the cornea over the graft using a broken razor blade. A small lid was cut open over the graft with a pair of microscissors so that the striatal portion of the nigro-striatal cograft was exposed. A plastic chamber was placed over the eye and agarose was used to seal the chamber to the rat. The chamber was superfused
with Earle’s balanced salt solution (EBSS, Gibco) at 37°C. A reference electrode (Ag/AgCl) was glued to the edge of the chamber. For measurements, the electrode and micropipette were inserted into the graft under a dissection microscope (Gerhardt et al. 1991; Johansson et al. 1995). KCl was injected using micropressure to achieve potassium-induced DA release (fig. 5). Registrations of maximal concentration of extracellular DA, half-time (the time it takes for the extracellular DA concentration to be reduced to half of the maximum value) and rise time (the time it takes to reach the maximal concentration) were performed in the LGE proximal and distal to the VM portion of the cografts. Proximal sites were defined as sites closer to the VM than half the length of the striatal graft, and distal sites were defined as sites more distal from the VM than half the length of the striatal graft.

After recordings the cografts were dissected from the eyes, and immersion fixed in 4% paraformaldehyde in phosphate buffer, rinsed in 10% sucrose, and processed for immunohistochemistry. It was possible to detect the trace of the injection- and recording-sites so that it could be concluded that recordings had been made in the striatal portion of the cografts.

**Organotypic primary cultures**

In paper IV, primary organotypic cultures were performed using the roller drum technique (Gähwiler et al. 1997). Tissue was taken from mouse embryos and cultured either as single VM cultures or as VM/LGE cocultures on sterile, poly-D-lysine (PDL, Sigma)-coated coverslips (12×24 mm) fitting into 15 ml Falcon tubes. Moreover, the VM was cultured as free-floating tissue blocks (Spenger et al. 1994). Instruments were sterilized after each dissected fetus using 70% ethanol and a glass bead sterilizer holding 250°C. This was made to prevent DNA contamination between embryos. Remaining parts of each fetus were saved and processed for DNA typing as described elsewhere (Zetterström et al. 1997).

The tissue piece dissected for culture was placed upon the coverslip in a drop of chicken plasma (Sigma) which was quickly stirred together with thrombin (Sigma) to achieve a plasma-clot covering the entire surface of the coverslip. For cocultures of VM and LGE the
tissue pieces were placed adjacent to each other. Fetal VM from E15.5 was cultured as two tissue pieces obtained from the same unilateral piece plated on the same coverslip (table 1). The glasses were allowed to dry for approx. 15 min. and were then placed in Falcon tubes with cell culture medium containing 10% heat inactivated fetal bovine serum (FBS, Gibco) and 1% antibiotics (10 000 units/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin; Gibco). The medium was prepared from DMEM (55%), Hank’s balanced salt solution (HBSS, Gibco, 32.5%), FBS, glucose (Gibco, 0.3%) and Hepes (Gibco, 0.2%). For experiments using free-floating tissue the VM tissue blocks were placed directly into 1 ml of the medium, which was either of the same type as for cultures plated on glass or serum-free Neurobasal (Gibco) medium supplemented with 0.5 mM L-glutamine, 25 µM glutamate, and 1% B27 supplement (Gibco). In addition, tissue cultured free-floating in serum free medium was supplemented with EGF (Sigma) and recombinant mouse FGF-8 (R&D Systems, UK) at a concentration of 10 ng/ml and 100 ng/ml respectively. The cultures were placed in a roller-drum holding an even rotational speed of 1 turn every second minutes in an incubator keeping the temperature at 37°C and the CO₂ concentration at 5%. After 3-4 days the media were changed, and after 7 days the cultures were fixed in 2% paraformaldehyde.

**Immunohistochemistry**

**Sections**

To fixate the brain and intraocular graft-tissue, intracardial perfusions were performed in all animals except the ones used for electrochemical recordings in paper I. The rats were intraperitoneally injected with an overdose of pentobarbital prior to perfusion with 100 ml cold Ca²⁺-free Tyrode solution containing 0.1 ml heparin (5000 IU/ml) followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.4% picric acid. After perfusion, the brains and intraocular grafts with underlying irides were dissected and postfixed in the same type of fixative for 1h. Then the tissue was rinsed in cold 10% sucrose in 0.1 M phosphate buffer for at least 24 h before cryostat sectioning. The tissue was rapidly frozen with gaseous CO₂ and sectioned (14 µm) in a cryostat at approx. -20°C. After thawing, the slides were rinsed thoroughly in 0.1 M phosphate buffered saline (PBS)
3x10 min before processing for indirect immunohistochemistry (Coons 1958; Hökfelt et al. 1973). The slides were incubated in primary antibodies for 48 h at 4°C in a humid chamber. When antibodies against GFAP were used, the slides were fixed 3 minutes in acetone before application of primary antibodies (table 3). After incubation in primary antibodies sections were rinsed in 0.1 M PBS and incubated in secondary antibodies for 1 h at room temperature. Then the slides were rinsed in PBS and mounted in 90% glycerol in PBS. To counteract fading of FITC, 0.1% p-phenylene diamine was used.

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Table 3. Primary and secondary antibodies used in papers I-V.
**Explants**

The primary cultures in paper IV were processed for indirect immunohistochemistry following the same protocol as described for sections with exception for the fixation procedure. The Falcon-tubes were emptied of medium and filled with 2% paraformaldehyde in 0.1 M phosphate buffer for fixation for 1 h in room temperature. Then the cultures were rinsed in PBS 3×10 min. and incubated in primary and secondary antibodies (table 3).

A fluorescence microscope was used to study the sections and the microscope was connected to a digital CCD camera and to a computer with OpenLab software (Improvision). The density of the nerve fiber network in the striatal part of intraocular cогrafts was measured using a Nikon system for image analysis. The fiber density was determined using a standardized area in which the number of pixels covered by innervation was counted. Background values were measured in TH-negative areas and subtracted. The nerve fiber density was calculated as percentage of the standardized area. Images were captured through a 10× lens and a ZVS-47EC CCD video camera system. Sections selected were blind-coded and randomly chosen during measurements.

**Cell counts**

Cell counts were performed in papers II-IV. In paper II the counts were performed on every fifth section throughout grafts and the total number of TH-positive neurons within a graft was calculated using the Abercrombie formula (Abercrombie 1946).

Counts were made using the same procedure in paper III but for fluorogold/TH-positive versus TH-positive/fluorogold-negative neurons in intraocular grafts. This was performed to determine the number of neurons innervating immature versus mature striatal target in cогrafts.

In paper IV, it was not possible to determine the absolute cell number since the density of TH-positive neurons within the cultures was too high to enable distinguishing of single neurons. Therefore an appreciation was made using a scale for approximate cell numbers. One (1) represented a total of up to 50 neurons, between 50-150 cells
were given a 2, 150-250 were given a 3, 4 represented 250-350 cells and 5 represented more than 350 cells.

Statistics

Statistical comparisons were made using one-way ANOVA followed by Fisher’s PLSD post hoc test and expressed as means ± S.E.M. in papers I, II and IV. Data was considered significant when p<0.05*, p<0.01**, and p<0.001***. In paper III unpaired, two-tailed Student’s t-test was used. In paper I, the data from electrochemical recordings were pooled from animals in the same graft-subgroup.

RESULTS AND DISCUSSION

Different growth patterns and their functional relevance

When does LGE reach maturity?

Before the first study of this thesis was initiated, it had been shown that the striatum of a rat host gains diffuse and widespread dopaminergic innervation from a fetal VM graft. Moreover, the reinnervation was demonstrated to terminate before the host target had been completely covered (Freed et al. 1980; Strömberg and Bickford 1996). Using the intraocular cografting model for VM and LGE, the same result was achieved when LGE was implanted and matured before fetal VM was allowed to innervate LGE (Strömberg et al. 1997). On the contrary, in a simultaneously implanted intraocular cograft of VM and LGE, LGE gained the robust and patchy type of TH-positive innervation also seen in the striatum during normal nigrostriatal development (Olson et al. 1972; Strömberg and Humpel 1995; Strömberg and Johansson 1994). Furthermore, the striatal target was innervated to a larger extent than that seen for sequentially grafting (Strömberg et al. 1997). Therefore, the difference in innervation density was concluded to depend on the degree of maturity of the striatal target; i.e simultaneous grafting mimics innervation of an immature striatal target and induces patchy growth, while sequential grafting mimics innervation of a mature striatal target and induces diffuse growth (fig. 6).

Our first goal was to investigate how old an intraocular LGE graft is when it reaches maturity, i.e. receives diffuse and widespread
instead of dense and patchy dopaminergic innervation from the VM. In paper I, LGE was allowed to mature for 14 or 26 days before receiving a cograft of fetal VM. Cografts of VM innervating 14-day-old LGE exhibited

![Diagram](image)

Fig. 6 Growth patterns induced when 13-mm VM innervates LGE of different ages was studied (a). LGE was allowed to mature for either 14 or 26 days before implantation of VM. A 14-day-old LGE received a patchy innervation pattern surrounded by diffuse growth, while a 26-day-old LGE received diffuse growth and in some cases patches were found in or near the VM. Growth patterns induced when VM of different ages innervated LGE of different ages was studied (b, c). In (b) VM and LGE from 13 or 18 mm fetuses were simultaneously grafted. When using 13-mm tissue patchy growth was induced, while when using 18-mm tissue both patchy and diffuse growth was found. In (c) LGE was allowed to mature for 4 mo before receiving a cograft of 13- or 18-mm VM. 13-mm VM induced diffuse growth in LGE, while 18-mm VM surprisingly induced patchy growth in the LGE.

a patchy and dense nerve fiber network in large areas of the LGE. The patchy areas were surrounded by either the diffuse and widespread growth pattern or by areas free from TH-immunoreactivity. The nerve fiber density was significantly higher close to the VM portion of the cograft than at distal sites.

When 26-day-old LGE had been innervated, the dominant type of dopaminergic innervation pattern found was diffuse and widespread. A few small islands of patchy growth could be found, but they did not show the same distinct borders as for younger innervated LGE. The nerve fiber density was significantly lower distal to the VM than near the border between the cografts. Interestingly, nerve fiber density in 26-day-old LGE was at the maximal level of the same density as that shown for the lowest values in the 14-day-old LGE-grafts, demonstrating the difference in innervation density. These experiments indicate that the maturation of LGE occurs sometime between 14 and 26 days of age (fig. 6a).
When LGE is grafted to normal striatum of an adult rat, the host dopaminergic neurons first innervate the graft in patches defining distinct areas (Björklund and Strömberg 1997; Isacson et al. 1985; Sandberg et al. 1986). These zones are called P-regions and express AChE (Graybiel et al. 1989; Pakzaban et al. 1993). In our simultaneous intraocular experiments, the same pattern with TH/AChE overlap was found (paper I). Interestingly, AChE-rich zones were found in mature LGE grafts although the TH-positive innervation was diffuse and sparse. When the striatal part lacked TH-positive patchy growth, there was often a higher abundance of patches within the VM portion of the cograft, indicating that patch-forming neurons were present in the VM but did not protrude processes into the target area. Since AChE-immunoreactivity occurred independently of the type of TH-innervation, it is rather the developmental stage of LGE than the division of AChE-rich versus -poor areas that influences DAergic nerve fibers to form patches in the LGE.

**VM of different developmental stages grafted to mature LGE**

A mature 4-month-old LGE attracted only the diffuse type of growth from 13 mm VM (E15), while 18 mm VM (E17) induced a few patchy LGE-structures close to the VM in 9 out of 12 cases (fig. 6c). This was a surprising and thought-provoking result. It has been suggested that the two different growth patterns are induced by different dopaminergic subpopulations (Gerfen et al. 1987; Jimenez-Castellanos and Graybiel 1987; Wright and Arbuthnott 1981) and since in vivo grafting to the striatum does not induce complete reinnervation, one explanation might be degeneration of the specific subgroup of dopaminergic neurons capable of producing patchy innervation. The conclusion drawn from our results is that the neurons with capacity to form patches can survive grafting when the tissue is dissected from up to CRL 18 mm, a stage which is considered as suboptimal for cell survival. It therefore seems that in the context of in vivo grafting experiments, these neurons are present, but do not project to the host target. It is, however, difficult to explain how VM of CRL 18 mm can induce patches in mature LGE when a younger and supposedly more immature VM of CRL 13 mm cannot. However, one explanation may be that VM at some time point between stages CRL 13 and 18 mm
receives a signal from the striatal anlage to target the LGE with patches. This ability might be preserved when dissecting the late stage of VM, but has not yet been established in the earlier stage.

As a control, simultaneous cocrafts using the same embryonic stages were made (fig. 6b). VM and LGE cocrafted from stage CRL 13 or 18 mm gave in both cases rise to a patchy TH-positive innervation of the LGE. However, it appeared that CRL 13 induced patchy innervation to a somewhat larger extent than for CRL 18 which also induced the diffuse type of growth pattern.

The functional significance of patchy versus diffuse growth patterns

_in vivo_ electrochemical recordings were performed to study the functional relevance of the two different TH-positive growth patterns. Recordings of potassium-evoked DA release, rise-time, and half-life were made in the striatal part of the cocrafts. The results clearly demonstrated that patchy growth had the potency to release higher concentrations of DA than diffuse growth. This confirmed that the innervation pattern to strive for in a functional context was the patchy type of growth. Moreover, the results showed that there was no difference in DA release proximal and distal to the VM for simultaneously and sequentially implanted grafts. This was, for the sequentially implanted grafts somewhat surprising, since there was a clear morphological difference in the density of TH-immunoreactivity which was higher near the VM. This might be explained by the fact that many of the measurements performed distal from the VM were below the detection limit of our method, and not included in the statistics. A difference in amplitude of release might therefore be masked. Another explanation may be that the distal terminals have undergone an upregulation of DA turn-over, a phenomenon not unknown in partially DA-denervated striatum. The mechanism for an upregulation, however, is not clear (Altar and Marien 1989; Castañeda et al. 1990; Zigmond et al. 1984).

Moreover, half-life and rise-time was studied at proximal and distal sites, but no difference could be detected in either of the subgroups. This might be explained by a balance between amounts of extracellular DA and efficacy in DA-reuptake, both clearly affected by the nerve terminal density. High nerve terminal density allows large
amounts of DA to be released from the terminals, and leads to a long half-life but short rise-time compared to areas with low terminal density. However, apart from a large release of DA, a high density of nerve terminals might also lead to a more efficient DA reuptake, which has the opposite effect on half-life and rise-time (van Horne et al. 1992).

**Administration of growth factors using bioactive rods**

The findings of paper I indicate that it might be of importance to make the striatal environment more immature in order to enhance the reinnervation from grafted VM. Therefore, in **paper II** this problem was challenged. Various growth factors have been shown to affect the outgrowth from, survival of, and functional restoration achieved by VM grafts in the *in vivo* parkinsonian animal model. In addition, some growth factors have properties suggesting a connection to immature zones of the CNS, housing precursor cells (Seroogy et al. 1995; Wahlberg 1997; Wanaka et al. 1991). This applies for, for instance, bFGF- and EGF-receptor expressing striatal precursor cells lining the lateral ventricles.

Bioactive rods have been designed and utilized with encouraging results in both experimental setups for release of NGF and DA (Hoffman et al. 1990; Winn et al. 1989), and clinical studies for treatment of gliomas (Brem et al. 1995). Using this method for delivery of growth factors, we wanted to investigate both the potency of the method in itself and the effect of bFGF, EGF, GDNF, and TGFβ on outgrowth from the VM graft. Moreover, the possibility of an additive effect between GDNF and TGFβ was investigated.

The VM grafts were placed close to the lining of the lateral ventricle, i.e they had a medial position whereas the rods were inserted to a more lateral position. In that way the graft would optimally be exposed to both the subventricular zone and the growth factors secreted from the rods, and hopefully the VM-derived striatal innervation would increase. Since GDNF and TGFβ could not be delivered from the same rod, two rods were implanted in cases where both factors were studied.
Cell survival

Basic FGF was the only factor that increased the number of TH-expressing neurons within the VM graft, but the cell survival was unexpectedly enhanced in comparison with the GDNF-treated animals and not with the VM-grafted, untreated control animals. However, cell counts in the GDNF-treated group were not significantly lower than that found in the untreated group. One explanation to why GDNF did not enhance dopaminergic cell survival might be that higher doses of this factor are needed to achieve measurable effects when using a slow-releasing method of this type. Indeed, previous studies have shown that GDNF might be required in 10-fold higher concentrations than bFGF (Giacobini et al. 1993; Strömberg et al. 1993).

Nerve fiber density in the striatal target

The distance that TH-positive reinnervation reached was increased for all of the neurotrophic factors used in this study since the nerve fibers reached all the way to the rod (approx. 1.5 mm). Nerve fiber density near the rod was increased for both EGF- and bFGF-treated animals. Surprisingly, GDNF- or combination of GDNF- and TGFβ-treatment did not have a positive effect on the nerve fiber density. However, the measurements were not performed in the immediate vicinity of the rod, and for these two growth factors the density of nerve fibers happened to be highest in tight junction of the rod and lower near the graft; i.e. GDNF or GDNF+TGFβ had the potency to promote elongation of the nerve fibers, and terminalization did not occur until the source had been reached. The opposite relationship was true for EGF and bFGF which seemed to potentiate the terminalization of fibers near the VM graft and had a tendency to exhibit a sparser nerve fiber density in the close vicinity of the rod.

Nerve fiber density within VM grafts

The nerve fiber density within areas of VM grafts that were devoid of TH-positive neurons was significantly increased for bFGF-treated grafts. It therefore seems that an increase of cell survival does not exclusively give the desired increase of outgrowth into the target, but also increases the nerve terminal density within the graft. Hence, our effort to induce an immature environment in the striatal target by the
addition of bFGF did not stimulate all the nerve fibers to protrude from the graft. The cause to this might be that there is a neuronal subpopulation that may not be induced to terminalize outside VM. This is in accordance with previous data from our group showing that the antioxidant tirilazad mesylate increased VM neuronal survival, and nerve fiber density within VM, but that TH-positive innervation of the striatal target was not changed (Björklund et al. 1998).

**Rotational behavior**

Both bFGF and GDNF caused a significant decrease in the number of apomorphine-induced rotations of the grafted rats in comparison with rats that had received only a VM graft.

The results from the above studied parameters demonstrate that bFGF was the only factor of the neurotrophic factors used that increased both dopaminergic cell survival, outgrowth from the VM graft to the striatal target, nerve fiber density within the graft, and in addition increased the functional restoration.

**Addition of TGFβ to GDNF-treatment**

In this experiment, it was not possible to confirm earlier data showing that TGFβ mediates the effects of GDNF on VM dopaminergic neurons. It therefore seems that in an *in vivo* context, the additive effect previously reported (Kriegstein et al. 1998) cannot be pointed out. However, since TGFβ-levels have been shown to be elevated in the striatum after DA denervation (Mogi et al. 1995) and also in cerebrospinal fluid from patients suffering from PD (Mogi et al. 1995; Vawter et al. 1996), it seems that there is a compensatory mechanism from endogenous TGFβ that will be activated if GDNF is required.

**Different subpopulations of grafted VM**

In paper II it was shown that although survival of TH-positive neurons and outgrowth to the striatal target from the VM graft could be increased by usage of the growth factor bFGF, still some of the terminalization occurred within the VM. This led us to the assumption that not all of the dopaminergic cells within a VM graft project to the host striatum. It is therefore logical to reason that this might be due to the division of dopaminergic neurons into subgroups possessing
different traits, some of them simply lacking the potential to innervate mature striatum.

We therefore decided to proceed by investigating this issue and to study the proportion of neurons innervating the mature versus immature striatal target using the retrograde tracer fluorogold. Furthermore, identification of grafted TH-positive cells by usage of markers known to be specific for subgroups of VM was performed. The data from this project resulted in paper III.

**Intraocular grafts**

The intraocular corgrafts displayed the expected growth patterns; i.e., simultaneously implanted grafts of VM and LGE (LGE innervated at an immature stage) exhibited a dense and patchy TH-positive innervation pattern in the LGE whereas sequentially implanted grafts (LGE innervated at a mature stage) had received a diffuse and widespread growth pattern (fig. 3).

The number of TH-expressing neurons in the VM was the same in both groups, but a larger proportion of these neurons were also positive for fluorogold in the corgrafts expressing patchy TH-positive growth. For simultaneously implanted grafts 53±13% of the TH-positive neurons were fluorogold-positive, i.e., innervating the LGE, while only 14±5% were fluorogold-positive for the sequentially implanted grafts. The results seem logical, bearing in mind that simultaneously implanted grafts exhibit both patchy and diffuse growth patterns.

**Intraventricular grafts**

The grafts were placed in the lateral ventricle to avoid leakage of fluorogold from the striatal injection sites into the VM. The injections had succeeded, and no leakage of the compound into the grafts could be detected.

The distribution of TH-positive neurons within the VM graft followed different patterns. Neurons could be scattered over the graft, preferably in the periphery, and sometimes a few cells had migrated into the striatum. Both scattered and migrated neurons were fluorogold-positive. Furthermore, TH-expressing neurons had formed spherical clusters. A high nerve fiber density was found within these spherical clusters. The center neurons of the clusters were fluorogold-
negative, while the peripherally located cells were expressing fluorogold. Moreover, the centrally located neurons were calbindin<sub>D28k</sub><sup>-</sup> immunoreactive. The opposite relationship was found in clusters exhibiting an elongated A9-resembling appearance (Dahlström and Fuxe 1964); centrally located TH-positive neurons were fluorogold-positive and calbindin<sub>D28k</sub><sup>-</sup> negative. The pattern of TH-positive cell group formations might of course be due to the angle of sectioning; i.e. an elongated cluster may be spherical or shaped otherwise if sectioned from another angle. However, the relative amount of TH/fluorogold/calbindin<sub>D28k</sub><sup>-</sup>-expressing neurons differing between two cell populations should be the same in either case.

*Subpopulations of dopaminergic neurons*

Since immature striatal tissue is innervated by both types of growth patterns, a larger number of TH-expressing neurons were expected to be fluorogold-positive than for innervated mature striatum which is innervated only by the diffuse type of growth. This was proved to be an accurate assumption. Moreover, since it has been shown that neurons in the A10 area apart from TH also express for example calbindin<sub>D28k</sub> (German and Liang 1993), we used this marker to distinguish between A9 and A10 neurons in the intraventricular VM grafts. Normally, a more robust innervation of the striatum is achieved by the A9-area, since the patch-forming neurons are located here (Haque et al. 1997), and when patchy growth is induced, using the intraocular grafting model, a larger volume of the target is innervated (Strömberg et al. 1997; Vidal et al. 1998). Moreover, TH-positive outgrowth from VM grafts into adult host has shown to be target-specific and thus the A9-area more accurately than A10 matches its normal target-area when grafting to the dorsal striatum (Hudson et al. 1994; Strömberg et al. 1992). Indeed, recent results have shown that when MPTP-sensitive A9-neurons die within a human VM graft transplanted to rat, ventral striatum is left reinnervated indicating that A10 neurons are not projecting to the dorsal but to the ventral striatum (Strömberg et al. 2001).

Calbindin<sub>D28k</sub> was expressed in TH-positive neurons that were negative to fluorogold, i.e not innervating the striatum, which is in accordance with the assumption that these neurons may be of A10-
origin. Moreover, neurons that were TH- and fluorogold-positive did not express calbindinD\textsubscript{28k} and might therefore be of A9-origin. However, since the innervation pattern that was induced in the striatum was diffuse and widespread, it seems that the population extending neurites into the target were of matrix- and not patch-forming A9-origin.

There is a weakness of this theory; the existence of a few calbindinD\textsubscript{28k}-expressing TH-positive neurons of the A9 dorsal tier (Gerfen et al. 1985; Gerfen et al. 1987). However, it is more likely that the calbindinD\textsubscript{28k}-positive neurons are of A10-origin, based on the relative occurrence of calbindinD\textsubscript{28k}-immunoreactivity in these two dopaminergic subpopulations. It was found, though, a few calbindinD\textsubscript{28k}/TH/fluorogold-positive neurons indicating the existence of A9 calbindinD\textsubscript{28k}-positive neurons.

Within the graft, were also present TH-negative, fluorogold-positive cells of unknown origin. This means that some VM cells were innervating the unappropriate target. The identity of these cells is still unknown, but it is well known that VM grafts might include cells of various origins, like 5-hydroxytryptamine (5-HT) neurons and cells immunoreactive for AChE, substance P, and glutamate decarboxylase (Lu et al. 1991; Mahalik and Clayton 1991; Wright et al. 1991).

Is Nurr1 absolutely required for development of the nigrostriatal system?

In paper IV, another aspect was added to this thesis. Nurr1 had recently been shown to be of major importance for the nigrostriatal dopaminergic system. Newborn mice devoid of the Nurr1 gene exhibited a total lack of TH-expressing neurons in the ventral midbrain and of DA in the striatum (Witta et al. 2000; Zetterström et al. 1997). Our initial idea was to isolate the VM from Nurr1 knockout mice by dissection at a stage prior to normal onset of TH-expression, and to culture the tissue for one week in the presence of wild-type LGE. From the results we would be able to decide whether normal LGE could induce TH-expression in the VM of Nurr1 knockout tissue. If so, it would suggest that an endogenous factor, present in the in situ environment, causes the malfunction of the nigrostriatal development in Nurr1 knockout mice.
VM-LGE cocultures and single VM cultures of different stages

When VM was cultured together with wild-type LGE, TH-positive neurons were found in VM of cultures from all three genotypes (-/-, +/-, +/+ ) and no difference in cell number between the groups could be observed. This was an interesting result suggesting that LGE indeed could induce TH-expression in the VM.

The obvious continuation was to culture VM without the presence of LGE. All cultures but one from the -/- group exhibited TH-expressing neurons suggesting a null-requirement of LGE. However, the heterozygous and wild-type groups had a significantly larger number of TH-positive neurons than that found in the knockout tissue cultures. Since the onset of TH-expression occurs at E11.5 in vivo (Foster et al. 1988; Solberg et al. 1993), we had used E10.5 for the co-and single cultures. The next step was to culture tissue dissected from the later stage E15.5, when RALDH1, a marker for midbrain dopaminergic neurons (A9- and A10-areas), has disappeared in Nurr1 mutants. TH-positive neurons were intriguingly found in all of the cultures from knockout tissue. Again, the cell numbers were significantly higher in cultures from +/- and +/+ groups. To confirm that the cultured neurons had the appropriate origin, RALDH1 immunohistochemistry was used. Co-existence of TH and RALDH1 was found in the knockout cultures, and the conclusion was drawn that our dissected neurons indeed were midbrain dopaminergic neurons.

Single VM cultures in serum-containing or serum-free medium

To study if the expression of TH in knockout tissue was due to the action of an unknown factor in the serum of the culture medium, we decided to exclude the serum. To avoid interference from putative factors in the plasma-thrombin clots that embedded the cultures, the tissue was cultured as free-floating explants. The presence of serum in the medium proved to be important for the induction of TH-expression since only a few (2 out of 16) of the knockout cultures harbored TH-expressing neurons and in those cases the neurons were very few (up to 30 cells). The cell counts for heterozygous and wild-type VM were normal and differed significantly from the knockout group. When serum-containing medium was used, TH-immunoreactivity was induced
in knockout cultures and the cell numbers were in the same range for all groups. The results suggest that serum indeed is of great importance for TH-expression in VM Nurr1 knockout tissue.

To further investigate which serum-derived factor might be of interest, FGF8, involved in the induction of TH-expression in the midbrain (Crossley et al. 1996; Hynes et al. 1995; Hynes et al. 1995; Ye et al. 1998) and EGF, known to create an environment permissive for proliferating progenitor cells (Vescovi et al. 1993), were added to the serum-free medium. However, these trophic factors did not exert any measurable effect on TH-expression in VM from knockout tissue.

The formation of nerve fiber bundles

An interesting finding was the formation of a distinct nerve fiber bundle in wild-type VM-LGE cocultures. Bundles were found in 80% of the wild-type cultures, 14% of heterozygous cultures and in none of the knockout cultures, where only a diffuse nerve fiber network could be found. Nerve fiber bundles were not as frequently found in single VM cultures although it was found in a few cases of wild-type tissue. These results imply that LGE stimulates the formation of the MFBB in the normal context, i.e. when wild-type VM is cultured with wild-type LGE, and to some extent when heterozygous VM is cultured with wild-type LGE. The conclusion that can be drawn is that knockout tissue, although it might undergo the induction of TH-expressing neurons, lacks the potential to form a nerve fiber bundle. This might indicate that the neurons induced to express TH in vitro are present in situ, but since not stimulated by the striatal target to form a connecting nerve fiber bundle between the ventral midbrain and the striatum, they never undergo normal development expressing known midbrain-specific markers. Therefore, the formation of a nigrostriatal connection may exert important cues for long-term survival of midbrain DA neurons.

Previous studies have shown both presence and absence of the dopaminergic pathway in Nurr1 knockout animals, using retrograde tracers (Wallén et al. 1999; Witta et al. 2000). These discrepancies might be due to different gene targeting. Our data are in accordance with the conclusion that Nurr1 is required for DA axon pathfinding. Indeed, the mice used came from the line of Nurr1 knockout animals previously shown to lack nigrostriatal connections (Wallén et al. 1999).
Do the extracellular matrix molecules keratan sulfate, tenascin, and N-CAM participate in the guidance of VM graft outgrowth to the striatum?

In paper IV, it was demonstrated that the formation of a connection between the nigral neurons and the striatal target may be of importance for the neuronal survival. The mechanism behind the axonal guidance during formation of the MFBB has, however, not been fully investigated. It is known, though, that glial cells affect the guidance via inhibiting/attracting cues in the extracellular matrix (ECM), and that such molecules are implicated in the nigrostriatal development (Charvet et al. 1998; Gates et al. 1993; Johansson and Strömberg 2002; O’Brien et al. 1992). In paper V, the presence of three well-known ECM molecules was mapped in intrastratal VM grafts and studied in relation to nerve fiber outgrowth from putative dopaminergic subpopulations, in order to achieve information about how graft-derived outgrowth is guided, and how to possibly increase striatal innervation density.

*TH/calbindin$_{D28k}$-immunohistochemistry*

The VM grafts displayed a similar pattern of two different clusters of dopaminergic neurons as that found in experiments using fluorescent to document neurons projecting to the striatal target (paper III). One type appeared as dense, TH-positive spherical clusters of neurons and neurites, forming a distinct border with low occurrence of neurites protruding into the surrounding graft. The other type of cell cluster was composed by more densely packed neurons and a lower density of nerve fibers within the cluster. Instead these cell formations exhibited a diffuse border-appearance regarding processes projecting into the surrounding graft tissue.

In dense, well-defined clusters, there appeared to be a higher colocalization of TH- and calbindin$_{D28k}$-expression both for neurons and neurites, in comparison with the diffusely organized cell clusters.

*ECM-immunohistochemistry*

Neural cell adhesion molecule (N-CAM)-immunoreactivity was evenly distributed over the entire volume of the grafts, and did not
appear to display any specific binding to distinct cell types. Nor could any expression be found within the host striatal tissue. This was in accordance with previous in situ data (Shults and Kimber 1992).

Tenascin was expressed within the well-defined cell clusters co-expressing TH and calbindin_{D28k}. Since these cell formations were strictly separated from the surrounding graft and host tissue, it was concluded that tenascin may act by hindering dopaminergic graft fibers, causing them to remain within the cluster instead of extending into the host.

The keratan sulfate (KS)-expression was high within grafts and host brains, and appeared associated with microglial structures. However, cell clusters co-expressing TH and calbindin_{D28k} were devoid of KS-immunoreactivity, while a sparse distribution of KS-positivity was found over diffusely organized cell formations lacking calbindin_{D28k}—reaction.

During nigrostriatal development, KS appears in striatal striosomes when patches are formed, and later parallels the formation of matrix in matrix compartments (Charvet et al. 1998). It has also been demonstrated that KS promotes dopaminergic sprouting (Batchelor et al. 1999). Indeed, it is clear from our results that areas with few TH-positive neurons that project into the host are accompanied by fewer microglial structures expressing KS. Applying the findings from paper III, it becomes possible to make the assumption that the neurons situated in the presence/absence of KS and tenascin have their origin in different VM dopaminergic subpopulations possessing different dispositions for host innervation. The expression of KS over projecting TH-positive neurons suggests a permissive role of this molecule.
CONCLUSIONS

• It is important to achieve a dense striatal innervation upon VM grafting, since patchy growth has the potency to release higher amounts of DA than the diffuse growth found in graft-innervated mature striatal tissue.

• Basic FGF not only increases neuronal survival and striatal innervation density, but also the nerve fiber density within the VM graft, suggesting that not all neurons have the potency to project into the target.

• A larger proportion of VM neurons was shown to project to an immature than to a mature striatal target. Projecting neurons within an intrastrial VM graft may be of A9-origin as demonstrated by calbindinD28k-negativity.

• TH-expression in VM tissue from Nurr1 knockout mice might be induced under in vitro circumstances. However, formation of nerve fiber bundles is not found in mutant tissue, suggesting an impairment of the development of the nigrostriatal connection in situ.

• The extracellular matrix molecule keratan sulfate might function as an attractant in the guidance of dopaminergic outgrowth, suggesting involvement in the development of the nigrostriatal connection. Tenascin may hinder outgrowth to the target by causing terminalization within the graft.

**Taken together**, this thesis has shown that in order to enhance intrastrial VM graft efficacy, it is important to gain more knowledge about which neuronal subpopulations of grafted VM that are taking part in the striatal dopaminergic reinnervation, and how to trigger surviving DA neurons to give rise to a dense and functionally more potent reinnervation.
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