TROPHIC ACTIONS OF MYENTERIC PLEXUS EXPLANTS ON STRIATAL NEURONS IN CELL CULTURE

by

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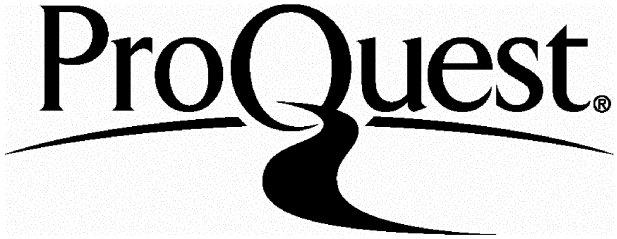

ABSTRACT

The aims of this work were to investigate the neurotrophic effect of the *myenteric plexus* on neurons of the *central nervous system* (CNS) and to determine which factors are involved in the events leading to increased neurite elongation of postnatal CNS neurons in coculture with *myenteric plexus* explants.

Previous investigations in the Department of Anatomy (UCL) of the use of enteric ganglia as a source of material for grafting into the CNS have revealed that the myenteric plexus produces a sprouting response by the host brain (Tew *et al.*, 1992, 1994). These observations suggest that the gut, and the myenteric plexus in particular, may be a source of growth promoting factors which are active on CNS neurons.

To investigate this further a novel coculture system of myenteric plexus explants with dissociated striatal neurons was developed as a model to determine if different cellular components of the myenteric plexus produce soluble factors active on striatal neurons and to analyse the effect of such factors on these neurons *in vitro*.

Neurotrophic effects were quantified by measuring neurite elongation of striatal neurons. Neuronal and glial cell numbers were counted to investigate correlations between neurite elongation and cell numbers. The factors investigated during my work included growth factors, extracellular matrix molecules, gangliosides and neurotransmitters.

The experimental results revealed that cells from the myenteric plexus promote neurite elongation of striatal neurons *in vitro*. This effect was independent of the elevation of striatal cell numbers and could be abolished by tetrodotoxin. A strong neuritogenic effect was only observed in the presence of myenteric plexus cells and could not be repeated by administration of several, different growth promoting

substances to striatal neurons alone. Adenosine and nitric oxide and also the second messenger cAMP were found to be involved in the events leading to increased striatal neurite outgrowth. Gangliosides promoted neurite outgrowth of striatal neurons but their involvement in the growth promoting effect of the myenteric plexus on striatal neurons *in vitro* could not be demonstrated.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
ADA	adenosine deaminase
ATP	adenosine-5'-triphosphate
αβ-meATP	α,β-methylene ATP
AGF1	mixture of bovine brain gangliosides
BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
ChAT	choline acetyltransferase
CNS	central nervous system
2-CA	2-chloroadenosine
CNTF	ciliary neurotrophic factor
DNase	deoxyribonuclease
DYN	dynorphin
ECM	extracellular matrix
ENK	enkephalin
ENS	enteric nervous system
FCS	fetal calf serum
FGF	fibroblast growth factor
GABA	γ-amino butyric acid
GAL	galanin
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
GTP	guanosine-5'-triphiphosphate
HBSS	Hank's balanced salt solution
HD	Huntington's disease
5-HT	5-hydroxytryptamine (serotonin)
NADPH	nicotinamide adenine dinucleotide phosphate hydride
NGF	nerve growth factor
NPY	neuropeptide Y
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
NO	nitric oxide
NOS	nitric oxide synthase
PNS	peripheral nervous system
PD	Parkinson's disease
PBS	phosphate buffered saline
p75NGFR	low affinity nerve growth factor receptor
PGP 9.5	protein gene product 9.5
8-PSPT	8-(*p*-sulfophenyl)theophylline
RT	room temperature
SOM	somatostatin
SP	substance P
SUR	suramin
TTX	tetrodotoxin
VIP	vasoactive intestinal polypeptide
EGF	epidermal growth factor
FGF-R	fibroblast growth factor receptor
PDGF	platelet derived growth factor
TGF	transforming growth factor

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PUBLICATIONS ARISING FROM THE WORK PRESENTED IN THIS THESIS

Abbracchio, M., Saffrey, M.J., Höpker, V.H. and Burnstock, G., Modulation of astroglial cell proliferation by analogues of adenosine and ATP in primary cultures of rat striatum. *Neuroscience* 59(1) (1994) 67-76.

Höpker, V.H., Saffrey, M.J. and Burnstock, G. Myenteric plexus explants promote neurite elongation and survival of striatal neurons *in vitro*. *Brain Res.* 648 (1994) 332-336.

Höpker, V.H., Saffrey, M.J. and Burnstock, G. Myenteric plexus explants promote neurite outgrowth of striatal neurons in coculture: involvement of purines. *Int. J. Dev. Neurosci.* 12 **(Suppl.)** (1994) 202.

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CHAPTER 1

1. INTRODUCTION

1.1.A. General Introduction

Transplantation techniques have been used as means of replacing neurons and supplying trophic factors in the treatment of neurodegenerative diseases (for detailed reviews see Iversen and Dunnett 1990, Gage and Fisher 1991, Dunnett and Svendsen 1993). These *in vivo* investigations of neuronal and non-neuronal tissue implanted in the central nervous system (CNS) have been performed to supply degenerating or injured CNS tissue with trophic support, or to elevate the reduced levels of neurotransmitters within these areas. Since the intrinsic nervous system of the gut, the enteric nervous system (ENS), shows many ultrastructural similarities to the CNS (see Jessen and Burnstock, 1982), cells from the myenteric plexus have recently been used as a source of material for grafting into the CNS (Jaeger 1993, Jaeger *et al.* 1993, Lawrence *et al.* 1991, Tew *et al.* 1992, 1993, 1994, Tew 1994). Grafts of intestinal muscularis externa containing ganglia of the myenteric plexus (Tew *et al.* 1992), and of the myenteric plexus free of smooth muscle (Tew *et al.* 1994) have been found to promote sprouting by CNS neurons after transplantation into the corpus striatum. These observations suggest that the gut, and the myenteric plexus in particular, may be a source of growth promoting factors which are active on CNS neurons.

The aim of the work described in this thesis was to examine the trophic interactions between the myenteric plexus and striatal cells using tissue culture techniques. Therefore, a novel culture system was developed in which dissociated striatal neurons were cocultured with the well characterized myenteric plexus explant system (see Bannerman *et al.* 1987, Saffrey *et al.* 1992a for reviews) as a model to determine if myenteric plexus cells produce factors which induce increased neurite elongation of striatal neurons *in vitro*. The main

objective was to develop a suitable *in vitro* system which could be utilized to identify which factors, cell types and cellular mechanisms are involved in the events leading to increased neurite elongation of striatal neurons in the presence of the myenteric plexus.

The work described in this thesis involves coculture of the CNS and the ENS. Therefore, in the Historical Introduction that follows, information about the morphology and neurochemistry of both the corpus striatum and the enteric nervous system is given. I have focussed on a description of neurotrophic factors and actions within the ENS and the striatum both *in vivo* and *in vitro*.

Chapter 2 contains a detailed description of the experimental procedures and materials used during this work.

Chapters 3 to 6 describe the experimental work and include a separate discussion specially related to each topic. In Chapter 3 the neurotrophic actions of myenteric plexus explants on postnatal striatal cells in coculture are presented and compared to effects of enriched enteric neuronal and glial cell preparations and other non-neuronal cell types on striatal cells. The chapter focuses on the difference between survival promoting (neuronotrophic) and neurite outgrowth promoting (neuritogenic) effects. Chapter 4 describes an investigation into the involvement of purines in the trophic interactions between myenteric plexus explants and striatal cells in coculture. Chapter 5 investigates the possible synergistic effect of purines (and adenosine in particular) in combination with several growth factors or gangliosides on striatal neurite outgrowth. Results of the investigation of the neuritogenic action of different extracellular matrix molecules are also presented. Chapter 6 contains

an investigation into the involvement of nitric oxide (NO) in the trophic interactions in myenteric plexus cocultures.

Chapter 7 consists of a General Discussion in which the experimental results are discussed in a broader context. Within this discussion I focused on synergistic modulation of neurotrophic factor actions.

1.1.B. Historical introduction

1.2. Corpus striatum

1.2.1. Anatomy, physiology and pathophysiology

The corpus striatum consists of the neostriatum (caudate nucleus or striatum) and the paleostriatum (globus pallidus) and, together with the subthalamic nucleus and the substantia nigra, forms the basal ganglia. The major physiological function of the striatum is the control and coordination of motor functions (De Long and Georgopoulos 1981), although it also has emotional and cognitive functions (Iversen and Dunnett 1990). The striatum can be subdivided into patch and matrix compartments on the basis of their specific neurotransmitter content and by differential patterns of connectivity (reviewed by Graybiel 1990, Gerfen 1992). The patch compartment (also referred to as 'striosomes') is characterized by dense μ-opioid receptor binding and low acetylcholinesterase (AChE) labelling (Herkenham and Pert 1981, Graybiel and Ragsdale 1978), by afferents from the deep frontal cortex (Gerfen 1989) and projections to dopaminergic neurons of the substantia nigra pars compacta and the pallidum (Gerfen *et al.* 1987). The matrix compartment can be identified by high levels of acetylcholine (ACh), calbindin and

somatostatin (SOM; Gerfen 1985), it receives afferents from dopaminergic neurons located within the ventral tegmental area, substantia nigra pars compacta and the retrorubal area (see Gerfen 1992 for review) and projects to the globus pallidus and to GABAergic neurons of the substantia nigra pars reticulata (Gerfen 1985).

The two (major) disorders associated with the corpus striatum are Parkinson's disease (PD) and Huntington's disease (HD). In PD, a marked degeneration of nigral dopaminergic neurons projecting to the striatum is observed. This results in a reduction in dopamine release in the striatum which induces an increased output activity of the striatum. The typical symptoms in PD patients are tremor and bradykinesia. HD is a genetically linked disease resulting in the degeneration of striatal inter- and projection neurons (The Huntington's Disease Collaborative Research Group. 1993). The symptoms associated with HD are chorea, dementia and death 15 or 20 years after onset. In the early stage of the disease, a loss of neurons containing γ-aminobutric acid (GABA) and projecting to the globus pallidus, is observed (see Ferré *et al.* 1992). Golgi-impregnations of striatal tissue from HD patients have revealed that medium-sized spiny neurons in particular, undergo degeneration (Graveland *et al.* 1985, Ferrante *et al.* 1991).

1.2.2. Cell types *in vivo*

1.2.2.1. Neurons

In the corpus striatum neurons can be classified into four major categories on the basis of cell body size and the development of dendritic spines, namely medium sized spiny neurons and large, medium

and small aspiny neurons. About 90-95% of striatal neurons are medium sized, spiny neurons (Graveland and DiFiglia 1985). These neurons are the primary source of striatal projections and utilize the inhibitory neurotransmitter GABA as their principal transmitter together with one or more of the neuropeptides dynorphin (DYN), met- & leu-enkephalin (ENK) and substance P (SP; see Graybiel 1990 for review). A colocalization of GABA and ENK is characteristic for striatal projections to the external segment of the globus pallidus, which projects to the subthalamic nucleus. The subthalamic nucleus projects back to the globus pallidus and the substantia nigra. This circuit is referred to as *indirect pathway*. The *direct pathway* is characterized by striatal projections to the internal segment of the globus pallidus and the substantia nigra pars reticulata which then project to the thalamus. The striatal projections of the direct pathway utilize GABA together with SP (see Alexander and Crutcher 1990 for review). The striatal medium spiny neurons project to the globus pallidus, to the substantia nigra, to the entopeduncular nucleus and to medium spiny neurons within the striatum (reviewed by Gerfen 1992). They receive input from large cholinergic and medium aspiny interneurons within the striatum, from the cortex, from the thalamus and from the substantia nigra (see Gerfen 1992). It has been found that dopamine D_2 receptors are colocalized with adenosine A_2 receptors on GABAergic medium sized neurons (Fink *et al.* 1992, Schiffmann *et al.* 1993). *In situ* hybridisation studies have revealed that adenosine A_2 receptors are expressed together with dopamine D_2 receptors on neurons containing ENK, but not on SP containing neurons or cholinergic neurons (Schiffmann *et al.* 1993). Adenosine A_2 receptors were found to be expressed by neurons within the striatum, whereas A_1 receptors were located on dopaminergic afferent neurons, suggesting that

adenosine has a regulatory role on dopamine action within the striatum (Moser *et al.* 1991). The density of A_2 receptors was reported to be dramatically decreased in both quinolinic acid-lesioned guinea pig striatum and in human striatum of HD patients, but not in PD patients, suggesting their localization on striatal output neurons (Martinez-Mir *et al.* 1991). A specific and antagonistic interaction between A_2 and D_2 receptors on these striatopallidal neurons has been reported and this was suggested to have regulatory function in the indirect striatal efferent pathway (Ferré *et al.* 1993, Fuxe *et al.* 1993, Pollack *et al.* 1993).

The second largest population of striatal neurons are large aspiny interneurons, which constitute about 2% of the total neuronal population (Phelps *et al*, 1985) and use ACh as their transmitter. Large aspiny neurons contain AChE (Bolam *et al.* 1984) and choline acetyltransferase (ChAT; Phelps *et al.* 1985). They receive synaptic input from the cortex and the substantia nigra (Dimova *et al.* 1993). The cholinergic striatal interneurons express messenger ribonucleic acid (mRNA) for a) both dopamine D_2 and SP receptors*) (76%) or b) only either SP receptors*) (16%) or D_2 receptors (2%); 6% express neither receptor (Aubry *et al*, 1993). This suggests that the activity of cholinergic striatal neurons is regulated to some extent by dopaminergic nigrostriatal neurons (see Stoof *et al.* 1992).

The medium aspiny neurons contain SOM and neuropeptide Y (NPY; DiFiglia and Aronin 1982, Vuillet *et al.* 1989). Furthermore, they contain the synthetic enzyme for NO, nitric oxide synthase (NOS) and NADPH-diaphorase (Dawson *et al.* 1991, Derer and Derer 1993, Hope *et al.* 1991). The medium aspiny neurons receive input from the cortex, from the substantia nigra, from the globus pallidus and from medium

*) neurokinin-1 receptor

spiny GABAergic neurons (Vuillet *et al.* 1989, Bennett *et al.* 1993).

The population of small aspiny neurons, which constitutes about 0.1% of the neuronal population, contains vasoactive intestinal polypeptide (VIP; Theriault and Landis 1987). They were reported to be striatal interneurons and associated with fibre bundles crossing the striatum (Theriault and Landis 1987).

1.2.2.2. Non-neuronal cells

Like other parts of the brain, the striatum contains different types of non-neuronal cells. One of the first to describe non-neuronal cells within the striatum was Ramón Y Cajal (1995 for English translation). He observed that the striatum contains a large population of astrocytes with short, spongy processes. Other non-neuronal cells, such as oligodendrocytes and microglia have been described *in vivo* (eg. Sturrock 1980) and can also be identified *in vitro* (see below).

1.2.3. Development and organization

The neurons of the rat striatum are generated between the embryonic days 13 (E13) and the postnatal day 2 (P2; Fentress *et al.* 1981, Bayer 1984, Marchand and Lajoie 1986). The large striatal neurons are generated mainly between E13 and E16 along a caudal to rostral gradient (Bayer 1984). Medium-sized neurons are generated in a ventrolateral to dorsomedial gradient during a period between E14-18 (ventrolateral) and E21-22 (dorsomedial, Bayer 1984, Marchand and Lajoie 1986). The neurotransmitter GABA has been detected within the rat striatum from E13 onwards (Lauder *et al.* 1986), cholinergic neurons are generated between E12 and E15 and SOM-containing neurons are generated at E15/16 (Semba *et al.* 1988).

Within the first postnatal week about 25-30% of rat striatal neurons undergo naturally occurring cell death (Fentress *et al.* 1981, Fishell and van der Kooy 1991). This period of cell death was found to be complete by P7 (Fishell and van der Kooy 1991). Interestingly, early born neurons (E13) which project early to the substantia nigra have been shown to be preferentially spared from cell death, suggesting a target-derived trophic support (Fishell and van der Kooy, 1991).

1.2.4. Striatal cell types *in vitro*

The morphology of dissociated monolayer cultures derived from both embryonic and postnatal rat striatum has been described in several studies (see below). Using phase contrast and fluorescence microscopy it has been shown that different cell types such as neurons and glial cells (astrocyte- and oligodendrocyte-like cells) are present *in vitro*, and that these cell types can be discriminated on the basis of their morphology.

1.2.4.1. Neurons

In cultures derived from mechanically dissociated newborn rat striatum Panula *et al.* (1979a) have described different cell types over a period of 28 days *in vitro*. Neuronal cells had a smooth, clearly defined and regular body contour. The majority of the neurons were bi- or tripolar, with branching neurites (Panula *et al.* 1979a). The cell bodies of small neurons were 10-20 µm and of medium-sized neurons 20-40 µm in diameter. After 2 weeks *in vitro*, neurons could be classified into 4 different neuronal types on the basis of ultrastructural observations (Panula *et al.* 1979b). In enzymatically

dissociated striatal cultures from newborn rat brain, five different neuronal types were classified by Messer (1981) using criteria like size, shape and location of the nucleus and later confirmed in a similar classification by Surmeier *et al.* (1988) in cultures from embryonic (E17) rat striatum. Bi- and tripolar neurons with scant cytoplasm and a smooth cell body margin were also numerous under these culture conditions (Messer 1981). The proportion of small to medium-sized bipolar neurons was about 80% in 2- to 3-week-old cultures (Surmeier *et al.* 1988). Medium-sized and large multipolar neurons were also found in these cultures although much less abundant (Messer 1981). At the light microscopic level these morphological characteristics correlated with the observations described earlier by Panula and coworkers, despite differences in preparation and culturing of the striatal cells.

1.2.4.2. Transmitter neurochemistry *in vitro*

Neurons from embryonic and postnatal striatum differentiate into neuronal subpopulations in culture which express distinct transmitter phenotypes. Although the neurotransmitter phenotypes in striatal interneurons and projection neurons as found *in vivo* can also be identified *in vitro*, the proportions can vary due to culture conditions and the lack of efferent neuronal connections in culture. Transmitters and neuroactive molecules that have been localized by immunocytochemistry in striatal neurons *in vitro* include ACh, GABA, Leu-ENK, SOM, SP and NO (Panula and Rechardt 1979, Panula *et al.* 1980, 1981, Messer 1981, Surmeier *et al.* 1988, Kessler 1986, Dawson *et al.* 1991).

1.2.4.3. Non-neuronal cells

The majority of non-neuronal cells in striatal cultures are

flattened, fibroblast-like cells with an irregular cell body margin (Panula *et al.* 1979a). Protoplasmatic, process bearing cells were also observed in these cultures after several days *in vitro*. These astrocyte-like cells could be distinguished from neurons by their large and irregular cell body. The second type of non-neuronal, process-bearing cells had a phase dark cell body with numerous processes revealing a regular branching pattern. They were recognized as oligodendrocyte-like cells (Panula *et al.* 1979a). This cell type was also observed in cultures derived from enzymatically dissociated striatum although they were not as numerous (Messer 1981). Other non-neuronal cell types such as macrophages, endothelial- and ependymal-like cells have also been described in cultures of dissociated rat striatum (Hansson *et al.* 1984).

1.2.5. Distribution of trophic factors and their receptors in the striatum *in vitro* and *in vivo*

Neurotrophic factors, such as the neurotrophins, regulate the proliferation and differentiation of neuronal progenitor cells (see Barde 1989, Davies 1994 for reviews), the survival and maintenance of neurons in the mature nervous system both under physiological conditions and after injury (see Brodkey *et al.* 1993). These trophic actions indicated that neurotrophic factors could be utilized to treat neurodegenerative diseases within the CNS (see Hefti 1994). With respect to the work presented here, a variety of neurotrophic factors and their receptors have been localized within the striatum and in targets of striatal projections and are summarized below (see table 1.1).

Table 1.1: List of trophic factors and their receptors associated with the striatum

Neurotrophins	
NGF	Martinez *et al.* 1985 Mobley *et al.* 1985 Korsching *et al.* 1985 Hartikka and Hefti 1988 Gage *et al.* 1989 Hagg *et al.* 1989, 1992 Matsuda *et al.* 1990 Vahlsing *et al.* 1991 Altar *et al.* 1992 Ebstein *et al.* 1993 Crowley *et al.* 1994 Strauss *et al.* 1994 Studer *et al.* 1994
BDNF and NT-3	Maisonpierre *et al.* 1990 Friedman *et al.* 1991 Lindsay *et al.* 1993 Engber *et al.* 1994 Mizuno *et al.* 1994 Nawa *et al.* 1994 Sauer *et al.* 1994 Zhou and Rush 1994
NT-4/5	Alexi *et al.* 1994 Ardelt *et al.* 1994
TrkA	Pioro and Cuello 1990 Altar *et al.* 1991 Ringstedt *et al.* 1993 Smeyne *et al.* 1994 Barbacid 1994
TrkB and TrkC	Ernfors *et al.* 1992 Lamballe *et al.* 1994
Fibroblast growth factors	
aFGF and bFGF	Walicke 1988 Abe *et al.* 1990, 1991 Matsuda *et al.* 1990 Hisajima *et al.* 1991 Tooyama *et al.* 1993 Weise *et al.* 1993 Zhou and DiFiglia 1993 Eckenstein 1994
FGFR-1 and FGFR-2	Wanaka *et al.* 1990 Asai *et al.* 1993

Other growth factors	
GDNF	Lin *et al.* 1993 Schaar *et al.* 1993 Strömberg *et al.* 1993 Springer *et al.* 1994
CNTF	Magal *et al.* 1991 Stöckli *et al.* 1991 Haag *et al.* 1992 Ardelt *et al.* 1994
PDGF	Pietz *et al.* 1994
EGF	Morrison *et al.* 1987 Abe *et al.* 1990 Lazar and Blum 1992 Reynolds and Weiss 1992 Reynolds *et al.* 1992 Alexi *et al.* 1994 Morshead *et al.* 1994
TGFa	Seroogy *et al.* 1991
Insulin	Kessler *et al.* 1986 Brass *et al.* 1992
Purines	Abbracchio *et al.* 1994a,b,c, 1995
Extracellular matrix/adhesion factors	
gangliosides	Skaper *et al.* 1985
laminin	Manthorpe *et al.* 1983
Cocultures	Bird 1989, 1991
Conditioned media	Varon *et al.* 1984 Barbin *et al.* 1984

1.2.5.1. Neurotrophins

The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) can bind with high affinity to the tyrosine protein kinase receptors TrkA, TrkB and TrkC, respectively, whereas neurotrophin-4/5 (NT-4/5) binds with high affinity to TrkB (see Barbacid 1994). All members of the Trk family, which include TrkA, TrkB and TrkC have been identified within the

caudate putamen (reviewed by Lindsay *et al.* 1993). TrkA has been detected in a small proportion of striatal neurons which are thought to be cholinergic interneurons (Pioro and Cuello 1990, Altar *et al.* 1991, see Lindsay *et al*, 1993 and Barbacid 1994 for reviews). *In situ* hybridisation studies for TrkA in the rat striatum have revealed weak labeling during the first postnatal week which increased in 2 and 4 week old animals (Ringstedt *et al.* 1993). Interestingly, in mice carrying an inactivated TrkA receptor gene (Smeyne *et al.* 1994) or a disrupted NGF gene (Crowley *et al.* 1994), the intensity in ChAT staining of the striatal cholinergic neurons is not affected, suggesting that trophic support might be provided by compensatory mechanisms (Crowley *et al.* 1994, Smeyne *et al.* 1994). Both NGF protein and mRNA have been localized in the developing and adult rat striatum (Korsching *et al.* 1985, Maisonpierre *et al.* 1990) and levels of NGF mRNA and protein were upregulated in kainic acid-lesioned rat striatum (Strauss *et al.* 1994). *In vivo*, NGF promoted recovery of cholinergic neurons and TrkA mRNA expression after injury (Gage *et al.* 1989, Altar *et al.* 1992), increased ChAT activity and cell body size during development (Mobley *et al.* 1985) and in adult rats (Gage *et al.* 1989, Hagg *et al.* 1989, 1992, Vahlsing *et al.* 1991). In cultures from embryonic rat striatum, NGF promotes ChAT activity (Martinez *et al.* 1985, Hartikka and Hefti 1988), upregulates the transcription of ChAT and m2 muscarinic receptor genes (Ebstein *et al.* 1993) and increases the number of AChE positive neurons (Hartikka and Hefti 1988), whereas there was no effect on neurite length and number of branching points of cholinergic neurons (Hartikka and Hefti 1988) or on neuronal survival (Matsuda *et al.* 1990). In cultures derived from postnatal rat striatum, NGF slightly promoted neuronal survival (Matsuda *et al.* 1990) and increased the complexity of

neurites (Studer *et al.* 1994).

Both TrkB and TrkC mRNA were detected as early as embryonic day E13 and E16 respectively, in the rat striatum (Ernfors *et al.* 1992) and were expressed on a large proportion of striatal projection neurons (Lamballe *et al.* 1994, see Lindsay *et al.* 1993). A transient mRNA-expression for both BDNF and NT-3 was observed during the first two postnatal weeks, but virtually no BDNF and NT-3 mRNA (Maisonpierre *et al.* 1990, see Lindsay *et al.* 1993) or NT-3-like immunoreactivity (Zhou and Rush 1994) were found in the adult striatum. BDNF mRNA has been identified in newborn rats within the substantia nigra, a target tissue for striatal projections (Friedman *et al.* 1991). Administration of BDNF into ventricles of newborn rats increased the levels of NPY-, SOM-, SP- and CCK-like immunoreactivity (Nawa *et al.* 1994) and the GABA content (Mizuno *et al.* 1994) within the striatum. Further, BDNF enhanced the expression of preprotachykinin and preproenkephalin mRNA in both 6-hydroxydopamine-lesioned and unlesioned rat striatal neurons but did not change striatal levels of *trk*B mRNA (Sauer *et al.* 1994). *In vitro* BDNF increased the content of GABA, NPY and SOM, increased GABA uptake and GAD-immunoreactivity and increased the frequencies of parvalbumin- and calbindin-immunoreactive neurons in cultures from embryonic (E18) rat striatum, but did not affect neuronal survival (Mizuno *et al.* 1994).

Radiolabelled BDNF and NT-3, but not NGF, are transported retrogradely after injection into the striatum and can be detected in brain areas projecting into the striatum like the substantia nigra pars compacta, frontal cortex and thalamus (see Lindsay *et al.* 1993). These observations correlate with the expression of TrkB and TrkC in

these regions and the absence of TrkA in neurons afferent to the caudate-putamen (see Lindsay *et al.* 1993). In quinolinic acid-lesioned striatum, a model for HD, the intrastriatal injection of NT-3 significantly reduced neuronal cell death of cholinergic and GABAergic neurons (Engber *et al.* 1994). NT-4/5 did not promote survival of striatal GABAergic neurons after quinolinic acid treatment, although a partial protection of calbindin-containing striatal neurons was observed (Alexi *et al.* 1994).

1.2.5.2. Fibroblast growth factors

Currently nine members of the fibrolast growth factor (FGF) family have been identified (see Baird 1994, Eckenstein 1994 for reviews). Basic FGF-immunoreactivity was reported during late embryonic development (E16/E17) in rat striatal neurons but no labelling was found before that stage (eg. at E14/E15) or in newborn rat striatum (Weise *et al.* 1993). However, reservations have been expressed about the localization of bFGF in embryonic CNS neurons using immunohistochemical techniques due to possible antibody cross-reactivity between different FGF's which share distinct sequence homologies (see Eckenstein 1994). In the adult rat striatum, FGF receptor (FGFR)-2 mRNA has been identified in the myelinated fiber tracts of the striatum and was suggested by the authors to be expressed by oligodendrocytes (Asai *et al.* 1993). No significant labelling was detected for FGFR-1 mRNA (Wanaka *et al.* 1990, Asai *et al.* 1993). *In vitro*, both aFGF and bFGF increased neuronal survival of embryonic (E16/E18; Walicke 1988, Abe *et al.* 1991, Hisajima *et al.* 1991) and postnatal (P15; Matsuda *et al.* 1990) striatal neurons. The survival promoting effect of bFGF in embryonic (E17/E18) striatal neurons was inhibited by the protein kinase blocker K252a (Abe *et al.*

1991). Whereas bFGF had no effect on neurite elongation of embryonic (E18) striatal neurons (Walicke 1988), an increased neurite elongation was observed in cultures of postnatal GABAergic neurons (Zhou and DiFiglia 1993). This effect, however, was observed when striatal cells were cultured for an extended period of 7 to 17 days in the presence of bFGF (Zhou and DiFiglia 1993).

In HD, aFGF has been reported to be upregulated in both neurons and astrocytes, whereas a moderate increase of bFGF was observed only in astrocytes (Tooyama *et al.* 1993).

1.2.5.3. Ciliary neurotrophic factor

Both ciliary neurotrophic factor (CNTF) mRNA and protein have been identified in the postnatal rat striatum (Stöckli *et al.* 1991). CNTF was found to up-regulate the low affinity NGF receptor (p75NGFR) within the striatum *in vivo*, but did not affect ChAT activity or induce hypertrophy and sprouting within the striatum (Hagg *et al.* 1992). In cultures from embryonic (E18) rat striatum, CNTF increased the number of p75NGFR-positive neurons. This trophic effect was not secondary to increased neuronal survival (Magal *et al.* 1991). The observation, that CNTF did not promote survival of striatal neurons *in vitro* was later confirmed in a study by Ardelt *et al.* (1994). In cultures from postnatal rat brain, CNTF immunoreactivity was detected in a subpopulation of type 1-like astrocytes (Stöckli *et al.* 1991).

1.2.5.4. Other growth factors

Messenger RNAs for both epidermal growth factor (EGF) and transforming growth factor (TGF)α have been identified within the adult mouse striatum (Lazar and Blum 1992). TGFα mRNA is expressed in

neurons within the striatum during early postnatal stages whereas in the adult striatum it is localized in glial cells more than in neurons (Seroogy *et al*, 1991; see Lindsay *et al*, 1993). Recently, TGFα has been reported to partially protect calbindin-containing neurons in quinolinic acid lesioned striatum, but did not rescue GABAergic neurons (Alexi *et al*. 1994). In culture, EGF has been found to increase the survival of striatal embryonic (E14) progenitor cells (Reynolds *et al*. 1992), neurons from embryonic (E17/18) rat striatum (Abe *et al*. 1990) and postnatal (P0-1) rat subneocortical nuclei (including striatal neurons, Morrison *et al*. 1987). EGF has also be identified to increase the survival of progenitor cells in the adult mouse striatum (Reynolds and Weiss 1992) and of neural stem cells within the subependyma of adult mouse forebrain (Morshead *et al*. 1994). The only member of the TGFβ family, which has been found in the striatum to date is glial cell line-derived neurotrophic factor (GDNF, Lin *et al*. 1993). GDNF is proposed to be a neurotrophic factor for dopaminergic neurons *in vivo* and *in vitro* (Lin *et al*. 1993, Schaar *et al*. 1993, Strömberg *et al*. 1993). GDNF mRNA has been detected in developing (E20-P7) rat striatum (Schaar *et al*, 1993; Strömberg *et al*, 1993), but not in the adult rat striatum (Strömberg *et al*, 1993). Springer *et al*. (1994) have reported that GDNF is expressed in two different forms in the human striatum and detected GDNF mRNA expression in the adult rat and human striatum.

Recently, platelet-derived growth factor (PDGF) was reported to be upregulated in striatal astrocytes after ibotenic acid lesion *in vivo* (Pietz *et al*. 1994). Further, PDGF-BB (which activates both PDGFα- and PDGFβ-receptor), but not PDGF-AA (which activates only the PDGFα-receptor), increased neuronal survival and neurite elongation in striatal cultures (Pietz *et al*. 1994). Insulin was found to be

required for the survival of SP-containing cells, but not for the survival of SOM- or ChAT-containing striatal neurons in cultures derived from embryonic (E13.5) rat brain (Kessler 1986). In cultures from embryonic (E15) rat striatum insulin has been reported to increase glutamate decarboxylase (GAD) activities (Brass *et al.* 1992). The ganglioside GM1 increased the proportion of neurite-bearing neurons in cultures from embryonic (E18) rat striatum (Skaper *et al.* 1985).

Several conditioned media from rat schwannoma, rat glioma, mouse Schwann, rat cerebral astroglial and chick embryo heart cell cultures have been found to promote survival of embryonic (E18) striatal neurons *in vitro* (Varon *et al.* 1984, Barbin *et al.* 1984). In cocultures, explants of the adrenal gland have been found to promote neurite elongation in dissociated cultures derived from embryonic rat striatum (Bird 1989, 1991). Few studies have examined the effects of extracellular matrix (ECM) molecules on striatal neurite outgrowth. Manthorpe *et al.* (1983) reported that laminin neurite outgrowth of embryonic (E18) striatal neurons *in vitro*.

In summary, the results reported previously by a number of groups indicate that there is no single neurotrophic factor promoting survival and/or neurite elongation of striatal neurons *in vitro* or *in vivo*. Since the striatum is a heterogeneous tissue containing different types of neurons (see above), specific neuronal subpopulations appear to require different neurotrophic factors.

1.2.5.5. Trophic effects on striatal non-neuronal cells

In vitro, bFGF increased the number of glial fibrillary acidic protein (GFAP)-positive astroglial cells in cultures from embryonic

(E18) rat striatum (Walicke 1988) and increased both process outgrowth and numbers of astroglial cells in cultures from postnatal (P1) rat striatum (Zhou and DiFiglia 1993). Recently, it was shown that both bFGF and the purine analogue, αβ-me ATP, increased process elongation and induces c-*fos* expression in GFAP-positive cells from postnatal (P7) rat striatum (Abbracchio *et al.* 1994a,b). An increase in c-*fos* mRNA induced by bFGF was also observed in GFAP-positive cells in embryonic (E17) striatal cultures, but not in neuronal cells (Simpson and Morris 1994). The induction of c-*fos* was abolished by the tyrosine kinase inhibitor genistein (Simpson and Morris 1994). *In vivo*, bFGF injection into mouse striatum induced astrogliosis (Zhang *et al.* 1994). Both ATP and the stable adenosine analogue, 2-chloroadenosine (2-CA) have been shown to modulate astroglial cell proliferation in primary cultures of postnatal day 7 (P7) rat striatum. Treatment with 2-CA reduced whereas αβ-meATP elevated the number of striatal astrocytes after 3 days *in vitro*. The effect induced by αβ-meATP was reversed by the P_2-purinoceptor blocker suramin whereas the effect of 2-CA was not antagonized by the P_1-purinoceptor antagonist 8-(*p*-sulfophenyl)theophylline (8-PSPT). Both analogues induced an increase in nuclear BrdU-incorporation (Abbracchio *et al.* 1994c). These reports suggest that purines which have trophic activities on various other neural systems (see below and chapter 4) also affect the proliferation and differentiation of striatal glial cells (Abbracchio *et al.* 1994a, 1995, see also chapter 7).

1.3. Enteric nervous system

1.3.1. Structure and function

The ENS is part of the autonomic nervous system and is responsible

*) These functions include enhancement of mucosal water and electrolyte secretion by stimulation of gut endocrine cells, both inhibition and contraction of intestinal longitudinal and circular smooth muscle and the modulation of the vascular calibre to regulate the blood flow during the process of digestion (see Furness and Costa 1987).

for the regulation of the intestinal functions. The enteric ganglia are located within the wall of the gastrointestinal (GI) tract. Although the ENS is connected with other autonomic ganglia outside the walls of the intestine and with the CNS, it contains complete reflex pathways controlling functions such as intestinal muscle contractions, blood flow and transport of water and electrolytes. The great number of different types of neurons, the complex integration of the neuronal network and the cytological and immunohistochemical characteristics of the ENS differ from those of other peripheral ganglia and show many ultrastructural similarities to the CNS (see below and reviews by Jessen and Burnstock 1982, Furness and Costa 1987, Gabella 1994).

The ENS consists of two major ganglionated plexuses which are interconnected. The myenteric (or Auerbach's) plexus is located between the longitudinal and the circular muscle layers and the submucous (or Meissner's) plexus is located within the submucosa.

The myenteric plexus consists of a network of enteric ganglia and interconnecting nerve strands. It can be subdivided into three components, a primary, a secondary and a tertiary plexus. The primary plexus consists of the myenteric ganglia (also referred to as nodes) and the internodal strands. The nerve fibres which arise from the internodal strands or the ganglia constitute the secondary plexus. These fibres run approximately parallel to the circular muscle bundles. The nerves of the tertiary plexus form a fine network in the spaces between the primary and secondary plexus (see Furness and Costa 1987). The myenteric plexus is present along the entire digestive tract from the oesophagus to the internal anal sphincter (Schofield 1968).

1.3.2. Cell types *in vivo*

1.3.2.1. Enteric neurons

Different types of enteric neurons can be discriminated on the basis of their physiological function, their cell shape and their neurochemical properties. With respect to their physiology they can be classified as both inhibitory and excitatory motor neurons, vasomotor neurons, secretomotor neurons, interneurons and sensory neurons. Morphologically, three types of enteric neurons were classified initially by Dogiel (1899): type I having short, broad and flattened processes and one long process, thought to be motor neurons of the circular muscle; type II neurons having several long processes (thought to be sensory neurons) and type III neurons which have one long and several shorter tapering processes.

The neurons of the ENS contain a large variety of neurotransmitters. This is a characteristic feature which differentiates enteric ganglia from other ganglia within the peripheral nervous system (PNS). Different types of neurotransmitters and neuroactive peptides have been found within enteric neurons by histochemical and immunohistochemical methods. The function of these molecules as neurotransmitters or modulators has been confirmed by localization of the transmitter synthesizing and degrading enzymes and with pharmacological and electrophysiological methods (Furness and Costa 1987). Neuroactive molecules that have been identified in enteric neurons are ACh, adenosine 5'-triphosphate (ATP), GABA, calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), DYN, ENK, galanin (GAL), gastrin-releasing peptide (GRP), 5-hydroxytryptamine (5-HT, serotonin), NPY, neurotensin, NO, SOM, SP and VIP (see Furness and Costa, 1987; Belai *et al.* 1992b; Furness *et al.* 1992; Llewellyn-Smith

et al, 1992; Nichols *et al.* 1992; Young *et al.* 1992). In culture, enteric neurons have been found to contain ACh, ENK, GABA, NOS, SOM, SP, VIP (Jessen *et al.* 1983b, Saffrey and Burnstock 1988, Saffrey *et al.* 1992b, Grider and Bonilla 1994).

1.3.2.2. Enteric glial cells

The glial cells of the ENS or enteric glia, show many features in common with CNS glial cells, particularly astrocytes (see Jessen and Burnstock 1982, Gershon and Rothman 1991 for reviews). They contain intermediate filaments (Cook and Burnstock 1976) and are immunopositive if stained for GFAP (Jessen and Mirsky 1980), glutamine synthase (Jessen and Mirsky 1983) and the Ca^{2+}-binding protein S-100 (Ferri *et al.* 1982). Individual enteric glia are not completely covered by a basal lamina like Schwann cells, but a basal lamina completely envelops each entire enteric ganglion. Furthermore, enteric glia enwrap bundles of axons in contrast to Schwann cells, which ensheath individual axons. No basement membranes or connective tissue are found inside enteric ganglia, but laminin, fibronectin and type IV collagen have been shown in the basement membrane covering individual ganglia (Bannermann *et al.* 1986). When enteric glia are grown in culture they have been found to express laminin-immunoreactivity on the surface and in the cytoplasm (Bannerman *et al.* 1988a).

1.3.3. Myenteric plexus explants *in vitro*

Explant cultures of myenteric ganglia, isolated from newborn guinea-pig have previously been well characterised (Baluk *et al.* 1983, Jessen *et al.* 1983a,b, see Saffrey *et al.* 1992a). The myenteric

plexus explant preparation used in this study consisted of groups of myenteric ganglia which had been completely separated from the smooth muscle of the gut wall of neonatal guinea-pigs (Jessen *et al.* 1983a). The development of the plexuses in culture follows a characteristic pattern (Jessen *et al.* 1983a). After the plexuses have attached to the coverslip, enteric glial cells form a continuous monolayer around the plexuses and in the spaces in between individual ganglia. Neurites grow out of the explants on top of the glial cell lining, referred to as outgrowth zone, and form a dense meshwork of fibers (Jessen *et al.* 1983a). When fibroblasts are present in the cultures, neurons begin to form compact aggregates which are interconnected by thick bundles of neurites after the first week *in vitro* (Jessen *et al.* 1983a). After appropriate treatment with mitotic inhibitors to eliminate the small number of adherent fibroblasts, myenteric plexus explants contain only enteric neurons and glial cells (Bannerman *et al.* 1988b, see Chapter 2). Since all myenteric plexus cultures utilized for this study were treated with a mitotic inhibitor, the formation of neuronal aggregates and fibre bundles did not occur. Myenteric neurons stain immunopositive for the glycoprotein Thy-1 and for gangliosides when stained with the antibody A2B5. All enteric glia are immunopositive for S-100 and 95% can be labelled with the antibody LB-1, which binds to the GD3 ganglioside (Bannerman *et al.* 1988a). The glial-specific antibody LB-1 and the neuronal specific antibody binding to Thy-1 can be used to generate highly enriched populations of enteric neurons or glial cells from such explant culture preparations (Bannerman *et al.* 1988b, see Chapter 3).

1.3.4. Growth factors and their receptors in the gastrointestinal tract

The development, growth and maintenance of the gut, including the ENS is regulated by tissue specific growth factors, although their functions have not yet been identified. The enteric neuronal and glial precursor cells are derived from the neural crest (see reviews by Gershon *et al.* 1993b, Saffrey and Burnstock 1994). Evidence has been produced that migrating precursor cells are pluripotent and that the differentiation of enteric neurons and glia is regulated by the enteric environment within the gut (see reviews by Gershon *et al.* 1993b, Saffrey and Burnstock 1994). Members of different growth factor families such as the FGF's, neurotrophins, EGF and cytokines have been identified within the gastrointestinal (GI) tract although the cellular localization remains to be determined in most cases (see Saffrey and Burnstock 1994 for review).

Table 1.2: List of trophic factors and their receptors associated with the GI tract

Neurotrophins

Gershon *et al.* 1983, 1993a,b
Baetge *et al.* 1990
Hohn *et al.* 1990
Maisonpierre *et al.* 1990
Berkemeier *et al.* 1991
Belai *et al.* 1992a
Pomeranz *et al.* 1993
Scarisbrick *et al.* 1993
Chalzonitis *et al.* 1994
Fariñas *et al.* 1994
Lamballe *et al.* 1994
Lewis *et al.* 1994
Saffrey and Burnstock 1994

Fibroblast growth factors

Eccleston *et al.* 1987
Finch *et al.* 1989
Nice *et al.* 1991
Saffrey *et al.* 1994

EGF and TGFα	Thompson 1988 Cartlidge and Elder 1989 Koyama and Podolsky 1989 Burgess and Sizeland 1990 Barnard *et al.* 1991 Pérez-Tomás *et al.* 1993 Hoffmann *et al.* 1994
TGFβ	Koyama and Podolsky 1989 Barnard *et al.* 1993
IGF I and II	Young *et al.* 1989 Lund *et al.* 1990 Mulholland *et al.* 1992b
Purines	Saffrey *et al.* 1994
Undefined trophic factors	Mulholland *et al.* 1992a

1.3.4.1. Neurotrophins

Transcripts of the neurotrophins NGF, NT-3 and NT-4/5 have been detected in the GI tract (Hohn *et al.* 1990, Maisonpierre *et al.* 1990, Berkemeier *et al.* 1991). Expression of NGF, BDNF and NT-3 mRNA have been identified in the submucosa of the embryonic rat stomach using *in situ* hybridisation (Scarisbrick *et al.* 1993). *In vitro*, NT-3-supported differentiation of immunoselected developing crest-derived cells from rat gut (Chalzonitis *et al.* 1994) but was not mitogenic for these cells (Gershon *et al.* 1993a).Together with the extracellular matrix protein laminin, NT-3 is likely to be involved in the early development and differentiation of the ENS (Gershon *et al.* 1993a, Pomeranz *et al.* 1993). Interestingly, in newborn mice carrying a null mutation in the gene encoding for NT-3 the myenteric plexus appeared to develop normally (Fariñas *et al.* 1994), suggesting the possibility of compensatory systems in the trophic support of

* The isoforms ($TrkC_0$, $TrkC_{14}$, $TrkC_{25}$, $TrkC_{39}$) show variable degrees of autophosphorylation. $TrkC_0$ is the only isoform which was found to promote neuronal differentiation *in vitro* and is predominantly expressed in the fetal gut. The authors suggest that the different isoform convey distinct regional and temporal responses to NT-3 during early development of the ENS (Lewis *et al.* 1994).

myenteric plexus cells. Immunohistochemical studies have shown that treatment of neonatal rats with NGF antiserum increased staining intensity for VIP, GAL and SP in the myenteric plexus (Belai *et al.* 1992), but had no effect on VIP and SP immunoreactivity in the embryonic myenteric plexus after anti-NGF treatment of pregnant guinea-pigs (Gershon *et al.* 1983). The low-affinity NGF receptor (p75NGFR) is expressed by enteric neurons (Baetge *et al.* 1990) and the high affinity NT-3 receptor (TrkC) mRNA has been located within these neurons using *in situ* hybridisation (Lamballe *et al.* 1994, see Barbacid 1994 for review). Recently, four TrkC isoforms were reported to be expressed in different regions and at different times in the developing ENS (Lewis *et al.* 1994).*

1.3.4.2. Other growth factors

The growth factors FGF (Finch *et al.* 1989, Nice *et al.* 1991), EGF (Cartlidge and Elder 1989), TGFα (Cartlidge and Elder 1989, Koyama and Podolsky 1989, Barnard *et al.* 1991, Pérez-Tomás *et al.* 1993, Hoffmann *et al.* 1994), tumor necrosis factor α (Murch *et al.* 1993) and interleukin 1 (Youngman *et al.* 1993) have been detected in the gut. Recently, a weak TGFα-immunoreactivity has been found in rat and mouse myenteric plexus neurons (Hoffmann *et al.* 1994). EGF, TGFα and TGFβ were mostly found in the salivary glands or Brunner's glands and the epithelial and mucosal layer (Cartlidge and Elder 1989, Barnard *et al.* 1993, Pérez-Tomás *et al.* 1993, see Saffrey and Burnstock 1994). These factors appear to be associated with trophic actions on mucosal epithelial cells and are thought to establish and maintain proliferation and differentiation during development and regeneration (reviewed by Burgess and Sizeland 1990). The receptors for FGF, EGF and TGFα (which both bind to the EGF receptor), TGFβ and

insulin-like growth factors I and II are expressed by mucosal epithelial cells (Koyama and Podolsky 1989, Lund *et al.* 1990, Thompson 1988, Young *et al.* 1989), but their expression within the myenteric plexus has not yet been investigated.

In vitro, insulin-like growth factors (IGF) I and II have been shown to increase neurite elongation from newborn guinea-pig myenteric plexus explants (Mulholland *et al.* 1992a). In cultures from dissociated myenteric plexus, neurite elongation and density was promoted by fetal calf serum in a dose-dependent manner (Mulholland *et al.* 1992b). Both the stable adenosine analogue, 2-chloroadenosine (2-CA) and bFGF have recently been reported to increase enteric neurite elongation in dissociated myenteric plexus cultures (Saffrey *et al.* 1994). The extent of neurite elongation was synergistically enhanced when both 2-CA and bFGF were added together (Saffrey *et al.* 1994). Basic FGF was also found to be mitogenic for enteric glial cells (Eccleston *et al.* 1987).

1.4. Trophic actions of purines and nitric oxide in the nervous system

Polypeptide growth factors, such as the neurotrophins, FGF's and CNTF, are well characterized neurotrophic factors in the CNS and the PNS. However, an increasing number of reports suggest that small neuroactive molecules have trophic actions in the nervous system. It has been shown that neurotransmitters and their receptors are expressed in the developing CNS prior to the establishment of synaptic contacts (eg. Lauder *et al.* 1986). Further, glial cells also express neurotransmitter receptors (see Leslie 1993). This suggests that neurotransmitters are involved in trophic interactions in the nervous

system and that trophic effects of neurotransmitters can be direct or mediated indirectly via glial cells (for reviews see Schwartz 1992, Leslie 1993).

A great variety of different neurotransmitters is expressed by neurons in both the enteric nervous system and the striatum (see above). This section focusses on the evidence of trophic actions of purines, particularly ATP and adenosine, and NO since the involvement of these factors in the neuritogenic actions of myenteric plexus cells on striatal neurons has been investigated in this study.

1.4.1. Purines

Purines and purine analogues have been reported to modulate the differentiation of PNS and CNS neurons and both the proliferation and differentiation of glial cells (Kim *et al.* 1991, Rathbone *et al.* 1992, Christjanson *et al.* 1993, Abbracchio *et al.* 1993, 1994c, Neary *et al.* 1994)*). ATP and the stable ATP analogue, αβ-methylene ATP, have been found to stimulate DNA synthesis in primary glial cell cultures and astrocytoma and neuroblastoma cell lines (Wang *et al.* 1990, Neary and Norenberg, 1992, Neary *et al.* 1994b)and induced an increase in astrocyte number in primary cultures of rat striatum (Abbracchio *et al.* 1994c). Further, ATP has been shown to stimulate mitogen-activated protein kinase activity, which is involved in signal transduction resulting in cell growth (Neary and Zhu 1994). Beside the effects of purines on proliferation, purines have also been shown to induce morphological changes in glial cells, such as hypertrophy, process elongation and an increased expression of glial fibrillary acidic protein (GFAP). In this respect, ATP has been reported to increase the GFAP content of astrocytes in culture (Neary *et al.* 1994a)and αβ-methylene ATP increased the process elongation of GFAP-

*) (Rathbone and Juurlink 1993)

positive cells (Abbracchio *et al.* 1994b). These changes are regarded as indicators of reactive astrogliosis. An increase in DNA synthesis which could be induced with both ATP or bFGF in cultured astrocytes was found to be synergistically increased when bFGF was applied together with ATP (Neary *et al.* 1994 b).A number of different polypeptide growth factors, such as bFGF, EGF and TGF-β, are produced by astroglial cells in response to CNS injury (see Hatten *et al.* 1991, Logan and Berry 1993). Moreover, the release of the neurotrophin NGF by cultured astrocytes can be induced by treatment with purines, such as GTP or guanosine (Gysbers *et al.* 1994). Both P_1- and P_2-purinoceptors were found to be expressed by astrocytes and the trophic effects of purines on these cells are likely to be mediated by cyclic AMP, Ca^{2+}-mobilization and arachidonic acid production (Gebicke-Haerter *et al.* 1988, Bruner and Murphy 1990, Neary and Norenberg 1992, Ogata *et al.* 1994, Peakman and Hill 1994).

In addition to their action on glial cells purines have also been shown to directly promote neurite outgrowth in different neural culture systems (Guroff *et al.* 1981, Huffaker *et al.* 1984, Abbracchio *et al.* 1989, Gysbers and Rathbone 1992, Saffrey *et al.* 1994). They were found to potentiate the effects of growth factors, such as nerve growth factor (NGF) or fibroblast growth factor (FGF) (Braumann *et al.* 1986, Guroff *et al.* 1981, Gysbers and Rathbone 1992, Huffaker *et al.* 1984, Rathbone *et al.* 1992, Saffrey *et al.* 1994).

In summary, the expression of purinoceptors by neurons and glial cells, the upregulation of growth factor production by astrocytes and the potentiation of growth factor effects on neurons by purines together with the observation that purines are released from CNS cells after injury or hypoxia support the idea that purines have

trophic effects in the nervous system.

1.4.2. Nitric oxide

Over the past ten years, increasing evidence indicates that the reactive free-radical NO is a neuronal messenger in the nervous system. NO is synthesized from L-arginine by NOS which has been shown to be an NADPH diaphorase (Dawson *et al.* 1991, Hope *et al.* 1991). In addition to the localization in neurons NOS has also been detected in glial cells (see Murphy *et al.* 1993). The localization of NOS containing neurons in the brain has revealed that these cells are uniquely resistant to excitotoxic and ischemic damage (Koh and Choi 1988, Dawson *et al.* 1991, Uemura *et al.* 1990) and are spared from degeneration in Huntington's, Parkinson's and Alzheimer's disease (Ferranté *et al.* 1985, Hyman *et al.* 1992, Mufson and Brandabur 1994). NO has also been suggested to have beneficial functions such as local vasodilitation and blocking of platelet aggregation following ischemia (Murphy *et al.* 1993). However, when released in large quantities NO was found to be involved in the glutamate mediated neurotoxicity (Nowicki *et al.* 1991, for reviews see Dawson and Snyder 1994, Schuman and Madison 1994). In culture, inhibition of NOS with arginine analogues has been found to protect CNS neurons from glutamate neurotoxicity (Dawson *et al.* 1994). A protective effect against glutamate neurotoxicity has also been reported in cultures pretreated with NO (Lei *et al.* 1992).

Elevation of intracellular cGMP was found to increase neurite elongation in a neuroblastoma cell line and in PC 12 cells (Zwiller *et al.* 1977, Hindley *et al.* 1993). NO is established to be a potent activator of soluble guanylate cyclase, inducing the elevation of cyclic GMP in target cells (see Schmidt *et al.* 1993 for review). Therefore, NO

has the potential to act as a trophic factor by its action on guanylate cyclase. Recently, nitric oxide was reported to synergistically increase NGF-induced neurite elongation in PC 12 cells by activating soluble guanylate cyclase (Hindley and Rathbone 1994). Several lines of evidence indicate the involvement of NO in early neuronal development. Neuronal NOS is localized in the developing rat nervous system and it has been suggested that the transient NOS expression might be associated with naturally occurring cell death (Bredt and Snyder 1994). Further, NO was suggested to be involved in modulating axonal pathfinding since it inhibited neurite elongation in dorsal root ganglion cells and PC 12 cells *in vitro* (Hess *et al.* 1993). The inhibition of NOS has been reported to block the differentiation of motor neurons in the spinal cord during early postnatal life, suggesting the involvement of NO in neuronal maturation and plasticity (Kalb and Agostini 1993). It has been reported that NO modulates transmitter release both *in vitro* (Hanbauer *et al.* 1992) and *in vivo* (Guevara-Guzman *et al.* 1994) probably through activation of guanylate cyclase (Guevara-Guzman *et al.* 1994, see Dawson and Snyder 1994). Further, in hippocampal granule cells NO has been found to upregulate the expression of dendritic microtubule associated protein-2 (MAP2) mRNA *in vivo* (Johnston and Morris 1994). NO appears to be involved in the maintenance and plasticity of synaptic connections. Numerous studies have suggested a role for NO in long term potentiation (see Schuman and Madison 1994 for review).

1.5. Neurite outgrowth and regeneration

The adult central nervous system (CNS) neurons show a limited capacity for neurite regeneration in comparison to peripheral neurons. This limitation of regenerative capacity may not appear to be an intrinsic feature of CNS neurons but is considered to be due to the extracellular environment (see Bunge and Hopkins 1990 for review). Both a lack of trophic support and an inhibition of neurite elongation are possible phenomena which may impair CNS neurons to reestablish their appropriate connections.

Cell culture studies provide a means to identify and characterize neurotrophic factors. They have been used extensively during the last 25 years to investigate the actions of neurotrophic factors and to understand the mechanisms of neurite elongation (see Bunge *et al.* 1987 for review).

The elongation of axons and dendrites, both referred to as neurites, occurs exclusively at their growth cone. The growth cone was first described by Ramón y Cajal in 1890 in the embryonic chick spinal cord (translated extracts in Jacobson 1991). In 1910, Harrison reported for the first time the movement of growth cones in tissue culture. The growth cone is a thickened tip of a neurite which constantly extends and retracts filopodia and lamellipodia (see Letourneau *et al.* 1994 for review). The mechanism of process extension is based on the synthesis of membrane and cytoskeletal components at the cell soma and the subsequent incorporation of membrane components and growth of the cytoskeleton at the level of the growth cone after axonal transport (Bray 1970, 1973, see Black 1994). The attachment of the growth cone to the substrate mediates the traction by which the process extends. The direction and the outgrowth of neurites is influenced by both neurite promoting and

inhibiting factors.

1.5.1. Tissue culture models

The large number of cellular phenotypes and their interactions within the nervous system makes investigations of mechanisms which control neurite elongation during development and regeneration a difficult task. One approach to simplify this complexity is the use of tissue culture methods. This allows control of experimental variables and simplifies quantitation of neuronal responses to treatment. In 1907 Harrison developed the *explant cell culture* technique and demonstrated that neurites are extensions of neuronal cell bodies. In *explant cell cultures* some of the histological features of the tissue *in vivo*, such as synaptic connections and gross anatomic relations are preserved since three-dimensional fragments of tissue are grown in culture (see Dreyfus and Black 1990, Freshney 1994). The outgrowth of processes from these explants *in vitro* allows identification of trophic factor actions on different neuronal cell types (eg. Levi-Montalcini 1987). The development of the roller culture system increased the survival of CNS neurons in explant culture and enabled investigation of the effects of growth factor treatments over relatively long culture periods (eg. Gähwiler *et al.* 1984).

With the aim of investigating the interactions and patterning of cells in a developing tissue, Moscona (1960) introduced the reaggregate cell culture system (see Honegger 1985). After dissociation of neuronal tissue and incubation of the cell suspension in rotating culture flasks, cells reaggregate spontaneously to form three-dimensional spherical structures. The long-term maintenance of

these cultures and the high degree of morphological differentiation within the reaggregates provides a useful means to investigate neurotrophic factor action (eg. the first demonstration that NGF is a trophic factor for cholinergic neurons within the CNS; Honegger and Lenoir, 1982).

A third tissue culture technique, the *dissociated cell culture* was developed in the 1960's (eg. the first description of cultures derived from dissociated fetal brain cells by Varon and Raiborn 1969). After enzymatical or mechanical dissociation of neuronal tissue, a single cell suspension is cultured on pretreated surfaces or a cellular feederlayer within a culture dish. The culture conditions and the age of the tissue can be selected to enable the detection of neurotrophic effects (see chapter 7 for details).

The *dissociated cell culture* technique in combination with the *explant culture* technique has been employed for this study. Previously, cocultures of dissociated striatal neurons and explants of the adrenal gland have been used successfully to detect and analyse neuritogenic effects on striatal neurons (Bird 1989, 1991). The advantages and limitations of these techniques in the investigations of neurotrophic effects are discussed in detail in chapter 7.

1.5.2. Factors that influence neurite elongation *in vitro*

There is a large number of factors with trophic actions on neurons and a wealth of data has been generated about their effects *in vitro*. Therefore, only a summary of the different families of trophic factors is presented here. The three major environmental factors promoting neurite elongation are diffusible neurotrophic molecules, ECM glycoproteins and neural cell adhesion molecules

(CAMs) (see Bixby and Harris 1991, Rathjen 1991, Taira *et al.* 1993, Loughlin and Fallon 1993 for selected reviews). Examples of diffusible neurotrophic factors are the classical trophic factors, such as the neurotrophins (see Davies 1994) and the FGF's (see Eckenstein 1994), and also other neuroactive molecules, such as neurotransmitters (see Schwartz 1992). Among the ECM glycoproteins, which have been found to promote neurite elongation are laminin, fibronectin and the collagens (see Bunge *et al.* 1987, Bixby and Harris 1991, Taira *et al.* 1993 for reviews). The CAMs are cell-surface-associated proteins and can be categorized into three major groups. These are the cadherins, the integrins and members of the immunoglobulin superfamily (see Rathjen 1991). Those factors which have previously been found to have trophic actions on striatal cells are described in chapter 1.2.5.

In addition, neurite elongation is also modulated by inhibitory factors. It is generally believed that non-neuronal cells within the CNS contribute to inhibit regenerative axonal growth within the CNS (for review see Hatten *et al.* 1991). Astrocytic scar formation after gliosis in response to injury and the presence of myelin-associated inhibitor produced by mature oligodendrocytes have been shown to impair regenerative growth in the mature CNS (Caroni and Schwab 1988, McKerracher *et al.* 1994, see Schwab 1990 for review). Although these factors are more important in regeneration *in vivo*, they can also modulate neurite outgrowth *in vitro* (McKerracher *et al.* 1994).

CHAPTER 2

MATERIALS AND METHODS

* Animals were anaesthetized with chloroform and the heads were cut off. The skin was sterilized with 70% ethanol and transferred into a laminar flow hood.

2.1. Cell culture

2.1.1. Striatal cultures

Monolayer cultures were prepared from dissociated rat striatum by a method similar to that described previously for basal forebrain neurons by Allen, Sim and Brown (1993)*. For each experiment, one or two brains of seven-day-old Sprague-Dawley rats were removed under sterile conditions and sliced into 400 µm thick coronal slices (McIlwain tissue chopper, U.K.). The slices were transferred into cold Hanks Balanced Salt Solution (HBSS, Life Technologies, U.K.) supplemented with 0.6% glucose. The caudate putamen was cut out from the slices and washed 3 x in HBSS without Ca^{2+} and Mg^{2+} (Life Technologies, U.K.) buffered with 10 mM Hepes (Life Technologies, U.K.). Double-strength Trypsin (Sigma, U.K.) and DNase I-Solution (Sigma, U.K.) were added in equal volume to the washed tissue to give a final concentration of 0.125% Trypsin and 10 µg/ml DNase I in 10 ml HBSS without Ca^{2+} and Mg^{2+}. The tissue was incubated for 45 min at 37°C and washed 4 x with 5 ml calcium-free HBSS, supplemented with 8 mM $MgCl_2$, 10% foetal calf serum (FCS, ICN Flow, U.K.) and 10 µg/ml DNase I, followed by 2 washes with the same solution supplemented with 0.02 mg/ml DNase I. Afterwards the tissue pieces were carefully triturated. The dissociates were centrifuged at 45 *g* for 15 min. The pellet was resuspended with a fire polished pipette in 1 ml medium 199 (Life Technologies, U.K.) supplemented with 5 mg/ml glucose and 10% FCS. The total cell number was determined in a haemocytometer using the trypan-blue dye exclusion test. The cell suspension was diluted with medium 199 supplemented with 5% glucose and 10% FCS to a final concentration of 1.8×10^5 viable cells/ml. Aliquots (1 ml) of the striatal cell suspension were inoculated into open Rose chambers

on poly-L-lysine-(PLL, Sigma, U.K.) coated glass coverslips and 1 ml of medium 199 supplemented with 0.5% glucose and 10% FCS was added giving a final cell density of 1.8×10^5 cells per chamber.

Dissociated striatal cells were set up at a density in which the cells were clearly separated from each other. Cells started to attach to the coverslip and to send out processes after a few hours. After 48 h *in vitro* neurons could be discriminated from non-neuronal cells, such as astrocyte- and oligodendrocyte-like cells, by morphological criteria under phase contrast microscopy in accordance with studies on the morphology of striatal cells *in vitro* by other groups (Messer 1981, Panula *et al.* 1979a, Surmeier *et al.* 1988) and our own group (Abbracchio *et al.* 1994c). Neurons typically had a phase bright, clearly defined body margin. The processes, mostly two or three, varied in thickness and some had started branching at this stage of culture (see Fig. 3.1; 3.2). Cells identified by these criteria were positive when immunostained for the neurospecific marker protein gene product 9.5 (PGP 9.5) at 4 days *in vitro* (Fig. 3.1) although occasionally, cells with a neuronal morphology were observed showing only very faint staining for PGP 9.5.

The majority of non-neuronal cells were flat, fibroblast-like cells. These cells were identified as type-1-astrocytes, since such cells have been shown to be glial fibrillary acidic protein (GFAP)-positive (Abbracchio *et al.* 1994c). These cells were PGP 9.5-immunonegative. Oligodendrocyte-like cells had a phase dark cell body and a greater number and thicker processes than the neurons. Type-2-astrocyte-like cells showed a large number of processes and could be discriminated from neurons by their flat, large cell body. They were PGP 9.5-immunonegative and GFAP-positive (Abbracchio *et al.* 1994c).

*) Animals were stunned by a blow to their head. The skin was sterilized with 70% ethanol. The following steps were performed under sterile conditions in a laminar flow hood. The skin was opened and the taenia coli of the caecum together with the underlying circular smooth muscle, containing the myenteric plexus, were dissected out of the gut wall. The tissue was cut into approximately 2-3 mm long segments and placed overnight at 4°C in HBSS containing collagenase A (1 mg/ml; Boehringer Mannheim, Germany). Afterwards, the tissue was incubated for 45 min. at 37°C. The myenteric plexus was completely freed from smooth muscle and connective tissue by microdissection under a dissecting microscope using fine forceps. The pieces of myenteric plexus were collected in fresh HBSS.

2.1.2. Myenteric plexus

Explants of myenteric plexus were obtained from beneath newborn guinea-pig taenia coli as described previously by Jessen *et al.* (1983a). Briefly, after removal of the taenia coli including the underlying circular muscle the tissue was treated with collagenase (1 mg/ml; Boehringer Mannheim, Germany) and the plexus was completely freed from smooth muscle by microdissection.* In some experiments, pieces of longitudinal muscle were kept to prepare dissociated smooth muscle cultures as described below.

The isolated enteric ganglia were explanted onto PLL-precoated glass coverslips and grown in Rose chambers in medium 199 supplemented with 10% FCS. Between four and six explants (depending on the size of the explants) were set up per chamber. Each explant contained about 30 to 100 ganglia. Three parallel chambers were prepared for each experiment. Myenteric plexus cultures were grown in the presence of 10^{-5}M cytosine arabinoside (Sigma, U.K.) for the first 3 days *in vitro* in order to eliminate residual fibroblasts. After approximately ten days *in vitro* the explants were set up in cocultures with dissociated striatal cells. As reported previously (Jessen *et al.* 1983a, Bannerman *et al.* 1988a)after 10 days *in vitro*, the myenteric plexus cultures contained only enteric neurons and glial cells.

Myenteric plexus explant cultures and all other types of cultures described below were incubated at 37°C in 97.5% air and 2.5% CO_2 and grown in medium 199 supplemented with 0.5% glucose and 10% FCS.

2.1.3. Enriched enteric glia

A modification of the method described by Bannerman *et al.* (1988b) was used to grow enriched enteric glia. Myenteric plexus explants

* The LB1 antibody recognizes the GD3 ganglioside (Bannermann *et al.* 1988a).

were prepared and grown as described above. After one week in culture the central neuronal area of the explants was excised. During the next two days the glial cells divided and migrated into the free space in the centre of the explant, forming a nearly confluent layer of glial cells with few remaining neurons. At this stage the cultures were used for coculture experiments. Enriched enteric glial preparations were not completely free of neurons. Immunolabelling of these cultures for PGP 9.5 showed a small number of single neurons in some explants sending out processes over a large area of the glial cell carpet.

2.1.4. Enriched enteric neurons

Enriched neuronal cell cultures were prepared as described previously (Bannerman *et al.* 1988b). Briefly, myenteric explant cultures were prepared as described above and grown in medium containing 2 x 10^{-5}M cytosine arabinoside for 2 to 3 weeks. The Rose chambers were then opened under sterile conditions and the coverslips were transferred into petri dishes where they were placed on sterile pedestals. Cells were treated with LB1 antibody (1:200; a gift from Dr.R. Reynolds, Imperial College, London, U.K.) and rabbit complement 1:20 (Serotec, U.K.) in 200 µl HBSS for 8 min at 37°C. The coverslips were then returned to Rose chambers containing fresh medium. Cultures were fed twice a week and retreated after one week with LB1 antibodies and complement if necessary. Enriched neuronal cultures contained aggregates of neurons which were interconnected by nerve fibres as described by Bannerman *et al.* (1988b). Due to the combined treatment of antimitotic agents and antibody complement-mediated cytolysis of the enteric glial cells after 2-3 weeks in culture, a

proportion of neurons were lost as some explants floated away from the surface of the substrate.

2.1.5. Rat heart fibroblasts

Primary cultures of heart fibroblasts were prepared by a method modified from that described by Hassall and Burnstock (1986). Briefly, the heart of a 7-day-old rat was removed under sterile conditions. The atria of the heart were washed in HBSS and cut into 1 mm^3 fragments in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS, Life Technologies, U.K.) supplemented with 5 mg/ml glucose. The tissue was transferred into 0.125% trypsin (Life Technologies, U.K.) and incubated overnight at 4°C followed by an incubation at 37°C for 20 min. Afterwards, the tissue was triturated and allowed to settle. The undissociated tissue fragments were resuspended in 2 ml modified DPBS and incubated at 37°C for 1 h. The cell suspension was transferred with a wide-bore Pasteur pipette into 1 ml FCS and stored at 4°C in a centrifuge tube. HBSS (2 ml) containing 0.2% collagenase A (Life Technologies, U.K.) was added to the residual tissue fragments which were then incubated at 37°C for 40 min. After trituration of the tissue fragments with a fine-bore Pasteur pipette this cell suspension was added to the first cell suspension in the centrifuge tube. The final suspension was centrifuged at 600 rpm for 5 min and the pellet was resuspended in medium 199. The cell suspension was diluted to a concentration of approximately 5×10^5 cells/ml and seeded onto glass coverslips in Rose chambers. Cultures were incubated at 37°C and after 10 min the Rose chambers were turned over. By that time only fibroblasts had attached to the former bottom coverslip, which was the top coverslip from then on. Cultures were fed after 24 h and every 3 days

subsequently. After 10 to 12 days *in vitro* the cultures were used for cocultures.

2.1.6. Smooth muscle cells

Cultures of dissociated smooth muscle cells were prepared from the longitudinal muscle of the guinea-pig taenia coli. Tissue was obtained from pieces of longitudinal muscle of the taenia coli kept after the microdissection of myenteric plexus as described above. Fragments of smooth muscle were washed 3 x with approximately 5 ml HBSS without Ca^{2+} and Mg^{2+} and incubated in 0.125% Trypsin and 10 µg/ml DNase I in 10ml HBSS without Ca^{2+} and Mg^{2+} for 45 min at 37°C. Afterwards the tissue was washed 4x with 5ml calcium-free HBSS, supplemented with 8 mM $MgCl_2$, 10% FCS and 10 µg/ml DNase I, followed by 2 washes with the same solution supplemented with 0.02 mg/ml DNase I. The tissue pieces were carefully triturated and the dissociates were centrifuged at 45 *g* for 15 min. The pellet was resuspended with a fire polished pipette in 1 ml medium 199 supplemented with 10% FCS and diluted to a concentration of approximately 10^6 cells/ml. Cultures were set up on 13 mm glass coverslips in 4-well-multidishes (Life Technologies, U.K.) and fed after 24 h and every 2 to 3 days subsequently. Cultures were used for cocultures after about 10 days *in vitro*. Smooth muscle cell cultures were immunonegative for smooth muscle myosin (see below; Gröschel-Stewart *et al.* 1985) and no spontaneously contracting cells were observed in living cultures during the coculture period. This suggests that the smooth muscle cells were de-differentiated.

2.1.7. Cultures in myenteric plexus-conditioned medium

Striatal cells were prepared as described above and set up in medium conditioned previously for 24 to 48 h by 10-day-old myenteric plexus cultures. The medium which was used to feed the striatal cultures at the first day *in vitro* had been conditioned for 48 h by a separate batch of myenteric plexus cultures.

2.1.8. Substrates pre-coated with extracellular matrix proteins

Fibronectin (33 µg/ml) or laminin (20 µg/ml) was applied to PLL-precoated coverslips (30-50 µl/coverslip). After 2 h at room temperature (RT) coverslips were rinsed 2x with HBSS before use. Laminin coated coverslips were not allowed to dry. A volume of 30-50 µl collagen (Vitrogen or prepared from rat tail; Bornstein 1958) was applied to glass coverslips and air-dried overnight at RT. When cells were grown on air-dried collagen attachment and growth of striatal cells was poor and neurite elongation could not be analysed.

2.1.9. Cocultures

Aliquots (1 ml) of the striatal cell suspension (prepared as described above) were inoculated into open Rose chambers on PLL precoated glass coverslips and 1 ml of medium 199 supplemented with 0.5% glucose and 10% FCS was added giving a final cell density of 1.8 x 10^5 cells per chamber. For experimental cultures the chambers were closed with a second coverslip on which myenteric plexus explants (or other cell types) were growing. As a result the central and peripheral cell cultures were facing each other at a distance of about 3.5 mm. For controls the chambers were closed with plain coverslips. Six separate chambers were set up per experiment, three controls and three experimentals. In cases in which the yield of

striatal cells after the dissociation was less than a total of 1.08 x 10^6 viable cells, five chambers were set up. This was taken into account in the evaluation of the data as described below.

The cultures were incubated and fed after 24 h *in vitro* with medium 199 supplemented with 0.5% glucose and 10% FCS. Striatal cells were always grown on the bottom coverslip during the first two days *in vitro*.

2.2. Evaluation of neurite outgrowth

2.2.1. Measurement of neurite length

The total length of processes per cell from striatal neurons was measured in living cultures after 48 h *in vitro* using phase contrast microscopy linked to an image analysis system (Seescan, U.K.). In each culture a total of 100 to 150 neurons each for control and experimental cultures were measured. Neurons were chosen randomly by scanning across the Rose chamber and were only measured if they were separated sufficiently to allow an accurate recognition and measurement of all processes (see Fig. 3.2).

In cases in which the yield of cells after the dissociation was not big enough to prepare six chambers, in either the two controls or two experimental chambers the total of 100 to 150 neurons per group was measured. The data of control cultures was pooled and compared with the pooled data of experimental cultures.

2.2.2. Evaluation of mean neurite length and statistics

To make a valid comparison between the total length of neurites of the cells measured in the experimental cultures and those in controls

using normal statistics, the lengths of processes would have to be normally distributed. The histograms of neuronal cell number versus their total process length showed a population slightly skewed toward shorter neurite length (for example see Fig. 2.1). This type of distribution in respect to neurite extension has also been reported by other groups in dissociated monolayer cultures of both retinal ganglion cells and hippocampal cells (Lipton *et al.* 1988, Walicke *et al.* 1986). By taking the natural logarithm of the process length the data was transformed into a normal distributed population as shown in Fig. 2.1. This enabled the mean natural logarithm of the neurite length of ~~each~~ neurons in the experimental condition to be compared to the controls using the Student`s *t*-test. The mean natural logarithm (± S.E.M.) was then retransformed into an absolute mean neurite length (± S.E.M.) and the increase of the mean neurite length is shown as % of control (controls = 100%). To compensate for systematic differences between individual experiments, each experiment was compared with its own control. The results of one experiment shown in Fig. 2.1 demonstrate that striatal neurons grew significantly longer processes in cocultures with myenteric plexus explants compared to controls.

2.3. Evaluation of cell numbers

2.3.1. Immunofluorescence staining

After 4 days *in vitro* cultures were fixed and stained for PGP 9.5 (Gulbenkian *et al.* 1987). Cells were briefly rinsed with HBSS and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 h at 4°C. All following steps were performed at room temperature. Cultures were washed 6 x with 80% ethanol (10 min each), washed 2 x 10 min with PBS and incubated for 10 min in 0.1% Triton X-100 in PBS,

followed by a final wash for 10 min with PBS. The cells were incubated overnight with the primary antibody, rabbit anti-PGP 9.5 (Ultraclone, U.K.) at a dilution of 1:2000, in antibody diluting solution (ADS) containing 0.1% Triton-X 100 (BDH, U.K.), 0.1% sodium azide (Sigma, U.K.), 0.01% bovine serum albumin (Sigma, U.K.) and 0.1% lysine (Sigma, U.K.) in PBS. Cells were washed 3 x 10 min with PBS and incubated for 1 h with biotinylated anti-rabbit secondary antibody diluted 1:250 (Amersham, U.K.) in ADS, followed by 3 washes (10 min each) with PBS. Afterwards the cultures were incubated for 1 h with streptavidin-fluorescein (1:100 in ADS, Amersham, U.K.) and washed 3 x with PBS before they were mounted in Citifluor (glycerol/PBS solution AF1; Citifluor, U.K.). Smooth muscle cells were fixed in 4% paraformaldehyde as described above and incubated at room temperature overnight with primary antiserum raised in rabbit against chicken gizzard smooth muscle myosin (1:200; a gift from U. Gröschel-Stewart; Gröschel-Stewart *et al.* 1985). Afterwards cells were washed 3 x 10 min with PBS and incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (1:100; Nordic, U.K.) for 1 h at room temperature. After 3 washes with PBS (10 min each) the cultures were mounted in Citifluor.

2.3.2. Quantitation of neuronal and non-neuronal cell numbers

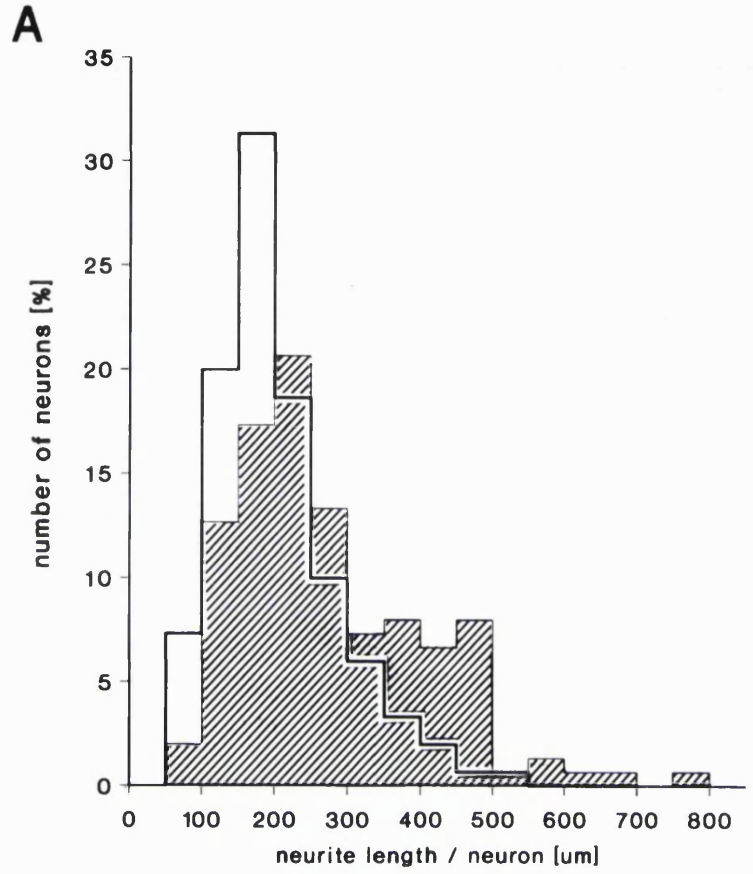
Quantitative estimation of the number of neuronal (PGP 9.5 - immunopositive cells) and non-neuronal cells (PGP 9.5 - immunonegative cells) after 4 days *in vitro* was performed by scanning 12 diameter strips altogether per condition under fluorescence microscopy (see Fig. 3.1), giving identical total surface areas in each condition. In one case 6 diameter strips in the controls were

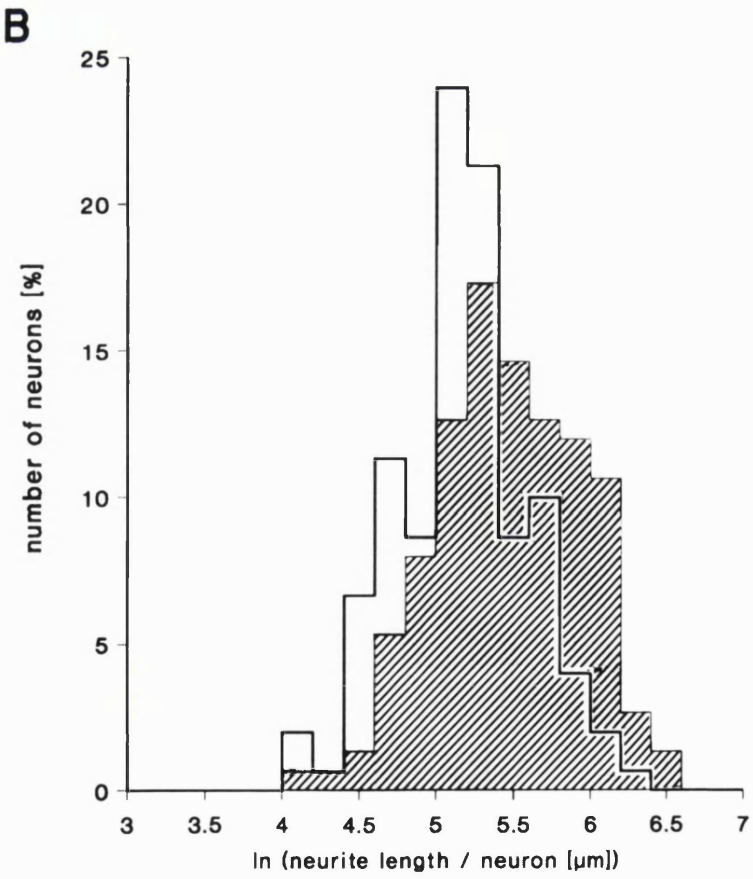
analysed. The total numbers of neurons and non-neuronal cells per experimental condition were compared to their own control and expressed as % control (control = 100 %). The changes in cell number of each group of experiments were pooled.

2.4. Materials

Adenosine-5'-triphosphate (ATP), α,β-methylene ATP (αβ-meATP), 2-chloroadenosine (2-CA), L-arginine, guanosine-5'-triphosphate (GTP), 8-(*p*-sulfophenyl)theophylline (8-PSPT), forskolin, N-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), and tetrodotoxin (TTX) were obtained from Sigma. Suramin was obtained from Bayer and adenosine deaminase (ADA) was obtained from Boehringer Ingelheim. Basic fibroblast growth factor (bFGF), nerve growth factor (NGF) and fibronectin were obtained from Sigma, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) were provided by Regeneron Inc. Laminin was obtained from Life Technologies , collagen was obtained from Vitrogen and a mixture of bovine gangliosides (AGF1; derived from bovine brain) was provided by Fida Abano Terme (Italy).

Fig. 2.1. Histograms representing the distributions of the *total neurite length* per cell of 150 striatal neurons scored for outgrowth under control conditions and in cocultures with myenteric plexus explant cultures (A). Empty bars represent control conditions; hatched bars represent striatal neurons in coculture. The natural logarithm (ln) of the total neurite length is displayed in (B) showing that the mathematical transformation of the data results in a normally distributed population of ln values plotted as a function of the neurite length. The mean ln value for the control cultures is 5.21 ± 0.03 S.E.M. and for the experimental cultures the mean ln value is 5.62 ± 0.04 S.E.M.. The mean ln value of the neurite length per neuron in the experimental condition was significantly longer compared to control ($P<0.0001$) using the Student's *t*-test.


A
35
30
25
20
15
10
5
0
number of neurons [%]
0
100
200
300
400
500
600
700
800
neurite length / neuron [um]


B
25
20
15
10
5
0
number of neurons [%]
3
3.5
4
4.5
5
5.5
6
6.5
7
ln (neurite length / neuron [µm])

CHAPTER 3

MYENTERIC PLEXUS EXPLANTS PROMOTE NEURITE ELONGATION AND SURVIVAL OF STRIATAL NEURONS *IN VITRO*

3.1. Summary

Dissociated striatal neurons exhibited increased neurite outgrowth when cocultured with myenteric plexus explants. Enriched enteric neurons or enriched enteric glia produced a less marked response, non-ganglionic cells had no effect. Increases in striatal neuron and glial cell numbers were seen in all cocultures. Tetrodotoxin abolished the neuritogenic response of myenteric plexus explants, but did not affect increases in cell numbers. These observations suggest that spontaneous neuronal activity within the myenteric plexus is involved in the release of a neuritogenic factor(s), possibly from glial cells, and that this is distinct from factor(s) affecting striatal cell numbers.

3.2. Introduction

Striatal neurons exhibited increased neurite outgrowth when cocultured with myenteric plexus explants. Enriched enteric neurons or glia produced a reduced response, non-ganglionic cells had no effect. Increases in striatal cell numbers occurred in all cocultures. Tetrodotoxin abolished the neuritogenic response, but did not affect increases in cell numbers. This suggests that spontaneous neuronal activity within the myenteric plexus is involved in the release of a neuritogenic factor(s), possibly from glial cells, which is distinct from factor(s) affecting striatal cell numbers.

The intrinsic nervous system of the gut, the enteric nervous system, shows many structural and functional similarities to the CNS (Jessen and Burnstock 1982). Investigation of the use of enteric ganglia as a source of material for grafting into the CNS has revealed that enteric ganglion cells survive when grafted into the striatum (Tew *et al.* 1992, 1993) and also produce a sprouting response by the host brain (Tew *et al.* 1992, 1994). These observations suggest that the gut, and the myenteric plexus in particular, may be a source of growth promoting factors which are active on CNS neurons.

To investigate this further a novel coculture system of myenteric plexus explants with dissociated striatal neurons was utilized as a model to determine if myenteric ganglia produce soluble factors active on striatal neurons and to analyse the effect of such factors on these neurons.

3.3. Material and Methods

Cultures from dissociated postnatal (P7) rat striatum were obtained as described in Chapter 2.1.1. These cells were cocultured

with either myenteric plexus explant cultures (see chapter 2.1.2.), enriched enteric glial cell preparations (see chapter 2.1.3.), enriched enteric neuronal preparations (see chapter 2.1.4.), rat heart fibroblast cultures (see chapter 2.1.5.) or smooth muscle cell cultures (see chapter 2.1.6.). The procedures for the preparation of cocultures are described in chapter 2.1.9. Striatal cultures were also grown in medium conditioned by myenteric plexus explant cultures (see chapter 2.1.7.). After 48 h *in vitro*, the neurite outgrowth of striatal neurons was analysed as described in chapter 2.2.1. The evaluation of the mean neurite length and the statistical analysis was performed as described in chapter 2.2.2. After 4 days in culture, striatal cells were fixed and stained for the neurospecific marker PGP 9.5 (see chapter 2.3.1.) and the numbers of neuronal and non-neuronal cells was quantitated (see chapter 2.3.2.).

3.4. Results

A marked increase in neurite length (149 ± 5%; Fig. 3.2 A,B; Fig. 3.3A) was detected in striatal neurons cocultured with the whole myenteric plexus. In each experiment (n = 6) the increase was significant ($P<0.0001$) compared to its own control. A less strong growth promoting effect was observed in cocultures with enriched enteric glia (126 %, three out of four experiments showed a significant increase, $P<0.0001$, Fig. 3.3A), while enriched enteric neurons had only a small neuritogenic effect (114 %, Fig. 3.3A). Tetrodotoxin (TTX; 1 µM) totally abolished the neuritogenic effect (Fig. 3.3A) while coculture with rat heart fibroblasts or de-differentiated smooth muscle cells and myenteric plexus conditioned medium had no effect (Fig. 3.3A).

In order to determine if increases in striatal neurite length were related to increased survival of striatal neurons or to increased numbers of striatal cells, the numbers of these cells were counted. Increases in neuronal survival and non-neuronal cell numbers, were promoted in all cocultures examined (Fig. 3.3B).

3.5. Discussion

These results indicate that the increases in neurite length observed in myenteric ganglion cell cocultures are unlikely to be due to the enhanced survival of a subpopulation of striatal neurons which extend longer processes, or to an indirect effect due to the presence of higher numbers of striatal non-neuronal cells in coculture, but rather that this is a specific neurite promoting effect of enteric ganglion cells. In addition, these differences suggest that separate molecules may be involved in mediating neurite promoting effects and effects increasing neuronal survival and non-neuronal cell numbers.

The reduced neuritogenic effect of enriched enteric glia suggests that a trigger mechanism from neurons to glial cells or vice versa might be involved in the release of neurite promoting factors. However, enteric glia could not be totally purified, so the few remaining neurons may have contributed to the neurite promoting effects of enriched enteric glia. The small neuritogenic effect of enriched enteric neurons may have been caused by a change in their state of differentiation or a reduction of neuronal number due to the procedure of enrichment (Bannermann *et al.* 1988b). However, the effect on neuronal survival remained as strong as that observed with whole myenteric ganglion explants, supporting the idea that different mechanisms and factors were affecting neurite outgrowth and/or cell numbers. Since the strongest neuritogenic effect was detected in

cocultures with whole myenteric plexus explants it seems likely that both enteric glia and neurons are in some way involved in a mechanism leading to increased neurite outgrowth. This neuritogenic effect of the myenteric plexus *in vitro* may correlate with the observation of a sprouting response of striatal neurons around and into grafts of myenteric plexus *in vivo* (Tew *et al.* 1992, 1994).

The observation that TTX abolished the neuritogenic effect indicates that substances released during spontaneous neuronal activity within the plexus are likely to be involved in the mechanisms leading to enhanced neurite outgrowth. Control experiments, in which TTX did not have any effect on the neurite length of striatal neurons cultured alone (see chapter 4; Fig. 4.1B, Fig. 4.4B), supported this idea. Spontaneous activity has been recorded from myenteric neurons in culture (Hanani and Burnstock 1985). The CNS cells themselves may have initiated a chain of events in the adjacent myenteric plexus cells which in turn released neurite promoting factor(s) acting on striatal neurons. Myenteric plexus conditioned medium was ineffective, possibly indicating the involvement of CNS cells. Alternatively, the neuritogenic effect may have been mediated by a substance with a short life time or several substances acting together of which at least one may be broken down quickly. Candidates in this respect are purine nucleotides and nucleosides, nitric oxide, neuropeptides and the classical transmitters acetylcholine and serotonin (noradrenaline is not found in rat enteric ganglia; see Furness and Costa 1987 for review), which are all considered to be enteric neurotransmitters (Costa *et al.* 1992).

It is also possible that a member of one of the established growth

factor families such as the neurotrophins or the recently described glial cell line-derived neurotrophic factor (GDNF; Lin *et al.* 1993) may be released by the cells of the enteric nervous system (Saffrey and Burnstock 1994). Transcripts of NGF and neurotrophin-3 have been found in the gut (Maisonpierre *et al.* 1990). In this respect, a number of different growth factors, such as NGF, FGF and EGF, have been shown to affect biochemical and morphological properties as well as cell survival of striatal neurons *in vivo* and *in vitro* (eg. Vahlsing *et al.* 1991, Walicke 1988).

On the basis of these results it is proposed that: (1) factors released from myenteric plexus are involved in a sequence of events which leads to increased neurite extension of striatal neurons; (2) a factor(s) released during spontaneous neuronal activity is likely to be involved; (3) all the cell types examined produce factor(s), which promote striatal neuronal survival and elevate glial cell numbers; (4) increased neurite extension and increased striatal cell numbers are independently affected and are thus likely to be mediated by different mechanisms.

Fig. 3.1 A, B: Fluorescence (A) and phase-contrast (B) micrographs showing the same field of a striatal culture immunostained for PGP 9.5 after 4 days *in vitro*. Neurons (arrow) were immunopositive for PGP 9.5 while astrocytes (asterisk) were immunonegative. Bar = 30 µm.

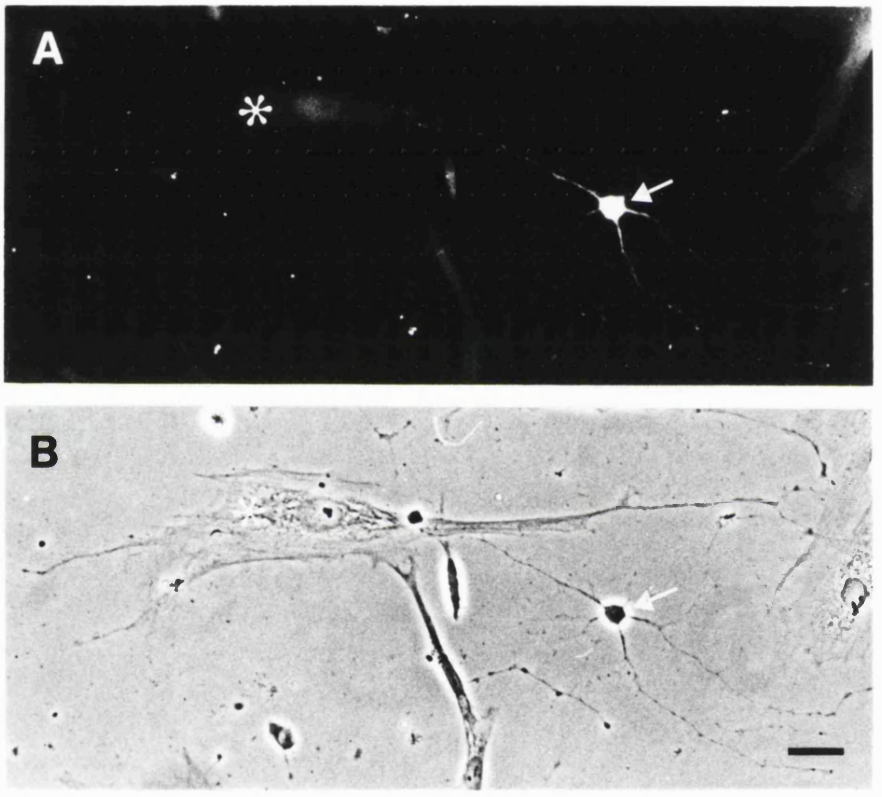

A
B

Fig. 3.2 A, B: Phase-contrast micrographs of living primary striatal cells cultured alone (A) or with myenteric plexus (B) after 2 days *in vitro*. Neurons (examples marked with arrows) were identified by morphological criteria and were only measured if all processes were clearly visible. Bar = 50 µm.

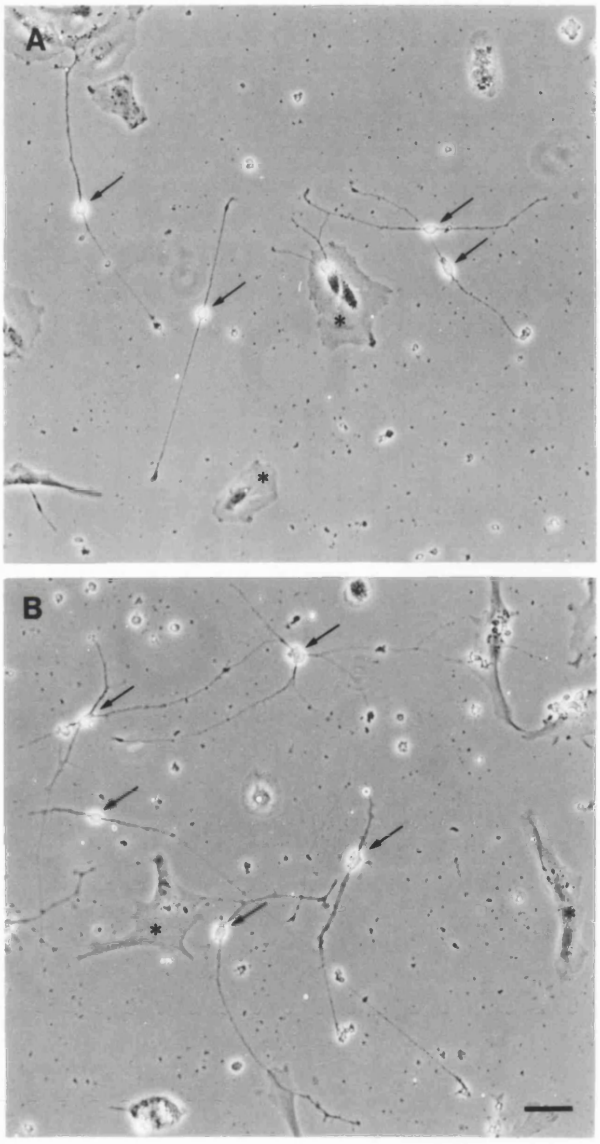
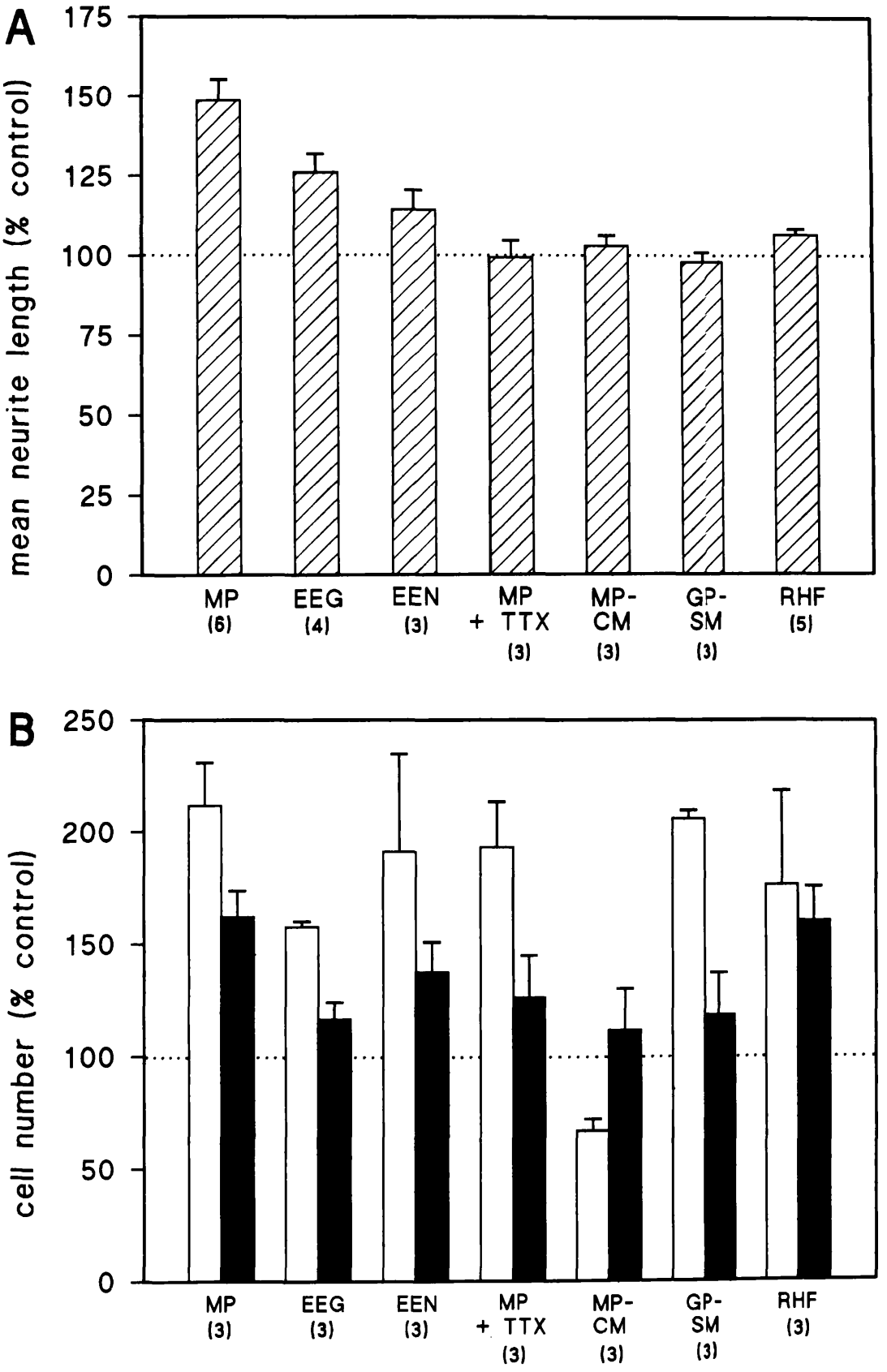

A
B

Fig. 3.3. Mean *neurite length* of striatal neurons after 2 days *in vitro* (A) and *neuronal survival* (open bars) and *glial cell numbers* (full bars) in striatal cultures after 4 days *in vitro* (B). Striatal cells were cocultured with different cell populations or grown in conditioned medium; MP = myenteric plexus explants; EEG = enriched enteric glial cells; EEN = enriched enteric neurons; TTX = tetrodotoxin (1 μM); MP-CM = myenteric plexus culture conditioned medium; GP-SM = guinea-pig smooth muscle cells (de-differentiated); RHF = rat heart fibroblasts; Each bar represents the pooled results of 3 to 6 experiments as indicated in parentheses (figures in parenthesis are the number of experiments).


A
mean neurite length (% control)
175
150
125
100
75
50
25
0
MP (6)
EEG (4)
EEN (3)
MP + TTX (3)
MP-CM (3)
GP-SM (3)
RHF (5)
B
cell number (% control)
250
200
150
100
50
0
MP (3)
EEG (3)
EEN (3)
MP + TTX (3)
MP-CM (3)
GP-SM (3)
RHF (3)

CHAPTER 4

NEURITE OUTGROWTH OF STRIATAL NEURONS *IN VITRO*: INVOLVEMENT OF PURINES IN THE GROWTH PROMOTING EFFECT OF MYENTERIC PLEXUS EXPLANTS

4.1. Summary

It has been shown that a soluble factor(s) released by the myenteric plexus promotes neurite outgrowth from postnatal striatal neurons, and that this effect was abolished by tetrodotoxin (see chapter 3). In the study described in this chapter the possible involvement of purines in the mediation of this neuritogenic response was investigated, by examining their effect on neurite length of striatal neurons both in coculture with myenteric plexus explants and cultured alone.

Both ATP and 2-chloroadenosine partially reversed the inhibitory effect of tetrodotoxin in cocultures with whole myenteric plexus, while the stable ATP analogue, α,β-methylene ATP, had no effect, suggesting that ATP was being broken down to adenosine before having its action. Further support for this view was that the ATP purinoceptor (P_2) blocker suramin did not reverse the effects of ATP, while the adenosine purinoceptor (P_1) blocker 8-(*p*-sulfophenyl)theophylline did antagonize the effects of ATP in tetrodotoxin-treated cocultures. Further, both 8-(*p*-sulfophenyl)theophylline and adenosine deaminase reduced the effect of the myenteric plexus on striatal neurons in the absence of tetrodotoxin; and the adenylate cyclase activator forskolin completely reversed the effect of tetrodotoxin in the coculture system utilized here. The neurite outgrowth promoting effect of 2-chloroadenosine in tetrodotoxin-treated cocultures was not further enhanced by a combination of neuropeptides. Serotonin and GTP were without effect on striatal neurons in the presence or absence of myenteric plexus explants. In experiments without myenteric plexus, both 2-chloroadenosine and forskolin caused a slight increase in

striatal neurite length; ATP and GTP were ineffective.

In summary, adenosine (probably acting through the A_2 subclass of the P_1-purinoceptors) leads to increased striatal neurite outgrowth in coculture with myenteric plexus and it is proposed that it does so either (1) by triggering the release of a neuritogenic factor possibly from enteric glial cells or (2) by acting synergistically with such a growth factor. Adenosine acts via P_1-purinoceptors, which leads to changes in cyclic AMP and the response to forskolin suggests that cyclic AMP is probably involved in the events leading to increased striatal neurite outgrowth.

4.2. Introduction

Previous studies in our laboratory have shown that the myenteric plexus promotes a striking sprouting response by host neurons when transplanted into the rat striatum (Tew *et al.* 1992, 1994). The fact that freeze-killed grafts of the myenteric plexus did not induce a sprouting response within the host brain strongly suggests that a soluble, growth promoting factor(s) released from myenteric plexus cells were responsible for the axonal sprouting within the striatum.

In order to analyse this further, a coculture system in which central and peripheral cells are cultured on separate sides of a Rose chamber has been developed allowing the diffusion of soluble factors while avoiding any cell to cell contact between the two cell populations. In chapter 3 it has been shown that soluble factors released by explants of the myenteric plexus promote neurite extension of postnatal striatal neurons *in vitro*. The observation that this effect is abolished by tetrodotoxin (TTX) indicates that a neurotransmitter(s) released from enteric neurons play a role in the events leading to increased neurite outgrowth of striatal neurons in coculture (see chapter 3).

One class of molecules, which may be involved in the neuritogenic effect of the myenteric plexus are purines. ATP is an enteric neurotransmitter (Hoyle and Burnstock, 1989) and recent studies have revealed that purines have trophic actions in the nervous system (see below). ATP is broken down to adenosine which also acts as an intercellular messenger. ATP and adenosine bind to specific classes of extracellular receptors, referred to as P_1- and P_2-purinoceptors, respectively (Burnstock 1978, see Burnstock 1993). Both classes can be subdivided further into receptor subtypes on the basis of ligand binding, molecular cloning and second messenger systems (reviewed by

Abbracchio and Burnstock 1994).

In the nervous system, purines have trophic actions on glial cells (Kim *et al.* 1991, Rathbone *et al.* 1992, Christjanson *et al.* 1993, Abbracchio *et al.* 1993, 1994a, Neary *et al.* 1994) and have also been found to be neuritogenic for neuronal cells. They directly promote neurite outgrowth in some neural culture systems (Gysbers and Rathbone 1992, Huffaker *et al.* 1984, Saffrey *et al.* 1994) and can act synergistically with growth factors, such as nerve growth factor (NGF) or fibroblast growth factor (FGF) (Braumann *et al.* 1986, Guroff *et al.* 1981, Gysbers and Rathbone 1992, Huffaker *et al.* 1984, Rathbone *et al.* 1992).

In the present study the possible involvement of purines in the neurite promoting effect of the myenteric plexus on striatal neurons has been investigated. The effect of purines, purine analogues and antagonists on neurite outgrowth of striatal neurons both in coculture with myenteric plexus explants and cultured alone was examined, in the presence and absence of TTX.

4.3. Material and Methods

Cultures from dissociated postnatal (P7) rat striatum were obtained as described in chapters 2.1.1. These cells were cocultured with myenteric plexus explant cultures (see chapter 2.1.2.) according to the procedures described in chapter 2.1.9. After 48 h *in vitro*, the neurite outgrowth of striatal neurons was analysed as described in chapter 2.2.1. The evaluation of the mean neurite length and the statistical analysis was performed as described in chapter 2.2.2.

4.4. Results

4.4.1. Effects of myenteric plexus on striatal neurite length in coculture

Myenteric plexus explants increased the mean neurite outgrowth of striatal neurons in coculture (149 ± 5% of control values in the absence of myenteric plexus; number of experiments (n) = 6; Fig. 4.2). This effect was totally abolished when cocultures were supplemented with 1 μM TTX (99 ± 5% of control; n = 3; Fig. 4.1A, 4.4A). The mean neurite length in striatal cultures alone in the presence of 1 μM TTX was 105 ± 2% compared to control (Fig. 4.1B, 4.4B).

4.4.2. Effects of purines on cocultures treated with 1 μM TTX

ATP, at a concentration of 50 μM, increased the mean neurite outgrowth in TTX-treated cocultures (125 ± 5% of control; Fig. 4.1A). This mean neurite outgrowth induced by ATP was significantly greater than that observed in TTX-treated cocultures ($P<0.05$). This increase in mean neurite outgrowth in TTX-treated cocultures as a result of the application of ATP (50 μM) was unaffected by the addition of the P_2-purinoceptor blocker suramin, at a concentration of 10 μM (124 ± 2% compared to control; Fig. 4.1A; $P<0.05$ compared to TTX-treated cocultures). When the hydrolysis-resistant ATP analogue, αβ-meATP (50 μM) was added to TTX-treated cocultures, there was no significant increase in mean neurite length (102 ± 2% of control; Fig. 4.1A). As shown in Figure 4.1A, the increase in neurite elongation induced by ATP (50 μM) was completely abolished (98 ± 2% of control) by the P_1-purinoceptor blocker 8-(*p*-sulfo phenyl)theophylline (8-PSPT; 10 μM). Addition of the stable adenosine analogue, 2-chloroadenosine (2-CA; 50 μM) resulted in an increase of the mean neurite

length to 139 ± 6% compared to control (Fig. 4.1A; P<0.01 compared to TTX-treated cocultures).

4.4.3. Effects of purines on striatal cultures alone in the presence of 1 μM TTX

In experiments without the myenteric plexus 50 μM ATP, both alone (101 ± 3% of control) and together with 10 μM suramin (102 ± 3% of control) did not affect neurite extension in TTX-treated striatal cultures, as shown in Figure 4.1B. Addition of either 50 μM αβ-meATP (103 ± 2% of control) or a combined treatment of ATP (50 μM) together with 10 μM 8-PSPT (107 ± 2% of control) did not significantly change neurite elongation in striatal cultures alone in the presence of 1 μM TTX (Fig. 4.1B). An increase in striatal neurite outgrowth was, however observed in the presence of 50 μM 2-CA (112 ± 5% of control; see Fig. 4.1B).

4.4.4. Effect of 8-PSPT and ADA on striatal neurons alone and in coculture in the absence of TTX

A significant reduction in the mean neurite length of striatal neurons in coculture with myenteric plexus was seen in the presence of 10 μM 8-PSPT (107 ± 2% of control; Fig. 4.2), when compared to untreated cocultures (P<0.01). When ADA was added to the cocultures, at a concentration of 2 U/ml, the neurite outgrowth was slightly increased (112 ± 3% of control; Fig. 4.2). This mean neurite length was significantly reduced in comparison with untreated cocultures (P<0.01). Neither 10 μM 8-PSPT (104 ± 4% of control) or 2 U/ml ADA (104 ± 2% of control) affected process elongation in striatal neurons alone, as shown in Figure 4.2.

4.4.5. Effects of purines on striatal cultures alone in the absence of TTX

ATP (50 µM; 102 ± 5% of control) and GTP (50 µM; 96 ± 2% of control) did not affect striatal neurite outgrowth in the absence of TTX, as shown in Figure 4.3. An increase in neurite elongation was observed in striatal cultures treated with 50 µM 2-CA (120 ± 4% of control; Fig. 4.3).

4.4.6. Effects of GTP, 5-HT, forskolin and a combination of peptides together with 2-CA in cocultures treated with 1 µM TTX

The neurite outgrowth promoting effect of 2-CA (50 µM) in TTX-treated cocultures (139 ± 6% of control; Fig. 4.4A; $P<0.01$ compared to TTX-treated cocultures) was not further increased, when 2-CA (50 µM) was added together with a combination of calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), somatostatin (SOM), substance P (SP) and vasoactive intestinal peptide (VIP) to TTX-treated cocultures (all peptides at 0.1 µM; 147 ± 7% of control; Fig 4.4A; $P<0.01$ compared to TTX-treated cocultures). There was no significant increase in mean neurite length, when either GTP (50 µM; 97 ± 1% of control) or 5-HT (1 µM; 96 ± 2% of control) was added to TTX-treated cocultures, as shown in Figure 4.4A. The adenylate cyclase activator forskolin (10 µM) completely reversed the blocking effect of TTX resulting in an increase in mean neurite length of 161 ± 5% (Fig. 4.4A; $P<0.01$ compared to TTX-treated cocultures).

4.4.7. Effects of GTP, 5-HT, a combination of peptides and forskolin on striatal cultures alone in the presence of 1 µM TTX

Addition of 2-CA (50 µM) together with a combination of CGRP, NPY, SOM, SP and VIP (all at 0.1 µM) resulted in an increased neurite

elongation in TTX-treated striatal cultures (115 ± 2% of control; $P<0.05$ compared to TTX-treated striatal cultures) as shown in Figure 4.4B. GTP (50 µM; 97 ± 2% of control) and 5-HT (1 µM; 99 ± 2% of control) did not affect neurite outgrowth in striatal cultures in the presence of TTX, whereas an increase in neurite elongation to 119 ± 4% compared to control was observed in the presence of 10 µM forskolin and 1 µM TTX (Fig. 4.4B; $P<0.05$ compared to TTX-treated striatal cultures).

4.5. Discussion

It has been shown that soluble factors produced by the myenteric plexus cells promote sprouting of striatal neurons *in vivo* (Tew *et al.* 1992, 1994) and *in vitro* (see chapter 3). Further, the neuritogenic effect of the myenteric plexus explants on striatal neurons in coculture was completely abolished by TTX (see chapter 3). This observation suggested that the action of neurotransmitter(s) released from enteric neurons may be involved in the trophic effects of the myenteric plexus on striatal neurons in coculture. In the present study evidence is presented that purines, particularly adenosine, formed by the breakdown of the enteric neurotransmitter ATP, are involved in the mediation of this neuritogenic effect. Adenosine acts through P_1-purinoceptors involving adenylate cyclase (Burnstock 1978) and it is demonstrated here that the second messenger cAMP is involved in the events leading to increased neurite elongation of striatal neurons *in vitro*.

Addition of ATP to TTX-treated cocultures partially reversed the blocking effect of TTX and an enhanced neurite outgrowth of striatal neurons was again observed; however, this effect of ATP could not be

antagonized with the P_2-purinoceptor blocker suramin. Moreover, the stable ATP analogue, αβ-meATP, did not restore the neuritogenic effect of the myenteric plexus in TTX-treated cocultures. These results suggested that the effect of ATP in TTX-treated cocultures are mediated by its breakdown product, adenosine. To explore this possibility, a selective P_1-purinoceptor antagonist was used in combination with ATP. It was found that the effect of ATP was antagonized by blocking P_1-purinoceptors with 8-PSPT. Further, the slowly-degradable adenosine analogue, 2-CA, induced an increase in striatal neurite elongation which was greater than that seen with ATP, and only slightly less than that observed in untreated cocultures (see chapter 3). Further evidence for this neuritogenic effect being adenosine-mediated was produced in coculture experiments in the absence of TTX, in which both 8-PSPT and ADA reduced the effect of myenteric plexus explants on striatal neurons to control levels. Also since adenosine acts on P_1-purinoceptors which lead to elevation in cAMP, the adenylate cyclase activator forskolin mimics the action of adenosine. In experiments without the myenteric plexus, in which the neuritogenic potentials of different combinations of supplements were analysed on striatal cells cultured alone, only 2-CA and forskolin had a stimulatory effect on neurite outgrowth. A slight neuritogenic effect of adenosine at similar concentrations has also been reported in PC12 cells and dissociated enteric neurons (Gysbers and Rathbone 1992, Saffrey *et al.* 1994).

Guanosine, a breakdown product of GTP, has been reported to have a neuritogenic effect on PC 12 cells (Gysbers and Rathbone 1992). However, unlike ATP, GTP did not reverse the blocking effect of TTX in our cocultures, therefore suggesting that GTP is not involved in the events leading to increased neurite outgrowth of striatal neurons

in this system.

ATP is a transmitter of non-adrenergic, non-cholinergic inhibitory neurons within the myenteric plexus (see Hoyle and Burnstock 1989) and modulates synaptic transmission on both the pre- and postsynaptic sites (Kamiji *et al.* 1994). Hanani and Burnstock (1985) have shown that ATP is released from myenteric neurons during spontaneous neuronal activity in tissue culture. Once it is released, ATP is rapidly broken down to adenosine by ecto-ATPases (Meghji 1993). Adenosine has been shown to be involved in presynaptic modulation of neuronal activity (Moody and Burnstock 1982). Since adenosine is also produced from extracellular ATP at the striatal cholinergic synapse (James and Richardson 1993), striatal cells could also have contributed to the formation of adenosine in our cocultures.

The observation that both ATP and 2-CA increased neurite extension of striatal neurons to a much greater extent in TTX-treated cocultures with myenteric plexus explants than in cultures of striatal neurons alone suggests that another molecule originating in the myenteric plexus is also involved in the neuritogenic effect. Adenosine may have triggered the release of a neuritogenic factor from cells within the myenteric plexus, or alternatively may have acted directly on striatal cells in concert with such a factor produced independently by myenteric plexus cells. The observation that enriched enteric glial cell preparations also have a neuritogenic effect on striatal neurons (see chapter 3) suggests that a neuritogenic trophic factor may have been released from enteric glial cells. Since forskolin completely reversed the blocking effect of TTX in our cocultures, increased cAMP could have been involved in the production and release of a growth factor(s) from enteric glial

cells. Christofi *et al.* (1993) have shown that forskolin elevated intracellular cAMP levels in myenteric plexus cultures from guinea-pig small intestine and that 80-90% of the cAMP was formed by enteric glial cells. Interestingly, increased cAMP has been reported to upregulate NGF-mRNA expression in cultured Schwann cells (Matsuoka *et al.* 1991).

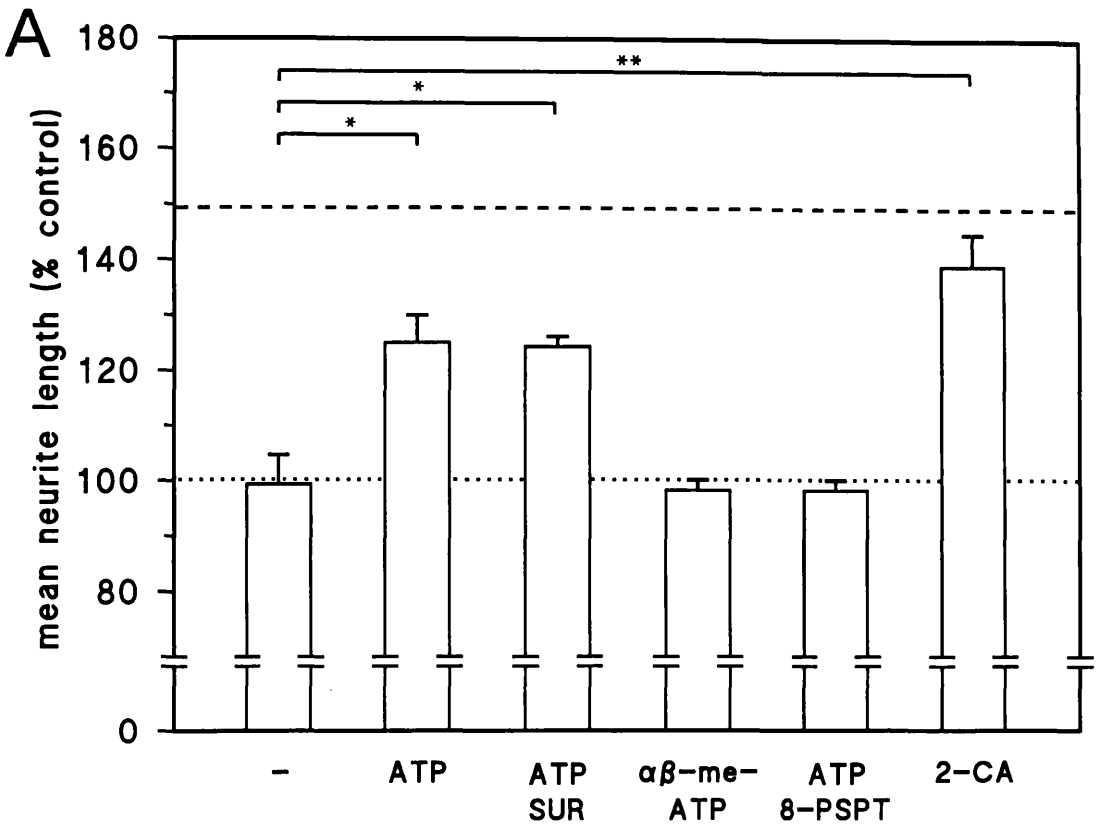
In support of this, 2-CA increased neurite extension in striatal cultures in the absence of the myenteric plexus. The P_1-purinoceptor subclass A_2-receptors have been found to be localized in spiny medium-sized striatal neurons (Ferré *et al.* 1992) and to be stimulatory coupled to adenylate cyclase in rat striatum (Moser *et al.* 1991). Adenosine, in micromolar concentrations, has been shown to increase intracellular cAMP in cells which express A_2-receptors (van Calker *et al.* 1979, Premont *et al.* 1983). Furthermore, increases in intracellular cAMP alone have been reported to modulate neurite outgrowth in different ways: increasing neurite elongation in embryonic *Xenopus* nerves (McCaig, 1990) and mouse neuroblastoma cells (Bolsover *et al.* 1992) and reducing neurite outgrowth in chick dorsal root ganglion cells (Lankfort and Letourneau 1991). Since a large proportion of striatal neurons express A_2 receptors (Ferré *et al.* 1992), the increase in neurite length in response to 2-CA seen in the present study could possibly have been a result of increased cAMP. In support of this, both 2-CA and the adenylate cyclase activator forskolin increased neurite elongation of striatal neurons cultured alone to the same extent. Forskolin specifically activates adenylate cyclase (Seamon *et al.* 1981), elevates the intracellular cyclic AMP (cAMP) concentration in neurons (Kilmer and Carlsen 1984) and enhances the rate of neurite elongation *in vivo* (Kilmer and Carlsen, 1984). Although serotonin (5-HT), which is also an enteric

neurotransmitter, has been found to activate adenylate cyclase in neuronal cultures of embryonic mouse striatum at micromolar concentrations (Premont *et al.* 1983) we observed no effect on neurite outgrowth with 1 µM 5-HT in postnatal striatal cultures in both the absence and the presence of myenteric plexus explants.

A neuritogenic trophic factor may be released from the myenteric plexus in coculture. Trophic factors such as the neurotrophins, FGFs, epidermal growth factor, insulin-like growth factors and transforming growth factors have been detected in the gastrointestinal tract (Saffrey and Burnstock 1994). Therefore, one of these factors may have been involved in the increase in neurite extension of striatal neurons observed in our coculture system. In chapter 5, the effects of a number of growth factors, gangliosides and extracellular matrix molecules together with 2-CA have been investigated to examine a possible additive or synergistic neuritogenic action on striatal neurons *in vitro*.

Taking the results presented here and those produced by other groups together, adenosine, in micromolar concentrations, acting on P_1-purinoceptors (probably A_2 subtype) expressed by striatal neurons could have resulted in an increase of intracellular cAMP in these cells. Together with a trophic factor, possibly released by enteric glial cells, this might have resulted in the strong neurite outgrowth response in striatal neurons in an additive or synergistic manner in these cocultures.

Fig. 4.1 A,B; Mean *neurite length* of striatal neurons after 2 days *in vitro*. Striatal cells were cultured in the presence (A; open bars) or absence (B; solid bars) of myenteric plexus explants supplemented with 1 µM tetrodotoxin (TTX). The dashed line at 149% represents the mean neurite length of striatal neurons in cocultures with myenteric plexus explants without TTX present. The dotted line represents the mean neurite length in control striatal cultures without the presence of myenteric plexus normalized to 100%; ATP = adenosine-5'-triphosphate (50 µM); SUR = suramin (10 µM); αβ-meATP = α,β-methylene ATP (50 µM); 8-PSPT = 8-(*p*-sulfophenyl)theophylline (10 µM); 2-CA = 2-chloroadenosine (50 µM); each experiment was performed in triplicate and bars represent mean ± S.E.; * = $P<0.05$; ** = $P<0.01$ (Student's *t*-test).


A
mean neurite length (% control)
180
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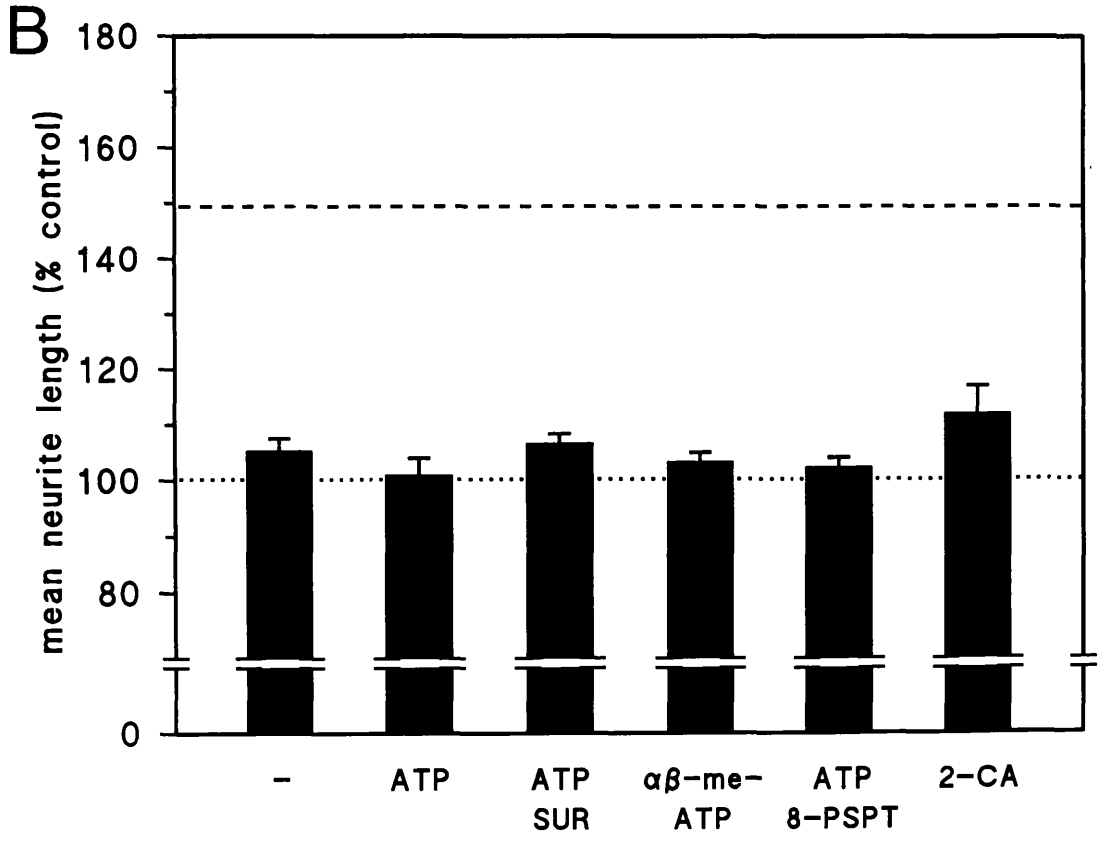
ATP
ATP SUR
αβ-me-ATP
ATP 8-PSPT
2-CA


B
mean neurite length (% control)
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160
140
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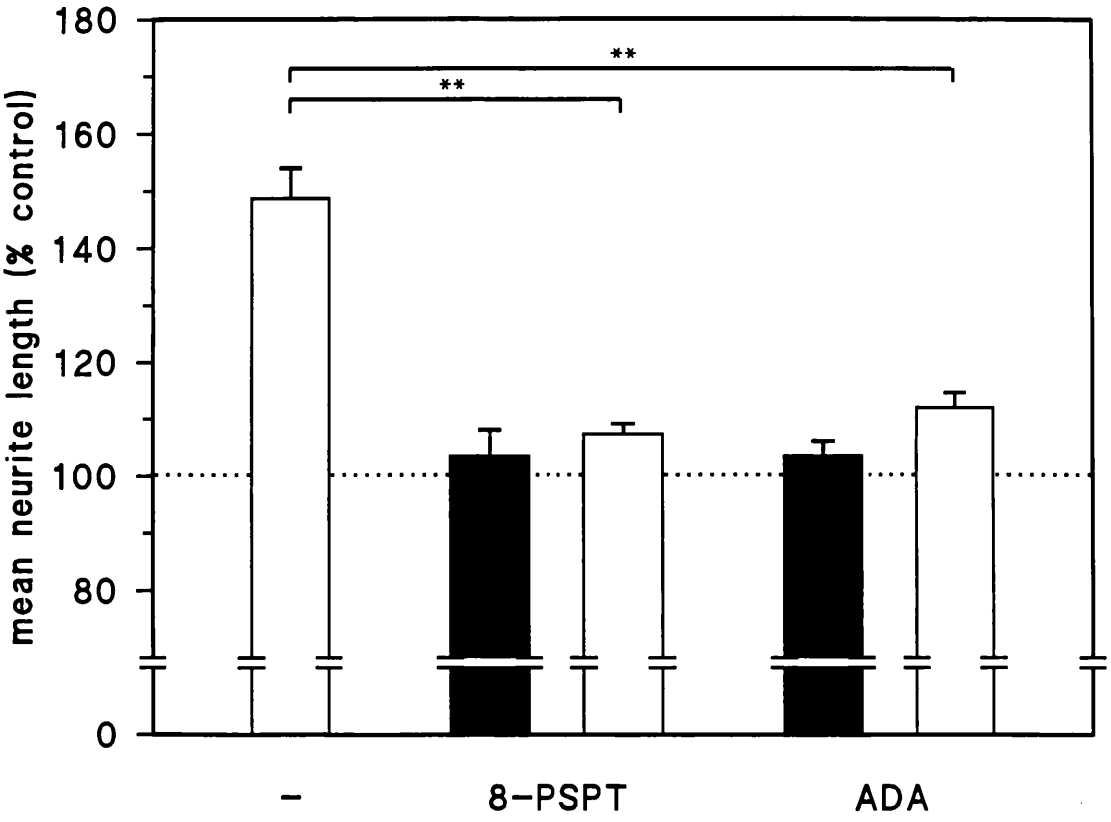
100
80
0
-
ATP
ATP SUR
αβ-me-ATP
ATP 8-PSPT
2-CA

Fig. 4.2; Effects of adenosine deaminase (ADA; 2 U/ml) and 8-(*p*-sulfophenyl)theophylline (8-PSPT; 10 µM) on neurite outgrowth of striatal neurons both in the absence (solid bars) and presence (open bars) of myenteric plexus explants after 48 h *in vitro*. For comparison first open panel shows the mean neurite length of striatal neurons in cocultures with myenteric plexus explants. The dotted line represents the mean neurite length in control striatal cultures (without the myenteric plexus present) normalized to 100%; results are means ± S.E. of three independent experiments; ** = $P<0.01$ (Student's *t*-test).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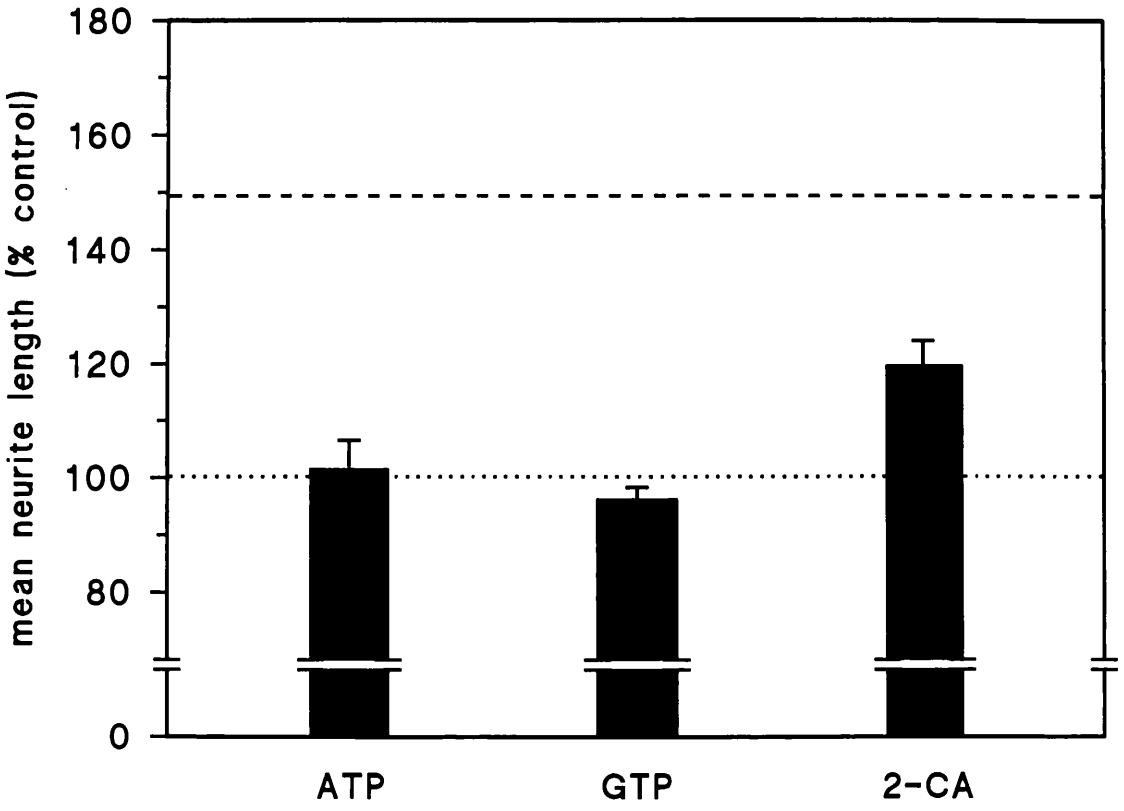
–
8-PSPT
ADA

Fig. 4.3; Mean *neurite length* of striatal neurons after 2 days *in vitro*. Striatal cells were cultured in the absence of tetrodotoxin; the mean neurite length of striatal neurons in cocultures with myenteric plexus explants is illustrated as dashed line (149%). The dotted line represents the mean neurite length in control striatal cultures (without the presence of myenteric pc plexus) normalized to 100%; ATP = adenosine-5'-triphosphate (50 µM); GTP = guanosine-5'-triphosphate (50 µM); 2-CA = 2-chloroadenosine (50 µM); each experiment was performed in triplicate and bars represent mean ± S.E..


180
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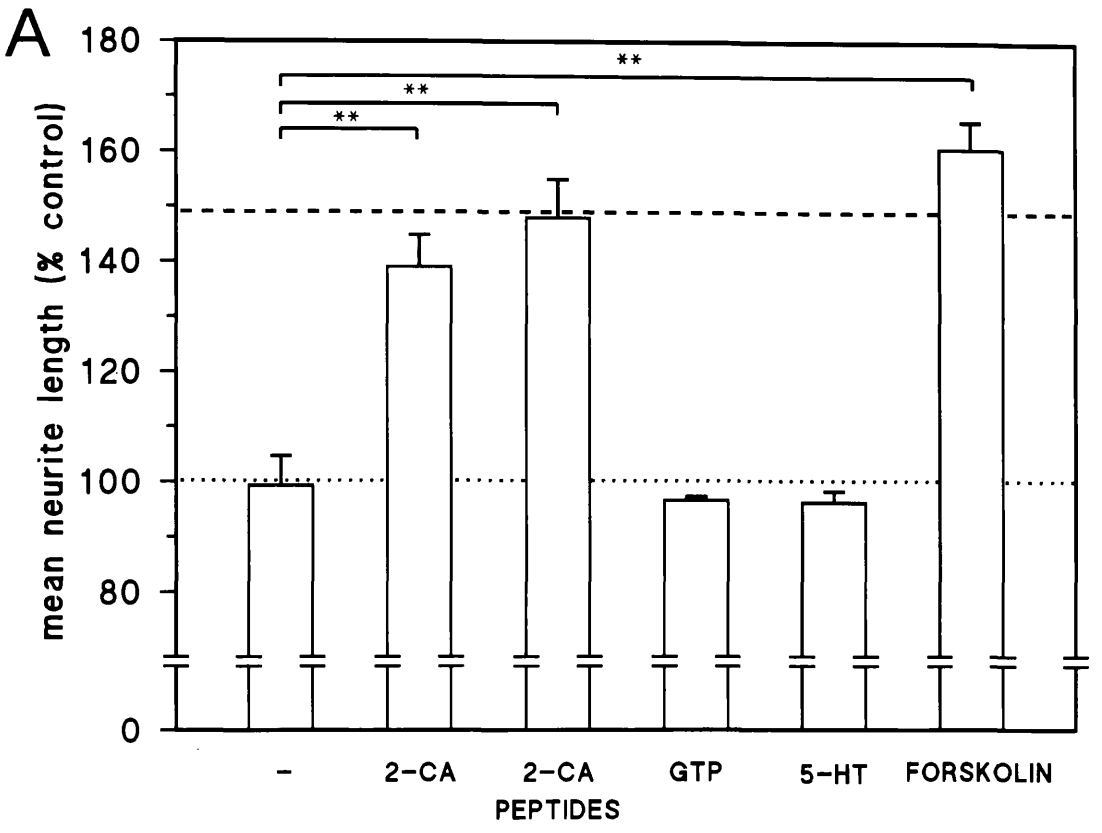
80
0
mean neurite length (% control)
ATP
GTP
2-CA

Fig. 4.4 A,B; Mean *neurite length* of striatal neurons after 2 days *in vitro*. Striatal cells were cultured in the presence (A; open bars) or absence (B; solid bars) of myenteric plexus explants supplemented with 1 µM tetrodotoxin. The dashed line (149%) represents the mean neurite length of striatal neurons in myenteric plexus cocultures. The dotted line represents the mean neurite length in control striatal cultures (without myenteric plexus present) normalized to 100%; 2-CA = 2-chloroadenosine (50 µM); PEPTIDES = combination of calcitonin gene-related peptide, neuropeptide Y, somatostatin, substance P and vasoactive intestinal peptide, all at 0.1 µM; GTP = guanosine-5'-triphiphosphate (50 µM); 5-HT = 5-hydroxytryptamine (1 µM); FORSKOLIN (10 µM); each experiment was performed in triplicate and compared to its own control (represented as a dotted line); bars represent mean ± S.E.; * = $P<0.05$; ** = $P<0.01$ (Student's *t*-test).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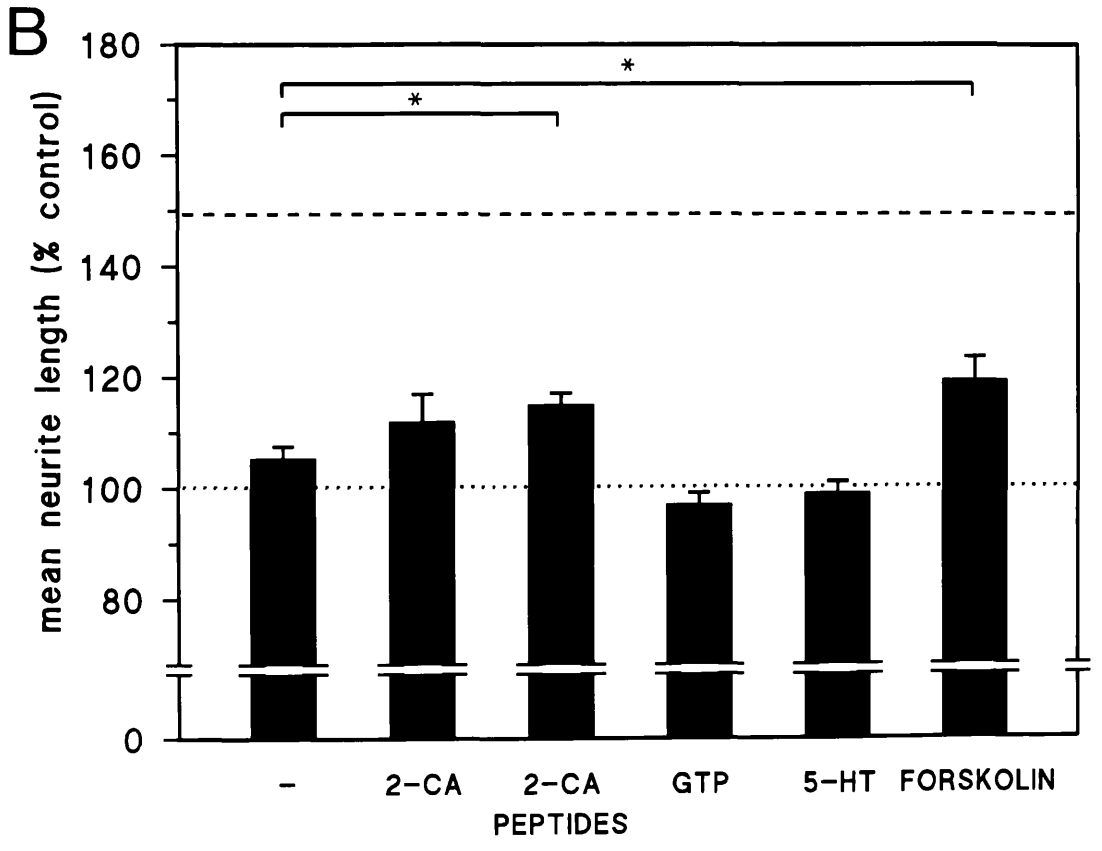
FORSKOLIN
PEPTIDES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2-CA
GTP
5-HT
FORSKOLIN
PEPTIDES

CHAPTER 5

NEURITE OUTGROWTH OF STRIATAL NEURONS *IN VITRO*: EFFECTS OF NEUROTROPHIC FACTORS, EXTRACELLULAR MATRIX MOLECULES AND GANGLIOSIDES

5.1. Summary

The neurite outgrowth promoting effect of various growth factors and gangliosides alone and in combination with the hydrolysis-resistant adenosine analogue, 2-chloroadenosine, was studied in cultures of dissociated striatal neurons from postnatal day 7 rats. In addition, we examined the neuritogenic effect of extracellular matrix molecules in these cultures.

In chapter 4 evidence was presented that adenosine increases neurite outgrowth of postnatal striatal neurons together with an unidentified factor(s) probably released from enteric glial cells. In this study it is demonstrated that basic fibroblast growth factor, nerve growth factor, neurotrophin-3 or neurotrophin-4/5 had no effect on neurite outgrowth in postnatal striatal cultures after 2 days *in vitro*. When these growth factors were added in combination with 2-chloroadenosine, the observed increase in mean neurite length did not exceed that induced by 2-chloroadenosine alone. Both 2-chloroadenosine and the ganglioside mix AGF1 increase neurite elongation of striatal neurons after 2 days *in vitro*, but an inhibition of enhanced neurite outgrowth was observed when both substances were added together. It is further demonstrated that both laminin and fibronectin were not neuritogenic for postnatal striatal neurons under our culture conditions. These observations suggest that a factor other than the growth factors tested here is involved in the promotion of striatal neurite outgrowth in coculture with myenteric plexus explants.

5.2. Introduction

A number of different growth factors have been shown to have trophic effects on both survival and neurite extension of striatal neurons in cell culture systems and/or *in vivo*. Such factors include the neurotrophins (Ardelt *et al.* 1994, Hartikka and Hefti 1988, Matsuda *et al.* 1990, Studer *et al.* 1994), members of the fibroblast growth factor (FGF) family (Abe *et al.* 1990, 1991; Hisajima *et al.* 1991, Matsuda *et al.* 1990, Walicke 1988, Zhou and DiFiglia 1993), epidermal growth factor (EGF) (Abe *et al.* 1990) and ciliary neurotrophic factor (Magal *et al.* 1991). Extracellular matrix (ECM) molecules (Manthorpe *et al.* 1983) and gangliosides (Skaper *et al.* 1985) have also been found to promote neurite outgrowth of striatal neurons. In coculture, explants of the adrenal gland (Bird 1989, 1991) and of the myenteric plexus (see chapter 3) have been shown to increase neurite elongation of embryonic and postnatal striatal neurons, respectively.

Recent evidence has shown that purines also have trophic effects in the nervous system, and can promote neurite outgrowth from both PC 12 cells (Gysbers and Rathbone 1992) and enteric neurons (Saffrey *et al.* 1994). Purines have also been shown to act synergistically with growth factors such as FGF and nerve growth factor (NGF) (Braumann *et al.* 1986, Guroff *et al.* 1981, Gysbers and Rathbone 1992, Huffaker *et al.* 1984, Rathbone *et al.* 1992).

In chapter 3 evidence was presented to suggest that a growth factor might be released from enteric glial cells to act together with a neurotransmitter to promote growth of striatal neurons in coculture with myenteric plexus. In chapter 4 evidence was presented that adenosine, following breakdown of ATP released from enteric neurons, is involved in the neurite promoting effect of the myenteric

plexus on striatal neurons in coculture.

In the present study, the effects of several neurotrophic factors, gangliosides and extracellular matrix (ECM) proteins on striatal neurite elongation were investigated and the possibility that adenosine may act in an additive or synergistic manner with such factors in inducing an increased neurite elongation of postnatal day 7 (P7) striatal neurons *in vitro* was examined.

5.3. Material and Methods

Cultures from dissociated postnatal (P7) rat striatum were obtained as described in chapters 2.1.1. These cells were cocultured with myenteric plexus explant cultures (see chapter 2.1.2.) according to the procedures described in chapter 2.1.9. After 48 h *in vitro*, the neurite outgrowth of striatal neurons was analysed as described in chapter 2.2.1. The evaluation of the mean neurite length and the statistical analysis was performed as described in chapter 2.2.2.

5.4. Results

5.4.1. Effects of myenteric plexus on striatal neurite length in coculture

In chapter 3, it was shown that myenteric plexus explants promoted neurite elongation of striatal neurons in coculture to 149% compared to control; this effect was totally abolished by TTX (99% of control). For comparison with the data shown here, this increase in mean neurite outgrowth shown in Figures 5.1 to 5.3 as a dashed line.

5.4.2. Effects of neurotrophic factors alone and in combination with 2-CA

No increase in striatal neurite length was measured in the presence of either 10 ng/ml bFGF (99 ± 2% of control), 100 ng/ml NGF (104 ± 1% of control), 10 ng/ml NT-3 (103 ± 3% of control) or 10 ng/ml NT-4/5 (97 ± 3% of control), as shown in Figure 5.1. As reported in chapter 4, the stable adenosine analogue, 2-CA (50 µM), promoted an increase in neurite elongation to 120 ± 4% of control. For easier comparison, this result is shown in Figure 5.2. This increase was unaffected by addition of bFGF (10 ng/ml; 119 ± 4% of control), and was slightly reduced on addition of NGF (100 ng/ml; 110 ± 3% of control), NT-3 (10 ng/ml; 109 ± 4% of control) or NT-4/5 (10 ng/ml; 102 ± 6% of control); see Fig. 5.2. In all cultures treated with bFGF, astrocyte-like cells displayed a stellate morphology (data not shown).

5.4.3. Effects of gangliosides

AGF1, at a concentration of 10 µg/ml, increased the mean neurite length to 107 ± 4% of control, as shown in Figure 5.3. At a higher concentration of 100 µg/ml neurite outgrowth was increased to 137 ± 5% (Fig. 5.3; $P<0.0001$ compared to control). A reduction of neurite outgrowth to 68 ± 2% (Fig. 5.3; $P<0.0001$ compared to control) was measured in cultures supplemented with 500 µg/ml AGF1. Under these conditions the cells did not appear healthy and the reduction in neurite elongation was probably secondary to a general cytotoxic effect. When AGF1 (100 µg/ml) was added in combination with the stable adenosine analogue, 2-CA (50 µM), a smaller increase in mean neurite length was observed (111 ± 3%; see Fig. 5.3; $P<0.05$ for one experiment, two not significant compared to control). This increase

was less than that seen with 2-CA alone and was significantly smaller in comparison to the increase observed with AGF1 (100 µg/ml) alone ($P<0.01$; see Fig. 5.3).

5.4.4. Effects of fibronectin and laminin

No significant increases in the mean neurite length of striatal neurons were seen when cultures were grown on substrate precoated with either laminin (102 ± 4% of control) or fibronectin (105 ± 3% of control); see Table 5.1.

5.5. Discussion

In chapter 3, it was demonstrated that the myenteric plexus promotes an increased neurite outgrowth of striatal neurons in coculture and suggested that a growth factor released from enteric glial cells acting together with a transmitter released from enteric neurons might be responsible for this effect. In chapter 4, evidence was presented that this effect is mediated, in part, by adenosine resulting from breakdown of ATP released from enteric neurons, but that another factor produced by the myenteric plexus is also involved. Purines have been found to act synergistically with trophic factors in some neural systems (Braumann *et al.* 1986, Guroff *et al.* 1981, Gysbers and Rathbone 1992, Huffaker *et al.* 1984, Rathbone *et al.* 1992). In the present study, therefore the effects of several different neurotrophic factors on striatal neurite growth, both alone and in combination with the stable adenosine analogue, 2-CA, was investigated.

Although expression of FGF's and EGF have been demonstrated in the gut, production of neurotrophic factors by cells of the enteric

ganglia has not been demonstrated (see Saffrey and Burnstock 1994). This study, therefore was focussed on neurotrophic factors which have previously been shown to be active on striatal neurons. None of the factors examined (FGF, NGF, NT-3 and NT-4/5) caused an increase in striatal neurite length in our culture system when applied alone and did not further enhance neurite elongation when added together with 2-CA. While these molecules have all previously been found to have trophic actions on cultured striatal neurons, their effects have either been demonstrated on neurons of a different developmental age, or after more prolonged periods in culture than those employed in the present study. For example, while neither acidic or basic FGF increased neurite elongation of embryonic striatal neurons (Walicke 1988), basic FGF induced increased neurite outgrowth of postnatal GABAergic striatal neurons, but this effect was only seen after 6 days *in vitro* (Zhou and DiFiglia 1993). This is consistent with the results presented here. NGF has been found to influence the complexity of neurites of postnatal cholinergic striatal neurons (Studer *et al.* 1994), but to have no effect on GABAergic striatal neurons. This observation corresponds with the expression of TrkA receptors by these cells (Ringstedt *et al.* 1993). Although NT-4/5 promotes the survival of neonatal mouse striatal neurons (Ardelt *et al.* 1994), and both TrkB and TrkC mRNA has been identified in several populations of striatal neurons (see Lindsay *et al.* 1993), its effects on striatal neurite extension have not previously been studied. The results presented here therefore indicate that the growth factors examined in this study may either have no effect on striatal neurite growth at this developmental stage, or that different concentrations, culture conditions (eg. serum-free medium) or more prolonged treatment is required to see an effect. However,

these results do indicate that these factors are unlikely to be involved in the mediation of the neuritogenic effect of the myenteric plexus on striatal neurons in coculture. Further studies to identify the neurotrophic factors which may be produced by enteric ganglia and act on striatal cells in culture are currently underway.

Gangliosides have also been reported to increase neurite elongation *in vitro* (Byrne *et al.* 1983, Ferrari *et al.* 1983, Facci *et al.* 1984, Katoh-Semba *et al.* 1984, Skaper *et al.* 1985, Leon *et al.* 1988) and to enhance regenerative repair *in vivo* (see Schengrund, 1990). The neurite promoting effect of gangliosides has been found to involve Ca^{2+}-influx *in vitro* (Wu and Ledeen, 1991; Hilbush and Levine, 1991) and gangliosides were suggested to enhance axonal growth *in vivo* by promoting cell adhesion molecule-induced Ca^{2+}-influx (Doherty *et al.* 1992). Skaper and coworkers (1985) have shown that the GM1 ganglioside can increase neurite outgrowth of embryonic (E18) rat striatal neurons *in vitro*. Here it is shown that the ganglioside mix AGF1, which also contains the ganglioside GM1, increased neurite elongation of postnatal striatal neurons in a dose-dependent manner, suggesting that this effect may not be restricted to an early developmental stage.

Although both gangliosides and 2-CA independently increased neurite outgrowth in our culture system, an antagonism was observed when both agents were applied together. An antagonism of intracellular cAMP-elevating agents and gangliosides has been reported previously, although in non-neuronal cells. An increase in cAMP has been shown to convert astroglial cells from a flat, fibroblast-like into a process-bearing, stellate (star-shaped) morphology and that this response was antagonized by gangliosides

(Skaper *et al.* 1986). Facci *et al.* (1987) further reported that this antagonizing effect of gangliosides was not accompanied with a reduction of elevated cAMP. With respect to the results presented here, neurite outgrowth in striatal cultures was increased by both 2-CA and the adenylate cylase activator forskolin to the same extent, suggesting the involvement of elevated intracellular cAMP in striatal neurons (see chapter 4). Further, the ganglioside-mix AGF1 counteracted the increase in neurite elongation, which was probably mediated by increased cAMP. These results suggest that gangliosides are unlikely to be responsible for the neuritogenic effect of myenteric plexus explants on striatal neurons (see chapter 3). Furthermore, it implies that neurite elongation of postnatal striatal neurons *in vitro* can be modulated by different molecules and possibly via separate mechanisms.

With respect to the involvement of cell adhesion molecules, the ECM molecules laminin, fibronectin and type IV collagen have been shown in the basement membrane covering individual ganglia *in situ* (Bannermann *et al* 1986). Laminin is also synthesized by enteric glia *in vitro* (Bannerman *et al.* 1988a) and has been reported previously to increase neurite extension of embryonic striatal neurons *in vitro* (Manthorpe *et al.* 1983). In the cultures used here, there was no increase in mean neurite length after 48 h *in vitro* when postnatal striatal cells were grown on either laminin- or fibronectin-coated substrates.

There are a number of possible interactions between the different neuronal and non-neuronal cells from the enteric and central nervous system in our coculture system. In an alternative model to this investigated here, adenosine might have triggered the release of an as yet unidentified neuritogenic factor from myenteric plexus cells.

In this respect, myenteric plexus conditioned medium was not neuritogenic for striatal cells, suggesting that striatal cells might have triggered the release of a neuritogenic factor or, alternatively, that these factor(s) were broken down quickly (see chapter 3).

Fig. 5.1.; Mean *neurite length* of striatal neurons after 2 days *in vitro*. For comparison the mean neurite length of striatal neurons in cocultures with myenteric plexus explants is illustrated as dashed line (149%). The dotted line represents the mean neurite length in control striatal cultures normalized to 100%; bFGF = basic fibroblast growth factor (10 ng/ml) ; NGF = nerve growth factor (100 ng/ml); NT-3 = neurotrophin-3 (10 ng/ml); NT-4/5 = neurotrophin-4/5 (10 ng/ml); each experiment was performed in triplicate and bars represent mean ± S.E..

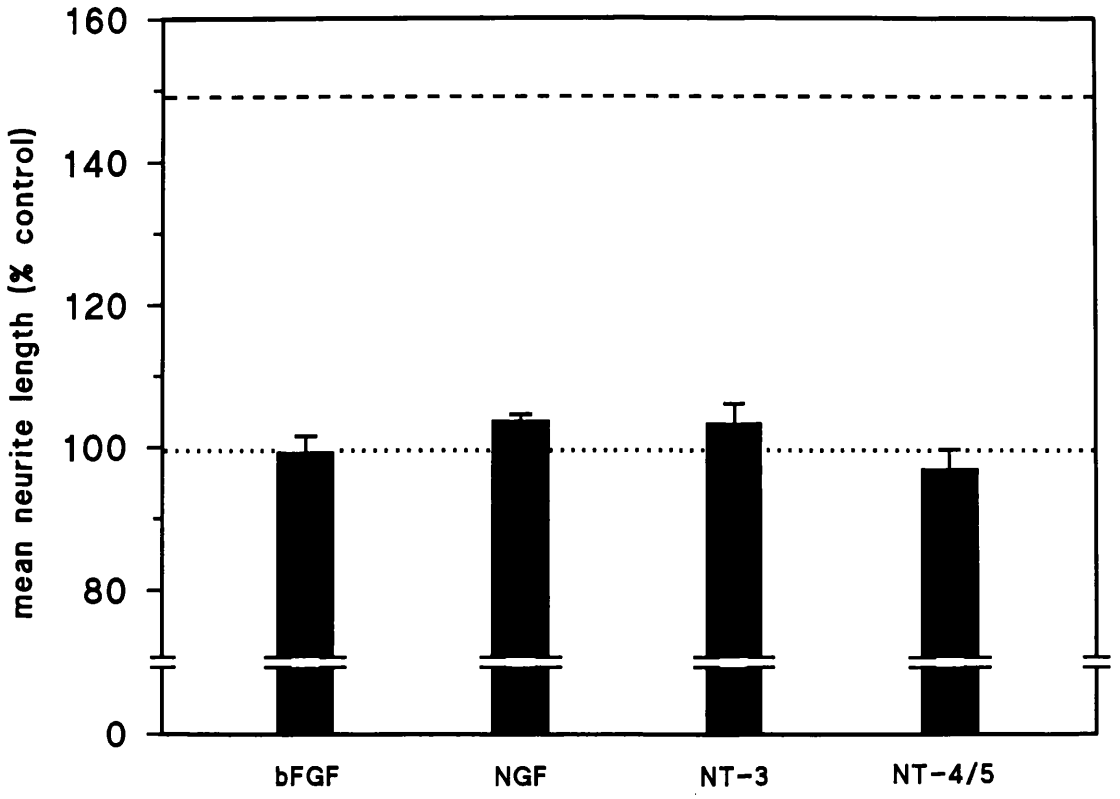

160
140
120
100
80
0
mean neurite length (% control)
bFGF
NGF
NT-3
NT-4/5

Fig. 5.2.; Mean *neurite length* of striatal neurons after 2 days *in vitro*. For comparison the mean neurite length of striatal neurons in cocultures with myenteric plexus explants is illustrated as dashed line (149%). The dotted line represents the mean neurite length in control striatal cultures normalized to 100%; 2-CA = 2-chloroadenosine (50 µM); bFGF = basic fibroblast growth factor (10 ng/ml) ; NGF = nerve growth factor (100 ng/ml); NT-3 = neurotrophin-3 (10 ng/ml); NT-4/5 = neurotrophin-4/5 (10 ng/ml); each experiment was performed in triplicate and bars represent mean ± S.E..

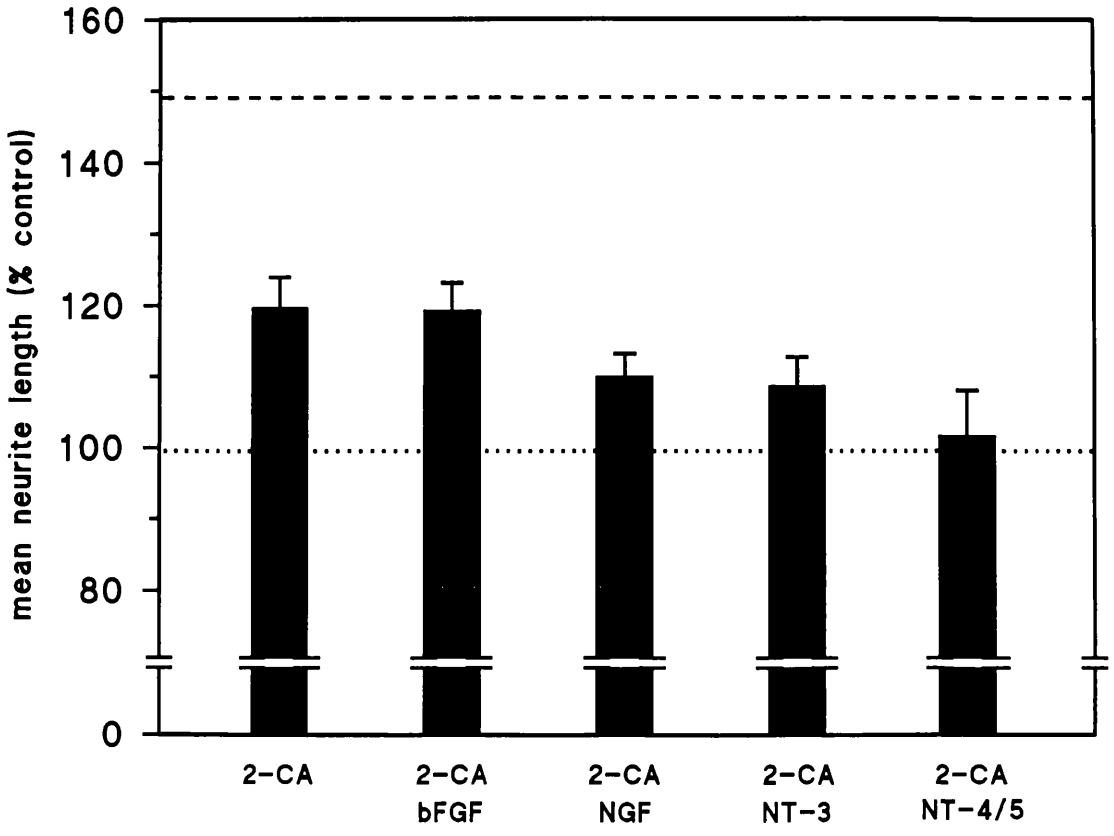
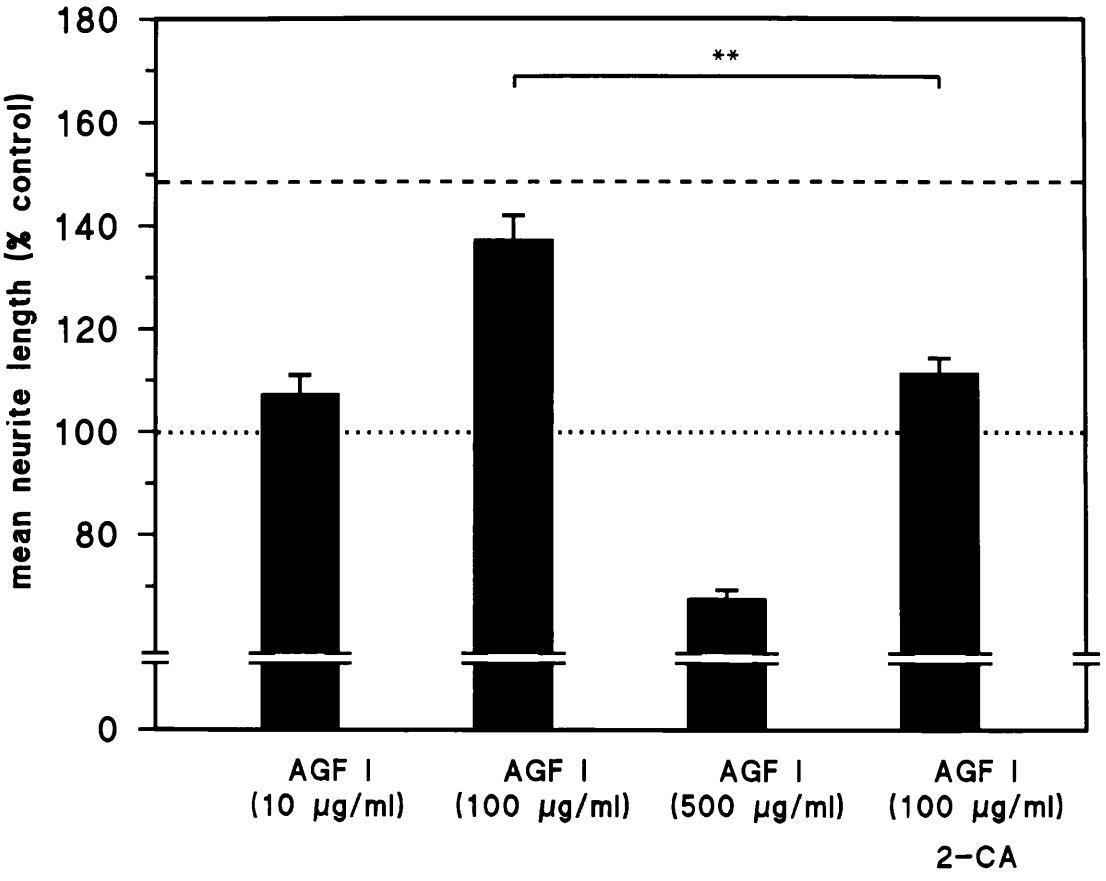

160
140
120
100
80
0
mean neurite length (% control)
2-CA
2-CA bFGF
2-CA NGF
2-CA NT-3
2-CA NT-4/5

Fig. 5.3.; Mean *neurite length* of striatal neurons after 2 days *in vitro*. The mean neurite length of striatal neurons in cocultures with myenteric plexus explants is illustrated as dashed line (149%). The dotted line represents the mean neurite length in untreated control cultures normalized to 100%; AGF1 = ganglioside mixture from bovine brain; 2-CA = 2-chloroadenosine (50 µM); each experiment was performed in triplicate and bars represent mean ± S.E.; ** = $P<0.01$ (Student's *t*-test).


180
160
140
120
100
80
0
mean neurite length (% control)
**
AGF I
(10 µg/ml)
AGF I
(100 µg/ml)
AGF I
(500 µg/ml)
AGF I
(100 µg/ml)
2-CA

	mean neurite length of striatal neurons (% control)
laminin	102 ± 4%
fibronectin	105 ± 3%

Table 5.1. Effects of laminin- and fibronectin-precoated substrates on process elongation of postnatal striatal neurons in culture. After 48 h *in vitro* the total neurite length of 100 cells was measured and compared to control. Results are means ± S.E. of three independent experiments.

CHAPTER 6

THE NEURITOGENIC EFFECT OF MYENTERIC PLEXUS ON STRIATAL NEURONS IN COCOULTURE INVOLVES NITRIC OXIDE

6.1. Summary

The results described in chapter 3 demonstrate that myenteric plexus explants promoted striatal neurite elongation in coculture and that this effect was abolished by tetrodotoxin. In this chapter results are presented which demonstrate that the nitric oxide synthase blocker N-nitro-L-arginine methyl ester significantly reduced the neuritogenic effect of the myenteric plexus whereas the nitric oxide donor, sodium nitroprusside, partially reversed the blocking effect of tetrodotoxin. 2-chloroadenosine, a stable analogue of adenosine, which is produced following release of ATP from enteric neurons, further enhanced the effect of sodium nitroprusside. Basic fibroblast growth factor or neurotrophin-3 in combination with 2-chloroadenosine and sodium nitroprusside were only marginally neuritogenic in striatal cultures alone. These results suggest that nitric oxide is involved in the trophic effects of myenteric plexus explants on striatal neurons.

6.2. Introduction

Grafts of the myenteric plexus have been found to induce a massive sprouting response within the host corpus striatum (Tew *et al.* 1992). Furthermore, it was demonstrated that myenteric plexus explants induced an increase in neurite elongation of dissociated striatal neurons from postnatal day 7 rats in coculture, and that this effect was totally abolished by 1 μM tetrodotoxin (TTX; see chapter 3). This observation suggested that an enteric neurotransmitter may be involved in the trophic interactions between myenteric plexus cells and striatal neurons. 2-chloroadenosine (2-CA), a stable analogue of adenosine, which is formed by the breakdown of the enteric neurotransmitter ATP, has been found to partially reverse the effect of TTX in myenteric plexus cocultures and was also found to be neuritogenic in striatal cultures alone (see chapter 4).

In the study described in this chapter the possible involvement of nitric oxide (NO), a putative messenger in the central and peripheral nervous systems (Stark and Szurszewski, 1992) was investigated. Many myenteric neurons from rat and guinea-pig have been found to contain the synthetic enzyme for NO, nitric oxide synthase (NOS), *in situ* (Belai *et al.*, 1992b) and *in vitro* (Saffrey *et al.* 1992b). Together with ATP and vasoactive intestinal polypeptide (VIP), NO has been found to be involved in neurotransmission from non-adrenergic inhibitory neurons in the guinea-pig intestine (Hoyle and Burnstock 1989, Shuttleworth *et al.* 1991, see Wiklund *et al.* 1993). In the corpus striatum, neurons using NO as a transmitter have been identified as medium to large aspiny interneurons in rat (Dawson *et al.* 1991), mouse (Derer and Derer 1993) and human (Egberongbe *et al.* 1994, Nisbet *et al.* 1994). It has been found that NO facilitates catecholamine release from presynaptic corticostriatal nerve

terminals *in vitro* (Hanbauer *et al.* 1992). *In vivo* NO has also been reported to induce the release of a number of different neurotransmitters within the striatum and this was shown to be mediated by activation of guanylate cyclase (Guevara-Guzman *et al.* 1994). The site of NO action is correlated with the localization of soluble guanylate cyclase mRNA throughout the striatum (Burgunder and Cheung 1994).

Evidence for the involvement of NO in long term potentiation has been produced in experiments in which NO induced upregulation of dendritic microtubule associated protein-2 (MAP2) mRNA in hippocampal granule cells *in vivo* (Johnston and Morris 1994). NO has also been implicated in axonal pathfinding during development since it inhibited neurite elongation in dorsal root ganglion cells and PC 12 cells *in vitro* (Hess *et al.* 1993).

In the present study it was examined whether NO is involved in the events leading to enhanced neurite elongation of striatal cells in myenteric plexus cocultures. It has been investigated whether the NOS-inhibitor N-nitro-L-arginine methyl ester (L-NAME) and the NO donor, sodium nitroprusside (SNP; $Na_2Fe(CN)_5NO$), affected the neuritogenic effect of the myenteric plexus on striatal cells in the absence and presence of tetrodotoxin (TTX), respectively. Further a possible synergism of SNP in combination with basic fibroblast growth factor (bFGF), neurotrophin-3 (NT-3) and 2-chloroadenosine (2-CA) has been investigated. 2-CA is a stable analogue of adenosine and has been found to promote neurite elongation of striatal neurons *in vitro* (see chapter 4).

6.3. Materials and Methods

Cultures from dissociated postnatal (P7) rat striatum were obtained as described in chapters 2.1.1. These cells were cocultured with myenteric plexus explant cultures (see chapter 2.1.2.) according to the procedures described in chapter 2.1.9. After 48 h *in vitro*, the neurite outgrowth of striatal neurons was analysed as described in chapter 2.2.1. The evaluation of the mean neurite length and the statistical analysis was performed as described in chapter 2.2.2.

6.4. Results

6.4.1. Effects of myenteric plexus on striatal neurite length in coculture

The investigations described in chapter 3 have revealed that myenteric plexus explants promote neurite elongation of striatal neurons in coculture (149 ± 5% of control values in the absence of myenteric plexus; number of experiments (n) = 6; Fig. 3.3; 6.1). This effect was totally abolished by 1 µM TTX (99 ± 5% of control; n = 3; Fig. 3.3; 6.2). For statistical analysis and comparison with the data shown here, the mean neurite outgrowth of striatal neurons in coculture with myenteric plexus, both in the absence and the presence of TTX is shown in Fig. 6.1 and 6.2.

6.4.2. Effects of L-NAME and arginine on striatal neurite length in the presence and absence of myenteric plexus explants

Addition of the NOS inhibitor L-NAME resulted in a concentration-dependent reduction of the neurite outgrowth promoting effect of myenteric plexus explants on striatal neurons in coculture, as shown in Figure 6.1. In the presence of 30 µM L-NAME the mean neurite

length of striatal neurons was reduced (132 ± 5% of control; Fig. 6.1) compared to untreated cocultures (149 ± 5% of control; Fig. 6.1). In the presence of 100 µM L-NAME, however, the mean neurite length was reduced to control levels (108 ± 8% of control; Fig. 6.1; $P<0.01$ compared to untreated cocultures). The addition of L-NAME to striatal cells cultured alone did not affect neurite extension of striatal neurons, either at 30 µM (102 ± 1%; Fig. 6.1) or 100 µM (101 ± 1%; Fig. 6.1). The blocking effect of L-NAME (100 µM) in coculture was not reversed with the addition of L-arginine, at a concentration of 500 µM (104 ± 2; data not shown). When L-arginine was added at a higher concentration (10 mM) cultures showed morphological characteristics of unhealthy cells so that analysis was not reliable.

6.4.3. Effects of SNP and 2-CA in TTX-treated cocultures

When SNP (0.1 µM), a source of NO, was added to TTX-treated cocultures a significant increase in striatal neurite elongation was observed (126 ± 5% of control; Fig. 6.2; $P<0.05$ compared to 99 ± 5% in TTX-treated cocultures). Neurite elongation was not affected by addition of TTX (1 µM) and SNP (0.1 µM) to striatal cultures alone (102 ± 2%; Fig. 6.2). Addition of SNP (0.1 µM) together with the stable adenosine analogue, 2-CA (50 µM), to TTX-treated cocultures resulted in a significant increase of mean neurite length (139 ± 6%; Fig. 6.2; $P<0.01$ compared to TTX-treated cocultures), whereas there was only a moderate increase in neurite outgrowth in the absence of myenteric plexus (109 ± 4%; Fig. 6.2). This increase could not be further enhanced by either the addition of 10 ng/ml bFGF (115 ± 4%; Tab. 6.1) or 10 ng/ml NT-3 (110 ± 4%; Tab. 6.1). In some experiments, SNP caused a marked reduction in striatal cell number, indicating

that it can have a toxic action, as reported previously (Chen *et al.* 1991).

6.5. Discussion

The neural messenger NO was found to be involved in the events leading to increased neurite elongation of striatal neurons in cocultures with myenteric plexus explants. SNP, an NO donor, enhanced striatal neurite elongation to a greater extent in TTX-treated cocultures than in striatal neurons cultured alone. This observation suggests that another molecule, released from the myenteric plexus, is involved in the neurite outgrowth promoting effect. In this respect, it has been reported that direct application of NO to the growth cones of elongating neuronal processes *in vitro*, inhibits neurite elongation (Hess *et al.* 1993). Together with the results shown here, this implies that NO itself may not be neuritogenic, but might induce neurite elongation together with another factor(s). Alternatively, NO could have induced the release of such a factor from other cells.

There are several different cell types which could produce NO, and possible sites at which NO could act leading to increased striatal neurite outgrowth in this coculture system. Both enteric and striatal neurons are capable of producing NO (Dawson *et al.* 1991, Belai *et al.* 1992b, Saffrey *et al.* 1992b). NO could have been released from non-adrenergic, non-cholinergic (NANC) inhibitory neurons within the myenteric plexus and triggered the release of a neuritogenic factor from enteric glial cells. Alternatively, NO might have acted together with such a factor directly on striatal neurons. In this respect, adenosine is produced following breakdown of ATP released from NANC inhibitory neurons within the myenteric plexus (Hoyle and Burnstock

1989) and the stable adenosine analogue, 2-CA, has been shown to be neuritogenic in striatal cultures (see chapter 4). Both bFGF and NT-3 in combination with SNP and 2-CA did not increase striatal neurite outgrowth to the same extent as observed in coculture with myenteric plexus. This suggests that the neuritogenic effect of myenteric plexus explants on striatal neurons in coculture may not be due to an additive or synergistic action of NO together with bFGF or NT-3 in combination with 2-CA. The trophic effects could have also been initiated by the striatal cells themselves.

In addition to the localization of constitutive NOS in striatal neurons, inducible NOS has also been identified in rat cortical glial cells *in vitro* (Demerlé-Pallardy *et al.* 1993). This suggests that striatal glial cells could also be involved in the trophic interactions in our cocultures both as a source of NO or by releasing a trophic factor in response to an NO action. Previous observations in which myenteric plexus conditioned medium was not neuritogenic for striatal neurons (see chapter 3) are further evidence for the involvement of a substance with a short half life (such as NO and/or adenosine).

In cultures from embryonic day 15 rat striatum, SNP, at a concentration of 10 μM, has been demonstrated to increase intracellular cGMP and to cause a significant reduction in neuronal cell number (Chen *et al.* 1991). In this study, there was also a marked reduction in neuronal cell number in some postnatal striatal cultures in the presence of 0.1 μM SNP. Interestingly, the neurite elongation of those neurons analysed after 48 h *in vitro* did not appear to be negatively affected by 0.1 μM SNP, since an increase in neurite outgrowth compared to controls was observed in some

experiments (Fig. 6.2). The possibility that a specific subpopulation of striatal neurons is selectively affected by SNP-toxicity can not be excluded and awaits further investigation (see future plans).

Myenteric neurons which express NADPH diaphorase/NOS have previously been shown to survive grafting into the striatum (Tew *et al.* 1993). The results presented here indicate that NO produced by these neurons may be involved in the dramatic sprouting response seen in the striatum of animals receiving such myenteric plexus grafts (Tew *et al.* 1992, 1994).

The NOS inhibitor L-NAME reduced the neuritogenic effect of myenteric plexus explants on striatal neurons in coculture in a dose-dependent manner. Moreover SNP, which releases NO while degrading in solution, partially reversed the blocking effect of TTX in these cocultures, resulting in an increased neurite extension of striatal neurons in TTX-treated cocultures. On the basis of these results it is proposed that the neurotransmitter NO may be involved in the events resulting in an increased neurite extension of striatal neurons in coculture with the myenteric plexus.

Fig. 6.1.; Mean *neurite length* of striatal neurons after 2 days *in vitro*. Striatal cells were cultured in the presence (open bars) or absence (solid bars) of myenteric plexus explants. Cultures were supplemented with the nitric oxide synthase blocker N-nitro-L-arginine methyl ester (L-NAME; 30 µM and 100 µM); the dotted line represents the mean neurite length in untreated control cultures normalized to 100%; each experiment was performed in triplicate and bars represent mean ± S.E.; ** = $P<0.01$ (Student's *t*-test).

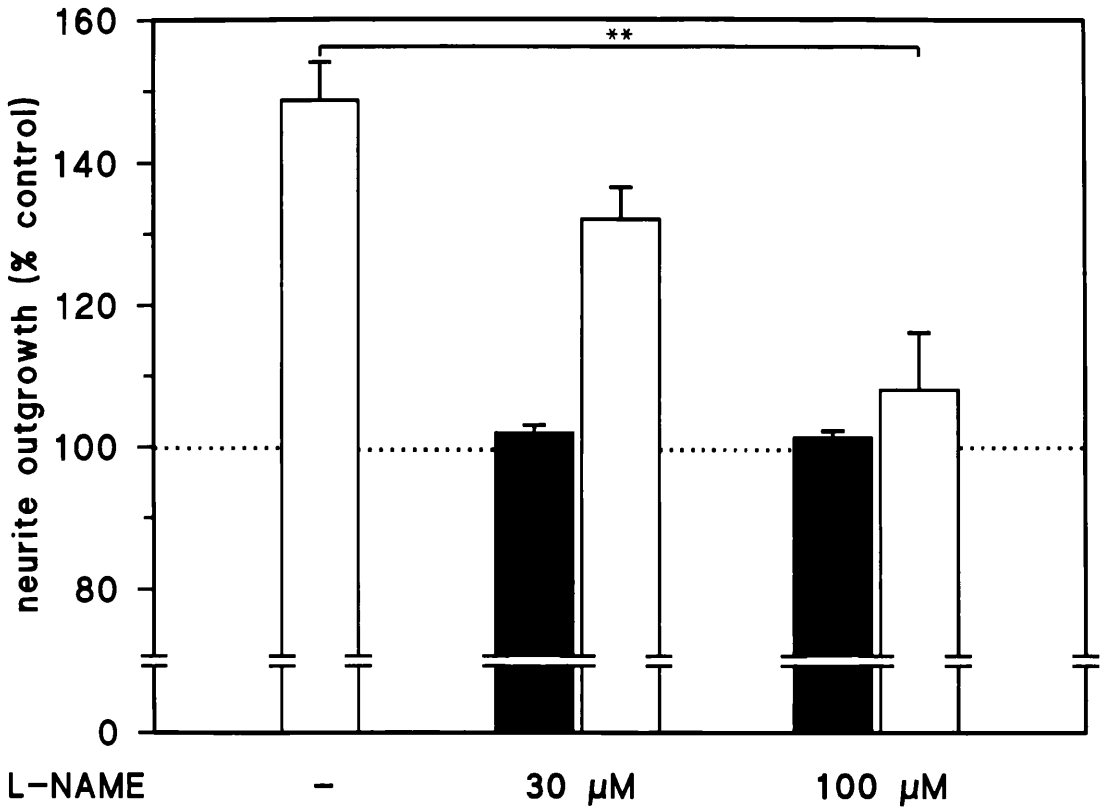
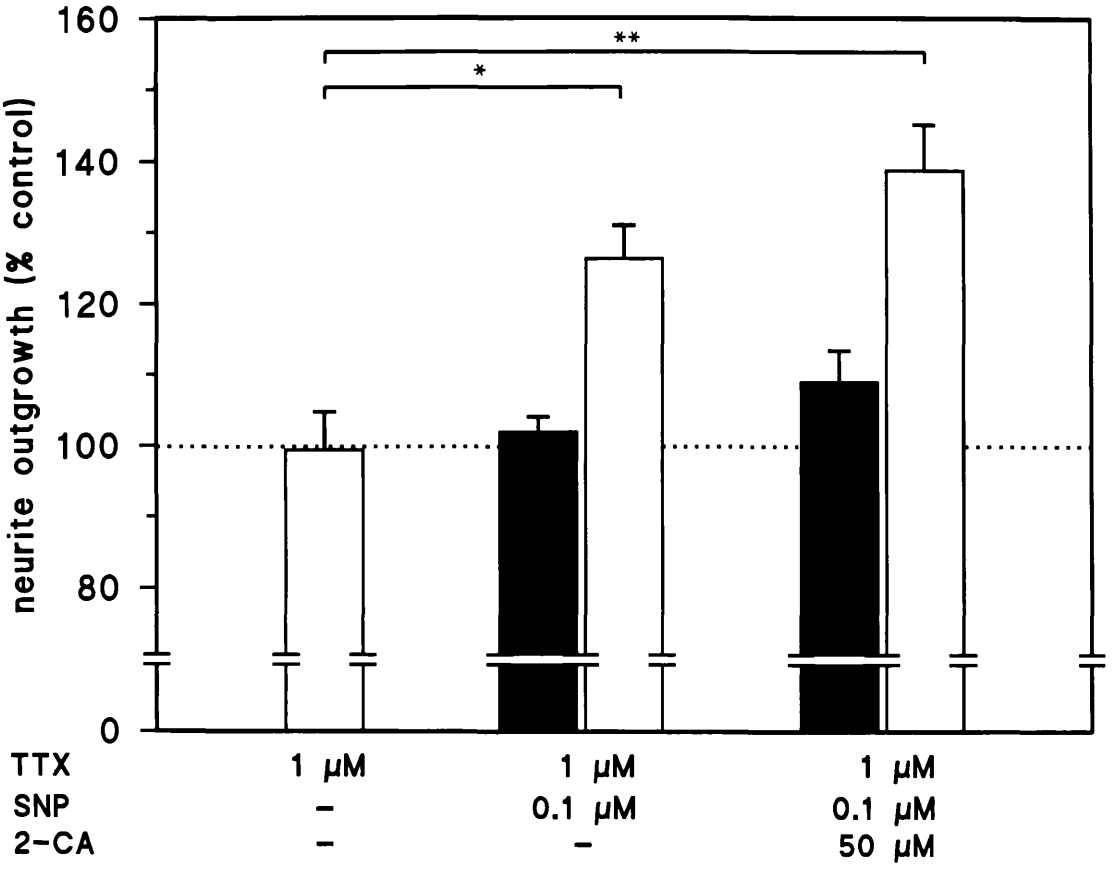

160
140
120
100
80
0
neurite outgrowth (% control)
**
L-NAME
-
30 µM
100 µM

Fig. 6.2.; Mean *neurite length* of striatal neurons after 2 days *in vitro*. Striatal cells were cultured in the presence (open bars) or absence (solid bars) of myenteric plexus explants supplemented with tetrodotoxin (TTX; 1 µM) and sodium nitroprusside (SNP; 0.1 µM); 2-CA = 2-chloroadenosine (50 µM); the dotted line represents the mean neurite length in untreated control cultures normalized to 100%; each experiment was performed in triplicate and bars represent mean ± S.E.; * = $P<0.05$, ** = $P<0.01$ (Student's *t*-test).


160
140
120
100
80
0
neurite outgrowth (% control)
**
*
TTX
SNP
2-CA
1 μM
–
–
1 μM
0.1 μM
–
1 μM
0.1 μM
50 μM

	mean neurite length of striatal al neurons (% control)
bFGF (10 ng/ml) SNP (0.1 µM)	100 ± 1%
bFGF (10 ng/ml) SNP (0.1 µM) 2-CA (50 µM)	115 ± 4%
NT-3 (10 ng/ml) SNP (0.1 µM) 2-CA (50 µM)	110 ± 4%

Table 6.1. Effects of basic fibroblast growth factor (bFGF) and neurotrophin 3 (NT-3) in combination with sodium nitroprusside (SNP) and 2-chloroadenosine (2-CA). 2-CA (50 µM) alone has been found to increase striatal neurite elongation to 120 % compared to control (see chapter 4). After 48 h *in vitro* the total neurite length of 100 cells was measured and compared to control. Results are means ± S.E. of three independent experiments.

CHAPTER 7

GENERAL DISCUSSION

It has previously been demonstrated that grafts from the myenteric plexus produce a massive sprouting response by host striatal neurons around and into the graft (Tew *et al.* 1992, 1994). It was concluded that the myenteric plexus is a potential source of material for treating neurodegenerative diseases (see chapter 1). The work described in this thesis was aimed at investigating the trophic interactions between the myenteric plexus and striatal cells further, using a tissue culture approach, and to study the cell types and molecules involved.

The results of the cell culture experiments described in this thesis demonstrate that the myenteric plexus promoted neurite elongation of postnatal striatal neurons in coculture. Evidence was produced that spontaneous neuronal activity within the myenteric plexus was involved in the events leading to the release of a neuritogenic factor(s), possibly from glial cells. Both adenosine, probably as a breakdown product of the enteric neurotransmitter ATP, and nitric oxide (NO) were shown to be involved in the increase of striatal neurite elongation. The adenylate cyclase activator forskolin induced marked striatal neurite elongation in tetrodotoxin (TTX)-treated cocultures. This is consistent with the modulation of cAMP-levels by adenosine via P_1-purinoceptors.

The trophic factors nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and basic fibroblast growth factor (bFGF) (alone and together with the stable adenosine analogue 2-chloroadenosine (2-CA) and the NO donor sodium nitroprusside (SNP)) were only marginally neuritogenic and neither laminin or fibronectin increased striatal neurite outgrowth in the cell culture system employed for this study. A ganglioside mix (AGF I) did increase

neurite extension in striatal cultures in a dose-dependent manner and this effect was antagonized by simultaneous application of 2-CA. This result suggested that gangliosides are unlikely to be involved in the neuritogenic effect of the myenteric plexus in coculture. Furthermore, it implies that striatal neurite elongation could be modulated via different mechanisms.

A study in collaboration with Dr. Maria Abbracchio (University of Milan, Italy) was performed in order to examine effects of purine analogues on glial cell numbers in striatal cultures. These experiments revealed that striatal glial cells are responsive to analogues of adenosine and ATP (see chapter 7.3.4. and Appendix for details).

7.1. Cell culture systems

Cell culture systems have been used extensively to study the actions of various factors on cells from the peripheral nervous system (PNS) and the central nervous system (CNS). The implications of the isolation of neuronal tissue from its natural environment, however, need to be critically considered in the discussion of results obtained by *in vitro* experiments.

7.1.1. Advantages and limitations of a tissue culture system

The major advantage of a tissue culture system is the direct control of the physical and chemical conditions of the cellular environment. The concentration of medium ingredients can be controlled very precisely and supplemented reagents have direct access to the target cells. Furthermore, the homogeneity and quantity of replicate cultures, the relatively easy processing and direct access to the cells are of advantage for the quantitation of cellular

parameters and experimental endpoints.

On the other hand, the dissection and processing of neuronal tissue for an *in vitro* system results in an immediate withdrawal of the interactions of neurons with their microenvironment. For example, neurons are deprived of their afferent neurotransmitters, postsynaptic feedback modulations and the actions of hormones. Further, mechanical and trophic support by glial and other neighbouring cells, such as target cells of neuronal projections, is interrupted. Mechanical forces and enzymatic treatment can damage the membrane integrity and membrane proteins such as cell surface receptors and adhesion molecules. As a result, the preparation procedure and the culture conditions can selectively support survival and differentiation of subpopulations of cells.

The dissection procedure, the choice of the culture system (eg. dissociated or slice culture) and growth medium, the hormone and growth factor supplements and the culture substrate all substantially influence the health and state of differentiation of a neuronal cell culture. As a direct consequence, the cellular responses (eg. activity of transmitter metabolizing enzymes, transmitter release, neurite elongation or cell survival) to externally applied factors are predetermined by the choice of culture conditions.

Therefore, results obtained in tissue culture experiments must be interpreted carefully in regard to these limitations. The direct control of experimental conditions is of great value for the investigation of cellular and molecular mechanisms, but the absence of interactions with the cellular environment (like *in vivo*) makes it difficult to compare observations made *in vitro* directly with those made *in vivo* and direct conclusions may be misleading.

7.1.2. Choice of tissue culture procedures

There are a large number of different tissue culture models due to the variety of possible combinations of materials and methods, such as species and age of tissue donor, type of cultured tissue, method of culture preparation, type of medium and added supplements, growth substrate and duration of culturing, analysed parameter and method of analysis (see Bunge *et al.* 1987). Varon and coworkers (1988) have proposed that the use of monolayer cultures of dissociated neurons is a most suitable system for the quantitative evaluation of neurotrophic and neurite-promoting actions on neurons, because morphological characteristics of individual cells (eg. neurite elongation and branching pattern, expression of transmitter metabolizing enzymes) are easy to visualize and relatively simple to quantitate.

The experimental procedures used in this study have been chosen for a number of reasons. The rationale of this study was to further investigate trophic interactions of myenteric plexus grafts within the host corpus striatum, as has been reported previously (Tew *et al.* 1992), using tissue culture techniques. Therefore, culture conditions were chosen to allow the detection of parameters such as process elongation and cell survival (see Varon *et al.* 1988). A combined analysis of these two parameters can provide evidence of the extent to which an increased degree of differentiation (eg. neurite elongation) is a specific effect or simply secondary to a larger number of more healthy neurons.

7.1.2.1. Species and age of animals

Dissociated neurons from postnatal day 7 rats have been chosen for a number of reasons. Neuronal cells from the mature CNS cannot be maintained in culture for longer than a few hours without a supplement of trophic factors (see Lindsay *et al.* 1993). In contrast, embryonic CNS tissue appears to be much less sensitive to a lack of external trophic support. For example, embryonic (E14/E15) striatal neurons have been reported to survive up to 50 days *in vitro* under selected culture conditions (Sebben *et al.* 1990). However, the results obtained in studies using developing embryonic neurons might not reflect responses of adult neurons (see Hefti 1994). Developmental outgrowth and pathfinding of embryonic neurons has been found to be regulated by growth promoting factors different from those responsible for regenerative growth of adult CNS neurons (see Jacobson 1991). Therefore, postnatal (P7) striatal cells have been chosen as a source of material and were regarded as a better model for regenerative neurite elongation than embryonic neurons. Since the period of naturally occurring cell death within the rat striatum was found to be completed by P7 (Fishell and van der Kooy 1991) an underlying effect on neuronal cell numbers by this developmental process was eliminated from the investigations described here. Rats have been chosen as CNS tissue donors in this study as this allowed easier comparison with other *in vitro* and *in vivo* studies using rats (see chapter 1).

Under the culture conditions used for this study (eg. fetal calf serum (FCS)-supplemented medium, relatively sparse cell density) striatal neurons gradually declined in number during the first week *in vitro*. Thus a trophic effect on these cells such as increases in both neuronal survival and neurite elongation, could be analysed in

these cultures. These parameters were chosen in order to determine whether a change in neurite elongation was secondary to an alteration in the number of surviving and healthy neuronal or non-neuronal cells (see chapter 3, see Varon *et al.* 1988). The sparse seeding density permitted the recognition of individual viable neurons and the evaluation of the total neurite length (for details see chapters 1 and 3). The use of FCS as a medium supplement has previously been shown to increase striatal neuronal viability *in vitro* (Kessler 1986) and was essential for neuronal survival in the culture method employed here.

Previous studies have shown that explants from the myenteric plexus obtained from two-week old rats produced a massive sprouting response in the host brain after grafting into the striatum of adult rats (Tew *et al.* 1992, 1994). For this study, myenteric plexus explants from newborn guinea-pig have been chosen as a putative source of a trophic factor(s). On the basis of the current literature, it appears that growth factors have no apparent species specificity (see Altin and Bradshaw 1993 for review). Further, explant cultures from newborn guinea-pig myenteric plexus explants have been well characterized in previous studies (for details see chapter 1) and since the dissection method is relatively easy compared to rat myenteric plexus, it is possible to obtain reproducible culture quality and experimental results. Therefore, myenteric plexus explants from newborn guinea-pig was cocultured together with dissociated cells from postnatal rat striatum.

7.1.2.2. Coculture system

A novel coculture system was developed for this study in which two cell populations face each other on separate sides of a Rose chamber (chapter 2). This enabled the investigation of the action of soluble trophic factors without any direct cell-cell contacts between the two different cell populations. The design of the Rose chamber also allowed microscopic investigations, such as the measurement of neurite elongation to take place on living cells.

A major disadvantage of this coculture system was that any medium supplement always affected both cell populations. In an attempt to improve the design of the coculture system, a peristaltic pump was used to drive medium through one Rose chamber containing myenteric plexus explants into a second containing striatal cells allowing a selective treatment of striatal cells without affecting myenteric plexus cells. Although different media, batches of FCS and perfusion rates were tested, striatal cells degenerated within hours in all experiments.

7.1.2.3. Evaluation of neurite outgrowth

Neurite elongation was measured by analysing living cells using phase contrast microscopy. This allowed an accurate measurement of all processes of those cells which were clearly recognized as neurons. Although the morphology of striatal neurons *in vitro* has been described in detail previously, a limitation of this method was that those cells which had no or only very short processes were not clearly identified as neurons. As a result of this, the mean neurite length of each culture was slightly overestimated. However, this overestimation was compensated by comparing each experimental value to a parallel control and by comparing relative changes in neurite

outgrowth rather than absolute values. In order to validate this method of analysis, striatal neurite elongation was analysed twice in the same cultures, both on living and on fixed and PGP-9.5 labelled cells. After neurite length had been measured in living cultures, the cultures were immediately fixed and stained immunocytochemically for the neurospecific marker PGP-9.5. Then neurite elongation was analysed a second time using fluorescence microscopy. Two sets of experiments were analysed in this way; one in which no increased neurite elongation had been observed previously and another with a strong increase in striatal neurite elongation. The relative change in neurite elongation compared to their own controls was the same in striatal cells treated with tetrodotoxin and αβ-meATP (living neurons: 105% of control; PGP-9.5 labelled neurons: 97% of control) and in coculture with myenteric plexus explants (living neurons: 149% of control; PGP-9.5 labelled neurons: 148% of control). This demonstrated that the mean neurite outgrowth measured in living cultures was a valid method of quantification provided that absolute experimental data was compared to its own control.

7.2. Possible sources of a trophic factor

The large variety of various neuronal and non-neuronal cell types, from both the striatum and the myenteric plexus (see chapter 1 for details), cocultured in these experiments gives rise to a large number of possible interactions. The majority of those cell types present in grafting experiments *in vivo* were also present in the coculture system. However, target tissues of striatal projections and those axonal processes projecting through or into the striatum were absent in coculture (discussed below).

7.2.1. Involvement of enteric glial cells

It is possible that a putative neuritogenic factor(s) released by enteric glial cells may have been involved in the events leading to striatal process elongation (for detailed discussion see chapter 3). In this respect, CNS regenerating sprouts have been reported to grow into myenteric plexus grafts transplanted into the host striatum and to become closely associated with enteric glial cells (Tew *et al.* 1992). Interestingly, however, purified enteric glial cells grafted into the corpus striatum did not induce a sprouting response in the host brain (Tew 1994). Poor viability, a low number of grafted enteric glial cells or the rejection of the graft were thought to be possible explanations for this (Tew 1994). However, the evidence produced in this study, indicates that the release of enteric neurotransmitter(s) is involved in the trophic interactions in cocultures of myenteric plexus explants and striatal cells (for detailed discussions see chapters 4 and 6). Therefore, enteric glial cells might be the source of a putative growth factor(s) and the production and/or release of such factors might be triggered by enteric neurotransmitters. Further, both enteric neurons and glia need to be present to induce a sprouting response by striatal neurons. Alternatively, a growth factor released by enteric glial cells might only induce neurite elongation by acting in concert with another factor, such as an enteric neurotransmitter. Although experiments have been performed to identify a possible additive or synergistic action of transmitters together with growth factors, they have produced no direct evidence to support this model (see chapters 5 and 6). A number of reports have shown that peripheral glial cells can produce trophic factors. In cultured Schwann cells, NGF and BDNF-mRNA could be upregulated by different regulatory mechanisms

(Matsuoka *et al.* 1991, Meyer *et al.* 1992). Further, Schwann cell-conditioned medium contained CNTF-like neurotrophic activity (Meyer *et al.* 1992). The production of any of these growth factors by enteric glial cells, however, has not been reported.

Extracellular matrix molecules have been found to stimulate neurite outgrowth and to be involved in axonal guidance (see Bixby and Harris 1991 for review). Enteric glial cells have been reported to produce laminin *in vitro* (Bannerman *et al.* 1988 a), but neither myenteric plexus-conditioned medium (chapter 3) or laminin-coated substrates (chapter 5) increased neurite elongation of postnatal striatal neurons *in vitro*. In an attempt to investigate the neurite promoting effect of collagen, striatal cells were cultured on air-dried collagen (chapter 2). Under the culture conditions employed here, attachment and growth of striatal cells on both commercially available collagen and freshly prepared rat tail collagen was poor and neurite outgrowth could not be quantified (Bornstein 1958).

7.2.2. Involvement of enteric neurons

Evidence has been presented in the work described here, that spontaneous neuronal activity within the myenteric plexus is involved in the neuritogenic effect of the myenteric plexus on striatal neurons in coculture; TTX completely blocked the neuritogenic effect in these cocultures (see chapter 3 for details). Furthermore, two enteric neurotransmitters, ATP (after being metabolized to adenosine) and NO partially reversed the blocking effect of TTX in cocultures, but did not affect neurite elongation when applied to striatal cultures alone (see chapters 4 and 6). In support of this, enriched enteric glial cell preparations also increased striatal neurite

elongation in coculture (see chapter 3). Therefore, these observations suggest that enteric neurons contribute to the increase in striatal neurite outgrowth in cocultures by triggering the release of a neuritogenic factor(s) (from enteric glial cells), rather than releasing such a factor(s) themselves. However, 2-CA slightly increased neurite elongation of striatal neurons alone (see Chapter 4). Adenosine is formed as a breakdown product of the enteric neurotransmitter ATP (Burnstock 1978). Therefore, ATP released from enteric neurons and broken down to adenosine could have acted together with another factor(s), released from enteric glial cells on striatal neurons. Coculture experiments with pure enteric glial cell preparations might produce results to answer this question. However, pure enteric glial cell cultures could not be obtained by the culture method employed here. The role of other enteric neurotransmitters (see chapter 1) is as yet unknown and awaits investigation.

Finally, a variety of different neuropeptides has been identified in enteric neurons (see chapter 1 for details) which have also been reported to have neurotrophic effects (see Hökfelt 1991 and Schwartz 1991 for reviews). Although a mixture of peptides did not significantly increase striatal neurite elongation of the stable adenosine analogue 2-CA in TTX-treated cocultures (see chapter 4), a detailed study of all enteric neuropeptides is necessary to determine their neuritogenic potential and possible involvement in the trophic interactions in myenteric plexus cocultures.

7.2.3. Interstitial cells of Cajal

Another type of cells which is closely associated with enteric neurons are the intestinal cells of Cajal (see Christensen 1992 for review). The interstitial cells are thought to have pacemaker

function in the rhythmic contraction of the intestine (see Christensen 1992, Torihashi *et al.* 1994). These cells might also be present in myenteric plexus explant cultures and could therefore be involved in the release of a trophic factor from within the myenteric plexus, although this was not examined in the present study.

7.2.4. Involvement of striatal glial cells

The cultures from postnatal rat striatum contained both neurons and glial cells (for details see chapter 1 and 3, Abbracchio *et al.* 1994a,b). These striatal glial cells, such as astrocytes and oligodendrocytes, could also have contributed to the promotion of striatal neurite elongation by the myenteric plexus in coculture. Astrocytes have been reported to modulate developmental and regenerative neurite outgrowth *in vitro* and *in vivo*. This neuron-glia interaction is due to diffusible factors (eg. Varon and Adler 1981) and/or cell surface interactions (eg. Noble *et al.* 1984, Bovolenta *et al.* 1993). Accumulating evidence indicates that both the state of maturation and the activation of astrocytes (eg. after CNS injury) play a role in the neurite promoting or inhibiting potential of these cells.

The levels of neurotrophins in astrocytes are very low under physiological conditions *in vivo* but are up-regulated during reactive gliosis after brain injury or inflammation (see Lindholm *et al.* 1994). NGF-like immunoreactivity was increased in GFAP-positive septal and hippocampal astrocytes following electrolytic lesion *in vivo* (Oberfeld-Nowak *et al.* 1992). *In vitro*, free radicals have been reported to increase NGF- and bFGF mRNA and to upregulate c*fos* mRNA in cortical astrocytes (Pechan *et al.* 1992). Cultured astroglial

cells have also been found to produce BDNF (Zafra *et al.* 1992), NT-3 (Rudge *et al.* 1992) and NT-4/5 (Condorelli *et al.* 1994). No increased neurite elongation, however, was observed in the striatal cultures used here in the presence of NGF, NT-3 or NT-4/5 (see chapter 5). Astroglial cell culture-conditioned medium has been reported to increase neuronal survival in cultures from embryonic (E18) rat striatum (Varon *et al.* 1984). Further, astrocytes have been reported to produce bFGF both *in vivo* and *in vitro* (eg. Hatten *et al.* 1988, reviewed by Baird 1994). Upregulation of ChAT expression was found in cortical cells when they were grown in medium conditioned by differentiated astrocytes and was thought to be due to bFGF or a bFGF-like compound (Gray and Patel 1992). With respect to this work, bFGF has been reported to increase neurite elongation in cultures from postnatal rat striatum (Zhou and DiFiglia, 1993) but had no effect under the culture conditions employed here (see chapter 5). Increased neurite outgrowth was also observed when striatal neurons were grown on a monolayer of striatal astrocytes (Chamak *et al.* 1987). This effect was only observed in a homotypic combination and was not observed when striatal neurons were grown on a mesencephalic astroglial feederlayer, suggesting astrocytes from different brain regions have different growth promoting properties (Chamak *et al.* 1987).

Within the striatum, the expression of growth factors such as the neurotrophins is much less evident than in other brain areas (Lindsay *et al.* 1993). CNTF mRNA and protein has been detected within the adult rat striatum and was shown to be confined to a subpopulation of GFAP positive type 1-like astrocytes (Stöckli *et al.* 1991). Messenger RNA transcripts for bFGF and the TGF-β's 1,2 and 3 have all been found in cultured neonatal rat astroglial cells (see Unsicker *et al.*

1992) and have recently been proposed to be involved in reactive gliosis, neuronal maintenance and neurite regeneration *in vivo* (Unsicker *et al.* 1994). Expression of TGFα has also been detected in striatal glial cells (see Lindsay *et al.* 1993).

In summary, these reports demonstrate that CNS glial cells can produce trophic factors and indicate that striatal glial cells may be involved in the trophic interactions in myenteric plexus cocultures. Any factor released by the myenteric plexus in coculture or supplemented to the cultures could have induced the production of a trophic factor by striatal glial cells. The results of this study, however, did not produce any direct evidence for this.

7.2.5. Modulation of astroglial cell proliferation by analogues of adenosine and ATP

A study in collaboration with Dr. Maria Abbracchio (University of Milan, Italy) was aimed at detecting effects of purine analogues on GFAP-positive astroglial cell numbers in postnatal day 7 striatal cultures. These experiments revealed that striatal glial cells are responsive to analogues of adenosine and ATP. Treatment with 2-CA decreased whereas αβ-meATP increased the number of striatal glial cells in a dose-dependent manner. The effect of αβ-meATP was antagonized by the P_2-purinoceptor blocker suramin whereas the P_1-purinoceptor antagonist 8-PSPT did not reverse the effect of 2-CA. Both analogues induced an increase in nuclear BrdU-incorporation (Abbracchio *et al.* 1994c; reprint included as Appendix).

The observation that αβ-meATP increased process elongation, BrdU-incorporation and cell numbers of striatal astrocytes *in vitro* suggests that an ATP-mediated effect might be involved in reactive

gliosis (Abbracchio *et al.* 1994a,b), a condition under which CNS astrocytes have been reported to up-regulate growth factor production (see above). However, αβ-meATP did not affect striatal neurite elongation in TTX-treated (cocultures or in striatal cultures alone (see chapter 4).

The results presented in chapter 4 demonstrate that adenosine is involved in the neuritogenic effect seen in myenteric plexus cocultures. Further, 2-CA was found to increase neurite outgrowth in striatal cultures alone (see chapter 4 and 5). Abbracchio *et al.* (1994a) have found that 2-CA induced an increase in the proportion of BrdU-incorporating astrocytes associated with a reduction of astrocyte cell numbers. Since 2-CA was found to modulate striatal astrocyte numbers, it could also have induced the production of a growth factor by these cells, although this has not yet been investigated. However, Unsicker and coworkers (1994) have recently reported that bFGF increased numbers of both astrocytes and BrdU-incorporating cells in astrocyte cultures, whereas TGF-β had no effect on numbers of GFAP-positive cells or BrdU-incorporation. Interestingly, TGF-β inhibited the effect of bFGF in a dose-dependent manner when both factors were supplemented together and a reduction of astrocyte numbers was observed. Regarding the results presented by Abbracchio *et al.* (1994a) it is tempting to speculate that purines, which have been proposed to be involved in reactive gliosis (see Abbracchio *et al.* 1995 for review), could induce the production of growth factors in the injured CNS.

In summary, it is possible that striatal glial cells may be involved in the trophic interactions in myenteric plexus cocultures, although there is no direct evidence for this.

7.3. Synergy in trophic regulation

It is becoming increasingly evident that the maintenance as well as the regulation of developmental and regenerative growth within the nervous system is a highly orchestrated process involving the action of diffusible factors and interactions with both the ECM and neighbouring cells. The responsiveness of neurons to trophic factors depends on the cell type, the developmental age or state of differentiation and their microenvironment (eg. Maisonpierre *et al.* 1990). The most economical way to regulate an integrated system, such as the nervous system, appears to combine a minimal number of signalling factors with a variety of possible ways to modulate the signal transduction pathway. Synergistic action of two or more factors has been proposed to be one of the mechanisms to facilitate the large variety of cellular responses to trophic factors (reviewed by Black 1993, Ip and Yancopoulos 1994).

There are several possible mechanisms by which the trophic potential of a growth factor can be modulated. The first possibility is that a modulating agent may bind to a growth factor, resulting in an alteration of its affinity for the growth factor receptor. For example, the affinity of basic fibroblast growth factor (bFGF) to the transmembrane FGF-receptor is increased after FGF binds to heparan proteoglycan, which induces a conformational change of FGF (see Eckenstein 1994 for review).

Secondly, the potentiation of growth factor actions has also been demonstrated by molecules which directly interfere with growth factor receptors (Knüsel and Hefti 1992). The alkaloids K-252a and K-252b, which are potent inhibitors of neurotrophin actions, have been found to potentiate the trophic actions of NT-3 on CNS neurons and PC 12

cells *in vitro*, at concentrations lower than those necessary to cause a direct inhibitory effect (Knüsel and Hefti 1992). This synergistic action has been suggested to be facilitated by the interaction of the K-252 compounds with the tyrosine-specific protein kinase domain of the receptor, rather than by interfering with the binding of the growth factor to its receptor (Knüsel and Hefti 1992).

As a third possibility, synergy was also reported between members of different growth factor families utilizing distinct signalling mechanisms (see Ip and Yancopoulos 1994). Recently, Ip *et al.* 1994 have demonstrated that CNTF together with FGF collaborate in promoting the differentiation of MAH cells (a sympathoadrenal progenitor cell line) into NGF-dependent postmitotic neurons. Further, the ECM protein laminin has been reported to potentiate NGF induced neurite extension from the trigeminal I motor nucleus of early chick embryos, suggesting that the effect of soluble growth factors can be modulated by components in the ECM (Heaton *et al.* 1989).

Synergy was also found between growth factors and small neuroactive molecules. In dissociated cerebellar Purkinje cell cultures simultaneous exposure of NGF together with the excitatory neurotransmitters glutamate or aspartate increased neuronal survival in a synergistic action (Cohen-Cory *et al.* 1991). This effect was proposed to be mediated via NGF-receptor upregulation (see Black 1993). Purines have also been reported to act synergistically together with different growth factors on neuronal survival and/or neurite elongation (for details see chapter 4). Evidence has been produced in this work (chapter 4) and by other groups (see below), that this may occur via modulation of second messenger systems, such as cAMP. In support of this, synergy between increased cAMP and

various growth factors has been demonstrated previously (eg. Togari *et al.* 1985, Ho and Raw 1992, see also chapter 4).

The results presented here also raise the possibility that there is an additive or synergistic action, between an enteric neurotransmitter (such as purines and NO) and another factor probably released from enteric glial cells, in the events leading to increased neurite elongation of striatal neurons in coculture (see chapters 4 and 6 for experimental results and discussion). Although this model is in agreement with the current literature, the results obtained during my work did not produce direct evidence to verify the model. To further investigate a possible synergy of various neuroactive molecules (such as growth factors and neurotransmitters) it would be of great advantage to employ a screening test system to analyse the large number of possible combinations of factors (applied at different concentrations). The use of primary CNS cultures combined with a more robust method of analysis (proportion of neurite bearing cells) may help to detect synergistic combination of neuroactive factors (see chapter future plans).

7.4. Comparison of results obtained in coculture with those produced in transplantation experiments

The neurite outgrowth promoting effects of myenteric plexus explants on striatal neurons in coculture (see chapter 3) are consistent with the observations in transplantation experiments (Tew *et al.* 1992, 1994). When freeze-killed explants of the myenteric plexus were grafted in the host striatum, no axonal sprouting was observed within the host brain (Tew *et al.* 1992). This observation suggested that a soluble neuritogenic factor(s) has been released

from living cells within the graft (Tew *et al.* 1992). This is consistent with the results presented here, since the coculture system employed during this study allows the diffusion of soluble factors between myenteric plexus and striatal cells. *In vivo*, striatal neurite elongation may also have been modulated by direct cell-cell interactions with myenteric plexus cells and ECM components within the graft and with enteric axonal processes growing out of the graft into the striatum as described by Tew *et al.* (1993).

Since monolayer cultures derived from the striatum do not contain any cells projecting to (eg. dopaminergic nigrostriatal neurons) or extending processes through the striatum (eg. corticospinal fiber tracts), the *in vitro* system employed here can exclusively detect effects on striatal projection or interneurons. In this respect, a sprouting response induced by myenteric plexus grafts has also been observed in the excitotoxin-lesioned host striatum, although the number of CNS sprouts was reduced compared to the response to grafts in unlesioned host striatum (Tew *et al.* 1994). This observation suggests that the sprouting response observed around myenteric plexus grafts within the host striatum may arise from striatal neurons as well as neurons originating in other brain areas and projecting into, or traversing through, the striatum (Tew *et al.* 1992, 1994).

A strong sprouting response was also observed after intrastriatal transplantation of rat colonic smooth muscle (Tew 1994). Since freeze-killed smooth muscle grafts did not promote sprouting within the host striatum, a soluble trophic factor(s) was suggested to be produced by smooth muscle cells (Tew 1994). In coculture experiments, monolayer cultures of de-differentiated smooth muscle did not increase neurite elongation of striatal neurons (chapter 3). There are several possible explanations for this apparent discrepancy.

Firstly, the sprouting response observed *in vivo* might have been induced in afferent axons projecting to the striatum or in fibers traversing the striatum (eg. corticospinal projections). Since those neurons projecting to the striatum are not present in dissociated striatal cultures, these responses would not be detected *in vitro*. More importantly, the smooth muscle cells in dissociated cultures were de-differentiated and therefore might have produced different factors than the freshly dissected pieces of smooth muscle used for grafting (Tew 1994). Finally, grafts have been transplanted into the striatum of adult rats whereas one week old animals were used for tissue culture. The analysis of the transplantation experiments was performed 3 to 6 weeks after grafting whereas striatal neurite outgrowth was measured after 48 h *in vitro*.

7.5. Future directions

This investigation was an initial *in vitro* study to further investigate trophic interactions observed in *in vivo* experiments. Although additional variations of the experimental procedures, such as different animal ages or other culture methods, might have produced interesting results, priority was given to investigate a trophic effect which was observed in myenteric plexus coculture during the early stage of this work. In this section a number of possible future projects are presented which represent some of the questions which have been left unanswered within this thesis.

1. Cell type specific staining of neuronal and non-neuronal subpopulations in striatal cultures. The immunocytochemical or histochemical labelling of striatal neurons (such as GABAergic or

NADPH-diaphorase positive neurons) may reveal to what extent the neuritogenic effect of the myenteric plexus affects certain neuronal subpopulations within these cultures, or alternatively, if the myenteric plexus releases a number of different neurotrophic factors, affecting various striatal neuronal subpopulations.

2. Effects of specific agonists or antagonists for purinoceptor subtypes. The use of agents which selectively activate purinoceptor subtypes might reveal which second messenger pathways are involved in the induction of neurite elongation.

3. Blocking of a growth factor action. The use of neutralizing antibodies for established neurotrophic factors (such as EGF, TGF-α or CNTF) aims to block the trophic interaction in the myenteric plexus cocultures.

4. Use of pure enteric neuronal or glial cells for coculture experiments. This approach would aim to reduce the complexity of the coculture system and probably reveal the source of a neuritogenic factor(s) within the myenteric plexus.

5. Trophic factors in the myenteric plexus. A different approach is the identification of growth factors and their receptors in myenteric plexus cells using *in situ* hybridisation and immunocytochemical techniques.

6. Neurotrophic factors on striatal cultures alone. Only a limited number of trophic factors could be tested during the period of this work and few studies have been published directly localizing growth

factors within cells of the myenteric plexus (eg. recently TGFα was identified in enteric neurons). Therefore, further studies are necessary to investigate the actions of the large number of growth factors, ECM proteins and neurotransmitters on striatal neurons. Of particular interest is the investigation of a possible synergistic action of two or more factors acting together.

7. Primary CNS cultures as screening system. The use of primary cultures combined with an uncomplicated and fast method of analysis (eg. percentage of process bearing cells after 24h *in vitro*) may provide useful means for the screening of a synergistic neuritogenic action of the great number of possible combinations of factors.

8. Investigation of striatal neurite elongation using striatal tissue from newborn or embryonic animals. An investigation of the neurite promoting effects of the myenteric plexus on striatal neurons from earlier developmental stages might reveal if striatal neurons are differentially responsive to the neuritogenic effects of the myenteric plexus.

9. Neurotrophic effect on striatal progenitor cells. Previous studies have shown that progenitor cells are present within the striatum and in the subependyma of the ventricle wall of adult rats (see chapter 1). A tissue culture study of myenteric plexus explants cocultured with dissociated cells from these brain areas may show if the myenteric plexus produces factors which support the proliferation and sprouting of striatal progenitor cells of the adult brain.

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APPENDIX

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MODULATION OF ASTROGLIAL CELL PROLIFERATION BY ANALOGUES OF ADENOSINE AND ATP IN PRIMARY CULTURES OF RAT STRIATUM

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Abstract—We have studied the possible purinoceptor-mediated modulation of astroglial cell proliferation in neuron–glia primary cultures obtained from rat corpus striatum. Cultures were grown for three days in the presence of either 2-chloro-adenosine or $\alpha\beta$-methylene-ATP (which behave as agonists of adenosine/P1 and ATP/P2 purinoceptors, respectively), and then immunostained with an antibody to glial fibrillary acidic protein. 2-Chloro-adenosine decreased and $\alpha\beta$-methylene-ATP increased the number of astroglial cells in culture. For both derivatives, the effect was dose-dependent. The effect of $\alpha\beta$-methylene-ATP was antagonized by the trypanoside suramin, suggesting the involvement of a suramin-sensitive P2 purinoceptor, whereas the effect of 2-chloro-adenosine was not reversed by the P1 purinoceptor antagonist *p*-sulphonyl-phenyl-theophylline, implying the activation of a xanthine-insensitive adenosine purinoceptor subtype. In order to evaluate the extent of astrocyte proliferation in the presence of these two analogues, some cultures were incubated with bromodeoxyuridine for 24 h before fixing, and then double-immunostained for glial fibrillary acidic protein and bromodeoxyuridine. The percentage of bromodeoxyuridine positive astrocytes was significantly increased after exposure to both agents.

It is therefore concluded that purines can modulate astroglial cells in opposite ways, inducing decreases or increases of cell number by activation of P1 and P2 purinoceptors, respectively. For the P2 purinoceptor-mediated effect, there was a quantitative correlation between the percentage of bromodeoxyuridine positive astrocytes and the cell number. For the P1 purinoceptor-mediated effect, no apparent correlation between these two parameters was found. This suggests the activation of independent effects, which involve other mechanisms besides the stimulation of DNA synthesis, and which eventually result in a reduction of cell number. The possible relevance of these findings to *in vivo* regulation of astrocyte cell function as well as in trauma- and ischaemia-associated hypergliosis is discussed.

Astroglial cells were originally thought to serve only as supporting structures for neurons, offering appropriate substratum and guidance to migrating neuroblasts and growing axons during CNS ontogenesis, and participating in the formation of the glial limiting membrane and of the blood–brain barrier (for review, see Ref. 34). More recent evidence, however, suggests that astrocytes may also play an active role in neurotransmission. Astroglial cells have been found to express membrane receptors for a variety of transmitters/modulators, and to regulate neurotransmitter concentrations in the synaptic cleft.[34] CNS astroglia have been shown to express region-specific neurotransmitter uptake and metabolizing systems, which are indicative of specialized functions.[34] Astroglia are also known to react to various types of injury by rapid and vigorous astrogliosis, characterized by rapid proliferation, hyperplasia and extensive hypertrophy, and by an increase in immunodetectable glial fibrillary acidic protein (GFAP).[7,18] Although astrogliosis has always been interpreted as a beneficial response by which injured brain might restore its damaged functions, the exact functional significance and precise mechanisms underlying this phenomenon are still a matter of discussion.[37]

These observations indicate the importance of identifying and studying the different endogenous modulators of astroglial cell function. The nature and exact role of the factors which modulate astroglial cell proliferative responses are not well elucidated. Besides a few characterized protein mitogens, a number of unidentified soluble factors, released from dying cells and activated microglia after CNS injury, are believed to play a role in regulating glial cell proliferation and initiating astrogliosis.[9]

Several lines of evidence implicate purines, namely adenosine and ATP,[4] as likely candidates for regulators of glial cell proliferation. First, purines can be

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Abbreviations: ADA, adenosine deaminase; $\alpha\beta$-meATP, $\alpha\beta$-methylene-ATP; BrdU, bromodeoxyuridine; 2-CA, 2-chloro-adenosine; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; PGP 9.5, protein gene product 9.5; 8-PSPT, 8-*para*-sulphonyl-phenyl-theophylline; TRITC, tetramethylrhodamine isothiocyanate.

released by injured or hypoxic cells in both the cardiovascular system[5,23] and in the CNS (for review, see Ref. 27). In the cardiovascular system, purines have been demonstrated to stimulate proliferation of capillary endothelial cells, as part of the angiogenic response in wound healing;[6] similar modulatory mechanisms on astroglial cell proliferation might also be operative in brain. Secondly, glial cells have been shown to express both adenosine/P1 and ATP/P2 extracellular purinoceptor subtypes, although their exact role has not yet been fully elucidated.[24,25] Thirdly, studies performed on chick astrocytes have suggested a modulatory role of purines on proliferation,[28] although to our knowledge no studies have been reported to date on possible modulatory effects of purines on proliferation of mammalian astrocytes in primary culture.

Based on this, we deemed it interesting to study the effects of purines on astrocyte proliferation in primary cultures obtained from neonatal rat brain. On the basis of ongoing studies from our laboratory on implantation of peripheral nervous system into the corpus striatum of adult rats,[30] we have chosen the corpus striatum as a first brain area to characterize the possible effects of purines on glial cells. Moreover, due to the profound and reciprocal interactions between neurons and glial cells in culture,[14] we deemed it appropriate to start these studies by utilizing primary cultures from dissociated rat striatum containing both neurons and glial cells, where both cell types can be reciprocally modulated.

EXPERIMENTAL PROCEDURES

Cultures

Cultures were prepared by a method similar to that described previously for the culture of basal forebrain neurons.[3] Briefly, after rapid decapitation, the brains of one or two seven-day-old Sprague–Dawley rats (University College London) were removed under sterile conditions and sliced into 400-μm-thick coronal slices (McIlwain tissue chopper). The slices were transferred to ice-cold Hanks balanced salt solution (HBSS, Life Technologies) supplemented with 0.6% glucose. The caudate–putamen was cut out of the slices and washed three times in HBSS without calcium and magnesium (Life Technologies). Double-strength trypsin (Sigma) and DNAase I solution (Sigma) were added in equal volumes to the washed tissue to give a final concentration of 0.125% trypsin and 10 μg/ml DNAase I in 10 ml solution. The tissue was incubated for 45 min at 37°C and washed four times with 5 ml HBSS without calcium supplemented with 8 mM $MgCl_2$, 10% fetal calf serum (FCS; ICN Flow) and 10 μg/ml DNAase I, followed by two washes with the same solution supplemented with 20 μg/ml DNAase I. The tissue pieces were carefully triturated and the dissociates were centrifuged at 45 g for 15 min. The pellet was resuspended with a fire polished pipette in 1 ml medium 199 (Life Technologies) supplemented with 10% FCS and 5 mg/ml glucose. The total cell number was determined in a haemocytometer using the Trypan Blue dye exclusion test. The cell suspension was diluted with medium 199 + 10% FCS and 5 mg/ml glucose to a final concentration of 2.4×10^5 viable cells/ml. Cultures were set up in four-well dishes (Nunc) on poly-L-lysine-coated glass coverslips. A cell suspension (6×10^4 cells in 250 μl) was inoculated into each well followed by 250 μl medium containing double-strength hydrolysis-resistant purine analogues 2-chloro-adenosine (2-CA, Sigma) or $\alpha\beta$-methylene-ATP ($\alpha\beta$-meATP, Sigma), which behave as P1 and P2 purinoceptor agonists, respectively. Cultures were maintained in a 2.5% CO_2 incubator at 37°C and fed 24 h after preparation.

In some experiments, agents such as adenosine deaminase (ADA, Boehringer Ingelheim) and selected P1 or P2 purinoceptor antagonists, namely 8-*p*-sulphonyl-phenyltheophylline (8-PSPT, Sigma) or suramin (Bayer), were also added to the culture medium.

Antibodies and immunofluorescence staining

To identify particular cell types in dissociated cultures, cells were labelled by indirect immunofluorescence using rabbit anti-GFAP immunoglobulin to identify astrocytes (1:500, DAKO A/S; see Ref. 31) and rabbit anti-protein gene product (PGP) 9.5 antibody to identify neurons (1:2000, Ultraclone; see Ref. 10). Briefly, after two to four days in culture, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C, followed by six washes (10 min each) with 80% ethanol. Cells were then treated with 0.1% Triton X-100 in PBS (10 min at room temperature) before overnight incubation with primary antibodies in antibody diluting solution containing 0.1% Triton X-100 (BDH), 0.1% sodium azide (Sigma), 0.01% bovine serum albumin (Sigma) and 0.1% lysine (Sigma) in PBS. After three washes (10 min each) with PBS, cells were incubated for 1 h at room temperature with biotinylated donkey anti-rabbit secondary antibody (1:250, Amersham) followed by incubation with streptavidin–fluorescein (1 h at room temperature, 1:100, Amersham). After three final washes with PBS, coverslips were mounted in Citifluor (glycerol–PBS solution AF1, Citifluor) and labelled cells examined with a Zeiss fluorescence microscope equipped with a fluorescein filter.

Classification of astroglial cells as either "type 1" or "type 2" astrocytes was based exclusively on their morphological appearance, since both types of astroglia are GFAP-positive. Type 1 (fibroblast-like) astrocytes were defined as GFAP-positive cells with abundant cytoplasm and virtually no processes. Type 2 astrocytes were defined as GFAP-positive cells with a small, round cell body and at least three processes, each of which was more than a cell body diameter in length (Fig. 1).

Bromodeoxyuridine incorporation assay

To determine the extent of cell proliferation under different experimental conditions, a bromodeoxyuridine (BrdU) incorporation assay was used.[13] BrdU is a thymidine analogue which is incorporated into the DNA of proliferating cells during the S-phase. Cells that have synthesized DNA in the presence of BrdU can then be specifically identified by indirect immunofluorescence using anti-BrdU antibodies.

After 48 h in culture, 10 μM BrdU (Sigma) was added to the culture medium and incubated with cells for an additional 24 h. Cells were then fixed with methanol (10 min at −20°C), washed twice with PBS and DNA was denatured by incubation with 2 N HCl (25 min at room temperature). Incubation with HCl was performed to render the DNA accessible to the anti-BrdU antibody and was followed by a 25 min incubation with 0.1 M sodium borate (Sigma; pH 8.5) before incubation with monoclonal mouse anti-BrdU antibody (1 h at room temperature, 1:40, DAKO A/S). Positive nuclei were detected by subsequent incubation with anti-mouse tetramethylrhodamine isothiocyanate (TRITC) conjugated immunoglobulin G (1:80, Nordic Immunology).

For double-labelling experiments with anti-GFAP and anti-BrdU antibodies, cultures were first processed for anti-BrdU labelling and subsequently processed for anti-GFAP immunofluorescence as described above. Cultures were then mounted and examined with the fluorescence microscope

using a fluorescein filter for anti-GFAP immunoreactivity and a rhodamine filter for anti-BrdU immunoreactivity in nuclei.

In initial experiments, we demonstrated that immunostaining with anti-GFAP was not affected by cell fixation with methanol and by HCl denaturation of DNA. Moreover, specificity of immunoreactivity and lack of cross-reactivity were checked in experiments in which application of mouse anti-BrdU antibody was followed by anti-rabbit secondary antibody + streptavidin–fluorescein, and by the converse, i.e. by incubating cells with rabbit anti-GFAP followed by anti-mouse TRITC. No immunolabelling was seen in either case.

Quantitation of labelled cells

Identical areas corresponding to approximately 100 optical fields and containing at least 200–300 cells were counted from triplicate coverslips for each experimental condition/experiment with a ×20 or ×40 objective. For single-labelling experiments, results were expressed as the number of GFAP- or PGP 9.5-positive cells/condition. For double-labelling experiments with anti-GFAP and anti-BrdU antibodies, cells of each optical field were first scored under the fluorescein filter for GFAP immunoreactivity in the cytoplasm and immediately afterwards under the rhodamine filter for BrdU immunoreactivity in nuclei. Results were expressed as the percentage of double-labelled cells with respect to the total number of GFAP-positive cells counted. To compare the different experimental groups, the "ratio" between these two parameters was also calculated ("labelling index", see also legends to Table 5 and Fig. 6).

RESULTS

Cell types in culture

After three days in culture, the majority of rat striatal cells displayed a strong immunofluorescence for GFAP (Fig. 1). Based on morphology, two different types of GFAP-positive cells could be detected: a first type, more abundant and characterized by a large, flattened shape, seemed to correspond to the previously defined "type 1 fibroblast-like astrocyte", whereas a second category of less numerous cells displayed a stellate, process-bearing morphology characteristic of "type 2 neuron-like astrocytes"[34] (Fig. 1; see also Experimental Procedures). In the rest of the paper, we will therefore refer to these two morphologies as "type 1" and "type 2" astrocytes, respectively. No immunoreactivity for GFAP was displayed by small to medium-sized bipolar and tripolar cells, whose cell body and processes were conversely strongly immunostained by the neuron-specific antibody PGP 9.5 (data not shown). A small number of highly-branched cells negative for both GFAP and PGP 9.5 could also be detected in our cultures. These probably represented oligodendrocyte-like cells which do not grow significantly under the culture conditions utilized here, i.e. in the presence of serum.[13] This last type of cell represented less than 5% of the total number of cells in our culture system.

Effects of purine analogues on cell number in culture

In a first set of experiments, to study the possible modulation of astroglial cell number by "endogenous" purines, we grew cells in the presence of either ADA, which is known to inactivate endogenous

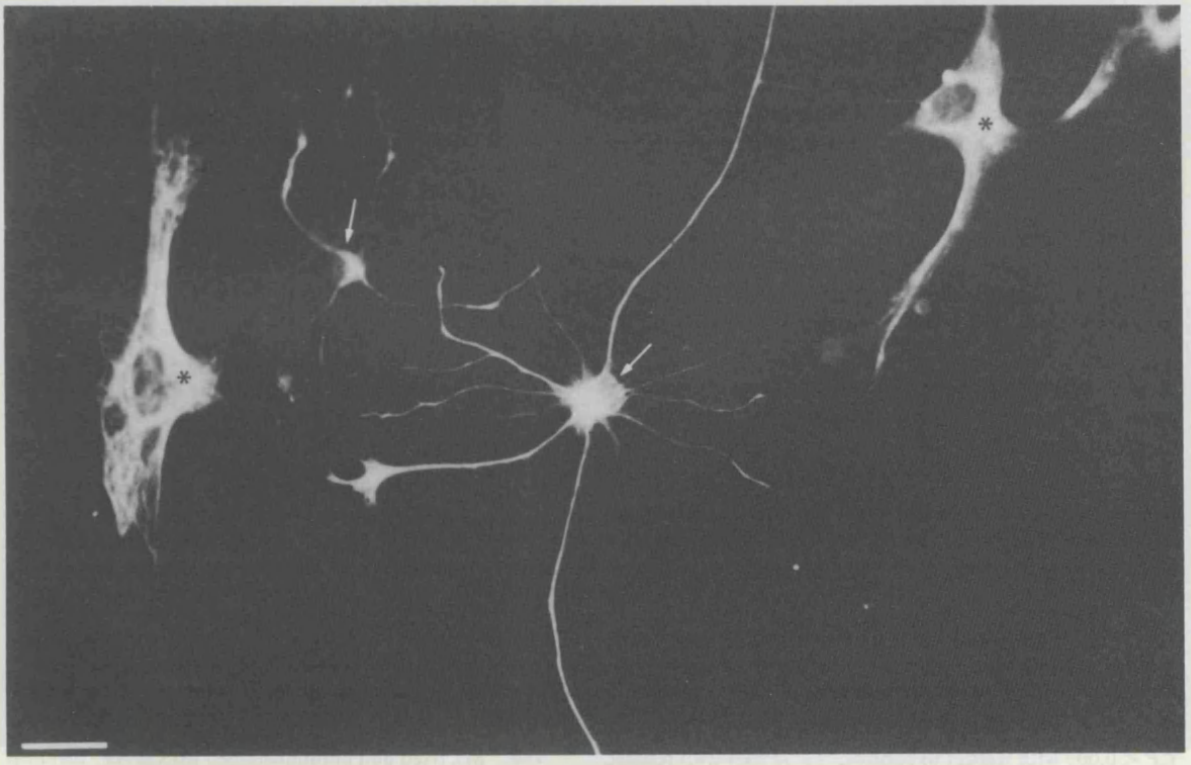

Fig. 1. Immunofluorescence micrograph showing GFAP-positive cells in a control culture (no additions) after three days *in vitro*. Two type 1 astrocytes (asterisks) and two type 2 astrocytes (arrows) are visible. Scale bar = 25 μm.

Table 1. Effects of adenosine deaminase, 8-PSPT and suramin on the number of glial cells in striatal cultures

Agent	No. of GFAP-positive cells (% of control cell number set to 100%)
Adenosine deaminase, 2 I.U./ml	100 ± 0.6
8-PSPT, 10^{-5} M	95 ± 7
Suramin, 10^{-5} M	94 ± 5

Rat striatal cells were cultured for three days in the absence or presence of the indicated agents, fixed and immunostained for GFAP. Results represent mean ± S.E. of six determinations/condition.

adenosine by degradation to inosine, or of purinoceptor antagonists such as 8-PSPT or suramin. No differences in the number of GFAP-positive cells with respect to control were detected in cultures grown in the presence of either agent (Table 1), ruling out any role of endogenous purines in regulation of cell number under our culture conditions. Similarly, no change in the number of PGP 9.5-positive cells with respect to control was induced by the same agents (data not shown).

In a second set of experiments, the effects of "exogenous" purine analogues on the number of GFAP- and PGP 9.5-positive cells were studied by growing cultures in the presence of 2-CA or $\alpha\beta$-meATP. The effects of these two agonists on the number of GFAP-positive cells maintained in culture for two, three or four days are shown in Fig. 2. Exposure of cultures to the P2 purinoceptor agonist $\alpha\beta$-meATP increased the number of astroglial cells, whereas 2-CA induced the opposite effect on the same parameter. Both the $\alpha\beta$-meATP-induced increase and the 2-CA-induced reduction of GFAP-positive cell number were maximal after three days in culture; all subsequent studies were therefore performed at this time point.

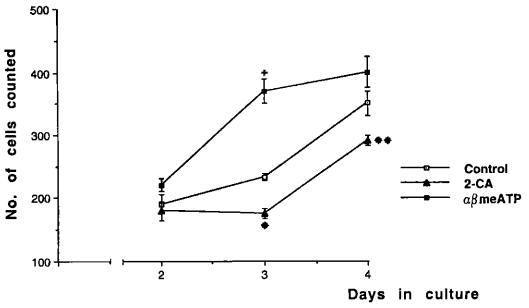

Fig. 2. Effects of $\alpha\beta$-meATP and 2-CA on the number of GFAP-positive cells at different days in culture. Cultures were grown in complete medium in the absence (control) or presence of either purine analogue (10^{-5} M). After two, three or four days in culture, cells were fixed, immunostained for GFAP immunoreactivity and counted. Results represent the mean ± S.E. of triplicate determinations. †: $P < 0.003$ with respect to control; *: $P < 0.04$ with respect to control; **: $P < 0.02$ with respect to control, Student's t-test. Similar results were obtained in three independent experiments.

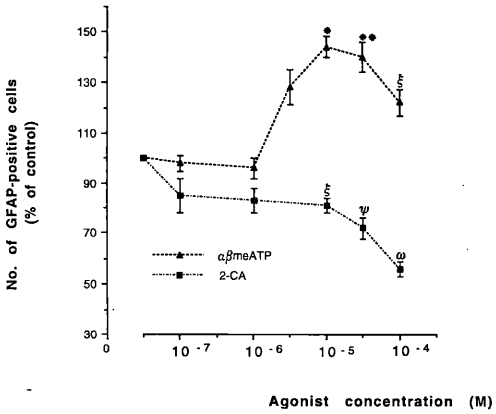

Fig. 3. Effect of graded concentrations of $\alpha\beta$-meATP and 2-CA on the number of GFAP-positive cells in culture. Cells were grown in the absence (control) or presence of the indicated concentrations of either purine analogue. After three days, cells were fixed, immunostained for GFAP and counted. Results are expressed as percentage of control cell number set to 100%. Each point represents the mean ± S.E. of a minimum of nine to a maximum of 21 replicates/condition. *: $P < 0.007$; **: $P < 0.003$; ξ: $P < 0.05$; ψ: $P < 0.002$; ω: $P < 0.0001$ with respect to control.

The dose dependency of the $\alpha\beta$-meATP- and 2-CA-induced effects are shown in Fig. 3. Increases in the number of GFAP-positive cells were detected with micromolar $\alpha\beta$-meATP concentrations; the effect was maximal at 10^{-5} M and still maintained at higher concentrations up to 10^{-4} M. Conversely, significant and dose-dependent decreases of astroglial cell number ranging from 10 to 45% were observed with 10^{-5}–10^{-4} M 2-CA. Agonist concentrations over 10^{-4} M were not tested because of the possibility of non-specific toxic effects.

The increases or decreases in the number of type 1 and type 2 astrocytes in response to $\alpha\beta$-meATP or 2-CA were comparable (Table 2), suggesting that purine analogues affect both astroglial cell types in culture similarly.

The $\alpha\beta$-meATP-induced increase of astroglial cell number was completely blocked by the concomitant exposure of cultures to the P2 purinoceptor antagonist suramin (Table 3); in contrast, 2-CA-induced reduction of GFAP-positive cell number could not be

Table 2. Effects of $\alpha\beta$-meATP and 2-CA on type 1 and type 2 astrocytes in rat striatal cultures

Condition	Type 1 astrocytes (mean ± S.E.)	Type 2 astrocytes (mean ± S.E.)
Control	395 ± 12	71 ± 9
$\alpha\beta$-meATP	525 ± 35 (+33%)	103 ± 15 (+45%)
2-CA	227 ± 15 (−43%)	31 ± 6 (−57%)

Rat striatal cells were cultured for three days in the absence (control) or presence of $\alpha\beta$-meATP (10^{-5} M) or 2-CA (10^{-4} M), fixed and immunostained for GFAP. Identical coverslip areas were scored with a fluorescence microscope for all the experimental groups. Results represent the mean ± S.E. of six replicates.

Table 3. Effect of suramin and 8-PSPT on $\alpha\beta$-meATP and 2-CA-induced effects on GFAP-positive cells in rat striatal cultures

Condition	No. of GFAP-positive cells (Mean ± S.E.)
Control	359 ± 13
$\alpha\beta$-meATP, 10^{-5} M	448 ± 2*
$\alpha\beta$-meATP, 10^{-5} M + Suramin, 10^{-5} M	362 ± 21**
2-CA, 10^{-4} M	185 ± 19***
2-CA, 10^{-4} M + 8-PSPT, 10^{-5} M	220 ± 23****

Rat striatal cells were cultured for three days in the absence or presence of the agents indicated, fixed, immunostained for GFAP and scored under a fluorescence microscope. Results represent the number of positive cells counted in identical areas for six experimental samples/condition.

*$P < 0.0001$ with respect to control; **$P < 0.01$ with respect to $\alpha\beta$-meATP, and $P = 0.9$ with respect to control; ***$P < 0.001$ with respect to control; ****$P < 0.03$ with respect to control, and $P = 0.22$ with respect to 2-CA.

reversed by the P1 antagonist 8-PSPT (Table 3). In experiments pooled in Table 3, 10^{-5} M 8-PSPT was tested against 10^{-4} M 2-CA; however, lack of antagonism was also found when equimolar concentrations (either 10^{-5} M or 5×10^{-5} M) of agonist and antagonist were used (data not shown).

Table 4. Lack of effects of $\alpha\beta$-meATP and 2-CA on neuronal cell number in striatal cultures

Condition	No. of PGP 9.5-positive cells (mean ± S.D.)
Control	290 ± 36
$\alpha\beta$-meATP, 10^{-5} M	277 ± 27
2-CA, 10^{-5} M	283 ± 2

Rat striatal cells were cultured for three days in the absence or presence of agonists, fixed and immunostained for PGP 9.5, and scored under a fluorescence microscope. Results represent the number of positive cells counted in identical areas for six experimental samples/condition.

Finally, purine analogue-induced effects were selective for astroglial cells, since at concentrations which were effective in modulating the number of GFAP-positive cells in culture, neither the P1 nor the P2 purinoceptor agonist significantly affected the number of neurons, as determined by immunofluorescence for the selective neuronal marker PGP 9.5 (Table 4).

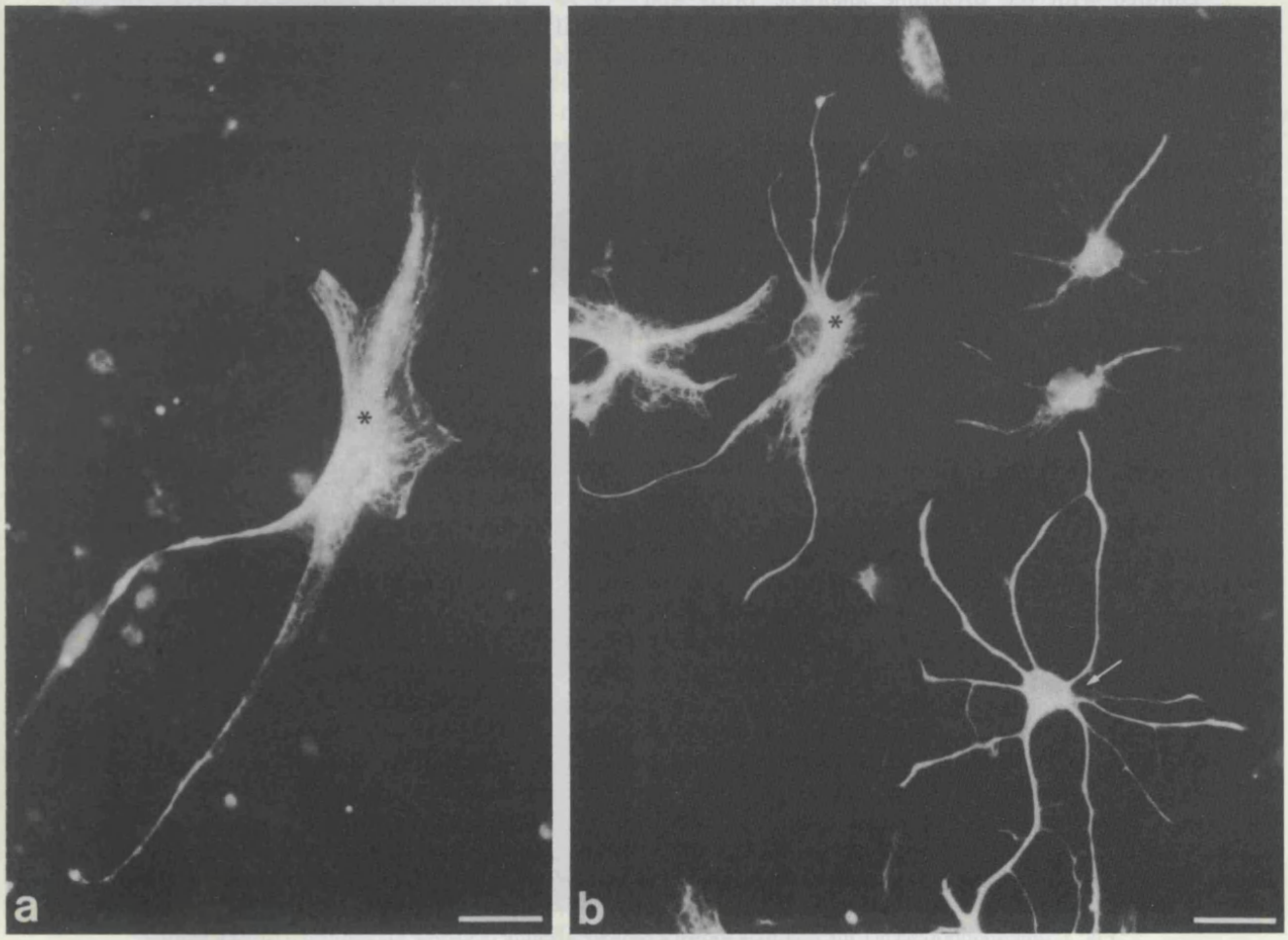

Fig. 4. Immunofluorescence micrographs showing GFAP-positive cells in cultures grown for three days in the presence of 10^{-5} M $\alpha\beta$-meATP. Type 1 astrocytes show stellation (asterisks in a and b). Type 2 astrocytes show increased branching (arrow in b). Scale bars = 25 μm.

Effects of purine analogues on the morphology of cultured astrocytes

Besides changes in cell number, purinoceptor agonists also seemed to induce morphological changes of glial cells in culture. In particular, as shown in Fig. 4, "stellation" of type 1 astrocytes (Fig. 4a) and increased "branching" of type 2 astrocytes (Fig. 4b) were observed in $\alpha\beta$-meATP-exposed cultures. Conversely, 2-CA induced "elongation" of astroglial cells which resembled the morphological changes induced by cAMP analogues in established cell lines.[16] Occasionally, in $\alpha\beta$-meATP-treated cultures, dense colonies of GFAP-positive cells could be detected.

Effects of purine analogues on bromodeoxyuridine incorporation into nuclei

The opposite modulation of astroglial cell number by P1 and P2 purinoceptor agonists could be due to effects on DNA synthesis and cell proliferation and/or to effects on cell survival in culture. To better understand the mechanisms underlying $\alpha\beta$-meATP- and 2-CA-mediated effects, we performed double-labelling experiments in which astroglial cells grown in the presence of either purine analogue were also cultured with the thymidine analogue BrdU and thereafter processed for anti-GFAP and anti-BrdU immunostaining visualized with fluorescein and rhodamine conjugates, respectively. As shown in Fig. 5, double-labelled cells showed GFAP immunolabelling in the cytoplasm and BrdU immunolabelling in the nuclei. A labelling index was obtained by calculating the ratio between the number of double-labelled cells and the total number of GFAP-positive cells counted.

This ratio was found to be changed in cultures grown in the presence of purine analogues (Table 5). For example, the $\alpha\beta$-meATP-induced increase in the number of GFAP-positive cells was accompanied by a significant increase in the number of double-labelled cells, which lead to a significant increase of ratio (0.58 ± 0.06 compared to 0.39 ± 0.03 in control cells). A similar effect was induced by 2-CA: in fact, although (consistent with data reported in Figs 2, 3 and Tables 2, 3) the number of GFAP-positive cells was significantly decreased with respect to GFAP-positive cells in control cultures, there were no changes in the number of double-labelled cells, which resulted in a significant increase of ratio (0.54 ± 0.04 with respect to 0.39 ± 0.03 in control cells).

These results were confirmed in other experiments where $\alpha\beta$-meATP- or 2-CA-treated cells were independently compared to controls. At a concentration of 10^{-5} M, $\alpha\beta$-meATP induced an increase of ratio equal to 145 ± 6 (percentage of control ratio set to 100%, mean $\pm$ S.E., $n = 12$, $P < 0.0001$), which was

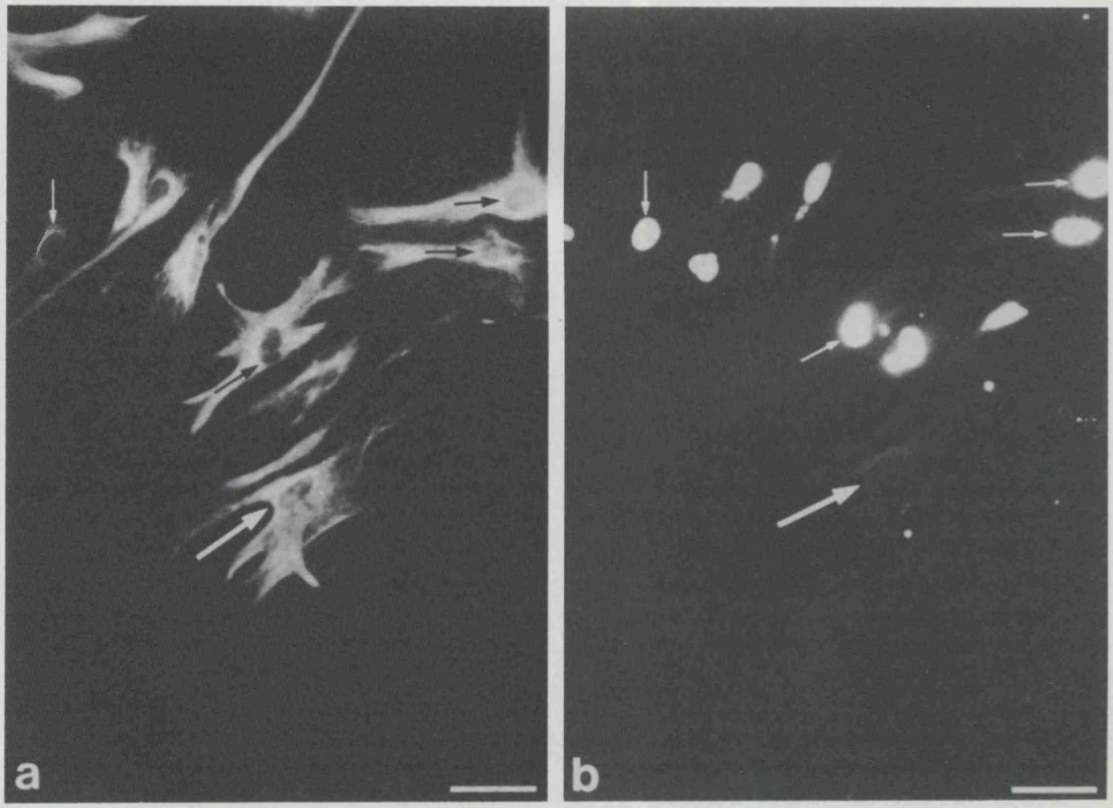

Fig. 5. Immunofluorescence micrographs showing double-labelling of GFAP (a) and BrdU (b) in a control culture after three days *in vitro*. Most of the GFAP-positive cells in the micrograph are also positive for BrdU (examples labelled with small arrows). An example of a GFAP-positive cell which has not incorporated BrdU is indicated by large arrows. Scale bars = 50 μm.

Table 5. Effects of $\alpha\beta$-meATP and 2-CA on BrdU incorporation into the nuclei of GFAP-positive cells in striatal cultures

	1. No. of GFAP-positive cells	2. No. of double-labelled cells	3. Ratio (2/1)
Control	233 ± 11	93 ± 9	0.39 ± 0.03
$\alpha\beta$-meATP	304 ± 16*	177 ± 13***	0.58 ± 0.06†
2-CA	155 ± 16**	85 ± 10****	0.54 ± 0.04‡

Cells were cultured in the presence of 10^{-5} M $\alpha\beta$-meATP or 10^{-4} M 2-CA as indicated. At day 2, 10 μM BrdU was added. After an additional 24 h (day 3), cells were fixed and double-immunostained with antibodies to GFAP and BrdU. Cells were then scored with a fluorescence microscope to visualize GFAP with fluorescein optics and BrdU-positive nuclei with rhodamine optics. Results are the mean $\pm$ S.E. of six replicates.

*$P < 0.05$ with respect to corresponding control; **$P < 0.03$ with respect to corresponding control; ***$P < 0.02$ with respect to corresponding control; ****$P = 0.65$ with respect to corresponding control; †$P < 0.01$ with respect to corresponding control; ‡$P < 0.003$ with respect to corresponding control.

quantitatively similar to the percentage increase of cell number induced by the same agonist concentration. At a concentration of 5×10^{-5} M, 2-CA induced an increase in ratio equal to 132 ± 4 (percentage of control ratio, which was set to 100%, mean $\pm$ S.E., $n = 10$, $P < 0.0001$), whereas at the same agonist concentration, the mean decrease of the number of GFAP-positive cells was $-45 \pm 4\%$. Besides being ineffective in antagonizing 2-CA-induced reduction of cell number (Table 3), 8-PSPT did not antagonize 2-CA-induced increase of the labelling index (data not shown).

In order to understand whether, for the 2-CA effect, there was any correlation between these two parameters (i.e. between the reduction of glial cell number and the increase of the labelling index), we performed double-labelling experiments in cultures grown in the presence of a range of 2-CA concentrations (10^{-7}–5×10^{-5} M). As shown in Fig. 6, significant decreases of cell number could be demonstrated at 2-CA concentrations which did not significantly affect the proliferation ratio (e.g. 10^{-6} M), apparently suggesting that the two effects are not related to each other.

DISCUSSION

In this study, we have investigated the possible modulation of astroglial cell proliferation by purine derivatives in primary cultures of mammalian

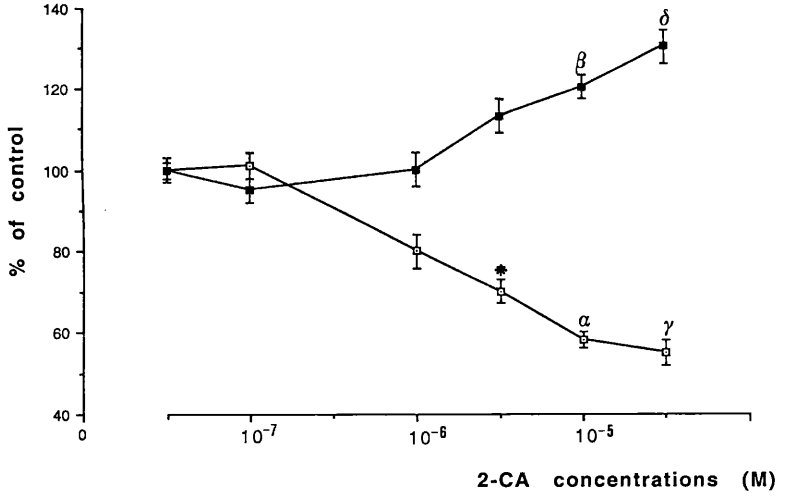

Fig. 6. Effect of graded concentrations of 2-CA on the number of GFAP-positive cells (□) and on the labelling index (■) in striatal cultures. Cells were grown for three days in the presence of the indicated concentrations of 2-CA. After two days, 10 μM BrdU was added for 24 h, cultures were then fixed and processed for anti-BrdU labelling followed by anti-GFAP immunostaining. At each agonist concentration, the labelling index was calculated as the ratio between the number of double-labelled cells and the total number of GFAP-positive cells. Results are reported as percentage of corresponding control set to 100%. Each point represents the mean $\pm$ S.E. of a minimum of six to a maximum of 12 replicates/condition. *$P < 0.03$; $^{\alpha}P < 0.003$; $^{\beta}P < 0.05$; $^{\gamma}P < 0.0001$; $^{\delta}P < 0.03$ with respect to corresponding control, Student's t-test.

striatum containing both neurons and glial cells. Our results indicate that cultured astrocytes are responsive to exogenously added hydrolysis-resistant analogues of adenosine and ATP, which induced a reduction and an increase, respectively, in the number of GFAP-positive cells. To our knowledge, this is the first demonstration of an opposite modulation exerted by these purine derivatives on astrocytic functions in the mammalian CNS.

In particular, exposure to the ATP/P2 purinoceptor agonist $\alpha\beta$-meATP resulted in a significant increase in the number of GFAP-positive cells in culture, which suggests a mathematical correlation with the concomitantly increased BrdU incorporation into DNA. These effects were blocked by the P2 purinoceptor antagonist suramin. It can therefore be reasonably concluded that $\alpha\beta$-meATP stimulates mitosis in astrocytes through the activation of a suramin-sensitive P2 purinoceptor, an effect which is directly related to the detected increase of the number of astroglial cells. These results are consistent with previous reports of mitogenic effects induced by ATP on chick astrocytes,[28] 3T3, 3T6 mouse fibroblasts, DDT1-MF2 cells and BALB/MK keranocytes.[15,32] The morphological astrocytic changes observed with $\alpha\beta$-meATP in our study also extend previous preliminary data reported by Norenberg *et al.*,[26] describing marked stellation of astrocytes after exposure to millimolar ATP concentrations.

Conversely, the effects induced by the purine analogue 2-CA, which acts by selectively activating adenosine/P1 purinoceptors,[35] seemed to be more complex and more difficult to interpret. Exposure to 2-CA resulted in an increase in astroglial labelling index which was, however, associated with a reduction in the total number of GFAP-positive cells in culture. There are several possible explanations for this apparent discrepancy. First, as suggested by the apparent lack of correlation between the two measured parameters, 2-CA might exert two different and independent effects on astroglial cells, i.e. a stimulatory effect on proliferation and a concomitant influence on cell adhesion and/or survival, eventually leading to a reduction in cell number. Future studies, focused on the possible effects of 2-CA on cellular factors directly involved in adhesion and cell survival, will determine if this hypothesis is correct.

Alternatively, astroglial subpopulations might exist, which are endowed with different proliferative responses to 2-CA. Recent data suggest that, within the same brain area and even within the same clonal clusters, morphologically similar astrocytes do show marked heterogeneity of immunological phenotype, suggesting that local factors control a wide range of antigen expression in glia.[20] Based on this, different astroglial subpopulations may express adenosine receptors at different levels and consequently respond to 2-CA with different proliferative responses.

In addition, there is evidence for adenosine-induced cell death in some immunological disorders, such as ADA deficiency syndrome.[8] This adenosine-mediated selective cell death affecting primarily T cells and, to a lesser extent, B cells[17] might occur through programmed cell death or apoptosis.[21] On this basis it could be hypothesized that subpopulations of glial cells may display different sensitivities to adenosine-induced cell death. If this latter hypothesis were true, exposure to adenosine analogues could result in selective killing of responsive cells and in a relative increase of the labelling index, actually due to the proliferation of 2-CA-insensitive astroglial cells. This would also be consistent with the growth inhibition reported previously for adenosine in the mammalian embryo[19] and in transformed mouse fibroblasts.[33] In this respect, it might be interesting to note that many apoptotic agents act through increases of intracellular cAMP,[22] which is likely to represent the second messenger utilized by 2-CA in the astroglial cells studied here. Two lines of evidence suggest that cAMP is involved in the actions of 2-CA. First, all the effects induced by the adenosine analogue in our study were detected at agonist concentrations in the high micromolar range, which are likely to activate the lower affinity adenosine P1/A2 purinoceptor which stimulates cAMP formation.[1,35] Secondly, 2-CA induced "elongation" of astrocytes, which is a typical morphological change associated with increases of intracellular cAMP in other cell types.[16] Interestingly, as suggested by the experiments with 8-PSPT, the astroglial adenosine receptor studied here shared the xanthine insensitivity already reported for cAMP-linked adenosine receptors in other cell systems.[2] Confirmation of the involvement of cAMP in 2-CA-mediated effects will come from the results of studies in progress in our laboratories.

It might also be hypothesized that only one of the two effects induced by 2-CA is directly exerted on glial cells, the other one being mediated by neurons. This hypothesis is supported by previous studies on neuron-free astrocytic cultures from chick brain, in which it was demonstrated that analogues of both ATP and adenosine could stimulate [^{3}H]thymidine incorporation into nuclei.[28] In our study, employing a neuron–glia culture system, we were able to demonstrate a similar stimulatory effect of 2-CA on astroglial DNA synthesis; however, in addition to a direct action on astrocytes, 2-CA might concomitantly act on neurons by stimulating the release of some, yet unidentified, substance with secondary independent effects on astroglial cells. In support of this hypothesis, adenosine analogues have been demonstrated to affect a variety of neuronal functions in culture, by favouring neuronal differentiation, by promoting neurite outgrowth and by inducing enzyme activities involved in neurotransmitter release.[1,11,36] Future studies employing neuron-free cultures will clarify this point.

The demonstration that purine derivatives can modulate astroglial cell proliferation has important biological implications. During brain anoxia, huge amounts of purine nucleotides, largely exceeding micromolar concentrations,[12] are released, due to both the loss of membrane integrity and to the hydrolysis of deoxyribonucleic acids from dying cells. Purines released in this way could participate in the initiation and modulation of reactive gliosis, a response which might be beneficial in the healing phase of injured CNS, by walling off areas of the CNS from non-CNS tissue environments following trauma, and by actively monitoring the extracellular molecular and ionic contents.[7] However, due to the rapid onset of the phenomenon, astrogliosis could result in interference with the function of residual neuronal circuits, remyelination and axonal regeneration.[29] In this respect, the effects exerted by adenosine on astroglial cells could be particularly useful in delaying or inhibiting this process, therefore giving neurons and oligodendrocytes the opportunity to re-establish a functional environment and regenerate.[37] It could also be hypothesized that the final outcome of ATP and adenosine effects on glial cells would depend on the relative ratio of these two derivatives, which would in turn depend on the functional state of the ischaemic tissue and on the ratio of conversion of ATP to adenosine. It will therefore be extremely intriguing in future studies to verify the role of these two purine derivatives in *in vivo* models of trauma- and ischaemia-associated hypergliosis.

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