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The role of glia during striatal ontogenesis: Convergence of the major developmental signals on astrocytes

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A mis Padres

For my Parents

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Abbreviations

AMPA	amino-3-hydroxy-5-mthyl-isoxazole-4-proprionic acid
Akt	Protein kinase B
APS	ammonium persulfate
BB	bromophenol blue
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumun
bp	base pair
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
Cy3	cyanine 3
DIV	days *in vitro*
D_1-R	dopamine 1 receptor
D_5-R	dopamine 5 receptor
dsDNA	double strand DNA
E	embryonic day
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid
ERK1/2	extracellular signal- regulated kinase 1/2
FACS	Fluorescence activated cell sorted
FCS	fetal calf serum
FITC	fluoresceine isothiocyanate
GABA	gamma-aminobutyric acid
GAD	glutamine decarboxylase
GFAP	glial fibrillary acidic protein
GLAST	glutamate/ aspartate transporter
GLT-1	glutamate transporter 1
GPe	external segment of the globus pallidus
GPi	internal segment of the globus pallidus
Hepes	N-(2-hydroxyethyl)-piperazine-N'-2 ethane

HPRT	hypoxanthine phosphoribosyl-transferase
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor-I
IP3	phosphatidylinositol-3-phosphate
kDa	kilodalton
L-GLU	L-glutamic acid
MAP-2	microtubule associated protein-2
MEK	mitogen-activated protein kinase or ERK kinase
MEM	minimum essential medium
min	minutes
MAPK	mitogen-activated protein kinase
M-MLV	reverse transcriptase
mRNA	messenger ribonucleic acid
MW	molecular weight
NBM	neurobasal medium
NMDA	N-methyl-D-aspartate
OD	optical density
P	postnatal day
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
RT-PCR	reverse polymerase chain reaction
SDS	sodium dodecyl sulfate
SN	substantia nigra
SNr	substantia nigra pars reticulata
STN	subthalamic nucleus
TAE	TRIS-acetate-EDTA buffer
TBS	TRIS-buffer saline
TEMED	N,N, N, N', -tetramethylethylenediamine
TH	tyroxine hydroxylase
TRIS	Tris (hydroxymethyl)-aminomethane
trkB	tyrosine kinase receptor B

1. INTRODUCTION

The striatum which is a major component of the basal ganglia plays a critical role in the regulation of sensorimotor and psychomotor behaviours as well as in fundamental aspects of attention and learning (in particular reward and punishment) and for the integration of cognitive and emotional responses. Abnormalities in the function of the striatum and its allied nuclei lead to neurological disorders of movement such as Parkinson's and Huntington's diseases and are implicated in drug addiction and major mental disorders including schizophrenia-like states, obsessive-compulsive disorders, and attention deficit hyperactivity disorder (ADHD). During development, the striatum undergoes a complex process that depends on the precise temporal and spatial action of developmental signals which is still not fully understood. This study attempts to contribute to a better understanding of the events involved in the striatum during ontogeny.

1.1. The striatum: a major component of the basal ganglia

The striatum consists of two subdivisions: the dorsal and the ventral striatum. The dorsal striatum, referred to as striatum, comprises the putamen and caudate nuclei which are incompletely separated by the internal capsule. The main afferents of the striatum arise from the cerebral cortex, particularly from the frontal lobe. These corticostriatal projections are mainly excitatory using the neurotransmitter glutamate. The striatum also receives important afferent projections from the substantia nigra pars compacta (nigrostriatal projections). These projections use the neurotransmitter dopamine. Minor projections arise also from the intralaminar nuclei of the thalamus and from the pedunculopontine nucleus. By contrast, the ventral striatum which comprises the nucleus accumbens, the olfactory tubercle, and the ventromedial parts of the putamen and caudate nuclei, receives afferents from areas that do not project to the dorsal striatum, notably the temporal, limbic, and orbitofrontal cortical areas as well as the basolateral amygdala.

At the cellular level, the striatum is organized into two compartments, such as striosome (patches) and matrix that are vividly demarcated by their differential expression of a variety of compounds ranging from neurotransmitters, neuropeptides, and their receptors (Graybiel, 1990; Holt et al, 1997). In particular in the mature striatum, striosomes are characterized by low levels of acetylcholinesterase (AChE) and calbindin-D28K, whereas the matrix expresses high levels of these compounds. This striosome-matrix

compartmentation represents an architecture that largely defines the "input-output" connections of the striatum. The matrix receives the striatal afferents mainly related to sensorimotor processing. By contrast, striosomes (including the entire ventral striatum) tend to receive inputs from neural structures affiliated with the limbic system, particularly the amygdala.

Like the dorsal striatum, the ventral striatum contains regions of tissue that have distinct chemical compositions and connections (Voorn et al., 1989, Berendse et al., 1992). The presence of multiple compartments in the nucleus accumbens has been well documented; however the precise boundaries of the nucleus accumbens are not easy to define in the human brain (Holt et al., 1997).

The major neuronal population in the striatum, almost 95 % of the total number of striatal cells, is represented by spiny projection neurons, most of them containing the neurotransmitter gamma-aminobutyric acid (GABA) (Kita and Kitai, 1988). GABA is synthesized by converting L-glutamic acid (L-GLU) to GABA by the enzyme glutamic acid decarboxylase (GAD) that occurs in two isoforms, GAD65 and GAD67, encoded by two different genes (Erlander et al., 1991). GABA can be co-localized alternatively with enkephalin and neurotensin or with substance P/ dynorphin (Beckstead, 1985). The expression of these neuropeptides depends on the pallidal segment to which the neurons project. The remaining 5 % of striatal cells consist of large aspiny interneurons containing the excitatory transmitter acetylcholine and smaller cells that contain somatostatin, neuropeptide Y or nitric oxide synthetase (reviewed by DeLong, 1995).

The complex cytoarchitecture of the striatum is reflected but not functionally related in the different projection pathways. In primates, the striatal projections of the medium spiny neurons are organized into two pathways; the "direct" and the "indirect" pathway. These two pathways connect and integrate functions between the basal ganglia nuclei, various regions of the cerebral cortex, and the thalamus for releasing or inhibiting movement. The "direct pathway" connects the striatum to the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulate (SNr) which are the two output nuclei of the basal ganglia and project to the brain stem and the thalamus. In the "indirect pathway" fibres of the striatum are connected to the external segment of the globus pallidus (GPe) (tonic inhibitory output) and from there to the subthalamic nucleus (STN) which itself project to the GPi. This circuits leads to an increase of the inhibitory output of the GPi. Activation of the direct pathway disinhibits the thalamus, thereby

increasing thalamocortical activity, whereas activation of the indirect pathway further inhibits thalamocortical neurons. As a result, activation of the direct pathway facilitates movement, whereas activation of the indirect pathway inhibits movement. These two striatal output pathways are affected differently by the dopaminergic projections from the substantia nigra pars compacta to the striatum. Striatal target neurons within the direct pathway have dopamine D1-like receptors (D_1 and D_5 receptors) that facilitate transmission, while those that belong to the indirect pathway express dopamine D2-like receptors that reduce transmission. However, it has also been suggested that D1-like receptors are significantly expressed in neurons of the indirect pathway (Surmeier et al 1996; Aizman et al 2000). The activity of D1- and D2-like receptors in these two output pathways also differentially controls neuropeptide expression in these efferent systems. Striatal expression of dynorphin and substance P is primarily associated with the D1-enriched neurons, whereas enkephalin expression is associated with the D2-enriched neurons (Graybiel, 1990).

The failure of this basal ganglia circuitry results in severe pathologies. The loss of striatal medium-spiny neurons leads to hyperkinetic movement disorder such as Huntington's disease, and the dopamine deafferentation of the striatum leads to a hypokinetic state comparable to that seen after chemical lesions of nigral dopaminergic projections and in Parkinson's disease.

1.2. Striatal development

1.2.1. Differentiation and compartmentation

During embryogenesis, the striatum is thought to be generated from two prominent elevations in the ventral telencephalon, the lateral and medial ganglionic eminence (LGE and MGE, respectively). In rats, the development of the striatum has been found to extend approximately from embryonic day 11 (E11) to the third week of postnatal life. The neurons within the patch and matrix compartments become postmitotic and make connections with the substantia nigra (SN) at distinct and sequential developmental time points. Patch neurons which make up 15 % of total striatal volume become posmitotic between embryonic days 12 (E12) and 15 (E15) and make a striatonigral connection prenatally (Gerfen et al, 1987; Fishell and van der Kooy, 1987; 1991). In contrast, matrix

neurons become postmitotic between E17 and E20 and do not form efferent connection to the SN until the first postnatal week (van der Kooy and Fishell, 1987).

The dopamine containing mesostriatal afferents reach the striatal anlage as early as E14 (Moon Edley and Herkenham, 1984; Guennoun and Bloch, 1991; Schambra et al., 1994) and first innervate the differentiated zone of the ganglionic eminence with a homogeneous pattern. At E18 of rat embryogenesis, however, the mesostriatal innervation becomes heterogeneous. Patchy zones and a lateral rim emerge with high number of tyrosine hydroxylase (TH)-immunoreactive neurons. These zones are transient and are named “dopamine islands”. Their emergence during striatal development is coincident with the developing striosomes and several studies have tested the possibility that dopamine-containing mesostriatal afferents may have an impact on the formation of the striatal compartments. Pharmacological blockade and embryonic lesions of mesostriatal afferents revealed that neither dopamine-mediated activity nor the mesostriatal afferents themselves are required for the establishment of compartments. However, the maintenance of striosome-matrix compartmentation is affected by striatal interconnections with the midbrain since early postnatal lesions of the substantia nigra result in disintegration of striatal compartmentation. Moreover, the early projection into the substantia nigra of striosomal cells, and also of a small population of matrix cells, has been reported to be correlated with cell survival during the cell death period that occurs in the striatum during the first postnatal week. These data suggest that the early innervation of the substantia nigra by striatal neurons may be important for striatal cell survival (reviewed by Liu and Graybiel, 1999).

GABA-immunoreactive neurons in the striatal anlage were not seen prior to E16 (Lauder et al., 1986; Meier and Jorgensen, 1986). An important aspect of the differentiation of GABAergic neurons is the developmental expression schedule of the striatal marker enzyme GAD. *In vivo* experiments have shown that the late prenatal period of the rat brain is critical with regard to the regulation of striatal GAD expression (Behar et al., 1994; Küppers et al., 2000). As indicated above, GAD is expressed in two isoforms, GAD65 and GAD67 (Erlander, 1991). The latter occurs in an embryonic and an adult splice variant. In the rat, between E14 and E17 both embryonic and adult splice variants of GAD67 are expressed in increasing amounts as proliferation of striatal precursors is in progress and postmitotic cells begin to differentiate into GABAergic neurons. Thereafter, the embryonic GAD67 isoform disappeared, whereas GAD65 remains unchanged

postnatally (Küppers et al., 2000). This correlates precisely with the time period for massive increase of nigrostriatal fibers (Specht et al., 1981), establishment of mature synapses (Trips et al., 1998), and acquisition of functional coupling of dopaminergic receptors possibly leading to activation of immediate early genes (Jung et al., 1996), suggesting that dopamine signalling might be involved in the regulation of the developmental expression and splicing of the GAD family mRNA.

Expression of dopamine receptors is first detected in the ganglionic eminence (striatal anlage) as early as E14 at which time the striatal primordium is starting to form. This raises the possibility that activation of dopamine receptors may regulate neurogenetic events leading to the generation of striatal cells (Liu and Graybiel, 1999).

The metabotropic (mGluR) and ionotropic glutamate receptors display distinct developmental expression patterns. Among the metabotropic receptors, the group I mGluR are particularly highly expressed during early striatal development and play an important role during the development of the nigrostriatal pathway (Plenz and Kitai, 1998). The ionotropic glutamate receptors are subdivided into N-methyl- D-aspartate (NMDA) and non-NMDA [α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionic acid (AMPA) and kainate (KA)] receptors. In particular, NR1, Nr2A and NR2B are the major NMDA receptor subunits expressed during development with NR1 and NR2B subunits being the predominant subtypes in the prenatal striatum (Monyer et al., 1994). Glutamate receptor diversity and differences in ontogenetic expression patterns may play an important role in determining the wide variety of responses to glutamate in the developing striatum (Monyer et al., 1994; LoTurco et al., 1995).

1.2.2. Developmental signals

The developmental fate of nerve cells is determined partly by lineage and partly by the environment. Developmental processes subject to regulation by environmental signals include the growth of dendrites and axons as well as synaptogenesis. Growth regulatory signals may be provided by the extracellular matrix or by way of synaptic or paracrine communication either among neurons or between glia and neurons. The development of striatal GABAergic neurons takes place perinatally and is regulated by the coordinated action of several ontogenic signals such as neurotransmitters, growth factors, peptides, and hormones (Liu and Graybiel, 1999).

1.2.2.1. Neurotransmitters

There is increasing evidence that classical neurotransmitters, typically mediating intercellular communication in adult neuronal circuits, also act as signals that influence neuronal differentiation during ontogenesis. These developmental actions involve activation of receptors and signal transduction mechanisms similar to those used in the adult brain (Lauder and Liu, 1994). Among the neurotransmitters that appear to exert prominent developmental roles are dopamine and glutamate.

Dopamine has been found to regulate axon outgrowth from target neurons in positive or negative ways. It increases neurite elongation in embryonic rat cortical neurons via activation of D2-like receptors (Todd, 1992) but inhibits growth cone motility and neurite outgrowth in avian retinal neurons through stimulation of D1-like receptors (Lankford et al., 1988). Furthermore, dopamine suppresses regenerative neurite outgrowth from invertebrate neurons (McCobb et al., 1988). This diversity of actions suggests that receptors and/or signal transduction pathways involved in dopaminergic regulation of neurite growth differ among species and neural systems (Lipton and Kater, 1989; Schwartz, 1992; Lauder, 1993).

Differentiative signals provided by dopamine are particular relevant for the developing striatum. A developmental role for dopamine is suggested by the temporal coincidence of the establishment of functional nigrostriatal dopaminergic synaptic connections with the onset of GABAergic cell differentiation and pattern formation within the striatal complex (Specht et al., 1981; Fishell and Van de Kooy, 1987; Lauder et al., 1986). The dopaminergic input from the midbrain appears to be required for normal development and/or maintenance of the organization and function of the striatum as shown by different experimental approaches including dopaminergic deafferentiation (Young et al., 1986; Vernier et al., 1988; Gerfen et al., 1991; Kerkerian et al., 1995; van der Kooy, 1996) as well as disruption of genes encoding enzymes or receptors involved in dopaminergic transmission (Xu et al., 1994; Drago et al., 1994; Zhou and Palmiter, 1995). The gene knockout studies collectively point out that dopamine signalling is important for regulating neuropeptide expression in the striatum which is thought to play a role in the establishment of the striosome/ matrix organization of the striatum (Liu and Graybiel, 1999).

In vitro studies have shown that dopamine promotes striatal cell survival (Jung and Bennett, 1996) and the stimulation of neurite growth and arborization of GABAergic

neurons (Schmidt et al., 1996). Moreover, dopamine increases the expression of BDNF in striatal neurons (Küppers and Beyer, 2001) and modulates the expression and splicing of the two subtypes of GABA-synthesizing enzymes, GAD65/67 (Küppers et al., 2000). These effects are mediated by selective D1-like receptor stimulation coupled to the activation of the cAMP/ protein kinase A pathway that converges on the phosphorylation of cAMP-response element binding protein (CREB) (Schmidt et al., 1998; Küppers and Beyer, 2001). It has also been proposed that differential expression and phosphorylation of CREB by dopamine and calcium may be part of the mechanism by which compartmental phenotypes are acquired in the striatum during development (Liu and Graybiel, 1996; 1998). In line with the assumption of a dopaminergic modulation of striatal development are observations which have shown that dopamine can stimulate the expression of its own receptors, in particular the D1-like receptor, in striatal target cells during the perinatal period (Frohna et al., 1995).

Glutamate, the most important excitatory transmitter (Watkins, 2000), is excitotoxic at high concentrations and implicated in CNS pathology (Choi, 1988). Increasing evidence suggests that glutamate plays also important roles during brain development. Among them, the proliferation of forebrain neuronal precursors has been indicated by recent studies (Lo Turco et al., 1995; Sadikot et al., 1998; Haydar et al., 2000). Glutamate is present in the telencephalic germinal zones during embryogenesis and it exerts neurogenic effects that vary with receptor subtype (Blanton and Kriegstein, 1991; Behar et al., 1999). In the ventral telencephalon glutamate promotes proliferation of striatal neuronal progenitors by an NMDA receptor-dependent mechanism (Luk et al., 2003). In contrast, the proliferation of cortical progenitors derived from the dorsal telencephalon is regulated by activation of AMPA-KA, but not NMDA receptors (Blanton and Kriegstein, 1991). These heterogeneous responses to glutamate in proliferation of neuronal progenitors suggest an important role of this neurotransmitter in the generation of neuronal diversity in the dorsal and ventral forebrain (Luk et al., 2003).

Besides its modulatory role in proliferation glutamate also regulates migration, survival, differentiation, and neuritogenesis of neurons (Mattson and Kater, 1989; Rossi and Slater, 1993; Lo Turco et al., 1995; Rakic and Komuro, 1995; Behar et al., 1996; Bhave and Hoffman, 1997; Dammerman and Kriegstein, 2000). This view is supported by the following findings (review by Meier et al., 1991): 1) during early stages of differentiation, neurons transiently express supersensitive glutamate receptors; 2)

glutamate receptors are present before synapses become functionally active, and 3) the vulnerability of neurons to the toxicity of glutamate agonists changes during development, depending on the type of neuron and glutamate receptor involved. Furthermore, recent evidences have shown that glutamate released from cortical afferents plays an important "trophic" role for the development of the nigrostriatal dopamine pathway by acting on group I mGluRs (Plenz and Kitai, 1998). On the other hand glutamate, by acting on NMDA receptors, influences striatal synapse density (Butler et al., 1998). Taken together, these evidences indicate that glutamate and its diverse receptors have a strong influence on the development of the striatum.

1.2.2.2. Neurotrophins

The neurotrophins which include nerve growth factor (NGF), neurotrophin 3 (NT-3) neurotrophin 4/5 (NT-4/5), and brain-derived neurotrophic factor (BDNF) are a family of small proteins that influence neural development by regulating the proliferation of neural precursors and enhancing the survival and differentiation of specific sets of neurons (Cabelli et al., 1996). In the striatum, only a relatively small population of striatal cholinergic neurons have been shown to be responsive to NGF and express tyrosine kinase A receptors (trkA), whereas BDNF represents a potent neurotrophic factor able to promote survival and phenotypic differentiation of most developing striatal neurons (Jones et al., 1994; Mizuno et al., 1994; Widmer et al., 1994; Ventimiglia et al., 1995; Ivkovic et al, 1997; Gratacos et al., 2001). In striatal cell cultures, BDNF promotes differentiation of GABAergic neurons by increasing the cellular GABA content, mainly resulting from upregulation of GAD67 and the neuronal GABA transporter, GAT-1 (Mizuno et al., 1994). BDNF also promotes the number of calbindin-immunoreactive neurons and parvalbumin-immunoreactive neurons, and the expression of neuropeptide Y and somatostatin (Ventimiglia et al., 1995). Null mutation of the BDNF gene also results in reduction of calbindin-D28K, parvalbumin, and dopamine- and cyclic adenosine monophosphate-regulated phosphoprotein 32 (DARPP-32) expressions in the striatum (Jones et al., 1994; Ivkovic et al., 1997). Since BDNF has been shown to regulate expression of phenotypic markers of neurons during development, the trkB receptor may influence the induction of specific phenotypes and in this way play a role in establishing innervation patterns. Analysis of the developmental expression of trkB mRNA has shown that trkB transcripts are uniformly expressed from E16 to E18 in the striatal anlage, and then increase in the late

prenatal period followed by a gradual decline to adult levels. Moreover, on E19 trkB-immunoreactive cells and fibers localize to patches, which co-localize with patchy dopamine fibers and glutamate receptors. This spatiotemporal concentration of trkB receptor, DA innervation and glutamate receptor on neurons of the developing striatal patches indicates a convergence of neurotrophins and afferent innervation that may act to regulate the phenotypic differentiation of patch versus matrix cells in the striatum (Costantini et al., 1999).

It is also of particular interest that adult striatal neurons express little BDNF mRNA, but nevertheless the striatum contains high levels of BDNF immunoreactivity associated with nerve terminals. Moreover, lesions of striatal afferents in adults results in a dramatic decrease of striatal BDNF proteins levels (Altar et al., 1997). As BDNF mRNA is expressed in the dopamine-containing neurons of substantia nigra pars compacta, striatal BDNF is thus likely to be derived from afferents including dopamine-containing mesosotriatal fibres (Altar et al., 1997). The possibility that BDNF anterogradely transported in mesostriatal afferents may affect the development of striatal neurons remains to be clarified.

1.3. Interaction between developmental signals

The development of the striatum represents a complex process that depends on the timely and spatially coordinated interaction of the above mentioned developmental signals. It deserves particular attention that the developmental effects of the neurotrophin BDNF and dopamine share many similarities. Moreover, there is also a strong coincidence between the developmental expression pattern of BDNF and the onset and establishment of functional dopaminergic synapses in the striatum (Reisert et al., 1990; Schambra et al., 1994; Küppers and Beyer, 2001). It is, however not clear at present whether these factors act synergistically. Recent observations have reported a complex scenario of gene expression induced by dopamine in the striatum (Berke et al., 1998; Fauchey et al., 2000). Since previous studies have shown that dopamine is implicated in the control of BDNF expression (Küppers and Beyer, 2001), it must be taken into consideration that some of these developmental effects are not directly attributable to dopamine but might rather be the consequence of an intermediate stimulation by BDNF. The control of BDNF expression by dopamine points to the possibility of an autocrine or paracrine BDNF loop in the striatum which is activated/ initiated by dopaminergic activity (Küppers and Beyer,

2001). Modulation of dopaminergic transmission at the pre- as well as at the post-synaptic level might represent a possible mechanism how BDNF can mediate dopaminergic effects. A recent study has reported an interaction between dopamine and BDNF in the nucleus accumbens where BDNF controls the expression of D_3 receptor and triggers behavioural sensitization (Guillin et al., 2001). However, the role of BDNF in the regulation of the dopaminergic transmission mediated by D1-like receptors, mainly responsible for the dopamine-induced developmental processes, is not yet known.

On the other hand, there is increasing evidence that dopamine modulates the glutamatergic transmission. It has been reported that dopamine can control the efficacy of striatal glutamatergic synaptic inputs to medium spiny neurons during early postnatal development (Tang et al., 2001). There is also indirect evidence from dopamine depletion experiments and pharmacological studies that the activity of dopaminergic transmission contributes to the regulation of glutamate transporters and the enzyme glutamine synthethase in striatal astrocytes (Hazell et al., 1997; Stadlin et al., 1998). Moreover, dopamine can regulate the intracellular trafficking of striatal NMDA glutamate receptors (Dunah and Standaert, 2001) and can differentially modulate the gene expression of AMPA and NMDA receptors in striatal neurons (Lai et al., 2003). Since glutamate also plays an important role in proliferation and differentiation of striatal neurons (Jelitai et al., 2002; Luk et al., 2003) the interactions of dopamine with the glutamatergic system could be important for the proper striatal and cell organization.

1.4. Astroglial cells as growth regulators in the brain

Glial cells have long been thought to play a purely passive role of structural support and maintenance of homeostasis for neurons. Mounting evidences however suggest that they have much more important and dynamic functions becoming major players in the development, survival and functioning of the CNS. There are two categories of glial cells in the CNS: oligodendrocytes and astrocytes. In the embryonic brain glial cells are thought to exist first as radial glial cells that later on become astrocytes or oligodendrocytes. Radial glia acts as a guide for neuronal migration (Rakic, 1971, 2003). In addition, radial glia may also have an instructive role in regional neuronal differentiation. For example, as lateral ganglionic eminence cells migrate along radial glial fibers into the developing striatum, retinoids from these glial cells exert an effect on striatal neuronal differentiation (Toresson

et al., 1999). When neuronal generation and migration are complete, in most regions of the mammalian brain, radial glial cells disappear or transform into astrocytes.

The perceived role of astrocytes in the developing brain has evolved from their providing a passive support mechanism for neurons (Rakic 1971) to their functioning actively in regulating neurite extension and patterning (Steindler et al 1989; Herrup and Silver, 1994). Evidence from both *in vitro* and *in vivo* experimental approaches suggests that astrocytes control neurite pathfinding, thus modulating pattern formation and establishment of neuronal circuits in the developing CNS. Various properties of astrocytes contribute to this function. Astrocytes may release a range of factors comprising cytokines, proteases, and protease inhibitors, which vary depending upon developmental stage and in response to external stimuli (Powell et al., 1997). Astrocytes have on their surface a number of adhesion molecules that can influence axon growth and produce a variety of extracellular matrix molecules that can be permissive or non-permissive to neurite extension. Properly located, combination of permissive and non-permissive cues may guide the neurite to its destination. The spatial organization of restrictive astrocytes can result in the formation of boundaries to neurites and thus may guide neurites by preventing infiltration into specific areas (Steindler et al., 1989, 1990). Examples of these boundaries are found within the developing striatum where patch and matrix boundaries appear to form the mosaic structure (Steindler et al., 1988; Sajin and Steindler, 1994).

Besides of guiding functions, astrocytes promote the differentiation and survival of neurons by synthesizing and secreting neurotrophic factors like interleukin-1, platelet-derived growth factor (PDGF), laminin, basic fibroblast growth factor (bFGF), and neurotrophins (Selak et al., 1985; Davis et al., 1985; Hetier et al., 1988; Houlgatte et al., 1989). In particular, astrocytes in the lateral ganglionic eminences appear to be a major source of retinoids that are known to enhance striatal neuron differentiation (Toresson et al., 1999). Astrocytes can also express neurotrophic factor receptors. It has been shown that astrocytes express the receptor of BDNF, the tyrosine kinase trkB receptor (Climent et al 2000; Küppers and Beyer, 2001). Stimulation of these receptors, increase intracellular Ca++ mobilization and induce glutamate release in astrocytes (Climent et al., 2000; Pascual et al., 2001). This mechanism could be particularly relevant not only for neuronal survival but also for the modulation of synaptic transmission and plasticity.

Due to the proximity of astrocytes to synaptic clefts, they are in prime location for receiving synaptic information via released neurotransmitters. Studies over the past years,

performed mainly in cell cultures, have revealed that astrocytes can express a large variety of neurotransmitters receptors, including receptors for dopamine (Hansson and Rönnback, 1988; Mudrick-Donnon et al., 1993; Zanassi et al., 1999) and glutamate (Kettenmann et al., 1984) which are coupled to various intracellular signalling cascades. Activation of both dopamine and glutamate receptors elevates intracellular Ca++ levels in astrocytes (reviewed by Finkbeiner, 1993). With the aid of ATP signalling, this elevation of astrocytic Ca++ can propagate/ spread to its neighbours in the form of a wave of elevated Ca++. Upon Ca++ oscillations, excitatory amino acids like glutamate and aspartate are released by astrocytes, which can cause sustained modulatory action on neighbouring neurons**.** Studies *in vitro* show that astrocytes via Ca++ waves play a powerful part in the formation and maintenance of synaptic connections between neurons during development (Ullian et al. 2001). The number of synapses a neuron forms may not be entirely intrinsic to the neuron; it may be influenced by extrinsic factors, notably continuous signalling from astrocytes. When astrocytes were excluded from retinal ganglion neuronal cultures, electrophysiological recording revealed little synaptic activity. Adding astrocytes back to the cultures increased synaptic strength through a presynaptic effect causing more neurotransmitter to be released (Pfrieger and Barres, 1997). This view supports the idea that glia cells are involved in synaptic plasticity (Ullian et al., 2001).

Astrocytes may also regulate synaptic transmission by uptake of glutamate from the synaptic cleft via membrane transporters. Glutamate uptake represents an important mechanism to keep physiological concentrations of glutamate in the extracellular space required not only for the normal neuronal function but also for survival. Glutamate uptake by astrocytes is mediated by Na+-independent and dependent systems. However, astrocyte Na+-dependent systems have high affinity for glutamate and are the predominant glutamate uptake mechanism in the CNS. Two of the five high-affinity glutamate transporters identified so far, GLAST and GLT-1, are predominantly located on astrocytes allowing them to be responsible for most of the glutamate uptake (Danbolt 2001). Indeed, glutamate is predominantly converted to glutamine in astrocytes and returned to neurons in order to replenish the presynaptic neurotransmitter pool and help maintain synaptic transmission (Sibson et al., 2001). Several lines of evidence using antisense oligonucleotides and targeted gene disruption showed that GLAST and GLT-1 play a pivotal role in maintaining low extracellular concentrations of glutamate, thereby

protecting neurons against excitotoxicity in vivo (Rothstein et al., 1996; Tanaka et al., 1997; Watase et al., 1998)

As the influence that astrocytes (and the various molecules they produce) have on the development of CNS neurons becomes more evident, it will be important to consider how this information can be exploited to bring about better protection, recovery and/or regeneration of circuits which are destroyed in the adult CNS due to trauma or common neurodegenerative disorders**.** The identification of astroglial mechanisms involved in the regulation of the genesis of neuronal circuitry could elucidate to what extent developmental processes might have an influence on the pathogenesis of neuronal diseases in the adult.

1.14 Aim of the study

The development of the striatum represents a complex mechanism involving the timely coordinated expression of a number of differentiation–regulating signals. Among them, neurotransmitters and growth factors appear to interact to guarantee the proper development and differentiation of striatal cells. There is general agreement that dopamine represents a major developmental signal for the striatum. Previous work in our laboratory has shown that dopamine influences differentiation and synaptic plasticity of GABAergic neurons. However, the mechanisms involved in these processes are not known. Since some of the observed dopaminergic effects revealed latencies from 12 to 24 hrs with respect to their maximal occurrence, it seems conceivable to assume that these effects are mediated-at least partially-indirectly, perhaps involving another cell population and/or other developmental factors. To address this concern, in the first part of this study, we attempted to identify striatal target cells for dopamine analyzing the expression of both members of D1-like receptor family *in vitro* using neuronal and astroglial cultures. Since there is scarce information concerning the ontogenic pattern of these receptors, a previous evaluation of the developmental expression should be performed *in vivo,* too. In the second part, we were particularily interested in the possible role of BDNF in the mediation of dopaminergic effects on the development of striatal GABAergic neurons. BDNF represents a potent neurotrophic factor able to promote survival and phenotypic differentiation of most developing striatal neurons. Moreover, we have recently shown that dopamine is implicated in the control of BDNF expression in the striatum during development. However, little is known about the influence of BDNF on the dopaminergic transmission.

It might be conceivable that BDNF modulates the dopaminergic transmission at the postsynaptic level by modulation of D1-like receptors, mainly responsible of dopamine developmental effects in the striatum. Therefore, we investigated the effects of BDNF on the expression of D1-like receptors in astroglial and neuronal cultures. In the third part of this study, we focused on dopaminergic effects on glutamatergic transmission. Previous reports have shown that glutamate represents an important developmental signal in the striatum. However, there is little known about the modulation of the glutamatergic transmission by dopamine in non-neuronal cells. Since astrocytes are important regulators of the glutamatergic transmission by glutamate uptake, we analyzed specifically the influence of dopamine on glutamate uptake from the synaptic cleft via the two-astroglial membrane transporters (GLT-1 and GLAST). To this end, we examined the expression of GLT-1 and GLAST and we evaluated the glutamate turnover.

2. Materials and Methods

2.1. Substances

Aprotinin	Sigma
Agarose	PeqLab
APS	Sigma
Chloral hydrate	Sigma
BDNF	PrepoTech
B-Mercaptoethanol	Fluka
B27	Gibco
Bromophenol Blue	Sigma
Dopamine	Sigma
Ethidium bromide	Sigma
Fetal calf serum (FCS)	Gibco
Fungizon	Gibco
Glutamine	Gibco
Glutathione	Sigma
HEPES	Serva
Ly294002	Calbiochem
Minimum Essential Medium, (MEM)	Gibco
Neurobasal Medium (NBM)	Gibco
PD98059	Calbiochem
Penicilline-Streptomicin	Gibco
Poly-DL-ornithine	Sigma
PMSF	Merck
TRIS	Merck
SDS	Sigma

2.2 Enzymes

M-MLV reverse transcriptase	Invitrogen
Taq-DNA polymerase	Invitrogen

2.3 Nucleotides

Hexanucleotides Mix, 10x conc.	Boehringer Mannheim
PCR oligonucleotides primers	Interactiva
DNA polymerization mix	Amersham Pharmacia Biotech

2.4. Standards

100bp DNA ladder	Gibco
Mark 12 MW Standard-protein ladder	Novex

2.5. Antibodies

Goat anti-rat D_5-receptor	Alpha Diagnostic
Goat anti-actin	Santa Cruz
Mouse anti- D_5-receptor	Chemicon
Rabbit anti-trkB	BD Biosciences
Rabbit anti-P44/42 MAP kinase	New England Biolabs, Inc
Rabbit anti-Phospho MAP kinase	New England Biolabs, Inc
Rabbit anti-Akt	New England Biolabs, Inc
Rabbit anti-Phospho Akt	New England Biolabs, Inc
Guinea pig anti- GLT-1	Chemicon
Guinea pig anti- GLAST	Chemicon
HRP conjugated anti-mouse	Jackson Immunoresearch
HRP conjugated anti-rabbit	Jackson Immunoresearch
HRP conjugated anti-guinea pig	Jackson Immunoresearch
HRP conjugated anti-goat	Jackson Immunoresearch
Alexa 488 anti- rabbit	Molecular Probes
Cy3 anti mouse	Jackson Immunoresearch

2.6. Specials

BSA™ Protein Assay	Pierce
ECL Western Blotting detection reagents	Amersham
ABI Prism BigDye Ready reaction Kit	Perkin Elmer
QuantiTect™ SYBR® Green PCR Kit	Quiagen
Amplex® Red Glutamic Acid Assay Kit	Molecular Probes

QIAquick Spin columns	Quiagen
Capillaries	Roche

2.7. Equipment

PCR equipment	PCR DNA thermal cycler 480, Perkin Elmer Eppendorf Master Gradient, Eppendorf LightCycler, Roche Molecular Biochemicals
Electrophoresis	Power pack 300, BioRad Sub-CellGT Agarose gel electrophoresis System, BioRad
Electroblotter	Transblot, BioRad
Documentation system	Image Master VDS Software, Pharmacia
Sequencing equipment	ABI Prism 310 Genetic Analyzer, Perkin Elmer
FACS system	BD Biosciences
Fluoroscanner	Ascent FL., Labsystems
Centrifuges	Eppendorf
Film developer	Hyperprocessor, Kodak
Confocal laser scanning microscope	Leica TCS-SP, Bensheim

2.8. Animals

All experiments were carried out with BALB/c mice. Animals were kept in 12 h dark/light cycle and fed a standard pellet diet with water provided *ad libitum*. Animals were mated during a 12 h period, and day 0 of pregnancy was defined as the day after copulation. For tissue preparation, mothers were anesthetized with 1ml/100g bodyweight of 25% chloral hydrate in 0.9% NaCl, and the embryos were taken out and decapitated. Postnatal and adult animals were anesthetized and then decapitated. For the experiments, striatal tissues from E15 and E17 embryos and individuals on P0, P7 and P15 were used.

2.9. Tissue dissection

From embryonic brains the striatal anlage was dissected. For the preparation of postnatal striata, a first coronal cut was made anterior and a second cut posterior to the optic chiasm. The excised region was confined dorsally and laterally by the corpus

callosum, ventrally by the anterior commissure, and medially by the lateral ventricle. Striatal tissues were immediately frozen in liquid nitrogen and kept at -70 ºC until RNA isolation.

2.10. Preparation of neuronal cell cultures

Cultures were prepared from mouse striatum on ED17 and kept for 6 DIV. Tissue pieces were dissected and collected in preparation buffer. After dissection, tissues were incubated for 15 min in 10 ml Versene containing 0.1% trypsine at RT. The enzymatic digestion was stopped by adding 10 ml dispersion buffer. Cells were mechanically dissociated by triturating through a plastic pipette. The obtained suspension was filtered through a 125 μm nylon mesh. Cells were centrifuged for 5 min at 1400 rpm at RT. The pellet was dissolved in 4 ml of MEM containing 5% FCS. The number of viable cells was determined by their ability to stain with trypan blue. Cells were plated at a density of 200.000 cells/cm^2 on poly-DL-ornithine-coated culture dishes (3.5 cm^2) and were incubated in a water-saturated atmosphere of 5% CO_2 and 95% air at 37 ºC. On DIV2, the medium was completely changed to NBM without FCS and was renewed every second day.

2.11. Preparation of astroglial cell cultures

Cultures were prepared from 7 newborn mouse pubs (P2). Meninges were removed carefully and pieces of tissue were stored in ice-cold preparation buffer. Collected tissue were subsequently chopped and incubated for 20 min at room temperature in Ca^{++} - and Mg^{++} - free PBS containing 0.1% trypsin and 0.02% EDTA. Trypsin action was terminated by transferring tissues pieces into MEM supplemented with 20% FCS. The tissue was dissociated by trituration, filtered through a 50 μm nylon mesh, centrifuged at 1400 rpm for 5 min, and resuspended in 20 ml of MEM supplemented with 20% FCS. Cells were seeded on 9.6 cm^2 culture dishes previously coated with poly-DL-ornithine. Upon reaching confluence, cells were trypsinized and replated at lower densities. Briefly, confluent cultures were incubated at RT with 0.1% trypsin solution (2 ml/ 9.6 cm^2). Upon detachment of the cells from the culture dish, trypsin action was quenched by addition of 8 ml of MEM containing 10% FCS and the cell suspension was centrifuged at 1400 rpm for 5 min. Cell pellet was resuspended in culture medium (MEM with 20%FCS) and cells obtained from one 9.6 cm^2 dish were routinely subplated into three 9.6 cm^2 dishes. When

replating for the second time, cell were either seeded into dishes or 24-well cluster plates containing coverslips. Second passage glial cultures were switched to serum-free NBM upon reaching confluence with a medium change every second day.

Versene, pH 7.2
0.9% NaCl, pyrogen-free.
200 mg EGTA

Preparation buffer, pH 7.2
145 mM NaCl
4 mM KCl
15 mM HEPES

MEM, Minimum essential medium, Earl's culture medium, pH 7.2-7.4
Per 100 ml
550 µl penicillin/streptomicine
100 µl Fungizon
100 ml MEM

Dispersion solution, pH 7.2
MEM-Earl's culture medium with
10% FCS
30 mM glucose
15 mM HEPES

NBM, pH 7.2
Per 100 ml
550 µl penicillin/streptomicine
100 µl Fungizon
200 µl B27
100 ml NBM

Petri dishes coated with poly-DL-overnight

One ml of poly-DL-ornithine (0.5 mg/ml) was added per dish and left overnight. After that, dishes were washed 3 times with ddH_2O and left to dry.

2.12. Treatment of cell cultures

Neurons and astrocytes were used for experiments after 3 DIV or 2 days after the second passage, respectively. They were treated with BDNF at a concentration 50 ng/ml for 72 h. To analyze the time-dependency of BDNF effects on dopamine receptor expression, cultures were exposed for 12, 24, 48 and 72 h. To establish a dose-dependency, cells were incubated with BDNF at concentrations ranging from 12 to 100 ng/ml. Signalling pathways were analyze by treating glial cultures with BDNF (50ng/ml) in combination with an inhibitor for MAP-kinase (PD98059, 20 μg/ml) or with an inhibitor of phosphatidyl inositol-3 kinase (LY294002, 20 μg/ml). For analyzing time-dependency of BDNF effects on MAP-kinase and Akt activation, cultures were exposed to BDNF (50 ng/ml) for 3, 7, 15 and 30 min. As a positive stimulation control for both kinases, cultures were treated with insulin-like growth factor I (IGF-1, 1 μM). To analyze dopamine effects on glutamate transporters expression and turnover of glutamate, astroglial cultures were treated with dopamine (100, 10 and 1 μM) daily for 72h. To protect dopamine against oxidation during treatment, glutathione was added at a concentration of 10 μM.

2.13. RNA isolation

Total RNA was isolated using PeqGold RNAPure solution according to the manufacturer's instructions. Briefly, tissue or cultured cells (pooled up to two 3.5 cm^2 dishes) were lysed with 1 ml PeqGold RNAPure solution. Then, phenol/chloroform extraction was performed by adding 0.2 ml chloroform, vortexing and centrifugation at 15000 g for 15 min at 4 ºC. The obtained aqueous phase was transferred to a new tube, precipitated in isopropanol for 10 min at RT, and centrifuged at 15000 g for 15 min at 4 ºC. The pellet was washed with ice-cold 70% ethanol to remove co-precipitated salt and centrifuged at 15000 g for 10 min at 4ºC. RNA was dried in a vacuum centrifuge for 5 min and reconstituted in 12 μl of sterile water. The concentration of isolated RNA was measured by spectrophotometry (an optical density (OD) of 1 corresponds to approximately 40 μg/ml RNA). Reconstituted RNA was stored at -70 ºC.

2.14. Reverse transcription and polymerase chain reaction (RT-PCR)

Random-primed reverse transcription was performed in a total volume of 25 µl containing 2-10 µg of total RNA, 1.5 µl 10x hexanucleotide mixture, 200 U of M-MLV reverse transcriptase, 1x M-MLV reaction buffer and 0.2 mM dNTP for 1 h at 37 ºC. The enzyme was inactivated by heating samples for 5 min at 95 ºC. Amplification was performed in total volume of 50 µl consisting 3 µl cDNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1x reaction buffer, 10-20 pM sense and antisense primers (see table 1) and 1.2 U Taq Polymerase.

Table 1. Primers applied in RT-PCR

Name of gene, Genbank accession number	**Position of primers (nt)**	**Primer sequence**	**Product length (bp)**	**Tm**
HPRT J00423	576-594 805-824	5'-GCT GGT GAA AAG GAC CTC T-3' 5'-CAC AGG ACT AGA ACA CCT GC-3'	249	61 ºC
MAP-2 M21041.1	253-273 796-816	5'-TGG ACA TCA GCC TCA CTC ACA-3' 5'-TGT GTC AGG AAC TAA GGC AGC-3'	563	56 ºC
GFAP X02801.1	1596-1616 2068-2088	5'- AGA GAA CAA CCT GGC TGC GTA-3' 5'-GGA GGG CTG CTA ACA TTT TCA-3'	493	64 ºC
D_1-R M35077	763-783 967-987	5'-CAG TCC ATG CCA AGA ATT GCC-3' 5'-AAT CGA TGC AGA ATG GCT GGG-3'	225	58 ºC
D_5-R XM-144308	782-801 990-970	5'-AGC ATG CTC AGA GTT GCC GG-3' 5'-ACA AGG GAA GCC AGT CTT TGG-3'	209	58 ºC
GLT AB007810.1	738-755 811-829	5'-GAG CCA AAG CAC CGA AAC-3' 5'-CCC AGG ATG ACA CCA AAC-3'	92	58 ºC
GLAST AF330257	226-244 365-384	5'-ACG GTC ACT GCT GTC ATT G-3' 5'-TGT GAC GAG ACT GGA GAT GA-3'	159	58 ºC

Tm, specific annealing temperature; nt, nucleotide position in the respective gene sequence; bp, base pair.

Amplification conditions were as follow: the first amplification cycle with 3 min denaturation at 95 ºC, 1 min annealing at specific temperature for each set of primers (see table 1), and 2 min elongation at 72 ºC. The remaining cycles were performed with 1 min denaturation.

2.14.1. Visualization of PCR products

2.14.1.1 DNA agarose gel electrophoresis

PCR products were separated in 1.5% agarose gels. Agarose gels were prepared by heating 1.5 g agarose in 100 ml of 1x TAE buffer until agarose was dissolved, then 3 µl EtBr (10mg/ µl) were added to the solution. After that, the solution was poured into a gel tray. The gel was allowed to set for 1 h. 10 µl PCR samples were loaded on the gel with 2 µl loading buffer (50% glycerol, 0.05% BB). A 100 bp DNA ladder was loaded along with the PCR samples and used to estimate the size of PCR products. The electrophoresis was carried out at 100 V for 45 to 60 min. The gel was examined on UV light (254 nm) and pictures of gels were taken using a fluorescent gel scanner. The specificity of the PCR products was tested by sequencing.

1x **TAE, pH 7.5**
44.5 mM TRIS
45.5 mM boric acid
1mM EDTA

2.14.1.2. Quantification of PCR products and linearity

Absolute ODs were measured for each PCR product. Pictures of gels were taken using the Image Master VDS and then ODs were calculated by using the Image Master VDS software (Pharmacia). For each individual sample, the OD of the corresponding HPRT band was used as a reference and set to 100%. In order to minimize inter-assay variations (≤ 5%), RNA isolation, RT-PCR and quantification of samples from each gestation stage were performed simultaneously. In order to determine optimal PCR conditions for each set of primers, cDNA amplification was carried out for 26, 28, 30, 32, 34 and 36/38 cycles. Since a linear relationship between the amount of PCR product and

the number of cycles was shown (see Figure 1), 32 amplification cycles were chosen for all further experiments.

2.15. Western blotting

2.15.1. Protein preparation

2.15.1.1. Total protein isolation

Cultured cell from striatum were rinsed with PBS and cells were lysed with 50 µl lysis buffer A per 3.5 cm dish. Viscous lysate was collected and ultrasonified on ice to increase protein solubility and DNA disruption. Protein solution was denatured at 95 ºC for 5 min and stored at -70 ºC.

2.15.1.2 Membrane preparations

For the purification of the membrane fraction cells were scrapped off in sucrose lysis buffer B, transferred to 1.5 ml Eppendorf tubes, and spun at 720g for 5 minutes at 4 ºC. The obtained supernatant was respun at 100,000 g for 45 min and the resulting pellet, which represents the membrane fraction, was resuspended in 40 µl of lysis buffer A.

Lysis buffer A
62.5 mM TRIS-HCL
2% SDS
10% sucrose
0.5 mM PMSF
10 µg/ml aprotinin

Lysis buffer B, pH 7.2
250 mM sucrose
5 mM TRIS
0.5 mM PMSF
10 µg/ml aprotinin

2.15.2. Protein determination

The total amount of protein was determined using the BSA^{Tm} protein assay. For protein estimation a fresh set of protein standards was prepared. BSA solution (2mg/ml) was diluted in lysis buffer to yield a working range of 50, 100, 150, 200, 250, 300, 350 and 400 µg of BSA per ml. Subsequently, 50 µl of each dilution was pipetted into separates tubes. Protein samples were diluted 10 and 50 times with lysis buffer A and 50 µl of both dilutions were aliquoted to the respectively labelled tubes. BSA working reagent was prepared by mixing 50 parts of reagent A with one part of reagent B from BSA^{Tm} protein assay. BSA standards and diluted protein samples were mixed with 1 ml of this solution and incubated at 60 ºC. After 30 min, tubes were cooled at RT and the absorbance was measured at 562 nm. The amount of protein was determined by comparing absorbance of the samples with the standard curve plotted from readings of the BSA standards.

2.15.3. Polyacrylamide gel electrophoresis and protein transfer

Protein was denatured in 1x denaturing loading buffer at 95 ºC for 5 min, chilled on ice, and separated by SDS-polyacrylamide gel electrophoresis (5% stacking gel/ 10% resolving gel). Electrophoresis was performed with 1x electrophoresis buffer at constant 120 V for 1 h. A standard-protein ladder (Mark 12 MW) was loaded along with the protein samples and used to estimate the size of proteins. Protein transfer was performed in 1x transferring buffer at 12V for 30 h to nitrocellulose membrane by semidry electroblotting. For visual verification of transfer efficiency, nitrocellulose blots were stained with 1x Ponceau S solution.

Stacking gel

0.25 M TRIS-HCl, pH 6.8
5% Acrylamide/Bisacrylamide 29:1
0.1% SDS
0.05% TEMED
0.1% APS

Resolving gel

1.5 M TRIS-HCl, pH 8.8
10% Acrylamide/Bisacrylamide 29:1

0.1% SDS
0.05% TEMED
0.1% APS

1x Electrophoresis buffer
5 mM TRIS-HCl, pH 8.3
50 mM glycine
0.1% SDS

1x Loading buffer
125 mM TRIS, pH 6.8
2% SDS
5% glycerol
0.006% BB

1x Transfer buffer, pH 8.3
39 mM glycine
48 mM TRIS
0.037% SDS
20% methanol

1x Ponceau
0.02% Ponceau
0.3% trichloracetic acid
0.3% sulfosalicilic

2.15.4. Detection

Non-specific binding sites were blocked by incubating the membranes for 30 min with blocking buffer and subsequently incubated overnight at 4 ºC with one of the following antibodies: anti-D_5-R (1:100), anti-ERK1/2, anti-pERK1/2, anti-Akt, and anti-pAkt (1:1000). Blots were washed three times (10 min) with washing buffer and then incubated 2 h with the respective secondary antibody conjugated to horseradish peroxidase: anti- mouse (1:5000), anti- rabbit (1:10000). Secondary antibodies were

washed out thoroughly by rinsing three times for 10 min with washing buffer. Both primary and secondary antibodies were diluted in 5 ml blocking buffer supplemented with non fat dry milk at a final concentration of 0.2%. In all cases, immunoreaction was detected using the enhanced chemiluminiscence kit from Amersham. Reagent A and B were mixed in a 1:1 ratio and incubated for 5 min at RT as recommended by the manufacturer. Blots were incubated with 1 ml of the mix per 100 cm^2 of blotting membrane in a sealed plastic bag. ECL optimized film was exposed in a cassette with the blots and developed in Hyperprocessor.

1x TBS, pH 7.6
150 mM NaCl
50 mM TRIS

Washing buffer, pH 7.6
1x TBS
0.05% Tween 20

Blocking buffer, pH 7.6
1x TBS
5% non-fat dried milk
0.05% Tween 20

Incubation buffer, pH 7.6
1x TBS
0.05% Tween
0.5% non-fat dried milk.

2.15.5. Quantification of MAPK phosphorylation

Three independent experiments were carried out for glial cultures. Absolute ODs were calculated for each band (see chapter 2.14.1.2) corresponding to the phosphorylated and non-phosphorylated MAPK 42 and MAPK 44. In each experiment, control values representing unstimulated cells were set to 100, and the values of the bands obtained from treated cultures were expressed as percentage of controls.

2.16. Fluorescence activated cell sorted (FACS) analysis.

2.16.1. Preparation of samples

Astroglial cells were isolated and dispersed by incubating with a ready to use dissociation solution for 5 min. After washing with blocking buffer (phosphate-buffered saline (PBS) Dulbecco containing 0.2% BSA and 0.1% sodium azide), cells were incubated with goat anti-rat D_5- receptor (1:100) for 1h on ice. Cells were then washed with the blocking buffer before labelling with anti-goat-IgG fluoresceine (FITC) conjugated (1:500) for 30 min on ice, protected from light. Thereafter, cells were washed and transferred to a FACScan flow cytometer with a 488 nm argon laser.

2.16.2. Data analysis.

Data analysis and graphic presentation were performed using Cellquest and the WinMDI software. Untreated cells were used to set the background fluorescence. The polygonal rate R1 was defined for this population of cells. Generally, 98-100% of unlabeled cells were gated. Subsequently, the polygonal rate R2 for FITC-labelling was defined. The number of D_5-R positive cells was counted and expressed as the number of cells shifted after fluorescence labelling from the polygonal gate R1 to the polygonal gate R2.

2.17. Immunocytochemistry

Cells coated in coverslips (13 mm) and after 72 h were fixed in acetone for 5 min followed by blocking with 1 % bovine serum albumin (BSA) for 1 h at room temperature (RT). Cells then were incubated with mouse anti-D_5-R and rabbit anti-trkB for 1 h at RT. After washing with PBS, cells were incubated with the corresponding secondary antibodies. Images were analyzed using Leica TCS-SP confocal laser scanning microscope. Controls for immunocytochemistry were done by omitting the primary antisera.

2.18. Quantitative real-time PCR

2.18.1. Preparation of external standards

External standards were prepared from cDNA containing the target sequence (HPRT, GLT-1, and GLAST). DNA fragments were obtained by purification of PCR products cut out from low melt-agarose gels using Quiaquick spin columns. The concentration of the standards nucleic acid was determined by measuring the absorbance at 260 nm according to standard procedures. To calculate the copy number of the standards the following mathematical correlation and formulas were used.

dsDNA MW = (number of base pair) × (660 Daltons/ base pair) = [g/mol]

$$\text{Amount of copies [copies/µl]} = \frac{6 \times 10^{23} \text{ [copies/mol]} \times \text{concentration [g/ µl]}}{\text{MW [g/mol]}}$$

Table 2: Main calculations for preparation of standards

	dsDNA MW [ng/mol]	**ng ds DNA/µl**	**copies/ µl**	**? µl= 10× 10^{10} copies**
GLT-1	6,072 × 10^{13}	13,2	1,3043 × 10^{11}	0.077
GLAST	10,494 × 10^{14}	21,7	1,2407 × 10^{11}	0.081
HPRT	16,434 × 10^{14}	46,5	1,6977 × 10^{11}	0.059

2.18.2. Absolute quantification using external standards

With the known concentrations, purified cDNAs were diluted in RNAse free water to yield the working dilutions ranging from 10× 10^{9} to 10× 10^{2} copies. Each standard curve was generated in one run using the second derivative maximum method, provided by the manufacturer's software, and stored as a file. The second derivative maximum method was applied to determine the maximum acceleration of the amplification process and the respective cycle number was used as crossing-point value. The file with the standard curve was loaded into the following experimental runs containing at least 3 samples of known concentration, thus allowing quantitative analysis in those run. The concentrations of unknown samples were determined by setting their crossing-points to the standard curve

and were normalized to HPRT. Since the LightCycler software calculates the slope for each standard curve, the PCR efficiency (E) may be calculated from the following formula: $E = 10^{-1/slope}$

This efficiency should be in the range of 1.6 and 2.

2.18.3. RT-PCR

Total RNA extraction and cDNA synthesis was performed as described in chapter 2.13. Expression of mRNA was analyzed by quantitative real-time PCR using the LightCycler system. PCRs were run in glass capillaries in a volume of 20 µl containing 10 µl of 2x QuantiTect SYBR Green PCR Master Mix, 0.5 µM of each primer, 2 µl of cDNA and RNase-free water. The mix was dispensed in the appropriate volume into PCR capillaries and subsequently the template DNA was added. The LightCycler was programmed according the programme outlined in Table 3.

Table 3. Experimental protocol to run a LightCycler PCR

Program	**Cycles**	**Segment number**	**Temperature Target (ºC)**	**Hold Time (sec)**	**Slope (ºC/sec)**
Preincubation	1	1	95	900	20
Amplification	45	1	94	15	20
		2	58	20	20
		3	72	20	20
Melting Curve	1	1	95	0	20
		2	65	30	20
		3	98	0	0.05
Cooling	1	1	40	30	20

2x QuantiTect SYBR Green PCR Master Mix

HotStar Taq DNA polymerase

QuantiTect SYBR Green PCR Buffer (Tris-Cl, KCl, $(NH_4)_2 SO_4$, 5 mM $MgCl_2$), pH 8.7

dNTP mix

2.19. Measurement of extracellular and intracellular L-glu concentrations

The L-glu concentration was measured by a fluorometric method using the commercially available Amplex Red Glutamic Acid assay kit. The assay was performed according to the manufacturer's instructions.

2.19.1. Principle of the assay

In the assay, L-glu is oxidized by glutamate oxidase to produce α-ketoglutarate, NH_3 and H_2O_2. L-Alanine and L-glutamate-pyruvate transaminase are included in the reaction to regenerate L-glu by transamination of α-ketoglutarate, resulting in multiple cycles of the initial reaction and a significant amplification of the H_2O_2. The hydrogen peroxide reacts with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) in a 1:1 stoichiometry in the reaction catalyzed by horseradish peroxidase (HRP) to generate the highly fluorescent product, resorufin (Fig. 14A, chapter 3.7.3). Because resorufin has absorption and fluorescence emission maxima of approximately 563 nm and 587 nm, respectively, there is little interference from autofluorescence in most biological samples.

2.19.2. Glutamic acid assay

1x reaction buffer without L-glu was used as negative control. Positive control was prepared by diluting 20 mM H_2O_2 working solution to 10 µM in 1x reaction buffer. The assay was performed in a total volume of 100 µl per microplate well. For each reaction a volume of 50 µl of samples (culture supernatant or protein) and controls were pipetted into separate wells of the 96-well microplate and mixed with 50 µl of substrate mixture consisting of 100 mM Amplex Red reagent, 0.25 U/ml HRP, 0.08 U/ml L-glutamate oxidase, 0.5 U/ml L-glutamate-pyruvate transaminase, 200 µM L-alanine, and 1x reaction buffer. The reactions were incubated for 30 min at 37 ºC, protected from light.

2.19.3. Estimation of L-glu concentrations

The concentration of L-glu in each sample was calculated from the standard curve, which was constructed in each assay using the appropriate amount of 200 mM L-glutamic acid stock solution diluted in 1x reaction buffer to produce L-glu concentrations of 1 µM to 10 µM. The fluorescence was measured at 4 different time points to follow the kinetics of the reactions (Fig. 14B, chapter 3.7.3).

2.20. Statistical analysis

To investigate BDNF effects on dopamine D_1 and D_5 receptor mRNA/protein levels, six independent culture experiments (neuronal or astroglial cultures) were performed. Each independent experiment was done with approx. 15-20 fetuses from 2-3 litters, which were pooled resulting in at least three wells per treatment. Time- and dose-dependency of BDNF effects were tested in four independent experiments. The effect of PD98059 and Ly294004 on BDNF action was also tested in six independent experiments. FACS analysis was performed in three independent experiments. For establishing BDNF effects on MAP-kinase and Akt phosphorylation, three independent experiments were done. All values given in the text and graphs represent the means ± SEM. Optical densities of amplification products are given as relative ODs normalized to the corresponding HPRT values. To investigate dopamine effects on glutamate receptor mRNA/ levels and glutamate concentrations, nine and three independent culture experiments (astroglial cultures) were performed respectively. Differences between experimental groups were tested by one-way analysis (ANOVA) followed by a posthoc Tukey-test or T-Test analysis with $p \leq 0.05$ as the criterion for statistical significance.

3. RESULTS

3.1 Optimizing of RT-PCR conditions

The expression of dopamine D_1 and D_5 receptor (D_1-R and D_5-R, respectively) mRNA was investigated using semiquantitative RT-PCR analysis. PCR oligonucleotide primers were designed using „oligo" DNA/ RNA primer selection software (for sequences see Table 1, chapter 2.14). To establish optimal PCR conditions, at first a PCR with a gradient of temperatures in the range ± 5 °C to the calculated annealing temperature (T_m) for each primer pair, was carried out. Table 4 shows the annealing temperatures that yielded one single band after PCR amplification and were consequently used for all experiments.

Table 4. Annealing temperatures and length of yielded RT-PCR products

Gene	**T_m**	**Length**
D_1- R	58 °C	225 bp
D_5- R	58 °C	209 bp
HPRT	61 °C	249 bp
MAP-2	56 °C	563 bp
GFAP	64 °C	493 bp

Additionally, the linear range of amplification was determined. PCR was performed for D_1-R and D_5-R cDNAs at cycle numbers ranging from 28 to 38 cycles. Figure 1 shows the cycle dependency for both receptor cDNAs. Since linear relationships between the amount of PCR products and the number of cycles were found for each cDNA between 28 and 38 cycles, 32 amplification cycles were chosen for all others experiments *in vivo* and *in vitro*.

To validate specificity of the yielded product, sequence analysis using ABI Prism 310 Genetic analyzer was performed for D_1-R and D_5-R RT-PCR products. Both analyzed RT-PCR products revealed sequence homologies from 98% to 100% to the known sequences obtained from Genbank (accession numbers are given in Table 1, chapter 2).

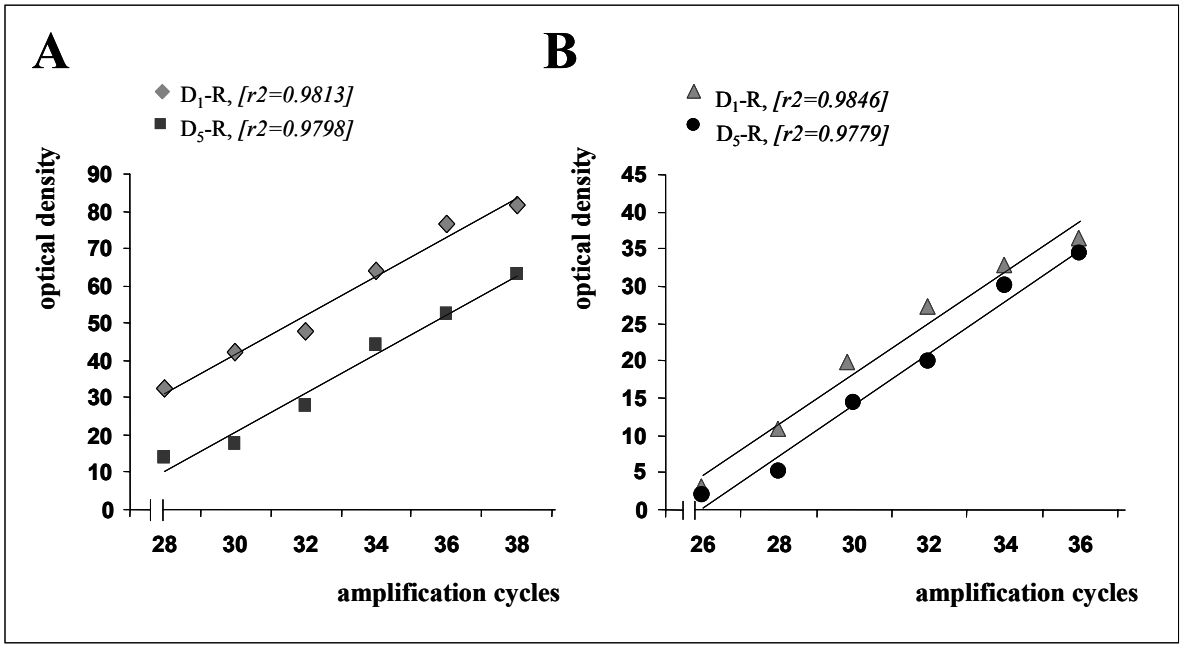

Figure 1. *Cycle dependency of RT-PCR for D_1-R and D_5-R.* ***(A)*** *striatal tissue and* ***(B)*** *cell cultures mRNA. Data were obtained by semiquantitative fluorescent gel scanning; r^2: correlation coefficient.*

3.2. Developmental expression of D1-like receptors in the striatum

The qualitative and quantitative analysis of the developmental expression of D_1-R and D_5-R mRNA in the striatum at different pre- (E15 and E17) and postnatal stages (P0, P7, P15) is shown in Figure 2. For each postnatal and embryonic stage, between 8 and 10 individuals were used. D1-like receptor transcripts were detectable as early as E15; however, D_5-R showed lower levels compared to D_1-R. From E15 to E17, expression levels of both receptors increased. From E15 to E17, there was a moderate increase of D_1-R and a more pronounced increase of D_5-R with a maximum around birth, (P0, $p<0.001$). Thereafter D_5-R expression remains at lower levels than D_1-R.

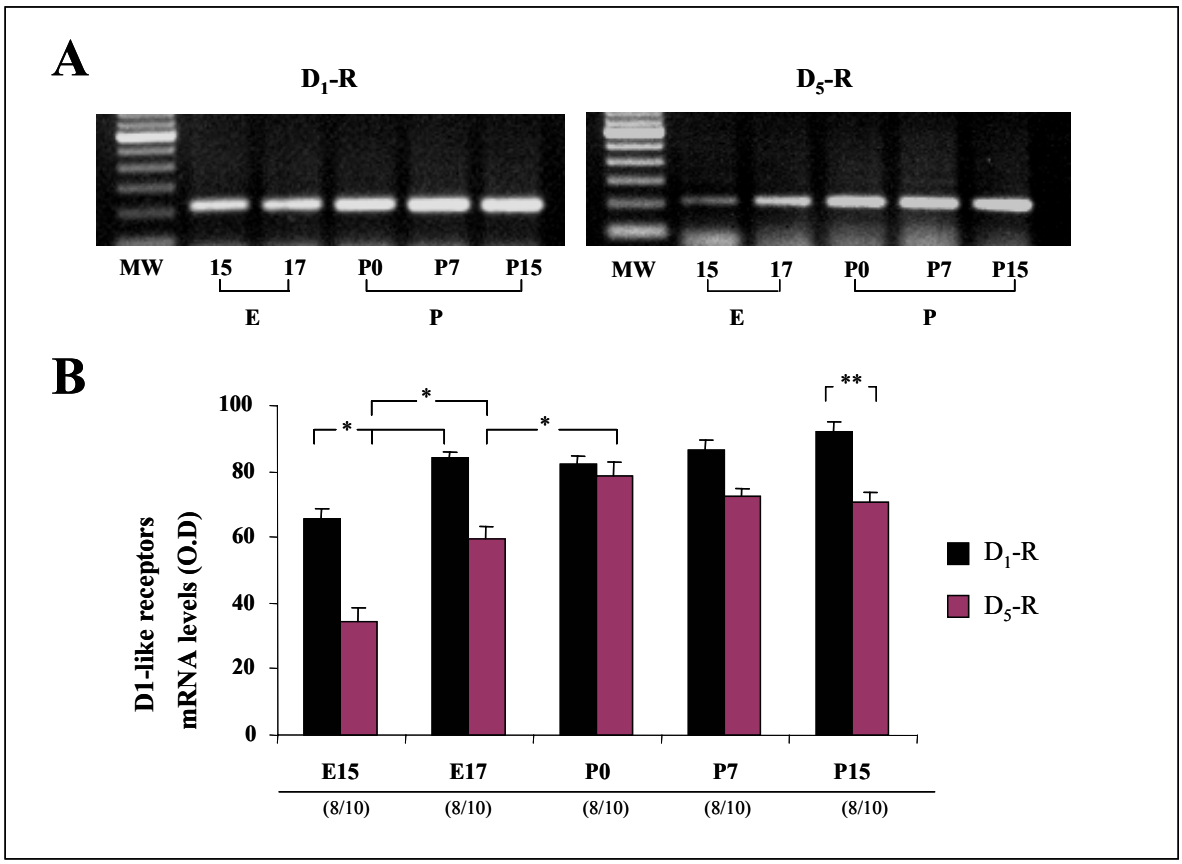

Figure 2. *Developmental profile of D1-like receptors in the striatum* ***(A)*** *Representative agarose gels of D_1-R and D_5-R RT-PCR products at different embryonic (E) and postnatal (P) stages.* ***(B)*** *Semi-quantitative evaluation of RT-PCR products. Data represent optical densities of bands normalized against the corresponding HPRT bands. The numbers of analyzed individuals are given in brackets. *p < 0.001, **p < 0.005*

3.3. Expression of D1-like receptors in neuronal and glia cultures

The expression of D_1- and D_5-R mRNA was investigated in primary embryonic neuronal and postnatal astroglial mouse cells cultures. Ethidium bromide staining of PCR products obtained from striatal neuronal cultures showed a strong band corresponding to the molecular size of 225 bp, which resembled the expected size of the D_1-R. In contrast, in striatal astrocytes the D_1-R oligonucleotide primers did not amplify any PCR product (Fig. 3A). On the other hand, D5-R mRNA was detected at the expected size, 209 bp, in both neuronal and astroglial cells although neurons appeared to have 2-3 times more D5-R mRNA compared to astrocytes (Fig. 3A). To characterize the cell culture system, PCR with oligonucleotide primers specific for neuronal or glial markers, respectively, was performed. RT-PCR analysis revealed the absence of transcripts for the neuronal marker MAP-2 but a strong signal for GFAP in glial cultures. In neuronal cultures, MAP-2 mRNA levels significantly predominated over GFAP levels (Fig.3B).

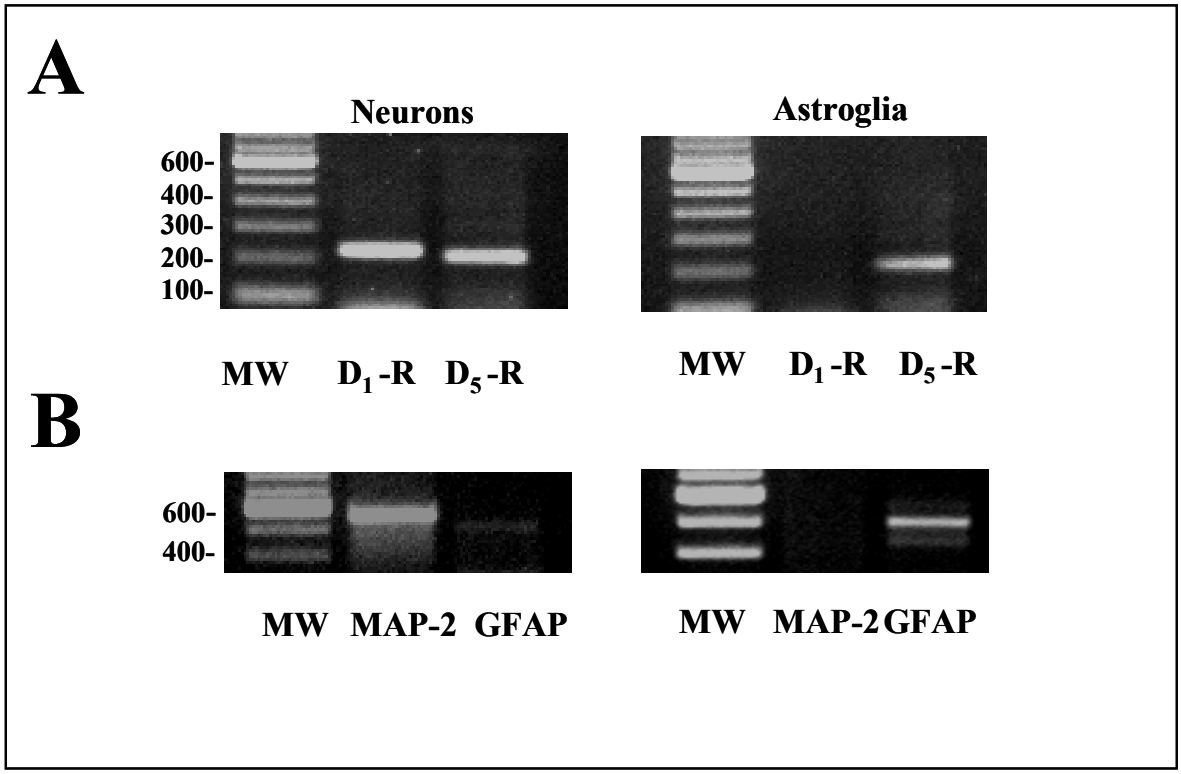

Figure 3. *RT-PCR analysis of neuronal and astroglial cultures.* ***(A)*** *Detection of mRNA encoding D_1- and D_5-R in 6 days old cultures of E17 striatal neurons and in second passage striatal glia maintained for 72 h, with serum free medium.* ***(B)*** *Detection of mRNA encoding neuronal (MAP-2) and astroglial (GFAP) markers in both cell cultures.*

3.4. BDNF effects on striatal cultures

3.4.1. BDNF effects on the expression of D_1- and D_5-R mRNA levels

To examine whether BDNF is involved in the regulation of the dopaminergic transmission at the postsynaptic level, the expression of D1-like receptor mRNA was studied in striatal neuronal and astroglial cultures treated with 50 ng/ml of BDNF. Application to both types of cultures for 72 h did not reveal any differences in the expression of D_1-R. In astrocytes, D_1-R mRNA was still not detectable, and in neuronal cells, there were no significant changes (Fig. 4).

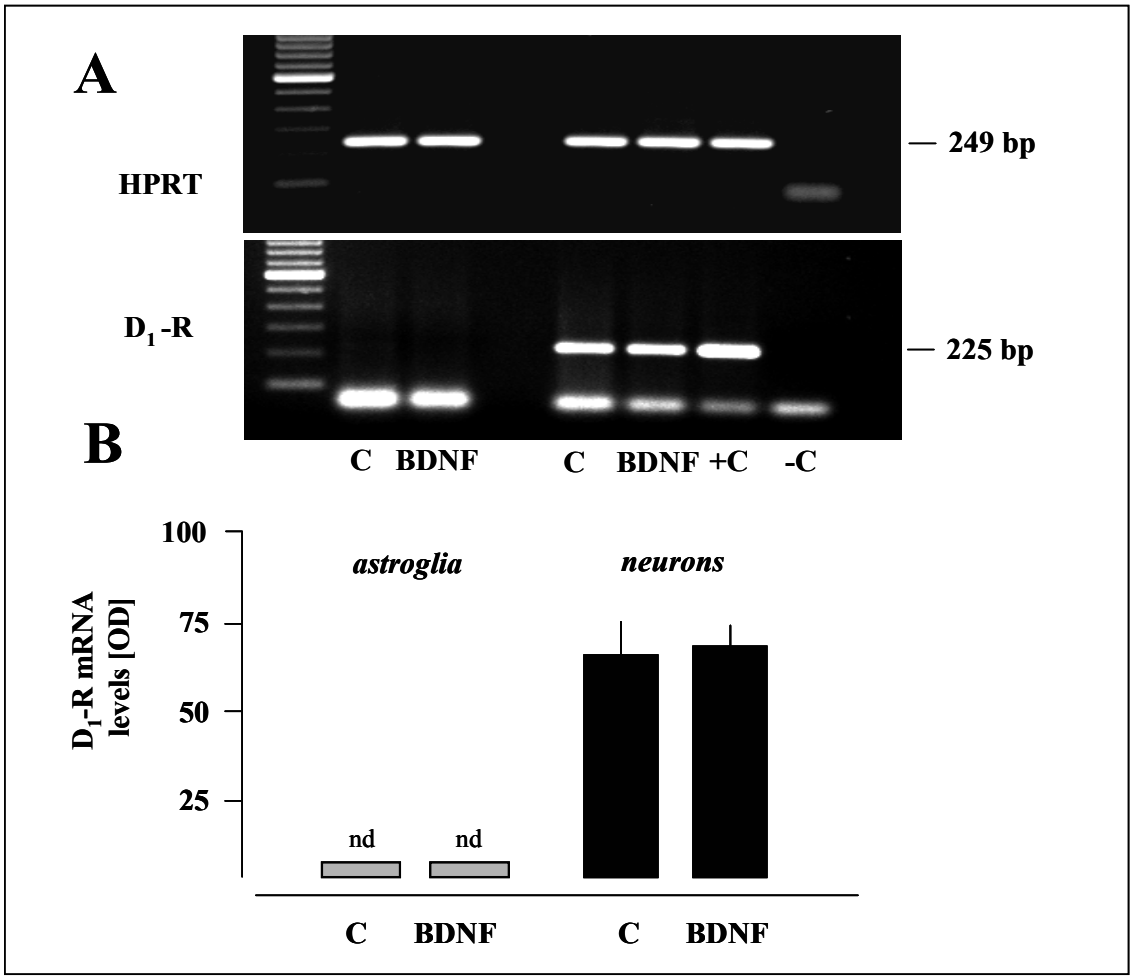

Figure 4. *Effect of BDNF treatment (50 ng/ml, 72h) on D_1-R mRNA levels in primary striatal astroglial and neuronal cell cultures.* ***(A)*** *Shows representative ethidium bromide-stained gels. The lanes were from left to right: molecular marker, control (astroglial culture), BDNF-treated (astroglial culture), empty lane, control (neuronal culture), BDNF-treated (neuronal culture), adult mouse striatum, negative control (without cDNA). In* ***(B)****, the quantitative data of these experiments are summarized (bright columns represent astroglial cultures, black columns are for neuronal cultures). nd, not detectable; C, untreated; HPRT, Hypoxanthine phosphoribosyl-transferase (internal standard); +C, adult mouse striatum tissue; -C, without cDNA.*

However, BDNF treatment significantly stimulated the expression of D_5-R mRNA in astrocytes resulting in a 105% increase ($p < 0.001$). In neuronal cultures, no obvious change in D_5-R mRNA levels was observed between control and BDNF-treated groups (Fig. 5).

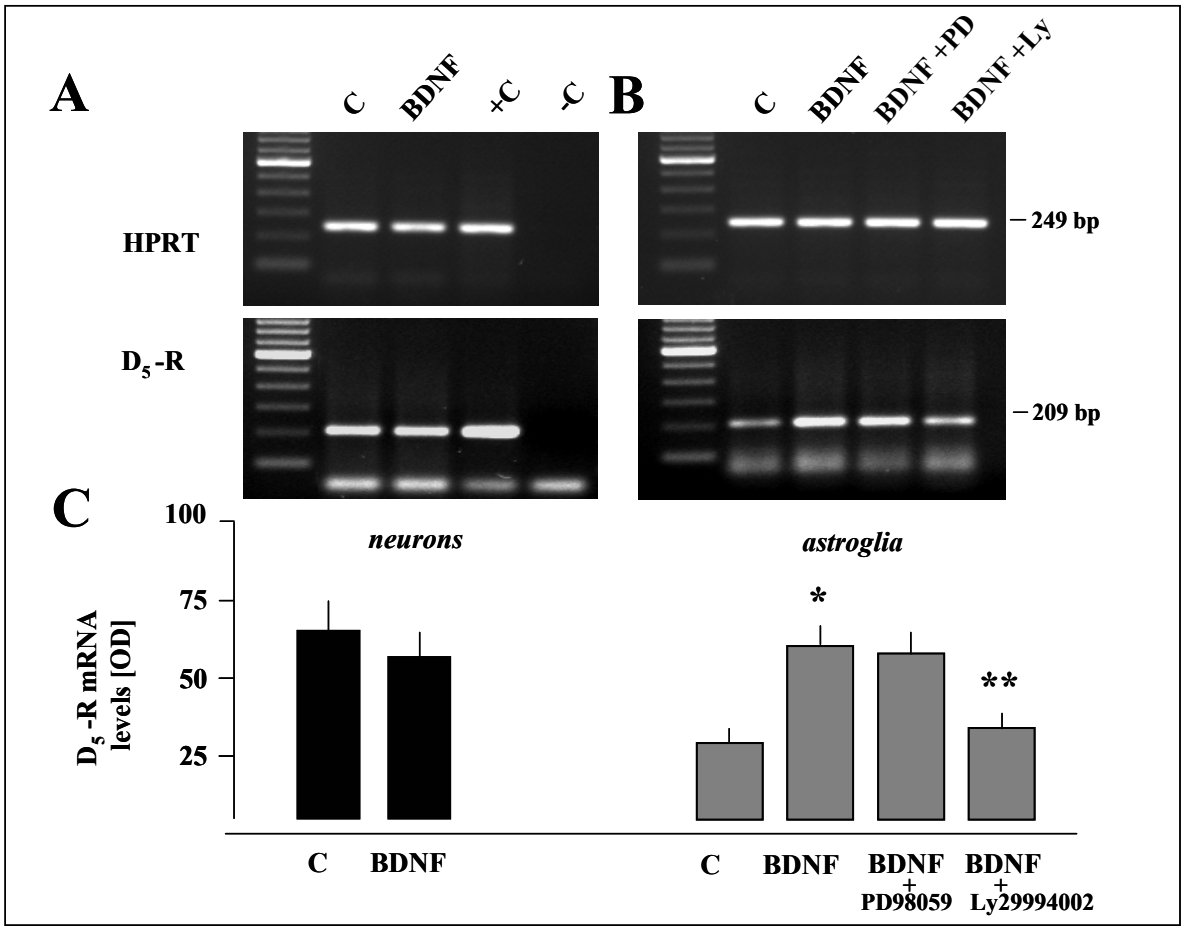

Figure 5. *Effect of BDNF treatment (50 ng/ml, 72 h) alone or in combination with inhibitors of the MAP-kinase and the PI3/Akt kinase-pathway on D_5-R mRNA levels in neuronal* ***(A)*** *and astroglial* ***(B)*** *striatal cell cultures.* ***(A)*** *and* ***(B)*** *show representative ethidium bromide-stained gels.* ***(C)*** *provides the cumulative data (gray columns represent astroglial cultures, black columns neuronal cultures). C, untreated, PD98059 (20 µg/ml, MAP-kinase inhibitor), Ly2994002 (20 µg/ml, PI3-kinase inhibitor), HPRT, hypoxanthine phosphoribosyl-transferase (internal standard); +C, striatal tissue; -C, without cDNA; *p < 0.001 C vs. BDNF, **p < 0.005 BDNF vs. BDNF + Ly2994002.*

Further experiments were performed to estimate a dose- and time- dependency of BDNF effects. A linear increase (r^2= 0.9693) in D_5 receptor mRNA levels in astrocytes after BDNF exposure up to 72h (Figure 6A) was found. Concordantly, BDNF effects were shown to follow a linear dose-dependent profile (r^2 = 0.9980) with a maximum increase at

a BDNF concentration of 50 ng/ml (Figure 6B). Higher BDNF doses or a longer treatment interval did not further increase D_5-R mRNA levels (data not shown).

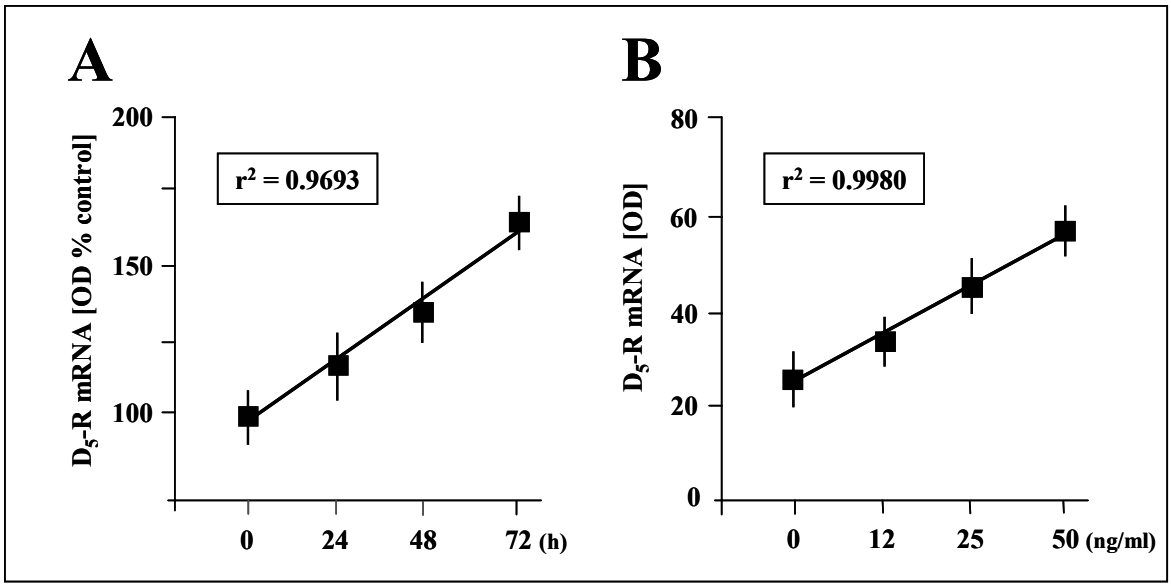

Figure 6. *Time- **(A)** and dose dependency **(B)** of BDNF effects on dopamine D_5-R mRNA levels in astroglial striatal cell cultures analyzed by densitometrical gel analysis. Data represent optical densities of bands normalized against the corresponding HPRT bands and are given as percentage of control. For time-dependency studies BDNF was applied at a concentration of 50 ng/ml, and for dose-dependency BDNF treatment lasted 72 h. r^2, regression coefficient*

In order to ascertain which intracellular signaling pathway is activated in astrocytes, and implicated in the transmission of BDNF effects, we have applied two inhibitors specific for the MAP-kinase (PD98059) and PI3/Akt-kinase (Ly294002). Both pathways have been described to represent downstream signaling pathways of BDNF. Treatment of astroglial cells with PD98059 (MEK inhibitor) and BDNF simultaneously did not affect the BDNF-dependent increase in D_5-R mRNA. However, the blockade of PI3-kinase by administration of Ly294002 completely prevented the BDNF-induced increase of D_5-R mRNA amounts ($p< 0.005$, Fig.5 B, C).

3.4.2 Effects of BDNF on D5-R protein expression levels in astrocytes cultures

For further investigations to which extent the newly synthesized dopamine D_5 receptor mRNA is translated into protein and integrated into the plasma membrane of astrocytes, western blotting and FACS analysis were performed. Immunoblot data revealed that BDNF increases the levels of D_5-R protein in cell lysates (Figure 7A) as well as in the membrane fraction (Figure 7B) of glial cells, thus pointing to an increase of receptor expression as well as to a higher degree of receptor allocation in the plasma membrane in BDNF-treated cultures. Again, this effect was specifically counteracted by the simultaneous application of Ly294002.

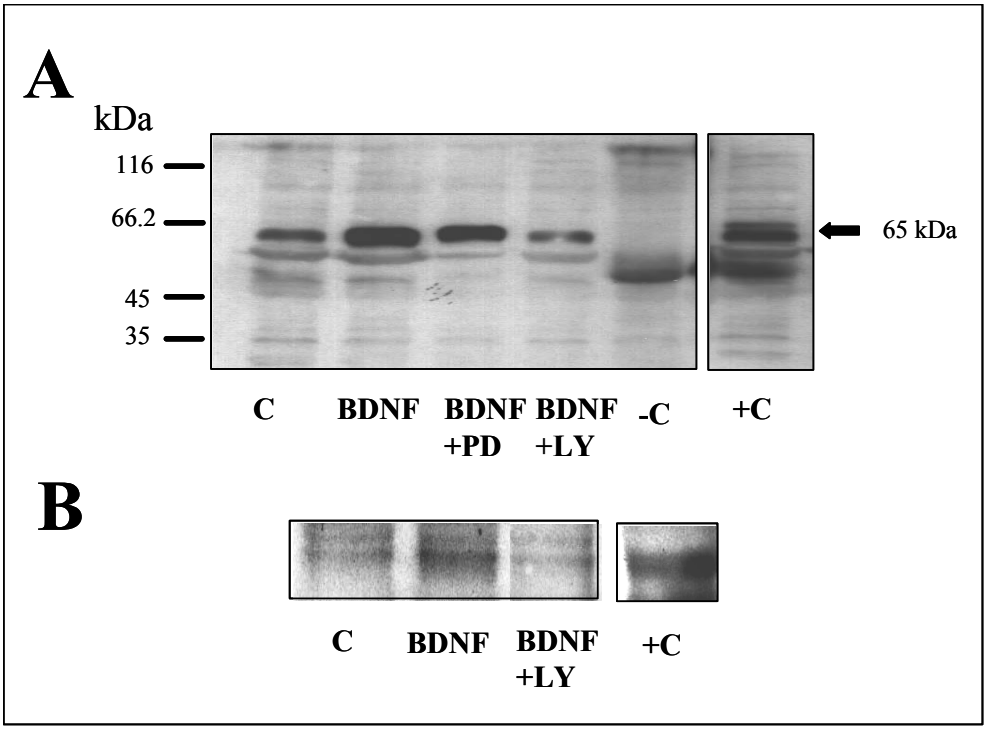

Figure 7. *Demonstration of protein translation of newly synthesized D_5-R in astrocytes after BDNF treatment,* ***(A)*** *shows a representative western blot with whole lysis extract demonstrating that BDNF exposure increased levels of dopamine D_5 receptor protein. This effect was specifically inhibited by the PI3-kinase antagonist Ly294002 but not by the MAP-kinase inhibitor PD98059.* ***(B)*** *Immunoblot of membrane fraction showing the increase of D_5-R allocation in the plasma membrane.C, control culture; BDNF, BDNF-treated; -C, skeletal muscle; +C, adult striatum.*

These observations are further substantiated by FACS studies. Exposure to BDNF resulted in a significant increase in the number of astrocytes expressing dopamine D_5 receptors at their surface (Figure 8). Under control (untreated) conditions, approximately 30% of all cells revealed dopamine D_5-R localized at the cell surface. The application of BDNF nearly doubled this number.

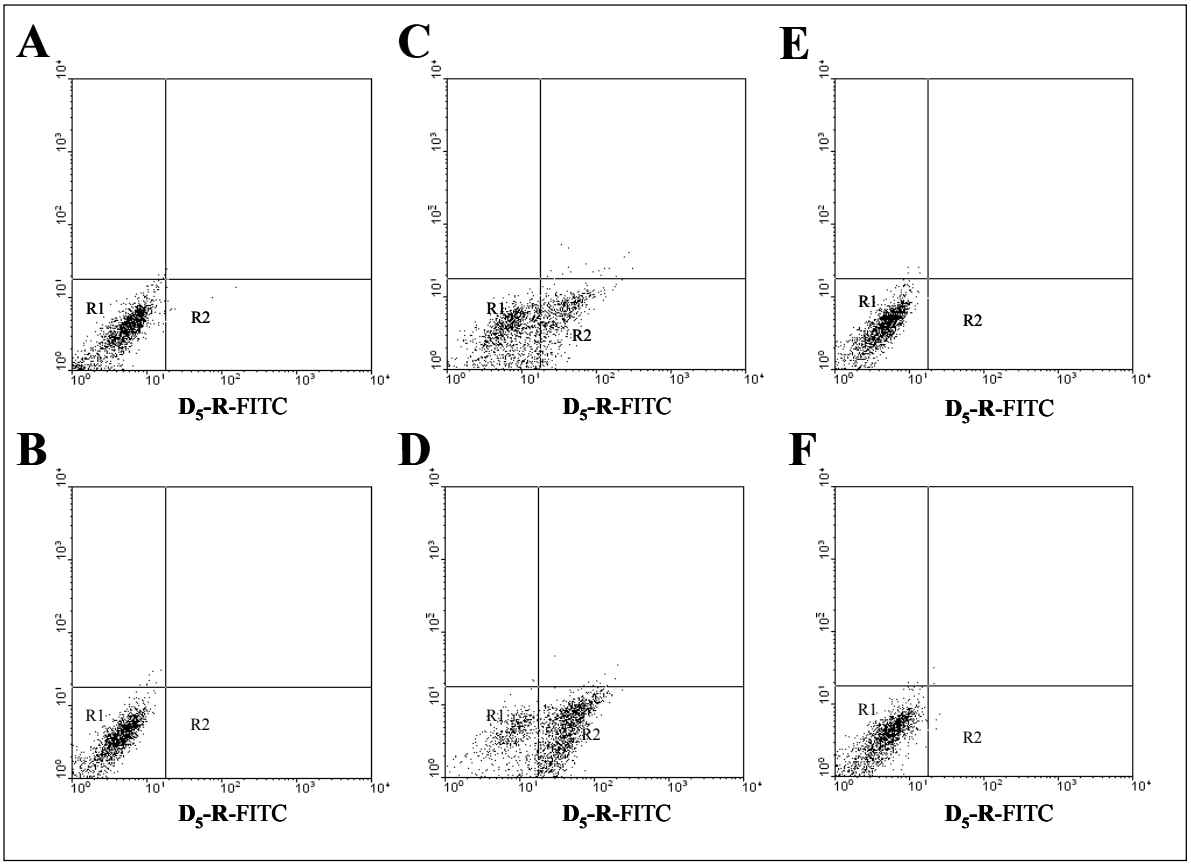

Figure 8. *FACS analysis showing the presence of D_5-R in striatal astrocytes. First graphs left show self- fluorescence of* ***(A)*** *untreated astrocytes and* ***(B)*** *astrocytes treated with BDNF. The two graphs in the middle reveal* ***(C)*** *non-increased fluorescence of untreated cells incubated with antibody directed against D_5-R (1:100) and fluorescent labelled secondary antibody (1:500),* ***(D)*** *increased fluorescence of cells treated with BDNF. Two last graphs in the right represent negative controls incubated only with secondary antibody,* ***(E)*** *untreated cells and* ***(F)*** *cells treated with BDNF. R1, polygonal gate representing self fluorescence of the astrocytes. R2, polygonal gate representing fluorescence of the astrocytes after incubation with anti- D_5 –R.*

3.4.3 Intracellular pathways activated by BDNF stimulation.

The pharmacological findings concerning the involvement of PI3/Akt-kinase in the BDNF-induced dependent upregulation of D_5-R is further supplemented analyzing the state of activation of the MAP kinase and the PI3/Akt kinase pathway in striatal astrocytes. Activation of these pathways is generally accompanied by phosphorylation of kinases. BDNF induced the phosphorylation of Akt within 30 min at its maximum (app. 90% increase vs. controls, Figure 9A). Similar effects were produced by the stimulation with IGF-I, a well-known activator of the PI3-kinase/Akt signaling system. Additionally BDNF stimulated the phosphorylation of ERK1 and ERK2 in astrocytes (Fig.9B). Similar to Akt phosphorylation, ERK1/2 activation was maximally after 30 min.

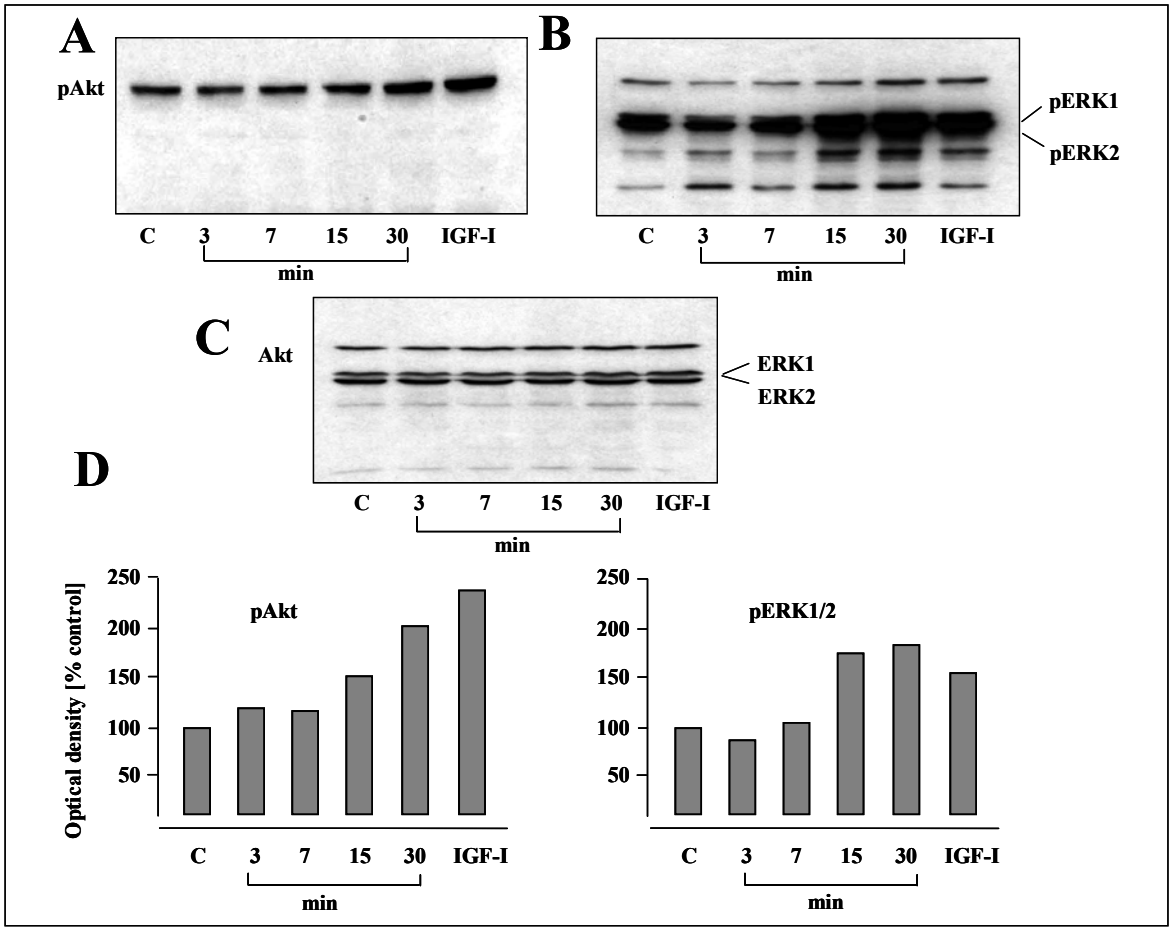

Figure 9. *Representative western blots demonstrating the time-dependent induction of Akt-* ***(A)*** *and ERK1/2 phosphorylation* ***(B)*** *in cultured striatal astrocytes after BDNF (50 ng/ml) application. For both kinases, maximum stimulation was seen after 30 minutes.* ***(C)*** *Represents the expression of the corresponding non-phosphorylated Akt (upper band) and ERK1/2 (lower band), respectively.* ***(D)*** *Shows the quantitative evaluation of the above experiments. Data are given as percent of control and represents the means of two independent experiments. C, control. IGF-1, 1 μM for 30 min, insulin-like growth factor-I.*

3.5. Region specificity of BDNF-induced increase of D_5-R mRNA

In order to establish whether the increase of dopamine D_5-R expression in astrocytes by BDNF is restricted to the striatum, glial cultures from cortex and hypothalamus were used to draw a comparison. The cultures obtained from these brain areas were treated with 50 ng/ml of BDNF for 72 h and the D_5-R mRNA levels were analyzed by RT-PCR. Representative ethidium bromide stained gels revealed that the up-regulation of the D_5-R mRNA by BDNF was restricted to striatal glia cells. In cortical and hypothalamic glial cultures, no effect of BDNF on the expression of the D_5-R was observed (Fig 10).

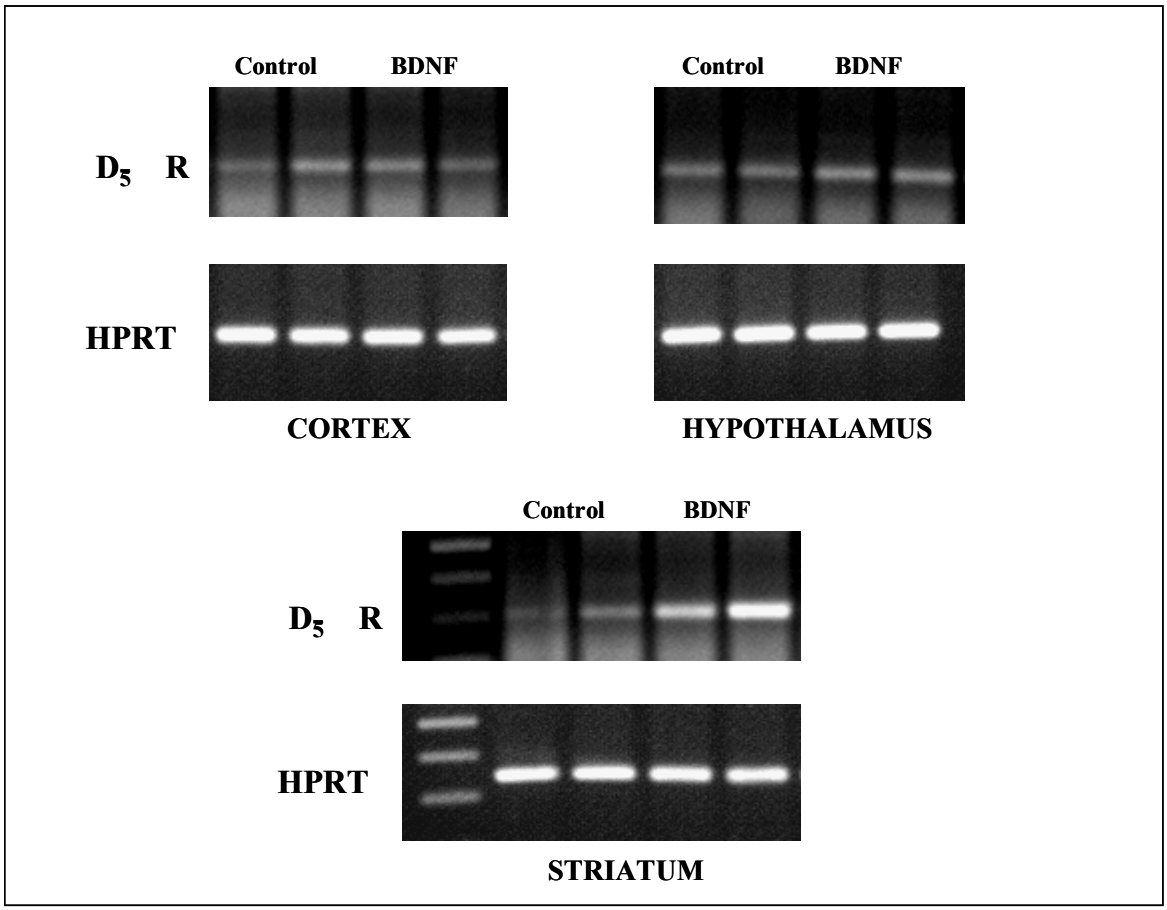

Figure 10. *Representative ethidium bromide stained gels showing the expression of D_5-R mRNA in glial cultures from striatum, cortex and hypothalamus in the absence or presence of BDNF (50 ng/ml). BDNF did stimulate the D_5-R mRNA only in striatal glial cultures.*

3.6 Immunocytochemical characterization of striatal glial cultures: heterogeneity with respect to D_5-R and trkB receptor expression and/or localization

Using a specific D_5-R antiserum, the presence of this type of dopamine receptor in astrocytes could be demonstrated by immunocytochemistry (Fig. 11). Double immunofluorescence stainings with antibodies specific for trkB and D_5-R displayed heterogeneity of cultured astrocytes with respect to D_5-R and trkB expression.

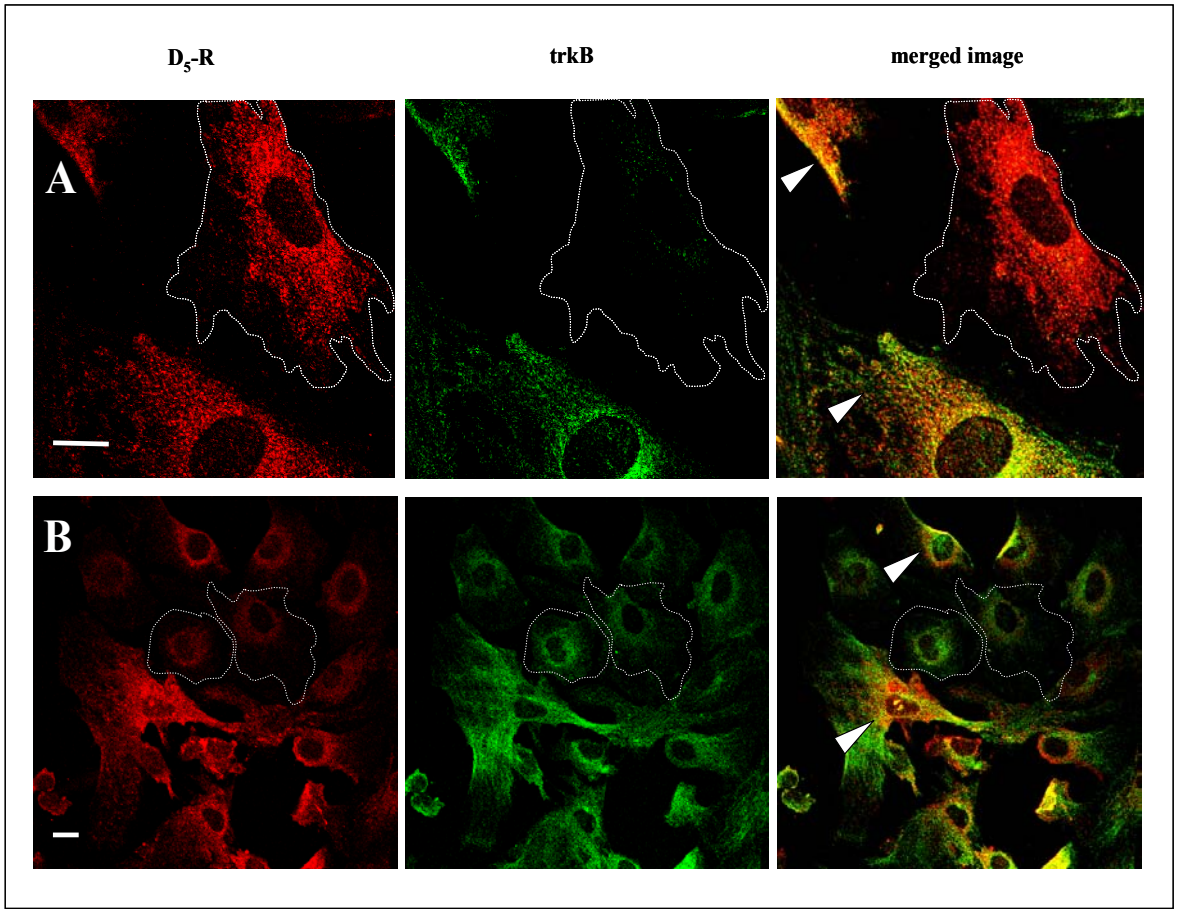

Figure 11. *Confocal laser microscopy of astroglial cultures from mouse striatum with antibodies specific for trkB (green) and D_5-R (red) at two magnifications (A), 100x; (B), 40x).*

In control cultures three different cell populations were observed: (i) cells with high expression of D_5-R and practically no trkB (about 6% of all cells), (ii) cells that were mainly positive for trkB with low or no co-expression of D_5-R receptors (about 60-70 % of the cells), and (iii) cells (about 25%) that expresses both receptors. Treatment with BDNF did alter neither the total number of cells nor the number of cells that predominantly express D_5-R. In contrast, BDNF treatment increased the number of double-stained cells

(70%) to the same extent as it reduced the number of cells expressing mainly trkB with no or only low amounts of D_5-R (20%).

3.7. Dopamine effects on glutamatergic transmission in striatal astroglial cultures

3.7.1. Establishment of the LightCycler RT-PCR

The expression of the glutamate transporter mRNAs (GLT-1 and GLAST), was investigated using quantitative real-time PCR analysis. Standard curves for the two genes of interest (GLT-1 and GLAST) and the housekeeping gene (HPRT) were generated by serial dilutions of PCR purified products ranging from 10^9 to 10^2 copies (see also chapter 2.18). Figure 12A illustrates the amplification of each standard in a representative LightCycler run. The number of cycles was plotted against the log concentration (copy number) of the standard DNA. The efficiency of all PCRs, given by the equation: efficiency = $10^{-1/slope}$, was between 1.8 and 1.95, which is fairly consistent with the theoretical PCR efficiency of 2.0 which theoretically can be achieved by a doubling of the original copy number in each cycle during the exponential PCR phase. The melting curve analysis was performed to confirm the specificity of the amplified sequence. Figure 12B shows the melting curve analysis of GLT, GLAST and HPRT PCR fragment on the LightCycler System. All melting curves show single melting maxima of 73 ºC (HPRT), 82 ºC (GLAST) and 83 ºC (GLT-1) indicates the specificity of the reaction. Specificity was also tested by extracting LightCycler PCR products from the capillaries and separating them on a 1.5 % ethidium bromide agarose gel. The length of the PCR products corresponded well with the predicted molecular weights of 249 bp for HPRT, 92 bp for GLT-1, and 159 bp for GLAST (data not shown).

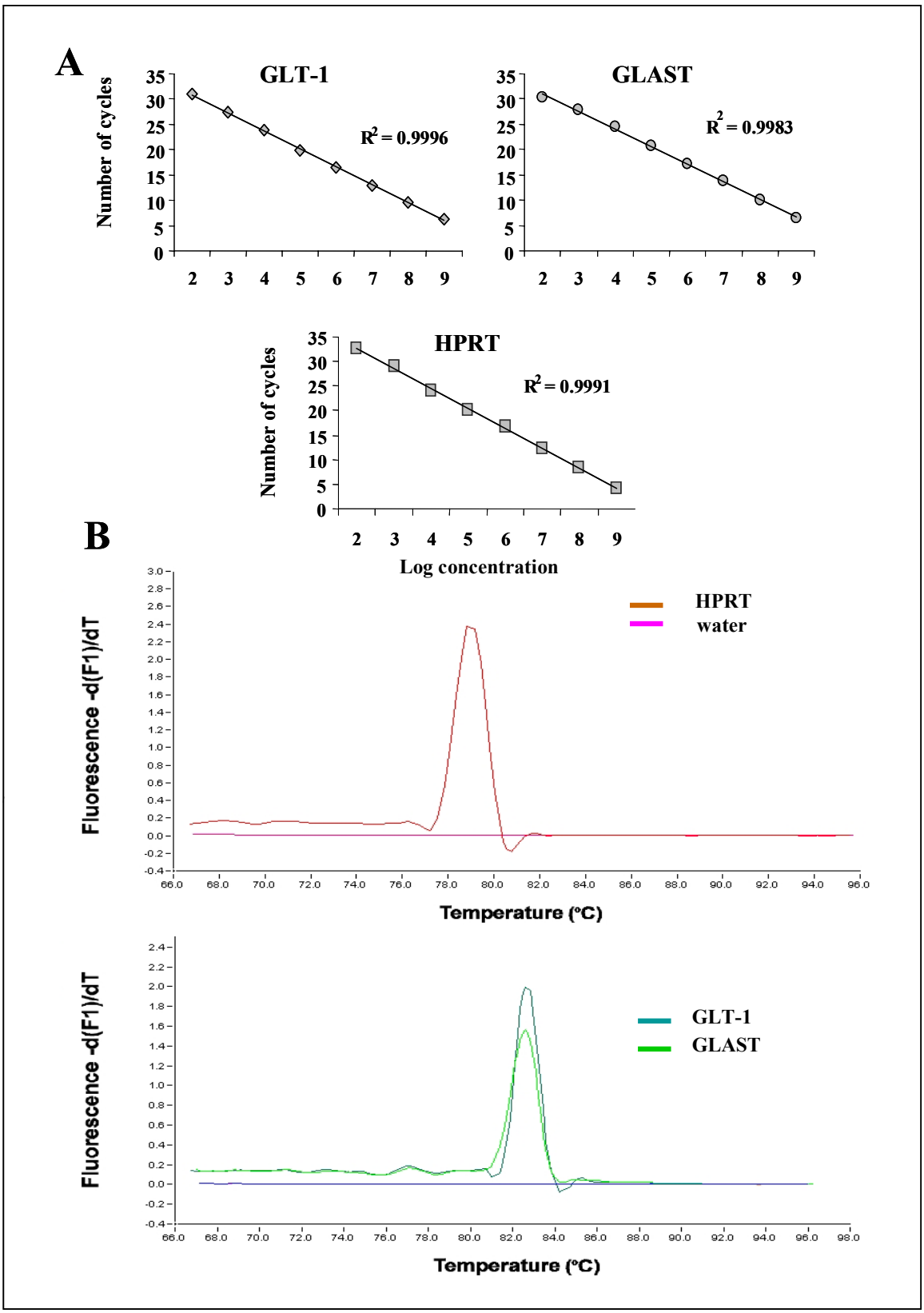

Figure 12. ***(A)*** *Standard curves for the different cDNAs at the range of 10^9 – 10^2 copies; the crossing point (cycle number) was plotted against the log concentration (copy number) of the standard DNA.* ***(B)*** *Melting peak analysis of the GLT-1, GLAST and HPRT primers. Melting temperatures of GLT-1, GLAST and HPRT products are between 73°C and 83°C.*

3.7.2. Influence of dopamine on glutamate transporters expression

To determine, whether dopamine affects the expression of GLT-1 and GLAST, mRNA and protein levels of both transporters were studied in glia cultures by real-time PCR and immunoblot analysis using GLT-1 and GLAST-specific oligonucleotide primers and antibodies. Under basal conditions the mRNA concentration of GLT and GLAST was 3.2×10^2 cDNA copies and 6.8×10^3 cDNA copies per 1×10^4 HPRT molecules. After treatment with dopamine (100 μM) a significant decrease of both transporter mRNA concentrations was observed (between 25 and 20 % of basal levels, $p<0.001$, Figure 13A).

In freshly prepared cell lysates, the GLT-1 and GLAST antiserum recognized a single protein with apparent molecular weights of 71 kD and 66 kD, respectively. Untreated striatal glia usually contained considerable amounts of both transporters. Prolonged treatment (72 h) with dopamine at the highest concentration (100 μM) resulted in a significant decrease in the expression levels of GLT-1 but not GLAST (Figure 13B).

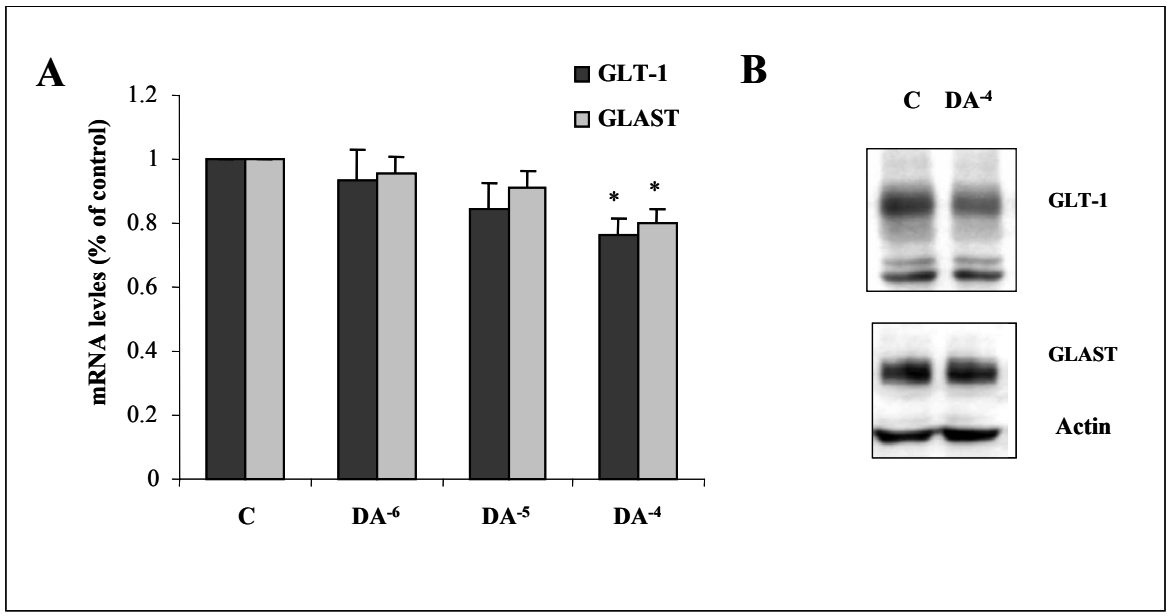

Figure 13. ***(A)*** *Effect of dopamine on GLT-1 and GLAST mRNA expression in astrocytes obtained from striatal tissue, maintained at different dopamine (DA) concentrations (1, 10 and 100 μM) for 72 h. Data represent values obtained by absolute quantification with external standard, normalized against the corresponding HPRT values and are given as percentage of control.* ***(B)*** *Representative western blot demonstrating the expression of glutamate transporters after dopaminergic treatment (100 μM) for 72 h. Actin was used as internal standard. *p<0.001*

3.7.3. Evaluation of intra- and extracellular L-glu concentrations in astroglial cultures under dopamine treatment.

To further characterize the effects of dopamine on glial glutamate turnover, astroglial cultures were treated with dopamine (100 µM and 1 µM) for 72 h. The concentrations of L-glu from medium at 0, 24, 48 and 72 h were measured using the Amplex Red reagent-based assay (see methods in chapter 2.19). The principle of the measurement is shown in Fig.14A. In medium from untreated astrocytes L-glu concentrations did not change significantly during the first 24 h (Fig. 14C). Thereafter a moderate increase of L-glu was observed in untreated cultures. Dopamine at 1 µM treatment did not alter the extracellular L-glu concentrations comparing to control. In contrast, after 48h, 100 µM of dopamine decrease the L-glu content in the cells and increase significantly the extracellular L-glu concentrations (about 25 % of control) (Fig. 14C and Table 5).

Table 5. Changes in L-glu concentrations after 72 h under dopamine treatment

	L-glu concentrations (µM)	
	Medium	**Cells**
Control	5.43 ± 0.71	8.73 ± 1.19
Dopamine 1 µM	5.62 ± 0.61	8.59 ± 1.15
Dopamine 100 µM	8.41 ± 1.26 *	5.29 ± 0.65 **

Data are the mean ± SD from three independent experiments.
*p < 0.01 vs. control and dopamine 100 µM;
**p =0.01 vs. control and dopamine 1 µM

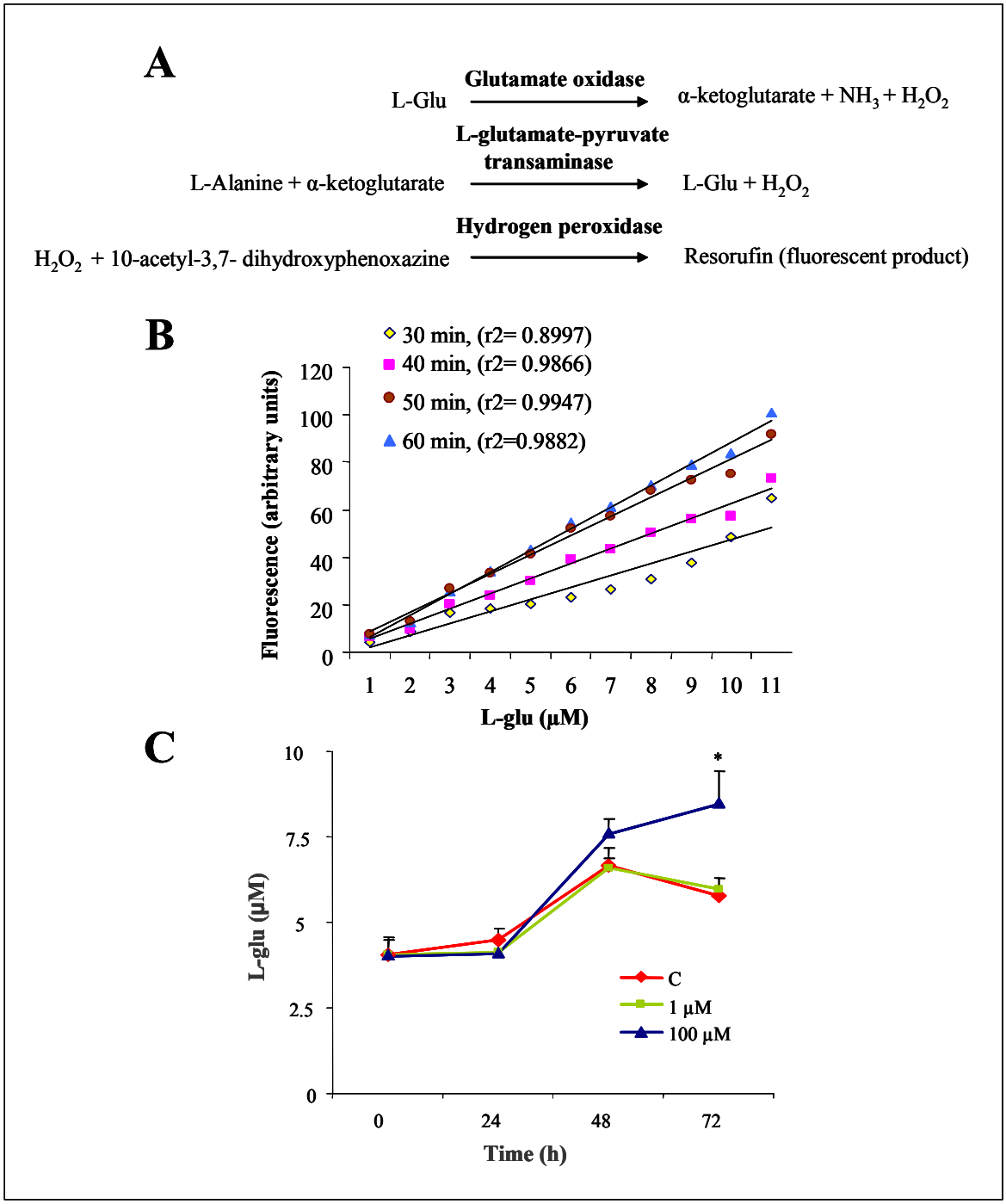

Figure 14. *Evaluation of extracellular concentrations of L-glu from cultured astrocytes. (A) The principle of the fluorometric method using AMPLEX RED reagent. (B) Standard curve and kinetic of the reaction. (C) Measurement of L-glu concentrations from cultured astrocytes treated with dopamine after 24, 48 and 72 h. Data represent the means of three independent experiments.*
**p < 0.01 dopamine 1 µM vs. C and dopamine 100 µM*

4. DISCUSSION

To achieve a better understanding of the mechanisms involved in dopamine-induced effects during striatal development, we have analyzed the expression of D_1- and D_5-R applying an *in vivo* an *in vitro* approach using RT-PCR, western blotting, and immunocytochemistry as well as FACS analysis. Moreover, we have investigated dopaminergic influence on the expression of glutamate transporters (GLT-1 and GLAST) and the glutamate turnover. The results of the present study demonstrate that in the striatum the developmental expression of the D_1-R steadily increased perinatally until it reached adults levels while the expression of D_5-R peaked around birth and then decreased to adult levels. *In vitro,* striatal astrocytes express D_5-R but not D_1-R, whereas both receptor subtypes were espressed in neurons. Furthermore, the expression of D_5-R in astrocytes was induced by BDNF by the activation of PI3-kinase. In addition, these studies point to an involvement of dopamine in the modulation of the glutamatergic transmission in astrocytes by regulation of the glutamate transporters expression (GLT-1 and GLAST). Here we postulate astrocytes to represent major targets for developmental BDNF and dopamine action.

4.1. Developmental expression of D1-like receptors in the striatum

Most of developmental dopamine effects have been shown to be mediated by a selective activation of D1-like receptors (Arnauld et al., 1995; Schmidt et al., 1996, Schmidt et al., 1998, Küppers and Beyer, 2001). Although it is clear that dopamine effects involve the participation of this receptor family, it was not possible so far to differentiate between D_1- and D_5-R dependent effects, as there are no specific pharmacological agents currently available to discriminate between them. Moreover, there are several reports concerning the pattern of expression of the D_1-R in relation to striatal ontogeny and compartmentation (Schambra et al., 1994, Arnauld et al., 1995, Caille et al., 1995, Jung and Bennett 1996). Although these reports are controversial concerning the time point of expression of D_1-R, they all show that D_1-R is early expressed during development. However in this regard, little is known about D_5-R. Therefore in the present study, the developmental profile of D_1-and D_5-R mRNA expression in the striatum was examined using an *in vivo* approach. Semi-quantitative RT-PCR analysis was used to examine animals from E15 until P15. In order to perform relative comparisons with low interassay

variations between samples at different development stages, RNA extraction, RT and PCR reactions were carried out under constant conditions at each step of the experiment. The analysis showed that D_1-R expression starts at E14, which is the time the striatal primordium is starting to form. This is in concordance with previous in situ hybridization and binding studies performed in rats (Schambra, 1994, Jung and Bennett, 1996). No information was previously available concerning the time of appearance of D_5-R receptor during striatal ontogeny. Here we show that D_5-R first is detectable at E14 and becomes more significant at E17 reaching a maximum around birth. At all other time points investigated the expression of D_5-R occurs at lower levels than D_1-R. The predominant expression of D_1-R seen in the mouse striatum is in agree with previous studies that reveal lower expression of D_5-R in the adult striatum from rat (Laurier et al., 1994; Luedtke et al., 1999). Despite the D_1-R subtype is much more abundant than the D_5 subtype, the presence of D_5-R in the striatum is not negligible because their affinity for dopamine is five times higher than that of D_1 (Grandy et al., 1991).

Our RT-PCR analysis attempted to clarify the developmental schedule of D1-like receptor expression in the striatum. However, conventional RT-PCR techniques with mRNA pooled from all the cell types in a brain region obviously lack the ability to resolve heterogeneity within a population of cells. Therefore, it is not possible with this technique to distinguish between mRNAs originating from neuronal and from non-neuronal sources as well as from different neuronal phenotypes within a region. Single-cell RT-PCR might overcome this problem. Surmeier et al. (1996) has adapted this technique to analyze the coordinated expression of dopamine receptors in adult striatal neurons (Surmeier et al., 1996). Immunocytochemical studies can also reveal the distribution of neurons containing D_5 and D_1-R and their molecular phenotype (Rivera et al., 2002). Further experiments should be performed to characterize *in vivo* the molecular phenotypes of striatal cells expressing these receptors during ontogeny.

Moreover, previous studies have demonstrated that the two compartments of the developing striatum display differential transcriptional and translational activity for the D_1-R gene and consequently two different and successive patterns of expression of D_1-R protein: patch neurons first express D_1-R intensely while matrix neurons express it later and in smaller amounts (Caille et al., 1996). Therefore, it would be interesting to study the pattern of distribution of D_5-R, which we consider is relevant to elucidate also the

functional role of this receptor in the setting of the striatal compartmentation during development.

4.2. Expression of D1-like receptors in neurons and astrocytes

It is still poorly understood whether the developmental effects of dopamine are exerted directly on GABAergic neurons or involve a different cell population, e.g. astroglial cells. In earlier studies, astrocytes from several brain regions have been reported to express dopamine receptors (Hansson and Rönnbeck, 1988; Bal et al., 1994; Zanassi et al., 1999). Although these studies are controversial with respect to the expression of D1-like receptors, they document the potential role of astrocytes in mediating dopaminergic effects. In the present study, we analyzed the expression of D_1- and D_5-R in neurons and astrocytes from the striatum using an *in vitro* approach. These experiments were performed using dissociated neuronal and glial cultures. In order to characterize unambiguously the results obtained with such cultures, we ascertain the neuronal and glial character of the studied cells using MAP-2 as neuronal marker and GFAP as astroglial marker. Neuronal cultures were composed mainly of neurons and a small fraction of glia cells due to the characteristic "feederlayer" these cell form underneath the neuronal cells. The highly enriched astroglial cultures were set up following an established protocol (Ivanova et al., 2001) and showed no contamination with neuronal cells as revealed by RT-PCR. In this study, we have shown that D_5-R is expressed in both neurons and astrocytes whereas D_1-R expression is restricted to neurons. Although in neuronal cultures both transcripts were present, it is important to remark that *in vivo* D_1- and D_5-R are primarily expressed in different striatal cell populations. D_1-R has been shown to be strongly expressed in projection neurons (Surmeier et al., 1996) whereas D_5-Rs are preferentially expressed in striatal interneurons and weakly expressed in projection neurons (Bergson et al., 1995; Rivera et al., 2002). The presence of D_5-R in cholinergic neurons in the striatum has been demonstrated by single-cell RT-PCR (Yan and Surmeier, 1997) and by double immunofluorescence experiments (Rivera et al., 2002) in which it was demonstrated that all cholinergic cells expressed D_5 receptors. In addition, Yan and Surmeier (1997) demonstrated that most of these cells do not express D_1 receptors. Our present observations in astrocytes that express D_5- but not D_1-R might point to a comparable differential pattern of expression. Despite that capacious information about this dopamine receptor subtype, the functional significance of D_5 receptors in striatal astrocytes is not yet known.

Dopamine has been shown to increase the expression of trophic factors in striatal astrocytes such as glial cell-line-derived growth factor (GDNF) and fibroblast growth factor 2 (FGF-2) (Reuss and Unsicker, 2000b; Kinor et al., 2001). Both developmental signals interfere with cellular differentiation and cell survival at the striatal level addressing either established dopaminergic connections or the morphological maturation of intrastriatal neurons, respectively (Tomac et al., 1995; Rosser et al., 2000). Thus, evidences condenses that dopamine plays a key role in the coordination of other important striatal growth-promoting signals not only in neuronal cells but also in astrocytes, non-neuronal cells that play pivotal roles during development.

4.3. Influence of BDNF on D1-like receptors in neurons and astrocytes

We have shown previously, that dopamine stimulates, via activation of D1-like receptors, neurite growth and expression of the GAD65/67 enzymes in striatal GABAergic nerve cells (Schmidt et al., 1996; Küppers et al., 2000). Interestingly, some of the effects of dopamine revealed latencies in the onset of 12h-24h after the treatment of the cells, thus possibly pointing to intermediate steps such as the cooperation with additional signals. In fact, previous studies have shown that BDNF and its receptor play an important role during striatal development. BDNF promotes survival and phenotypic differentiation of most developing striatal neurons (Jones et al., 1994; Mizuno et al., 1994; Widmer et al., 1994; Ventimiglia et al., 1995; Ivkovic et al, 1997; Gratacos et al., 2001) and the BDNF receptor protein, trkB, is highly expressed on striatal patch neurons and fibers during development (Constantini et al., 1999). Furthermore, we recently could demonstrate that dopamine upregulates the expression of BDNF via D1-like receptors in striatal neurons (Küppers et al., 2001). Taken together, this point to the possibility that the developmental effects of dopamine on striatal GABAergic neurons (Schmidt et al., 1996; Küppers et al., 2000) are mediated –at least partially- by BDNF. In order to pursue this line, we have examined whether BDNF potentially regulates postsynaptic dopamine signaling, focusing on the expression of D1-like receptors not only in neurons but also in astrocytes as possible target cells. Putative effects of BDNF treatment on D1-like receptor expression were examined by RT-PCR. The BDNF concentration used in these experiments was 50 ng/ml. This corresponds to ED_{50} as determined by the manufacturer by the dose dependent induction of choline acetyl transferase activity in rat basal forebrain primary septal cultures. Nevertheless, experiments were performed to establish a dose- and time-dependency of

BDNF effects. We observed a significant increase in D_5-R expression in astrocytes in presence of 50 ng/ml of BDNF after 72 h. This impact on the dopaminergic transmission at the receptor level was not seen in neurons. Although the RT-PCR technique was capable of detecting the presence and regulation of mRNAs for D_5-R, there was no guarantee that functionally significant levels of receptor protein were generated from these transcripts. Therefore, the protein expression of D_5-R was determined by western blotting using membrane fractions as well as whole cell lysates and FACS analysis. Both approachs revealed a significant increase of D_5-R protein in astrocytes under BDNF treatment. Thus, these observations suggest that one of the intermediary actions of BDNF in dopaminergic effects on striatal neuronal differentiation might be to regulate the sensitivity of astrocytes towards dopamine by inducing the expression of a distinct type of dopamine receptor. This hypothesis is supported by in vivo studies that have demonstrated a spatial and temporal expression of trkB that coincides with the establishment of the patch/matrix compartmentation in the developing striatum (Constantini et al., 1999). Moreover, trkB expression was co-localized with patchy dopamine fibers, substance P- immunoreactive neurons and glutamate receptors. TrkB expression occured perinatally and changed to a more scattered pattern at the same time that the innervation by nigrostriatal afferents becomes homogeneous throughout the striatum. This suggests strongly the convergence of BDNF and dopamine signaling.

Interestingly, comparable results regarding the expression of dopamine receptor subtypes have been reported in the shell division of the nucleus accumbens recently by Guillin and coworkers (2001). Performing lesion experiments and using gene-targeted mice lacking BDNF they demonstrated that BDNF, probably released from dopaminergic neurons arising in the ventral tegmental area, increased the expression of the dopamine D_3 receptor subtype, a member of the D2 receptor family. Moreover, the application of levodopa in the striatum of 6-OHDA-lesioned animals resulted in an overexpression of D_3 receptors, which is normally not or only to a low extent detectable in this brain region. This effect was blocked by the concurrent administration of BDNF antagonists indicating an intermediate role for BDNF. This observation is in line with our findings, that BDNF induces the expression of a distinct dopamine receptor subtype, and supports the hypothesis that this trophic factor might be implicated in the mediation of dopaminergic effects by eliciting changes in dopaminergic neutransmission. However, the functional importance of D_5-R upregulation in astrocytes remains unclear. In neocortical neurons, the

activation of D_5-R is causally linked to a rapid increase in intracellular calcium levels likely through an opening of L-type calcium channels (Lezcano and Bergson, 2002; Baufreton et al., 2003). Although this activation has not been shown in astrocytes yet, it is known that dopamine induce changes in intracellular calcium concentrations in astroglia (Verkhratsky et al., 1998), and such changes have been demonstrated to influence gene transcription (Hardingham et al., 1998; Ginty, 1997) and moreover to affect the glutamate release (Hansson and Rönnback, 2003). Assuming that such a signal coupling observed in neocortical neurons exist in astroglia, dopamine signaling through D_5-R might thereby supply glutamate to adjacent nerve cells and contribute to developmental processes like proliferation, survival and differentiation of GABAergic striatal cells.

4.4. Intracellular pathways activated by BDNF stimulation

There is a general agreement that trkB uses multiple signaling pathways to control specific effects (Atwal et al., 2000). Indeed, BDNF binds to trkB and may activate the PI3-kinase/Akt a well as the MAP-kinase/MEK pathway, mainly responsible for the promotion of neuronal survival (Kaplan and Miller, 2000). In order to understand the intracellular processes involved in BDNF-mediated regulation of dopamine D_5 receptor expression in astrocytes, we have analyzed these two signaling pathways. Pharmacological experiments using specific inhibitors for MEK and PI3-kinase revealed that BDNF effects on dopamine D_5-R induction depend on a rapid activation of PI3-kinase signaling. It is interesting that BDNF stimulated not only the PI3-kinase pathway but also the MAP-kinase signaling cascade as the phosphorylation of Akt and ERK1/2 revealed clearly. Activation of any of these pathways may cause activation of CREB in response to BDNF, which is implicated in the control of gene expression (Wu et al., 2001). However, our results indicated that only the activation of PI3-kinase pathway is involved in the observed BDNF effects on D_5-R expression. Whether the activation of MEK, alone or together with Akt, is associated to other astroglia responses to BDNF remains to be determined. The MEK/ERK cascade has been reported to be involved in the induction and maintenance of astrocyte stellation mediated by PKC (Abe and Saito, 2000). Moreover, inhibition studies indicate that activation of the MAPK pathway is required early in the differentiation process (Rajan and McKay, 1998). In addition, the stimulation of MAP-kinases in astrocytes causes the phosphorylation of CREB and leads to NF-kappaB activation (Zaheer et al., 2001); hence, it is conceivable that BDNF is also involved in the control of apoptotic process related to

astrocytes. The latter effect may also be in relationship with the well-established role of BDNF as a regulator of cell fate of different progenitors in the neostriatum (Lachyankar et al., 1997). Further data are required to substantiate this hypothesis.

4.5. Characterization of striatal glial cultures

As we discussed in the previous chapter, BDNF may elicit diverse cellular responses, like the control of apoptosis, promotion of survival and cell death. This study has shown that BDNF induces rapid activation of two signaling pathways known to be involved in survival events. Akt is reported to directly inhibit activation of the apoptotic machinery involved in cell death, while MAPK promotes the expression of anti-apoptotic proteins such as Bcl-2, as well as the transcription factor CREB, which is thought to be a major mediator of neuronal survival (reviewed by Kaplan and Miller, 2000). Although there is scarse information of these intracellular processes in astrocytes, the observed D_5-R upregulation in striatal glial cultures by BDNF might be due to a selective stimulation of survival or/and death of a distinct cell population rather than an increased expression of the receptor per cell. We have addressed this problem by performing immunocytochemistry to characterize our astroglial cultures with respect to the expression of D_5 and trkB receptors. Indeed, our studies revealed three subpopulations of astroglial cells with respect to the expression of D_5 and trkB receptors. A small population of cells predominantly expressed D_5-R. In these cells was no or only very low expression of trkB visible. Therefore, we think that these cells do merely represent targets for BDNF in our culture system. On the other hand, we found cells that expressed trkB in considerable amounts but no D_5-R expression was detectable. The third population consists of cells that expressed both trkB as well as D_5-R at various intensities. BDNF did not alter the total number of cells or the number of cells that express predominantly D_5-R. In contrast, BDNF shifted the ratio of cells expressing mainly trkB and cells being double-immunopositive for trkB and D_5-R toward the latter. Therefore, we conclude that the increase of double-immunopositive cells was not due to proliferation, nor to cell death or survival of a selective subpopulation but to an increase in D_5-R expression per cell. This data are also supported by FACS analysis revealing an increase in the number of immunopositive cells for D_5-R after BDNF treatment.

The *in vitro* approach we chose, does not allow to draw direct conclusions regarding the situation *in vivo*. Nevertheless, our data suggest that the heterogeneity of

astrocytes with respect to BDNF- and dopamine – responsiveness may be an important pre-requisite for the establishment of functional striatal compartmentation. This is supported by the hypothesis of Constantini et al., (1999) who described a spatial overlap in the expression of trkB and dopamine connections in the developing striatum. This again indicates a distinct role of BDNF for the genesis of the patch/matrix compartmentation. Moreover, the fact that striatal astrocytes are an important source of trkB expression points to that astrocytes might play a crucial part in this process.

4.6. Brain-region specific response to BDNF

Studies over the past years performed mainly in cell culture systems have revealed that astrocytes can express almost any known neurotransmitter or hormone receptor (Verkhratsky and Kettenmann, 1996). Some studies on glial cells *in situ*, however, indicated that astrocytes in defined regions of the brain are characterized by a restricted set of such receptors. Bergmann glial cells express functional receptors for glutamate, GABA, or histamine, but not for glycine or dopamine (Kirischuk et al., 1996; Müller and Kettenmann, 1995). Glycine receptors are found in glial cells of the spinal cord, a tissue well described for glycinergic transmission (Pastor et al., 1995). Such findings support the concept that there is a good match between the receptors expressed by the astrocytes and adjacent neurons, respectively (Reuss et al., 2000a). Previous studies have shown that D_5-R localizes in neurons from hypothalamus, striatum and cortex (Khan et al., 2000). Thus, our observations of D_5-R in astroglia from areas being targets of dopamine pathways support this concept of a synchronous expression pattern of neighboring astrocytes and neurons. However, the BDNF-induced increase of D_5-R was restricted to the striatum and did not occur in cortex or hypothalamus. This observed failure of BDNF to promote D_5-R expression in astroglia derived from CNS areas other than the striatum is not surprising given the fact that CNS astrocytes do not represent a homogenous cell population, but show region-specific differences with respect to their lineage, gene expression, and regulation (Wilkin et al., 1990). Unlike as in the hypothalamus and cortex, in the striatum BDNF represents a strong trophic factor for neuronal differentiation (Liu and Graybiel, 1999) and the trkB receptor plays a relevant role in establishing innervation patterns thus influencing the induction of specific neuronal types. Therefore, it is reasonable that only striatal astrocytes might be important targets for BDNF modulating dopaminergic responsiveness. Comparable results concerning brain region-specific responses of glia to

extracellular factors were reported for fibroblast growth factor (FGF)-2, -5 and -9 on the expression of the gap junction protein, connexin43 (Reuss et al., 1998, 2000a). Whereas FGF-9 attenuates connexin43 expression in glia cultured from the cortex, striatum and mesencephalon, FGF-2 exerts similar inhibitory effects only on glia of the neocortex and the striatum, but not on mesencephalic glia. Moreover, with FGF-5 such inhibitory effects on connexin43 expression are restricted to mesencephalic glia (Reuss et al., 1998, 2000a)

4.7. Dopamine influence in glutamatergic transmission in striatal astrocytes

Intensive research has been recently focused on the interactions of dopamine with the glutamatergic system in the striatum and further in the implications of dopamine/glutamate imbalance during Parkinson's disease. Electrophysiological and biochemical studies have shown that the striatal dopaminergic afferents exert an inhibitory control over the glutamatergic corticostriatal transmission through pre-synaptic and post-synaptic mechanisms (Kerkerian et al., 1987; Garcia-Muñoz et al., 1991; Cepeda et al 1993). Most of the studies so far have reported a modulation role of dopamine on the glutamatergic transmission of striatal neurons including regulation of expression and trafficking of striatal glutamate receptors (Dunah and Standaert, 2001; Lai et al., 2003). However, at present there is no direct evidence whether dopamine interferes with the glutamatergic transmission in astrocytes. The most important mechanism used by these non-neuronal cells to regulate the glutamatergic transmission is the uptake of glutamate from the synaptic cleft via the two astroglial membrane transporters, GLT-1 and GLAST. The observation that striatal astrocytes represent target cells for dopamine taken together with the above mentioned evidence suggesting a role of dopamine in the modulation of the glutamatergic transmission in neurons point to a dopaminergic regulation of GLT-1 and GLAST during striatal development. Therefore, we have investigated the possible regulatory role of dopamine in the expression of glutamate transporters. We observed that in striatal astrocytes dopamine downregulates the expression of GLT-1 and GLAST at the transcriptional level. This downregulation in mRNA was accompanied by a decrease in protein levels in the case of GLT-1. The inconsistency between the transcriptional and translational regulation of GLAST may be attributable to the duration of the dopamine treatment. A decrease in GLAST protein levels may require prolonged dopamine stimulation. Moreover, Gegelashvili and Schousboe (1997) previously reported that the metabolic turnover rates of the glutamate transporters (i.e. translation versus degradation

rates) and turnovers rates of the corresponding mRNAs (i.e. transcription versus degradation) do not necessarily change in concert and are probably regulated differentially depending on cell phenotype, environmental cues, and signalling pathways used. For example, in amyotrophic lateral sclerosis patients, dramatic loss of GLT1 protein was not matched by downregulation of the corresponding mRNA. Some regulatory signals like glutamate and kainate can induce GLAST or GLT protein but not mRNA (Gegelashvili et al., 1996). The precise molecular events involved in the utilization rates of glutamate transporter mRNAs have yet to be explored.

Furthermore, our evaluation of extracellular concentrations of L-glu showed that cultures exposed to dopamine show higher extracellular and lower intracellular concentrations of glutamate after 72 h whereby the decrease in intracellular L-glu concentration matches the increase in the extracellular. This observation may be due to a reduced amount of GLT-1 protein available for L-glu uptake activity, either by internalization of the transporter or by a lower translation rate. The latter supports our PCR data according to which dopamine decreases GLT-1 mRNA expression. However, the increase in extracellular levels of glutamate induced by dopamine may not necessarily result from a downregulation of GLT-1 but can result of an increase release. Analyses of supernatants from astrocytic cultures have shown that any ligand able to evoke Ca2++ elevations can cause the release of glutamate (Hansson and Rönnbäck, 2003). Since activation of dopamine receptors elevates intracellular Ca2++ levels in astrocytes (Reuss et al., 2000b) it is also possible that the increase in the extracellular glutamate found in our cultures is the result of an increased release of glutamate. The experiments performed so far do not enable us to discriminate if dopamine is associated with a decrease in the L-glu uptake or with the stimulatory release of L-glu. Even the induction of both mechanisms could be particularly relevant during striatal ontogeny when glutamate is critical for several developmental processes. Therefore, further experiments are necessary to elucidate which mechanism triggered by dopamine increases the extracellular concentrations of glutamate. First, evaluating the L-glu uptake activity of cultured astrocytes requires monitoring the changes in the concentration of exogenously applied L-glu. Second, to see whether dopamine treatment results in an increased release of L-glu, cells should be incubated in the presence or absence of Ca++. Absence of Ca++ can be achieved by treating the cells with the membrane-permeable Ca++ chelator BAPTA-AM.

Our results point to a dopaminergic influence on the glutamatergic transmission in astroglia with dopamine increasing the extracellular glutamate concentrations. Since in astrocytes, glutamate transporters -in contrast to neurons- are located on cell membrane that surrounds synapses (Chaudhry et al., 1995) dopaminergic modulation of GLT-1 directly influences the concentration of glutamate in the synaptic cleft. Elevated levels of glutamate may cause neurotoxicity. Therefore, astrocytes may play a dual role in assuring neuronal survival, not only by protecting neurons from excitotoxic levels of glutamate but also by supplying glutamate when endogenous levels fall below those that are optimal for neuron survival. Evidence indicates that reducing the astroglial glutamate transporters results in an increased vulnerability of neurons to death, as it has been shown in specific neurodegenerative processes underlying sporadic amyotrophic lateral sclerosis (Bristol and Rothstein, 1996). In line with this assumption, the inhibition of glutamate transporters expression *in vivo* was found to induce neurodegeneration and progressive paralysis (Rothstein et al., 1996). However, during striatal ontogeny glutamate plays an important role for many developmental processes. Indeed, it has been previously reported that in the period when plasticity is greatest and synaptic connections are formed an overproduction of glutamate coincides with astroglia differentiation (McDonald and Johnston, 1990). Glutamate might be involved in cell death that occurs in the striatum during the first postnatal week as well as in the proper differentiation and proliferation of striatal cells. Striatal cells might repond to glutamate depending on the developmental processes that take place during ontogeny.

Our study provides the first evidence that dopamine influences glia in the regulation of the glutamatergic transmission and might thereby supply glutamate to adjacent nerve cells and contribute not only to survival but also to the well known phenomenon of glutamate-mediated proliferation and differentiation of neostriatal neurons (Luk et al., 2003). It is important to consider astroglial responses in this kind of interaction for a better understanding about the signalling mechanisms involved during development and furthermore during restorative processes.

5. SUMMARY

The development of the striatum is a complex process that depends on the precise temporal and spatial action of developmental signals. In particular, dopamine represents a major developmental signal for the striatum that has been shown to influence the differentiation and synaptic plasticity of GABAergic cells by activation of D1-like receptors. However, the mechanisms by which dopamine can exert these effects are presently not well defined. They can be mediated directly on GABAergic cells or indirectly, either via another cell population or via other developmental factors. There is growing evidence that interactions between different developmental signals rather than singular actions are critical for cellular differentiation and organization of the striatum. Since previous works have shown that dopaminergic effects exhibit significant latencies, it is hypothesized here that additional factors and/or another cell population are required to serve an intermediate role.

The first part of this study is concerned with the expression of D1-like receptors in striatal cells. The expression analysis was performed by applying semiquiantitative RT-PCR. Our *in vivo* studies showed that both members of the D1-like receptor family are expressed already at E14. Whereas the developmental expression of the D_1-R steadily increased perinatally until it reached adults levels, the expression of D_5-R peaked around birth and then decreased to adults levels. The *in vitro* studies revealed expression of D_5-R in neurons as well as in astrocytes while D_1-R expression was restricted to neurons. These data clearly demonstrate that astrocytes represent target cells for dopamine and therefore might play an intermediate role in developmental processes induced by dopamine.

The second part focuses on the intermediate role of BDNF in dopaminergic effects via modulation of the dopaminergic transmission at the postsynaptic level in striatal cells. RT-PCR analysis showed that BDNF selectively up-regulated D_5-R expression in astrocytes. Pharmacological approaches indicated that this effect was mediated by activation of the PI3- but not MAP-kinase cascade. Furthermore, FACS analysis, western blot, and confocal laser microscopy revealed that the newly synthesized D_5-R was integrated into the plasma membrane of astrocytes. Our data support the hypothesis that BDNF might be implicated in the mediation of dopaminergic effects by eliciting changes in dopaminergic neutransmission in astrocytes. Our immunocytochemical characterization also revealed three subpopulations of astroglia cells with respect to the expression of D_5-R

and trkB. This heterogeneity of astrocytes with respect to BDNF-dopamine responsiveness may be an important pre-requisite for the establishment of functional striatal compartmentation.

The third part of this study is concerned with modulation of the glutamatergic transmission by dopamine in non-neuronal cells. Dopamine has been shown to be involved in the modulation of the glutamatergic transmission during development. Thus, we followed the idea that dopamine might regulate the glutamate uptake system in astrocytes. In order to study this, we have evaluated *in vitro* the expression of the astroglial glutamate transporters, GLT-1 and GLAST and the glutamate turnover after dopamine treatment. We observed that dopamine downregulates the expression of GLT-1 at transcriptional and translational levels. Moreover our evaluation of extracellular concentrations of L-glu showed that cultures exposed to dopamine reveal higher extracellular and lower intracellular concentrations of glutamate after 72 h. These data clearly show for the first time that dopamine can influence astroglial glutamatergic transmission and thereby might indirectly contribute to proliferation, differentiation and survival of GABAergic neurons.

In conclusion, our findings clearly suggest a potent intermediate role of astrocytes in developmental processes induced by dopamine. First, astrocytes express the D1-like receptor subtype (D_5-R) that display higher affinity for dopamine. Second, BDNF increases the expression of D_5-R thereby possibly increasing the sensitivity of astrocytes toward dopamine. Third, dopamine is able to regulate important developmental function in astrocytes such as the glutamatergic transmission by modulation of the glutamate transporters expression. Taken in consideration the importance of dopamine for striatal ontogenesis, we propose a new model of dopaminergic action where signals like dopamine and BDNF converge on astrocytes coordinating the availability of other developmental signals and establishing a network of interaction with neurons in a spatial and temporal correct fashion

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