NOTE TO USERS

Novel Roles of Amino Acid Neurotransmitters in Mammalian Forebrain Development

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Abbreviations used

5-HT	5-hydroxytryptophan
AANTS	amino acid neurotransmitters
ABC	avidin biotin complex
AC	anterior commissure
AKT	protein kinase B
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	analysis of variance
AP-5	D-2-amino-5-phosphonopentanoate
ARND	alcohol related neurological disorder
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblastic growth factor
bHLH	basic helix-loop-helix
BMI	bicuculline methiodide
BrdU	bromodeoxyuridine
CGS 19755	cis-4-(phosphonomethyl) piperidine-2-carboxylic acid
CNS	central nervous system
CR	calretinin
CSA	corticostriatal angle
CSF	cerebrospinal fluid
DAB	diaminobenzidene
DIV	days in vitro
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	embryonic day
EC	external commissure
EGF	epidermal growth factor
ERK	extracellular regulated protein kinase
FAS	fetal alcohol syndrome
FBS	fetal bovine serum
GABA	γ-amino butyric acid
GAD	glutamic acid decarboxylase
HBSS	Hank's balanced salt solution
i.p.	intraperitoneal
KA	kainic acid
LAMP	limbic system-associated membrane protein
LGE	lateral ganglionic eminence
MAP-2	microtubule associated protein 2
MAPK	mitogen activated protein kinase
MK-801	hydrogen maleate (dizocilpine)
mRNA	messenger ribonucleic acid
NA	numerical aperture
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NT 3/4	neurotrophin 3/4
PCNA	proliferating cell nuclear antigen
PHA	phytohemagglutinin
PI3	phosphatidyl inositol 3
PV	parvalbumin
PVE	periventricular epithelium
RNA	ribonucleic acid

RT-PCR	reverse transcriptase-polymerase chain reaction
MGE	medial ganglionic eminence
SNK	Student-Newman-Keuls test
SPP	secondary proliferative population
SS	somatostatin
SVZ	subventricular zone
TGF	transforming growth factor
VZ	ventricular zone

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Abstract

Neurotransmitters play multiple roles in the development and maturation of the central nervous system (CNS). In particular, glutamate and gamma-amino butyric acid (GABA) have been shown to influence the proliferation and survival of a variety of CNS cell types. Here, these two properties have been investigated in the developing rodent telencephalon. An *in vivo* model whereby specific subpopulations of neuronal progenitors are targeted according to their time of appearance was utilized in conjunction with stereological quantification techniques. Results demonstrate that while glutamate has a positive proliferative effect in the developing germinal zones, the role of GABA may be more restricted to survival. Moreover, administration of various amino acid receptor antagonists revealed complimentary differences between dorsal and ventral telencephalic compartments with respect to the glutamate receptors mediating the proliferative response. Proliferative responses to glutamate in the ventral and dorsal telencephalon are respectively mediated by the NMDA and AMPA/kainate subclasses of receptors. Cultured embryonic striatal and cortical neuronal progenitors also show responses to amino acid neurotransmitters which reflect observations made *in vivo*. Therefore, the neurotransmitter microenvironment in the developing brain may contribute to generating neuronal diversity via differential responses of proliferating progenitors.

Les neurotransmetteurs jouent de multiples rôles dans le développement et la maturation du système nerveux central (SNC) et en particulier, le glutamate et l'acide gamma-amino butyrique (GABA), qui ont été démontré capable d'influencer la prolifération et la survie d'une variété de types neuronaux dans le SNC. Dans cette étude nous avons fait l'investigation de ces deux propriétés au niveau du téloncephale de rongeurs en phase de développement. Nous avons utilisé un modèle, *In vivo*, où une sous-population de progénitures neuronales est ciblée en fonction du temps de son apparition, conjointement avec une technique de quantification stéreologique. Nos résultats démontrent que le glutamate renforce la prolifération des cellules au niveau des zones germinales en développement, alors que le GABA semble plutôt jouer un rôle dans la survie neuronale. Par ailleurs, l'administration de différents antagonistes des récepteurs d'acides aminés, révèle des différences complémentaires entre les compartiments télencéphaliques dorsale et ventrale. Les récepteurs au glutamate assurent la médiation de la réponse proliférative. De plus, dans le télencéphale ventral et dorsal, les réponses prolifératives au glutamate semblent être médiées par la sous-classe des récepteurs NMDA et AMPA/kainate. Nous avons par ailleurs, observé, *In vitro*, le même type de réponses aux acides aminés dans des cultures de progénitures neuronales issues du striatum ou de cortex, confirmant ainsi nos observations *In Vivo*. Le microenvironnement des neurotransmetteurs dans un cerveau en developpment semble contribuer dans la génération de variétés neuronales via une réponse différentielle des progénitures neuronales en prolifération.

Contribution of Authors

Experimental work for all papers on which I am first author were completed by myself under the supervision of the senior author(s). Conceptualization and planning (e.g. experimental design) were done with the help of senior authors, while all pertinent practical work including animal procedures, tissue culture, histology, quantification, and biochemistry were completed by myself, with the exception of paraffin embedded material used in Chapters 3 and 4. These were prepared with the assistance of the Neuropathology Unit staff at the Montreal Neurological Institute.

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Chapter 1: Introduction and Literature Review

1.1 Introduction

The final form and size of individual tissue systems are determined by the interplay between progenitor proliferation, exit from cell cycle, migration, differentiation into various cell types, and cell death. In the mammalian central nervous system (CNS), sophisticated orchestration of these cellular events is required to achieve a normal, operational structure (reviewed by Sanes, 2000). In turn, such cellular processes are regulated according to both intrinsic programs and extrinsic cues. While development and maintenance of phenotype is largely governed at the level of gene expression (e.g. positioning and cell specification by segmentation or homeobox genes), local trophic factors, cell contact, and activity may also activate new genes (Lillien, 1998; Edlund and Jessell, 1999). Such extrinsic variables in the extracellular milieu include physical parameters as well as the presence of signaling molecules. For example, dozens of soluble proteins have been demonstrated to modulate various cellular processes and have therefore been termed growth factors (reviewed in Cameron *et al.*, 1998; Weiss *et al.*, 1998). In the developing CNS growth factors have diverse and often pleiomorphic functions ranging from mitogen (bFGF, EGF*) (Reynolds *et al.*, 1992; Vescovi *et al.*, 1993), survival factor (NGF, BDNF) (Ghosh *et al.*, 1994)), and differentiation factor (TGFβ, NT3/4) (Constam *et al.*, 1994; Knusel *et al.*, 1994).

1.2 Neurotransmitters as morphogens

* Abbreviations: basic fibroblastic growth factor (bFGF); epidermal growth factor (EGF); nerve growth factor (NGF); brain-derived neurotrophic factor (BDNF); transforming growth factor (TGF); neurotrophin 3/4 (NT3/4)

There is now growing consensus that small molecules classically known for their neurotransmission properties in mammals may also have additional functions during development (Cameron *et al.*, 1998; Nguyen *et al.*, 2001). Indeed, it has been shown that primitive organisms utilize monoamines, acetylcholine, and GABA as endogenous growth regulatory mechanisms (Lauder, 1988). These molecules are released into the extracellular surroundings following intracellular Ca^{2+}elevation, and provide a local paracrine mechanism for eliciting their morphogenic activities. For example, diazepam, a GABA receptor ligand, increases the growth rate of the protozoan *Tetrahymena,* suggesting that GABA may act as a mitogen in its early development (Darvas *et al.*, 1985). Larvae of other species produce catecholamines that regulate metamorphosis (Kolberg and Martin, 1988). In somewhat higher multicellular organisms such as flatworms, serotonin, dopamine, and norepinephrine play roles in regeneration (Franquinet and Martelly, 1981). Both 5-HT* and dopamine act via specific receptors linked to G-proteins, thus facilitating the activation of signaling pathways involved in a number of cellular processes (Weiss *et al.*, 1998). Here, the presence of 5-HT appears to inhibit RNA synthesis while increasing DNA synthesis. Addition of dopamine restores RNA synthesis. *Planaria* express D1 and D2-like dopamine receptors that, unlike mammalian receptors, upregulate cAMP levels (Franquinet and Martelly, 1981). In nematodes and *Drosophila*, mutants with a deficiency in ChAT exhibit severe growth defects, and small size, in addition to uncoordinated behavior, suggesting that acetylcholine may be important in normal CNS development (Rand and Russell, 1984).

* Abbreviations: 5-hydroxytryptophan (5-HT); cyclic AMP (cAMP); choline acetyl transferase (ChAT)

Increasing evidence suggests that a number of neurotransmitters and their receptors are present in the embryonic mammalian CNS. Furthermore, it as been suggested that neurotransmitters may allow neuronal populations that mature first to provide feedback to populations still developing (McConnell, 1988; Barker *et al.*, 1998). Lastly, neurotransmitters may also provide a mechanism by which the embryonic CNS could respond indirectly to maternal signals and changes in its extracellular environment (Cameron *et al.*, 1995). For example, neurotransmitters such as norepinephrine and vasoactive intestinal peptide are known to cross the placenta from mother to embryo (Phillippe, 1983).

A significant body of evidence also suggests that amino acid neurotransmitters (AANTs) play a major developmental role in the CNS. During embryonic and postnatal development, the concentrations of free amino acid in brain undergo dramatic fluctuations. Such changes in the level of amino acids seen during early brain development likely reflect structural and metabolic maturation of the developing brain. Both mature CNS cells and progenitors are therefore surrounded by a developmentally regulated cocktail of AANTs throughout the period of their generation and maturation. Thin layer chromatography of blood, cerebrospinal fluid (CSF), as well as frontal and optic lobe tissue in the chick and mouse has revealed a number of candidate AANTs likely to influence CNS development. Specifically, CSF levels of glutamate, γ-amino butyric acid (GABA), glycine, taurine and aspartate are low throughout life, remain constant in serum, but transiently increase in brain tissue during the course of embryogenesis (Huether and Lajtha, 1991). For example, taurine,

known to act as a trophic factor during brain development is, perhaps incidentally, highest at day 8 in the chick embryo when neuronal proliferation is maximal.

In the mouse, the levels of GABA, glutamate, and aspartate in the developing cerebellum, olfactory bulb, hypothalamus, and spinal cord, all increase during embryogenesis and peak before birth (Miranda-Contreras *et al.*, 1998; 1999; 2000). Similarly, immunohistochemical staining of embryonic day (E) 10 murine cortical explants with antibodies against GABA and glutamate reveal a considerable number of cells reactive for these neurotransmitters in the neuroepithelium and pial layer (Haydar *et al.*, 2000). The intensity of GABA and glutamate staining diminishes progressively during the course of neurogenesis and is barely detectable at birth. Similar patterns of GABA expression are observed in the developing rat, monkey, and human cortex where the first GABAergic cells are detected in the plexiform primordium at the onset of neurogenesis and disappear from the ventricular and subventricular zones just after neurogenesis ceases (Van Eden *et al.*, 1989; Meinecke and Rakic, 1992; Yan *et al.*, 1992). The corresponding receptors to these neurotransmitters also display developmentally regulated patterns of expression in a region specific manner (Lauder *et al.*, 1986; Blanton and Kriegstein, 1992; Monyer *et al.*, 1994; Ritter *et al.*, 2001).

1.3 Glutamate and morphogenesis

Glutamate and aspartate are two non-essential amino acids synthesized from the transamination of α-ketoglutarate and oxaloacetate, respectively. Together, they represent the most widely distributed excitatory amino acid neurotransmitters in the

CNS (Curtis *et al.*, 1972). Glutamate is highly potent, capable of depolarizing neurons at sub-picomolar concentrations while aspartate acts at higher concentrations. These properties, in addition to its pattern of distribution in the developing CNS, make glutamate a likely candidate for regulating brain development.

1.3.1 Glutamate receptors

Receptors for glutamate can be divided into ionotropic and metabotropic subtypes. Secondary messenger systems, e.g. G-proteins, mediate signaling following stimulation of metabotropic receptors, whereas ionotropic receptors act as ligand-gated ion channels. The latter group can be further subdivided according to binding preferences and other pharmacological properties. Three such families exist: the AMPA*-, kainate-, and NMDA binding receptors (Schoepfer *et al.*, 1994). Of particular significance is that a number of functional ligand-gated ion channel receptors, including the NMDA and AMPA subclasses, have been reported in neural progenitors before synapse formation (Flint *et al.*, 1999; Maric *et al.*, 2000b; Jelitai *et al.*, 2002).

1.3.2 NMDA receptors

In the rat, NMDA receptors (NMDARs) are encoded by the highly regulated *NR1* and *NR2A-D* genes (*GluRδ1* and *GluRε1-4* in the mouse). The *NR1* mRNA is also alternatively sliced in different CNS regions, leading to eight isoforms of this subunit. The NR1 subunit has been shown to be required for functionality (Monyer *et*

* Abbreviations: N-methyl D-aspartate (NMDA); alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)

al., 1992) while the complementing NR2 subunits influence specific gating and membrane organizational properties (Varju *et al.*, 2001). *In vivo*, functional receptors are thought to be heteromeric tetra- or pentamers, although their precise stoichiometry is still debated. While homomeric NR1 receptors are functional, NR2 subunits appear to be necessary to elicit native scale response to agonists (Monyer *et al.*, 1992).

NMDARs exhibit ligand-gated conductance to Na^+, K^+, and Ca^{2+}, the latter distinguishing it from other ionotropic glutamate receptors with the exception of AMPA receptors lacking the GluR-B subunit (Ascher and Nowak, 1988; McBain and Mayer, 1994). Ionic permeability is positively modulated in the presence of glycine (a co-agonist) and certain polyamines such as spermine (Johnson and Ascher, 1987, 1990). An additional functional property of NMDARs is that of blockage by Mg^{2+} ions in a voltage-dependent manner. Therefore, channels may be closed at resting membrane potentials under normal conditions and transiently opened during depolarization (Johnson and Ascher, 1990). The receptor is also sensitive to excess Zn^{2+} ions and protons (Choi and Lipton, 1999).

Most studies on the functions of NMDARs have been performed using pharmacological agents which activate or antagonize receptor function. Commonly used antagonists include the MK-801* (non-competitive channel inhibitor)(Foster and Wong, 1987), CGS-19755 (competitive NMDA analogue)(Bennett *et al.*, 1989), and AP-5 (competitive NR2 antagonist)(Olverman *et al.*, 1984). One notable disadvantage of this approach is the lack of specificity that may occur given that a number of antagonists interfere with a putative aspartate receptor (Yuzaki *et al.*, 1996) whereas

* Abbreviations: MK-801 (hydrogen maleate; dizocilpine); CGS 19755 (cis-4-(phosphonomethyl) piperidine-2-carboxylic acid); AP-5 (D-2-amino-5-phosphonopentanoate).

some agonists may affect N-type calcium channels (Chernevskaya *et al.*, 1991). Nonetheless, NMDARs have been shown to orchestrate a wide variety of developmental events in addition to mediating synaptic transmission. Included in this list are migration of immature neurons (Komuro and Rakic, 1993; Rossi and Slater, 1993; Behar *et al.*, 1999), neurite outgrowth (Lipton and Kater, 1989), differentiation of neural cells (Ciccolini *et al.*, 2003), neuronal survival (Balazs *et al.*, 1988; Bhave and Hoffman, 1997), and apoptosis (Ikonomidou *et al.*, 1999; 2000). In addition, a growing body of evidence suggests that NMDARs may also be important in the proliferation of a number of CNS subpopulations such as the dentate gyrus granule cells (Cameron *et al.*, 1995), cerebellum granule cells (Fiszman *et al.*, 1999), and basal forebrain interneurons (Sadikot *et al.*, 1998; Luk *et al.*, 2003). In line with this view is the observation that expression of NMDARs is developmentally regulated both with respect to spatial-temporal distribution and subunit composition in the telencephalon (Williams *et al.*, 1993; Dunah *et al.*, 1999; Standaert *et al.*, 1999; Ritter *et al.*, 2001). Furthermore, knockout mice lacking the NR1 subunit die shortly after birth , underlining the importance of NMDARs in proper development (Forrest *et al.*, 1994). Interestingly, the majority of labeled cells from $NR1^{-/-}$ embryos survive when transplanted into wildtype recipients, suggesting that this subunit may not be vital to all neural cells (Maskos *et al.*, 2001).

When functional NMDARs first appear during *in vivo* development remains unclear. However, cultured neuronal progenitors derived from embryonic neocortex contain mRNA for the NR1 subunit before the appearance of AMPA receptor message (Maric *et al.*, 2000b) while non-differentiated cells belonging to the NE-4

neuroectodermal progenitor cell line express the NR1, NR2A, NR2B and NR2D subunits in the absence of retinoic acid induction (Jelitai *et al.*, 2002). In the turtle cortex, NMDAR expression also precedes AMPA/KA receptors (Rossi and Slater, 1993) although this pattern appears to be reversed in the primate cortex (Lidow and Rakic, 1995). In the rat, the NR2A and NR2C subunits also appear before or during the neonatal period (Dunah *et al.*, 1999) and functional receptors shift from being composed primarily of NR1/NR2B to NR1/NR2A subunits as indicated by a dramatic drop in affinity for ifenprodil, an inhibitor of the NR2B subunit polyamine binding site (Williams *et al.*, 1993). Most adult striatal neuron populations express NR1 and NR2A with the exception of ChAT-positive interneurons which preferentially express NR2B (Standaert *et al.*, 1999). In humans, NR1, NR2B, and NR2D transcripts are strongly expressed in the telencephalic ventricular zone from gestational week 10 onwards (Ritter *et al.*, 2001).

The wide range of cellular processes mediated by NMDARs suggests a sophisticated association to signal transduction machinery within the cell. Recent proteomic studies have revealed that NMDAR exists as a 2000kDa complex containing over 75 different proteins (Husi and Grant, 2001). These include putative neurotransmitter receptor subunits, cell adhesion proteins, adaptors, signaling enzymes, and associated cytoskeletal proteins (Husi *et al.*, 2000). Many of these proteins can be correlated to NMDAR function such as postsynaptic density protein 95 (PSD 95), which activates pathways regulating synaptic plasticity (Migaud *et al.*, 1998). Other pathways which appear to be coupled include the Ras-activated MAPK*

* Abbreviations: mitogen-activated protein kinases (MAPK); phosphatidylinositol-3-kinase (PI3k); protein kinase B (akt)

pathway, the PI3/Akt pathway, and the Rac pathway (Perkinton *et al.*, 2002). Together, these pathways have been implicated in the regulation of transcription, cell growth and survival, messenger trafficking, and cytoskeletal reorganization in forebrain neurons (Suzuki *et al.*, 2002; Barnabe-Heider and Miller, 2003) .

1.4 GABA

Like glutamate, GABA is abundant and widespread within the CNS. A product of the decarboxylation of glutamate via glutamic acid decarboxylase (GAD), GABA is considered the major inhibitory neurotransmitter along with glycine (Sieghart, 1995; Bormann, 2000). GABAergic synapses, identified via immunohistochemistry for GAD and GABA have been found in most regions of the brain, including the striatum where the majority of neurons are positive for this enzyme (Lauder *et al.*, 1986; Kubota *et al.*, 1993; Kawaguchi *et al.*, 1995). In the embryonic rat CNS, expression of GABA is already detectable in the CNS by E13 and GABAergic cells begin to appear in the developing striatum and cortex at E14 (Lauder *et al.*, 1986; Van Eden *et al.*, 1989).

Receptors for GABA can be subdivided into three subclasses: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are closely related ionotropic receptors whereas the $GABA_B$ subfamily is metabotropic and appears to be located presynaptically (reviewed in Bormann, 2000). $GABA_A$ receptors are the most commonly implicated subclass in normal GABA function. The majority of studies on the developmental and proliferative effects of GABA have also indicated a role for

this subclass, although there is little evidence for involvement of the other two subclasses (Cameron *et al.*, 1998; Nguyen *et al.*, 2001).

The $GABA_A$ receptor consists of a pentamer from a possible 15 subunits, each coded by a different gene (Backus *et al.*, 1993; McKernan and Whiting, 1996). While each subunit confers specific pharmacological and gating properties, the majority of GABAergic cells contain subunits of the β and γ subfamily. In the striatum, the majority of cells, presumably GABAergic projection neurons based on morphology, express β2,3 and γ2 subunits (Fritschy and Mohler, 1995; Riedel *et al.*, 1998) while the α1 subunit is found in the less numerous interneurons (Waldvogel *et al.*, 1997; Fujiyama *et al.*, 2000).

Under normal physiological conditions, the inhibitory effect of GABA is exerted by altering the Cl^- permeability of $GABA_A$ receptor ion channels, thereby hyperpolarizing the membrane (Bowery *et al.*, 1984; Chesnut and Swann, 1989). However, in immature neurons where the Cl^- gradient is reduced, opening of $GABA_A$ channels may actually result in hypo- or depolarization of the neuronal membrane (Yuste and Katz, 1991; Reichling *et al.*, 1994). $GABA_A$ receptors are also activated by muscimol and antagonized by bicuculline (Bowery *et al.*, 1976).

Based on binding studies of ^{3}H-muscimol, it appears that $GABA_A$ receptors are widely distributed throughout the perinatal CNS, often appearing in regions that are non-binding in the adult (Xia and Haddad, 1992). In addition, many areas exhibit binding that is multi-fold higher than in the adult, especially in rostral and cortical regions. As the affinity of receptor for ligand does not vary significantly with age or brain region (Coyle and Enna, 1976), it is suggested that such observations reflect

changes in receptor number or subunity composition. In the prenatal rat, $GABA_A$ receptor subunits have been reported in the cortex as early as E16 (Cobas *et al.*, 1991). Like the NMDARs, mRNA expression patterns for $GABA_A$ receptor subunits are also developmentally regulated with the β and γ isoforms most abundant prenatally (Van Eden *et al.*, 1995). Following birth, mRNA for the α1 subunit becomes predominant. Interestingly, cells dissociated from the proliferative cortical ventricular zone have been found to initially express α4, β1 and γ1 subunits before converting to α3, β3 and γ2 subunits (Maric *et al.*, 2001). Furthermore, precursors derived from cells in this region have shown proliferative responses to exogenously added GABA (Shaffer *et al.*, 2001; Nguyen *et al.*, 2003). In the striatum, the prenatal pattern of $GABA_A$ receptor expression remains largely unexplored though dissociated striatal cells from late stage rat embryos respond to GABA (Misgeld and Dietzel, 1989).

1.5 A role for glutamate and GABA in CNS proliferation

In vitro studies have provided the most direct evidence for a correlation between AANTs in the microenvironment and mammalian CNS development. In particular, GABA has been implicated in a variety of morphogenic events including cell migration (Behar *et al.*, 2001), differentiation and neurite outgrowth (Lipton and Kater, 1989; Belhage *et al.*, 1990; Borodinsky *et al.*, 2003), and cell survival (Ikeda *et al.*, 1997). Similar influences have been demonstrated with respect to glutamate (reviewed by Cameron *et al.*, 1998; Contestabile, 2000). With regard to the proliferative effects, a number of studies have indicated a strong role for these two

AANTs in a number of mammalian CNS regions. However, it is important to note that diverse responses are observed depending on the CNS region examined.

In cultured cerebellar neuronal progenitor cultures, the activation of $GABA_A$ receptors increases proliferation (Fiszman *et al.*, 1999). In contrast, both $GABA_A$ and AMPA/KA receptor activation appear to downregulate proliferation, as measured by ^{3}H-thymidine and BrdU* incorporation, in E16 and E19 rat cortical explants (LoTurco *et al.*, 1995). $GABA_A$ receptor activation also reduces proliferation in E16 dissociated cortical cell cultures (Antonopoulos *et al.*, 1997). A similar phenomenon is observed in cultured O-2A cells. Glutamate downregulates proliferation via non-NMDA receptors in these oligodendrocyte progenitors (Barres *et al.*, 1990; Gallo *et al.*, 1996). Considering that such cultures are relatively homogenous and express functional AMPA/KA receptors (Barres *et al.*, 1990), it is likely that glutamate has a direct influence on these cells rather than indirectly through another cell type.

In the embryonic CNS, both GABA and glutamate can depolarize immature neurons, the latter owing to the high concentration of Cl^- ions present in these neurons (Yuste and Katz, 1991; Blanton and Kriegstein, 1992). Therefore, depolarization leading to Ca^{2+} entry may be responsible for activating mitogenic pathways following exposure to neurotransmitter. In fact, application of a depolarizing concentration of K^+ (60mM) elicits similar decreases in DNA synthesis to glutamate and GABA (LoTurco *et al.*, 1995). In contrast, furosemide, a blocker of $Na^+/K^+/Cl^-$ co-transport, negates the effect of exogenous neurotransmitter. Ca^{2+} entry following exposure to common neurotransmitters is also detected in cortical precursors expanded *in vitro* (Li *et al.*, 2001). While such primitive cells respond first to ATP, they quickly

* Abbreviations: bromodeoxyuridine (BrdU)

develop sensitivity to GABA, glutamate, and acetylcholine (Maric *et al.*, 2000a). The observation that cells in the proliferative cortical ventricular zone are arranged in clusters with tight gap junctions which allow depolarization, and thus control of cell division, to spread across these coupled units also supports this perspective (LoTurco *et al.*, 1991; LoTurco *et al.*, 1995). Furthermore, BrdU and retroviral marker studies suggest such clusters are composed of clones with synchronized interkinetic nuclear movement and cell cycle duration (Cai *et al.*, 1997).

GABA also appears to enhance the survival and/or migration, but not proliferation of a number of cortical and striatal GABAergic neuron populations (Behar *et al.*, 1996; Ikeda *et al.*, 1997; Barker *et al.*, 1998; Behar *et al.*, 1998). These effects are reversibly blocked by the $GABA_A$ antagonist bicuculline methiodide (BMI) and Cl^- channel blocker picrotoxin, indicating activation of this receptor subclass helps mediate this phenomenon. Interestingly, blockade of the NMDA receptor during the neurogenesis period of parvalbumin-positive striatal interneurons also reduces their final number, suggesting that glutamate influences proliferation of this subpopulation (Sadikot *et al.*, 1998).

Further studies in an explant culture model similar to the one used by Kriegstein and colleagues have revealed that differences may exist between proliferating subpopulations in the developing neocortical VZ and SVZ (Haydar *et al.*, 2000). Direct application of either GABA or glutamate to explants results in up regulation of BrdU labeling in the VZ but down regulation in the SVZ, while the corresponding receptor antagonists elicit the opposite responses.

Administration of the NMDA antagonist MK-801 to adult rats increases ^{3}H-thymidine and BrdU uptake and cell number of hippocampal dentate gyrus granule cells, suggesting that receptor activation may act to downregulate proliferation in the postnatal brain (Cameron *et al.*, 1995). Proliferation in this population can also be reduced directly by injection of NMDA into the peritoneum. However, rats reared in an enriched environment and exposed to more physical activity show an almost two-fold increase in BrdU incorporation for this subpopulation (Kempermann *et al.*, 1998). Furthermore, in the medial cerebral artery occlusion rat model of stroke, MK-801 and other NMDA antagonists appear to suppress the normally observed injury-induced proliferative response (Arvidsson *et al.*, 2001). Therefore, other factors including the location, magnitude and context of the AANT stimulus may modulate the proliferative response. Indeed, GABA appears to attenuate the mitogenic potential of bFGF on cortical progenitors *in vitro* (Antonopoulos *et al.*, 1997), despite promoting cerebellar granule cell growth (Fiszman *et al.*, 1999). Activation of the NMDA receptor is also known to induce the release of striatal bFGF (Roceri *et al.*, 2001) and BDNF (Marini *et al.*, 1998), two growth factors known to affect the proliferation and survival of various neuronal populations, including cerebellar granule cells (Bhave and Hoffman, 1997; Bhave *et al.*, 1999). Messenger RNA levels of TGFβ, a pro-differentiation cytokine, are also reduced in developing cortical neurons following ionotropic glutamate receptor activation (Dobbertin *et al.*, 2000).

1.6 The Striatum

1.6.1 Striatal anatomy and neurogenesis

The mammalian striatum is a periventricular structure located ventral to the cortex, belonging to the telencephalon. It forms the principal component of the basal ganglia and comprises the caudate, putamen, nucleus accumbens, and the deep layers of the olfactory tubercle. This region receives inputs from virtually all cortical areas and is thought to process and regulate behavior initiated by the cortex (Kemp and Powell, 1971; Webster, 1975). Along with the globus pallidus, the striatum and basal forebrain arise from the ventral (subpallial) portion of the primitive telencephalon (Smart and Sturrock, 1979). In contrast, dorsal (pallial) domains generate projection neurons and glia of the neocortex, hippocampus, and piriform cortex (Bayer, 1991), while amygdala formation is thought to involve both pallial and subpallial contributions (Puelles *et al.*, 2000).

In rodents, the striatum arises from two distinct, adjacent populations known as the ganglionic eminences (GE). In addition to basal telencephalic glial populations, the lateral and medial ganglionic eminences (LGE and MGE) are thought to respectively generate striatal projection neurons and GABAergic interneurons, the latter of which populate both the striatum and cortex (Smart, 1976; Smart and Sturrock, 1979; Lammers *et al.*, 1980; Fentress *et al.*, 1981; Anderson *et al.*, 1997). In fact, the majority of cortical GABAergic interneurons appear to have origins in the GE (de Carlos *et al.*, 1996). The appearance of the LGE at E12 (E11 in the mouse), an event that is preceded by the formation of the MGE approximately one day earlier (Smart, 1985; Sheth and Bhide, 1997), represents the first visible

compartmentalization of the striatum (Bhide, 1996). Proliferating progenitors in both the LGE and MGE are located within the ventricular and subventricular zones, also known as the pseudostratified ventricular epithelium (PVE) and secondary proliferative population (SPP), respectively (Smart and Sturrock, 1979; Takahashi *et al.*, 1995; Bhide, 1996)). Cytokinetic studies in the mouse have demonstrated that both zones contribute roughly equal proportions of postmitotic cells in the striatum (Sheth and Bhide, 1997). The actual rate of this postmitotic output (i.e. number of cells exiting the cell cycle from these populations) is estimated to be 30-35% of PVE and SPP cells, and is approximately twice the rate of the neighboring cortical counterparts (Takahashi *et al.*, 1995; Sheth and Bhide, 1997).

In the rat, a common progenitor produces both striatal neurons and glia according to a strict chronological sequence, which then migrate radially to their final position (Misson *et al.*, 1988; Halliday and Cepko, 1992). Striatal neurogenesis extends from E12 through E22 in the rat, peaking at E15-16 (Fentress *et al.*, 1981; Bayer, 1984; van der Kooy and Fishell, 1987). This is followed by gliogenesis which commences at approximately E18, peaking just prior to birth (Das, 1979). A number of studies indicate that progenitors switch from neurogenetic to gliogenetic as they make the transition from VZ to SVZ so that glial cells are generated almost exclusively from the SVZ (Privat, 1975; Burrows *et al.*, 1997).This correlates with an increase in EGF receptor (EGFR) expression, a key regulator of cellular fate decisions (Seroogy *et al.*, 1995; Kornblum *et al.*, 1997) and migration (Caric *et al.*, 2001). Supporting this view, E14 neuron-producing progenitors from rat cortex or

striatum can be induced to prematurely give rise to astrocytes following infection with EGFR-bearing retrovirus particles (Burrows *et al.*, 2000).

While the striatum lacks the distinctive cytoarchitectural patterning found in the layer-bound cortex, striatal neurons are distributed within functional compartments (Gerfen *et al.*, 1985). For example, striatal output neurons are segregated according to their patch or matrix compartment distribution, as determined by the calcium-binding protein calbindin-28kD (Graybiel, 1983; Gerfen *et al.*, 1987; van der Kooy and Fishell, 1987) or other markers such as μ-opioid receptors , LAMP*, and the presence of cholinesterase (Graybiel and Ragsdale, 1978; Desban *et al.*, 1993; Cote *et al.*, 1995). Also known as striosomes, patches differ from their matrix counterparts in a number of properties including birthdates and projection targets (van der Kooy and Fishell, 1987).

The medium spiny neurons, so-called due to their medium-sized cell bodies (20-25μm diameter), form over 90% of the striatal neuron population (Spencer, 1976; Kawaguchi *et al.*, 1995). Through their spine-dense dendrites, they receive the majority of extrinsic cortical and thalamic glutamatergic afferents to the striatum (Kitai *et al.*, 1976; Spencer, 1976; Somogyi and Cowey, 1981; Bouyer *et al.*, 1984; Sadikot *et al.*, 1992). In addition, these spines receive significant input from midbrain dopaminergic neurons (Freund *et al.*, 1984; Smith *et al.*, 1994), as well as neighboring medium spiny neurons (projection neurons) and striatal interneurons (Wilson and Groves, 1980).

* Abbreviation: limbic system associated membrane protein (LAMP)

Striatal interneurons project to targets restricted within the striatum. These intrinsic neurons represent approximately 10% of the neuronal population which can be further subdivided according to their morphological and immunochemical properties (Kawaguchi *et al.*, 1995). These include the large aspiny cholinergic neurons (Bolam *et al.*, 1984; Semba and Fibiger, 1988), medium spiny neurons containing somatostatin (DiFiglia and Aronin, 1982) and neuropeptide Y (Vincent and Johansson, 1983). Two other medium spiny GABAergic subpopulations can be identified by the presence of the EF-hand calcium-binding proteins parvalbumin (Gerfen *et al.*, 1985; Cowan *et al.*, 1990) and calretinin (Kubota *et al.*, 1993; Parent *et al.*, 1995) with little overlap. Interestingly, neuronal populations in regions other than the striatum may contain multiple EF-proteins as is the case with Purkinje neurons and selective cells in the substantia nigra (Celio, 1990; McRitchie *et al.*, 1996). Although the precise function of such calcium-binding proteins remain unknown in neurons, it is believed that they play a role in regulating Ca^{2+} homeostasis and signaling (Baimbridge *et al.*, 1992). In particular, parvalbumin has been correlated with neurons exhibiting rapid firing properties, e.g. hippocampal interneurons (Kawaguchi *et al.*, 1987). There is also evidence that such proteins may act as Ca^{2+} buffers as neurons expressing PV and calbindin-D28K are relatively spared in Alzheimer's and Parkinson's disease, respectively (Yamada *et al.*, 1990; Hof *et al.*, 1991).

In addition to their morphology, distribution, and chemical and physiological properties the neurogenetic timetables of striatal interneurons have been documented using either ^{3}H-thymidine or BrdU incorporation methods. These studies have

revealed restricted, yet distinct periods of neurogenesis for each subpopulation. Thus PV-immunoreactive (ir) interneurons predominantly become postmitotic between E14-17 (Sadikot and Sasseville, 1997), CRir interneurons between E14-17 (V. Rymar et al, unpublished results), and ChAT interneurons from E12-14 (Semba and Fibiger, 1988; Phelps *et al.*, 1989). In contrast, the neurogenesis period for striatal projection neurons span from E12-19, peaking between E14-18 (Bayer, 1984; Marchand and Lajoie, 1986). These characteristic neurogenetic patterns have been exploited in a number of studies focusing on interneuron development (Sadikot *et al.*, 1998; Luk and Sadikot, 2001). By restricting treatment to specific gestational periods, specific neuron subpopulations are targeted according to their birthdate. However, since certain markers such as PV are not expressed until the postnatal period, these populations are examined after animals treated *in utero* reach adulthood.

1.6.2 Dorsoventral diversity within the developing telencephalon

Both the developing and mature mammalian forebrain display a number of dorsal-ventral structural and chemical differences during and following embryonic development. For example, the dorsal telencephalon is compartmentalized into six distinctive cortical layers absent in its ventral counterpart which contains striosomal compartments instead (Gerfen *et al.*, 1987). Another notable contrast is that the cortex contains primarily glutamatergic pyramidal neurons whereas GABA is predominant in the striatum.

Despite sharing a common telencephalic origin, the corticostriatal boundary appears to be strictly respected (Fishell *et al.*, 1993). At the level of the corticostriatal

* Abbreviations: calretinin (CR); parvalbumin (PV)

angle (CSA), cells belonging to either side, as indicated by adhesion molecule and transcription factor expression do not migrate outside of their native zone (Inoue *et al.*, 2001). Thus, Emx2, Pax6 and Neurogenin (Ngn) 1/2 gene products, and R-cadherin are expressed exclusively in dorsal domains while Gsh2, Mash1, and cadherin-6 are found only in the LGE (Caric *et al.*, 1997; Mallamaci *et al.*, 1998; Warren *et al.*, 1999; Fode *et al.*, 2000; Toresson *et al.*, 2000; Yun *et al.*, 2001). While projection neurons ultimately populate regions immediately adjacent to their neurogenetic zone, GABAergic interneurons which arise primarily from the MGE migrate tangentially over large distances to targets in the cortex (Marin *et al.*, 2000; Pleasure *et al.*, 2000). Also present at the CSA is a morphologically identifiable "palisade" of radial glial fibres that are thought to aid the selective migration of such interneurons while restricting that of other cells (Stoykova *et al.*, 1997; Gotz *et al.*, 1998; Chapouton *et al.*, 1999). Interestingly, a large number of GABAergic interneurons in the mature cortex continue to express MGE genes and fail to migrate in transgenic animals lacking the critical ventral telencephalic transcription factor Dlx1/2 (Marin *et al.*, 2000).

In addition to delineating and maintaining CSA integrity, the abovementioned transcription factors appear to regulate activation of either corticogenesis or striatogenesis. Specification appears to be decided by the precise combination expressed. Emx2, Pax6, and the homeobox gene Lhx2 direct cortical specification, probably by downstream activation of Ngn1/2 and Gli3 whereas specification towards striatum is mediated by Gsh2 activation of Mash1 and other basic helix-loop-helix (bHLH) transcription factors (Mallamaci *et al.*, 1998; Fode *et al.*, 2000; reviewed in

Schuurmans and Guillemot, 2002). Most of these factors are expressed in their respective telencephalic regions from early development and are profoundly involved in various processes including neuroblast proliferation, radial glial development, and cortical lamination (Caric *et al.*, 1997; Warren *et al.*, 1999).

Cortical specific factors also inhibit the expression of striatal genes and vice versa, therefore maintaining strict adherence to the CSA and dorsoventral distinctiveness. For example, Pax6 inhibits the action of Gsh2 while Mash1 inhibits Ngn1/2 (Corbin *et al.*, 2000; Toresson *et al.*, 2000; Yun *et al.*, 2001). Interestingly, deletion of either Emx2 or Pax6 in mice results in only partial conversion of presumptive dorsal telencephalon to striatal structures as identified by Gsh2 and GAD65/67 expression, suggesting a redundant role for these molecules individually. However, animals with mutations at both the Emx2 and Pax6 loci exhibit silencing of a number of cortical genes with almost complete conversion to a striatal gene profile in the cortical primordium (Muzio *et al.*, 2002). Ectopic expression of striatal or cortical specific adhesion molecules can also allow cells to cross the CSA (Inoue *et al.*, 2001).

1.7 Amino acid neurotransmitters and disease in the CNS

In contrast to their function in CNS development imbalances in AANT activity are thought to underlie the pathogenesis of wide number of neurological and psychiatric conditions including excitotoxicity following trauma or ischemia, certain spasticities and epilepsies, anxiety, and depression (Olney *et al.*, 2002). Interestingly, neuronal responses to such imbalances vary greatly according to their developmental stage.

In infant mice, rabbits, guinea pigs and monkeys, oral or subcutaneous administration of glutamate destroys neurons (Olney, 1969, 1976). While no immediate dysfunction is observed, alterations in body shape and behavior become apparent in adults, suggestive of a disturbance in the functional maturation of systems. The window of CNS susceptibility to glutamate toxicity is restricted primarily to the period of synaptogenesis (brain growth spurt) which spans from E21 to postnatal day 14 in rats, and corresponds approximately to the third trimester of gestation in humans (Dobbing and Sands, 1979). Unlike apoptosis (programmed cell death), which commonly occurs during CNS development (Oppenheim, 1991), cell death resulting from excessive glutamate exposure (termed excitotoxicity) is distinguished by its ultrastructural features, dependence on Ca^{2+} influx, and rapidity (Ishimaru *et al.*, 1999), resembling the classical "necrosis" process seen in non-neuronal tissues (Wyllie *et al.*, 1980). Neurons in the adult CNS are also susceptible to excessive glutamate following trauma or ischemia (Choi, 1987; Lipton and Rosenberg, 1994), though they appear to less sensitive than those in the perinatal brain. In both *in vitro* and in vivo models, addition of NMDA receptor antagonists (e.g. MK-801) attenuate glutamate excitotoxicity, suggesting that the process is mediated via this subclass of receptor (Ikonomidou *et al.*, 1996; Pohl *et al.*, 1999). Agonists to the $GABA_A$ receptor (e.g. diazepam) exhibit similar neuroprotective properties, indicating that reducing excitatory activity (hyperpolarization) may also prevent excitotoxicity (Farber *et al.*, 1993).

Interestingly, administration of MK-801 or diazepam during the synaptogenesis period also results in dramatically increased rates of apoptosis,

suggesting that NMDA receptor activation is required for the survival of certain neurons (Ikonomidou *et al.*, 1999). Indeed, different CNS regions vary in their sensitivity to NMDA receptor blockade with the cerebellum, caudate nucleus, and parietal cortex being most vulnerable. Together, these findings suggest that a physiological range of activity must be maintained in order for neurons to survive and mature. This view is supported by the observation that the Na^+ channel blocker tetrodotoxin replicates the pattern of cell death produced by NMDA antagonists (Mennerick and Zorumski, 2000).

Such results have significant clinical implications given that a high proportion of abused substances have NMDA-blocking or GABA-mimetic properties. Common examples include phencyclidine, ketamine, and "angel dust" which act as NMDA-antagonists. In contrast, barbiturates and benzodiazepines potentiate $GABA_A$ receptors. It should also be noted that such properties are shared with commonly used anesthetics and anticonvulsants including isoflurane, propofol, valproate, phenytoin, carbamazepine, lamotrigine, and nitrous oxide.

Alcohol (ethanol), perhaps the most widely abused substance, has both NMDA receptor-blocking and $GABA_A$ activating properties (Lovinger *et al.*, 1989; Harris *et al.*, 1995). *In vitro*, ethanol can kill cerebellar, thalamic, cortical, and hippocampal neurons (Bhave and Hoffman, 1997). When administered to postnatal day 7 rats, ethanol is more potent than either MK-801 or diazepam alone at inducing apoptosis (Ikonomidou *et al.*, 2000). Indeed, the elicited cell death occurs in regions and at a magnitude equivalent to the sum of that found in animals treated singly with MK-801 or diazepam.

Among humans, in utero exposure to ethanol results in a neurotoxic and dysmorphogenic syndrome termed fetal alcohol syndrome (FAS). In North America, FAS is the most common cause of mental retardation. Low-level exposure to alcohol during pregnancy is thought to result in milder symptoms collectively known as alcohol-related neurodevelopmental disorder (ARND). Similar to animals exposed to NMDA antagonists during synaptogenesis, ARND patients exhibit disproportionately high incidences of psychosis (40%), adult-onset major depression (44%), and attention deficit/hyperactivity disorder (72%) (Famy *et al.*, 1998). Anatomically, FAS is characterized by limb and craniofacial deformities along with microencephaly. MRI studies have revealed particular reduction in striatum volume and abnormalities in the cerebellum and corpus callosum (Archibald *et al.*, 2001), supporting the hypothesis that abnormal neuron numbers underlie ARND. A similar hypothesis has been postulated for schizophrenia (Pakkenberg, 1987), although correlations to both increased or decrease neuron number have been made (Thune and Pakkenberg, 2000). Interestingly, schizophrenia has also been linked to aberrant NMDA receptor signaling (Goff, 2000).

More recently, AANTs have been detected along with their receptors in non-CNS cells including osteoclasts, keratinocytes, megakaryocytes, pancreatic islet cells, lymphocytes, lung, liver, heart, kidney, and adrenal glands (Skerry and Genever, 2001). In these regions, especially in the case of immune cells, glutamate and GABA appear to have direct roles in influencing proliferation. For example, GABA influences proliferation in circulating T-lymphocytes (Tian *et al.*, 1999) while PHA-induced proliferation of infiltrating microglia from multiple sclerosis patients is

reduced by glutamate via NMDA mechanisms (Lombardi *et al.*, 2003). Glutamate has also been found in a variety of tumors and exposure of tumor-derived cell lines to either NMDA or AMPA antagonists appear to have cytostatic effects *in vitro* (Rzeski *et al.*, 2001; Takano *et al.*, 2001). In addition to proliferation, agents such as MK-801 and NBQX also reduce motility as measured by migration assays and morphological changes (e.g. reduced pseudopodia and membrane ruffling).

1.8 Research goals

1.8.1 Aims

An increasingly large body of evidence suggest that, in addition to their classical roles in neurotransmission, the amino acids glutamate and GABA profoundly influence CNS development. Multiple cellular processes fundamental to the formation of the brain appear to be regulated by the activation of their native receptors, including cell proliferation, migration, and survival. Moreover, the effects of AANTs appear to act at various developing regions of the CNS and upon a surprisingly wide range of cells.

Much of the data available on the role of glutamate and GABA in CNS proliferation has been built upon the work of Kriegstein and colleagues from *in vitro* studies on the developing neocortex of rats and turtles. Data from *in vivo* studies in mice and rats are also available. While a handful of studies have addressed the same theme in the cerebellum, rostral migratory stream of the olfactory cortex, and hippocampus, little data concerning the basal ganglia existed when we first embarked on this research. Those that were available had focused primarily on neuronal

survival in the context of AANT excitotoxicity. Our initial goal was to therefore investigate the proliferative role of glutamate and GABA on neuroblasts giving rise to the rat striatum. This particular region was chosen for a number of reasons. Firstly, the rat striatum (or caudate-putamen complex) is a prominent component of the basal nuclei. Like the cortex, it also derives from the telencephalic anlage during embryogenesis and provided us with the opportunity to compare AANT effects in developing dorsal and ventral telencephalon. Secondly, the striatum is a predominantly GABAergic region with minimal intrinsic glutamate. The rat striatum is well documented with well defined tissue and chemical anatomy available in the literature. Furthermore, the neurogenesis timetables for major striatal subpopulations were available from our previous work and that of others. Lastly, striatal tissue containing precursors are easily accessible, facilitating the establishment of primary striatal tissue culture for *in vitro* studies.

1.8.2 Hypotheses

We hypothesized that the AANTs glutamate and GABA influence the proliferation of neuroblasts. This was based largely on its significant effects in the neighboring cortex. In addition, an *in vitro* study on striatal neurons also demonstrated $GABA_A$ receptor activation increases total cell number, although this was attributed to cell survival rather than proliferation (Ikeda et al, 1997). Previous work from our group indicated that proliferation of striatal parvalbumin interneurons may be regulated by NMDA receptor activation (Sadikot et al, 1998). These data suggested that $GABA_A$ and NMDA receptors may be the main subtypes mediating

GABA and glutamate effects, respectively. The latter observation was in contrast to data showing the AMPA/KA receptor as primarily responsible for glutamate (down) regulation of proliferation (LoTurco et al, 1995). This led us to hypothesize that due to intrinsic differences between the dorsal and ventral telencephalon, glutamate regulation of neuroblasts proliferation may be mediated by different receptor classes in either the developing cortex or the striatum. We have employed both *in vitro* and *in vivo* methods in order to examine these two hypotheses.

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Chapter 2: GABA promotes survival of striatal parvalbumin neurons

GABA Promotes Survival but not Proliferation of Parvalbumin-Immunoreactive Interneurons in Rodent Neostriatum: An in vivo Study with Stereology. ***Neuroscience*** **104:93-103, 2001.**

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Abbreviations: AC, anterior commissure; CR, calretinin; BMI, bicuculline methoiodide; DAB, diaminobenzidine; DMSO, dimethyl sulfoxide; E, embryonic day; EC, external capsule; GABA, γ-amino butyric acid; i.p., intraperitoneal; N.A., numerical aperture; P, postnatal day; PBS, phosphate buffered saline; NPY, neuropeptide-Y; PV, parvalbumin; SNK, Student-Newman-Keuls; SS, somatostatin.

2.1 Abstract

Amino-acid neurotransmitters regulate a wide variety of developmental processes in the mammalian central nervous system including neurogenesis, cell migration, and apoptosis. In order to investigate the role of γ-amino butyric acid (GABA) in early development of forebrain interneurons, we determined the survival of parvalbumin-immunoreactive (PV-IR) GABAergic interneurons in the adult rat striatum following prenatal exposure to either $GABA_A$ receptor agonist or antagonist. Unbiased stereology was used to quantify PV-IR neuron number in the neostriatum of adult rats exposed to the drugs *in utero*, and the results were compared to pair-fed or vehicle controls. Embryos were exposed to the $GABA_A$ antagonist (bicuculline) or agonist (muscimol) during previously defined proliferative or post-proliferative periods for PV-IR interneurons. Unbiased stereology using the optical fractionator was used to estimate the total number of PV-IR neurons in neostriatum of experimental and control rats. No significant alteration in PV-IR neuron number was observed in rats treated with either bicuculline (1 or 2 mg/kg/day) or muscimol (1mg/kg/day) during the proliferative phase. Administration of bicuculline during the post-proliferative phase significantly reduced PV-IR neuron number in the neostriatum. A concomitant decrease in neostriatal volume was also observed, suggesting that the effect is not restricted to PV-IR interneurons. Positional analysis revealed loss of normal regional distribution gradients for PV-IR neurons in neostriatum of rats exposed to bicuculline in the embryonic post-proliferative phase. This data collectively suggests that GABA promotes survival but not proliferation of PV-IR progenitors. GABA may also promote migration of subpopulations of interneurons that ultimately populate the ventral telencephalon.

Key Words: $GABA_A$ receptor, neostriatum, morphogenesis, cell survival, cell migration, calcium binding proteins

2.2 Introduction

The final number of neurons in the adult mammalian forebrain is determined by neurogenesis, cell migration to appropriate domains, and developmental cell death. [12, 49] A growing body of evidence from cell culture, explant, and *in vivo* studies suggests that the microenvironment plays an important role in these processes. [4, 31, 40, 42, 43, 63, 69] γ-amino butyric acid (GABA) and other neurotransmitters are implicated in a variety of morphogenetic events in the mammalian and non-mammalian central nervous system (CNS). [40, 42, 58] Recent studies suggest that the amino acids glutamate and GABA play an important role in determining final neuron number in the developing telencephalon, including the cerebral cortex [4, 15, 40, 43], and striatum. [31, 69]

GABA is the major inhibitory neurotransmitter of the CNS. [9, 75] It exerts its physiological inhibitory effect by altering the chloride ion permeability of GABA receptor channels, thus hyperpolarizing the cell membrane in the majority of adult neurons. In early embryonic forebrain development, GABA possesses the ability to depolarize subpopulations of immature neurons at a time when the chloride gradient across the cell membrane is reduced. [4, 5, 65] GABA receptors include ionotropic $GABA_A$ and $GABA_C$ subtypes, and the metabotropic $GABA_B$ receptor. [9, 75] $GABA_A$ receptors may mediate a variety of developmental CNS disorders, including some genetic epilepsies (e.g. Angelman syndrome), fetal drug and alcohol syndromes, motor disorders and psychiatric disorders. [21, 36, 53] $GABA_A$ receptor-mediated depolarizing responses may have an important influence on several calcium-dependant developmental processes, including cell proliferation, migration, differentiation, apoptosis, neurite outgrowth, and synapse formation. [4, 31, 43, 55, 79, 90]

In cerebral cortex anlage derived from the dorsal telencephalon, varying concentrations of GABA differentially promote migration of distinct GABAergic or non-GABAergic neuronal populations. [4] Little information exists on the influence of GABA in early morphogenesis of the striatum, a ventral telencephalon derivative

arising mainly from the lateral ganglionic eminence [19]. Recent evidence suggests that the lateral ganglionic eminence not only gives rise to GABAergic interneurons of the ventral telencephalon, but is also a source of a large proportion of GABAergic interneurons that ultimately populate the cerebral cortex, a dorsal telencephalon derivative.[1, 20, 80] Thus, factors determining proliferation and migration of GABAergic interneurons in the lateral ganglionic eminence may not only influence the final number of striatal interneurons, but may also determine GABAergic interneuron density in the dorsal telencephalon. We hypothesize that GABA influences cell proliferation or post-proliferative events of different neuronal subpopulations that ultimately comprise the adult striatum. *In vitro* studies show that activation of $GABA_A$, but not $GABA_B$, receptors results in increased number of embryonic striatal neurons. [30] We investigate the effects of GABA in prenatal striatal development by utilizing agonists and antagonists to the $GABA_A$ receptor *in vivo*.

The mammalian striatum contains a chemically heterogeneous mosaic of medium spiny GABAergic projection neurons, and subclasses of interneurons. [33, 57, 76] In the rodent, interneurons comprise less than 10% of striatal neurons, and include GABAergic aspiny neurons which are mainly small to medium-sized, [10, 33, 57, 76] and a population of large aspiny cholinergic cells. [11, 51, 61] Distinct GABAergic interneuron subpopulations may be identified by co-localization with specific chemical markers, including the calcium binding proteins calretinin (CR) and parvalbumin (PV), and the peptide somatostatin (SS). [7, 13, 16, 17, 33, 35, 37, 38, 67] In comparison to projection neurons [47, 82], interneuron subtypes have restricted neurogenesis periods. [68, 73] Their well-defined morphological chemical characteristics [33, 37, 57] and relatively restricted periods of proliferation make interneurons a well-suited model for determining factors that influence cell morphogenesis in the prenatal basal telencephalon.[45, 69] We determine whether GABA regulates proliferation, migration or survival of striatum PV-IR interneurons. Separate groups of embryos are exposed *in utero* to $GABA_A$

receptor agonist or antagonist during or immediately after the main proliferative period for neuroblasts giving rise to PV-IR cells. [68, 69] In order to evaluate effects of $GABA_A$ receptor modulation, we employ stereological analysis [29, 56, 86] to provide unbiased estimates of the number of PV-IR interneurons in the striatum of control and experimental animals. Our findings suggest that GABA has little effect on cell proliferation. Rather, a $GABA_A$ receptor-mediated mechanism promotes survival of striatum PV-IR neurons during the prenatal post-proliferative phase.

2.3 Experimental procedures

Animals

Female Sprague-Dawley rats (Charles River, LaSalle, Quebec) were coupled with males between 3 p.m. and 5 p.m. The first 24 hours after coupling was designated as embryonic day (E) zero. A second group of females was coupled 48 hours later in order to provide control animals matched for food and water intake with experimental groups.

Drugs

Either the $GABA_A$ receptor agonist muscimol, or antagonist bicuculline methoiodide (BMI, Research Biochemicals International, Natick, MA), was administered to separate groups of rats. Drugs were administered by intraperitoneal (i.p.) injections for 4-day periods from either E15-18 or E18-21, corresponding to mainly proliferative or post-proliferative periods for PV-IR neurons. Muscimol (1 mg/kg/day) was dissolved in dimethyl sulfoxide (DMSO, 1 mg/ml) and administered daily by i.p. injection during proliferative or post-proliferative periods. BMI (either 1 or 2 mg/kg/day) was dissolved in normal sterile saline (1 or 2 mg/ml) and

administered daily during identical 4-day periods by i.p. injection. Each animal's food and water intake, and weight were recorded daily.

As controls, age-matched pregnant females were given daily i.p. injections of vehicle (saline or DMSO, 1 ml/kg/day) over identical 4-day periods as drug-treated dams. Pair-fed control groups were given access to the amount of food and water consumed by their drug-treated counterparts. A separate control group was given saline injections during the period of interest, without food restrictions. After birth, 5 males were randomly chosen from each litter and sacrificed between postnatal days (P) 35-42 for histology and immunohistochemistry. All animal protocols were previously approved by the McGill University Animal Care Committee.

Sectioning and Immunohistochemistry

Rats were perfused transcardially, under deep pentobarbital anesthesia (75 mg/kg, i.p.), with an initial wash of 0.9% saline (50-100 ml, 4°C) followed by 4% paraformaldehyde in phosphate buffer (300 ml, 0.1M, pH 7.4, 4°C). Brains were immersed in the same fixative for 12 hours, followed by immersion in cold 30% phosphate buffered sucrose solution (pH 7.4) for an additional 48 hours. Brains were then cut into 50 μm coronal sections encompassing the entire striatum using a freezing microtome. Upon identifying the rostral pole of the striatum, section collection was started at random, between the first and sixth section, as determined by the role of dice. Serial free-floating sections were collected in phosphate buffered saline (PBS, 0.1M, pH 7.4) as separate sets so that each set contained every sixth serial section. One set of sections was immunostained for PV, and another set was processed using 0.1% Cresyl Violet as a Nissl stain.

Drug-treated and control animals were then processed simultaneously for PV immunohistochemistry using a modified avidin-biotin complex (ABC) method as previously described. [30, 68] Briefly, free-floating sections were preincubated for 1 h in

PBS containing 0.3% Triton-X 100 and 5% bovine serum albumin. This was followed by overnight incubation with a primary monoclonal antibody against PV (1:5000 in PBS, Sigma, St. Louis, MO), followed by rinsing with PBS (3 X 5 minutes each). Next, sections were incubated in PBS containing secondary antibody (biotinylated anti-mouse IgG, 1:200, Vector, Burlinghame, CA). After rinsing again in PBS (3 X 5 minutes each) sections were incubated for 1 hour in ABC (1% in PBS, Vector). The final reaction product was revealed by exposure to a solution containing 3,3'-diaminobenzidine (DAB, 0.25mg/ml, Sigma) dissolved in Tris buffer (0.05M, pH 7.6) containing 1% imidazole (1.0M, Sigma) and 0.06% hydrogen peroxide. After 10-15 minutes exposure to DAB, sections were thoroughly washed with PBS, mounted out of distilled water, air-dried, and dehydrated using a graded series of ethanol concentrations. Sections were then cleared in xylene substitute, and coverslipped with Permount (Fisher, Fair Lawn, NJ).

Unbiased Stereological Estimate of Total Number of PV-IR Neurons in the Neostriatum

An unbiased stereological technique, the optical fractionator, was used to estimate the total number of PV-IR neurons in the neostriatum. The apparatus consisted of a light microscope (BX40, Olympus, Japan) coupled with a video camera (DC200, DAGE, Michigan City, IN, USA), motorized X-Y stage (BioPoint XYZ, LEP, Hawthorne, NY), Z-axis indicator (MT12 microcator, Heidenhain, Traunreut, Germany), and a computer running Stereo Investigator software (Microbrightfield Inc., Colchester, VT). The rostral and caudal limits of the reference volume were determined by the first and last coronal sections with visible caudate-putamen (approximately Bregma 2.20 to -2.60 mm, Paxinos and Watson atlas). [60] Every sixth serial section within this zone was examined, that is at 300 μm intervals through the reference volume. The corpus collosum, external capsule, lateral ventricle, globus

pallidus, and anterior commissure were used as boundaries (Fig. 1 A-E). In the most rostral sections, the ventral striatum was excluded from analysis by a line drawn from the ventral tip of the lateral ventricle to the dorsal border of the piriform cortex, corresponding to an angle of 20-30° below the horizontal axis. In more caudal sections, the caudate putamen was bordered by the external capsule, globus pallidus, bed nucleus of the stria terminalis, the substantia innominata, and the dorsal amygdala. Surface areas of each region of interest were estimated from tracings of the neostriatum generated using a 4X objective and the Stereo Investigator software. All tracings derived from the same animal were used to estimate the reference volume using the Cavalieri method.

Systematic random sampling of the neostriatum was performed by randomly translating a grid with 500 X 500 μm squares onto the section of interest using the Stereo Investigator software (Figure 1, A-E). Each intersection represented a sample site where an 80 X 80 μm counting frame with exclusion lines [29, 86] was then applied (Figure 1F). All randomly distributed computer generated sample sites were then examined using a 100X objective (oil, N.A. 1.3). Only PV-IR cell bodies falling within the counting frame without contact with the exclusion lines were enumerated. PV-IR cell bodies were included in the count provided they had a visible dendritic process. Objects seen in the counting frame were only counted if they came into focus within a predetermined 10 μm thick optical dissector positioned 2 μm below the surface of the mounted section as indicated by the Z-axis microcator. Each optical dissector therefore consisted of an 80 X 80 X 10 μm brick. [29, 86] Estimates of the total number of PV-IR cells in each animal were generated using the Stereo Investigator software. Mean estimates of the total number of PV-IR cells in the neostriatum in treatment or control groups were compared using one-way analysis of variance (ANOVA, α=0.05) with Student-Newman-Keuls (SNK) post-hoc test.

The proportion of PV neurons with respect to the total number of neostriatal neurons was also determined from Nissl-stained sections obtained from the saline control animals. Total neostriatal neuron number was estimated using the same unbiased stereological method described for PV neurons, except that the brick size (optical dissector) was 60 X 60 μm with an 8 μm thickness. Neurons were distinguished using the nucleus as a unique identifier, and glia were excluded on the basis of morphology [56] and by only counting profiles greater than 7 μm in diameter.

Image Analysis of Spatial Distribution of PV-IR Neurons

In order to investigate possible regional differences in PV-IR cell distribution induced by prenatal modulation of $GABA_A$ receptors, the striatum was divided into 4 equal quadrants at a coronal level at which the anterior commissure forms its ventral boundary (Bregma -0.26 mm in the Paxinos and Watson atlas, Figure 1). [60] The spatial distribution of PV-IR neurons was plotted using a light microscope (Leica Orthoplan, Wetzlar, Germany) equipped with an X-Y movement-sensitive stage and video camera coupled to a computer running image analysis software (Biocom, Les Ulis, France). Cell profile counts were obtained from the entire section thickness within the defined neostriatal area. The surface area of the region of interest was determined. Results are expressed as cell profile density per unit of striatal surface area. The mean cell profile densities for each treatment group were compared using the one-way ANOVA with the SNK post-hoc test ($\alpha = 0.05$). Each mapped neostriatum section was further subdivided along dorsal/ventral and medial/lateral axes into representative quadrants. Mean PV-IR cell profile densities were obtained for each quadrant in different treatment groups and compared using the one-way ANOVA test.

2.4 Results

Stereological Estimate of the Total Number of PV-IR Neurons in the Neostriatum

PV-IR neurons appeared as mainly medium-sized light to dark brown DAB-stained cell bodies with multiple aspiny processes (Figure 3A,B). In keeping with other studies, [13, 16, 17, 35, 68] PV-IR cell density is higher in the lateral aspect of the pre-commissural striatum compared to the medial part (Figure 3C). Highest PV-IR neuron densities are noted in the dorsolateral neostriatum, and lowest densities are observed in the ventromedial sector (Figure 3C). In the post-commissural striatum, PV-IR cell density is higher in the dorsal part, compared to the ventral part. A 300 μm interval separated coronal sections used for analysis, and a median average of 10 sections was analyzed per brain, representing 3,000 μm along the rostro-caudal axis. Mean section thickness after immunohistochemical processing, mounting, and coverslipping was 16 μm, as measured by the microcator using a 100X (oil) objective. [29, 86] A counting frame of 80 X 80 μm coupled with a 500 X 500 μm sampling grid at 100X (oil) magnification was applied to all sections. Our particular scheme examined 10-12 sections per neostriatum, with each section containing 10-64 sampling sites depending on its surface area. Mean coefficient of error (CE) of all PV-IR neuron number estimates was 0.12. The optical fractionator method revealed a total of 16,875 $\pm$ 203 (SEM, n = 4) PV-IR neurons in the neostriatum of (E18-21) saline control animals.

A similar sampling scheme was applied to Nissl stained sections. The sections had an average thickness of 12 μm. The mean CE of all Nissl-based

neostriatum neuron counts was 0.06. The optical fractionator estimated the total number of neostriatal neurons as 2.54 ± 0.13 million (SEM, n=4). Thus, approximately 0.7% of dorsal striatum neurons are PV-IR in young adult male Sprague-Dawley rats.

Stereological Counts of Neostriatal PV-IR Neurons after Prenatal Exposure to $GABA_A$ Receptor Antagonist or Agonist

Stereological analysis was performed on all experimental and control animal groups in order to obtain an unbiased estimate of the total number of PV-IR neurons in the neostriatum. Section thickness and sampling scheme (including characteristics of the sampling grid, dissector size, number of sections sampled and coefficient of error) were similar in animals derived from control and experimental groups (see previous section). Sections from a minimum of 4 animals were analyzed from each group treated with a $GABA_A$ receptor agonist/antagonist or vehicle.

Analysis of Variance (one-way ANOVA) revealed statistically significant differences in total PV-IR cell number between groups treated during the proliferative ($F_{6,21}$: 6.91; $p < 0.001$) or post-proliferative phase ($F_{4,15}$: 8.62; $p < 0.001$). The mean total number of PV-IR neurons in the neostriatum of animals treated with BMI (1 mg/kg/day) between E18-21 (10547 ± 1331, n=4) was significantly lower than that of pair-fed (14854 ± 967, n=4; $p < 0.01$, SNK post-hoc test) and saline control groups (16875 ± 203, n=4; $p < 0.01$, SNK post-hoc test) (Figure 2C). Administration of BMI (1 mg/kg/day) during the proliferative phase (E15-18) also resulted in a decrease in the total number of neostriatal PV-IR neurons (11777 ± 300, n=4), but the difference was

not statistically significant in comparison to pair-fed controls (12920 ± 439, n=4; Figure 2A). Even when the dose of BMI administered during the proliferative phase was doubled (2 mg/kg/day), no significant difference in total number of PV-IR neurons was seen in comparison to the pair-fed control group (11699 ± 1694 *vs.* 14150 ± 1039, n=4).

Groups exposed to the $GABA_A$ agonist muscimol during the prenatal proliferative phase showed an increase in total neostriatal PV-IR neuron number compared to pair-fed controls (14149 ± 390 vs. 12832 ± 545, n=4), as did those treated during the post-proliferative phase (14890 ± 310 vs. 14063 ± 517, n=4). However, statistical analysis revealed no significant difference in PV-IR neuron number after $GABA_A$ agonist exposure during either period (Figure 2B, D). Of note, in the case of both the E15-18 and E18-21 groups, mean PV-IR cell number in neostriatum of experimental or pair-fed animals was lower than in corresponding saline controls (E15-18 saline control: 18517 ± 915, n=4; E18-21 saline control: 16875 ± 203).

Mean neostriatal volume for each group of animals analyzed was as follows (mm^3, mean ± SEM): BMI (1 mg/kg/day, E15-18) 18.37 ± 1.58; BMI pair-fed (E15-18) 19.98 ± 1.57; muscimol (E15-18) 18.06 ± 1.21; muscimol pair-fed (E15-18) 19.51 ± 0.62; saline (E15-18) 19.00 ± 0.69; BMI (E18-21) 17.62 ± 0.87; BMI pair-fed (E18-21) 20.11 ± 0.73; muscimol (E18-21) 18.65 ± 0.67; muscimol pair-fed (E18-21) 19.62 ± 0.38; saline (E18-21) 22.60 ± 1.02. Neostriatal volumes were largely comparable except for the E18-21 BMI treated group, which had a significantly lower neostriatal volume compared to both pair-fed and saline control groups (ANOVA: $F_{11,34}$: 10.28; $p < 0.01$ for both SNK post-hoc tests). This reduction

in volume likely reflects a reduction in total number of neostriatum GABAergic projection neurons, the principal neurons of the striatum. However, quantification of this population is not performed here since the present experimental model is dependent on the relatively restricted neurogenesis period of PV-interneurons, compared to GABAergic projection neurons. Since total cell count obtained using the optical fractionator method does not vary with changes in size of the reference space, estimates of total number of PV-IR neurons in the neostriatum remain valid regardless of changes in neostriatal volume. [29,86]

Single Section Quadrant Analysis of PV-IR Cell Density

In order to determine the spatial morphology of neuronal loss in the striatum, we examined sections corresponding to coronal level Bregma -0.26 mm, from animals treated with BMI (1mg/kg/day) and their control groups. [60] At this level, the anterior commissure forms the ventral border of the striatum, and can be used as a reference for selecting comparable coronal sections in experimental and control groups (Figure 3 C, D). Mean PV-IR cell profile numbers and cell profile densities are shown in Table 1.

PV-IR neurons were categorized according to their position in quadrants (dorsolateral dorsomedial / ventrolateral / ventromedial) from plots obtained from image analysis (Biocom, Les Ulis, France). BMI administered during E18-21 resulted in a significant reduction in PV-IR cell profile density compared to pair-fed controls (ANOVA: $F_{1,8}$:11.34 , $p < 0.01$, SNK post-hoc test; Table 1 and Figure 3C, D). Visible gradients in cell density were evident between quadrants in control groups. The dorsolateral region had a significantly higher PV-IR cell density than the ventromedial region in both saline (ANOVA: $F_{1,8}$:19.04, $p < 0.01$, SNK post-hoc test)

and pair-fed control groups (ANOVA: $F_{1,8}$:12.01, $p < 0.01$, SNK post-hoc test; Figure 3C). This observed gradient in PV-IR cell density is not visible in progeny of dams treated with BMI between E18-21 (1 mg/kg/day; Table 1, Figure 3D), and statistical analysis revealed no significant difference in cell density between dorsolateral and ventromedial sectors.

2.5 Discussion

The main findings of the present study are: 1) Prenatal rodents exposed to the $GABA_A$ receptor antagonist BMI during early forebrain development, show significant reduction in the total number of parvalbumin-immunoreactive (PV-IR) GABAergic interneurons in the adult neostriatum. The effect is seen when BMI is administered during the prenatal post-proliferative phase, but not during the proliferative period for PV-IR neurons. These *in vivo* findings are compatible with prior *in vitro* evidence suggesting that GABA increases the number of striatal neurons by a $GABA_A$ receptor-mediated mechanism. [31] Our study extends these observations to a subclass of striatal interneurons with a well-defined period of neurogenesis. [68] The data suggests that GABA influences survival, but not proliferation of neuronal progenitors giving rise to PV-IR striatal neurons. 2) Prenatal treatment with BMI is also associated with loss of normal medial/lateral and dorsal/ventral distribution gradients of PV-IR interneurons in the neostriatum. This observation suggests that GABA may influence migration of PV-IR interneurons. 3) Finally, we provide a stereological estimate of the total number of PV-IR interneurons in the neostriatum.

2.5.1 Unbiased Estimate of the Total Number of PV-IR Neurons in the Neostriatum

The number of PV-IR neurons in the striatum has not been previously determined. The use of stereology allowed unbiased estimates of total PV-IR cell number in the neostriatum from control and experimental groups. Optical fractionator-based estimates of cell count within the entire volume of interest are preferred over non-stereological estimates based on profile counts, since the latter method is subject to bias resulting from changes in volume of either the reference space or the object counted. [29, 86]

Non-cholinergic aspiny interneurons include at least three groups of largely distinct GABAergic cells.[10, 33] The calcium binding proteins calretinin (CR) and PV identify two subclasses. [6, 7, 13, 16, 17, 35, 37, 38, 64, 67] A third class of aspiny neurons contains nitric oxide synthase (NOS), and co-localizes the peptides somatostatin (SS) and neuropeptide Y (NPY). [14, 22, 83] In the absence of colchicine pre-treatment, antibodies to GABA or its synthesizing enzyme glutamic acid decarboxylase (GAD) fail to immunostain most NOS-IR cells, reveal weak to intense staining in CR-IR neurons, and show strong staining in PV-IR neurons. [37] Following colchicine pre-treatment, all three subclasses of interneurons immunostain intensely for the GAD_{67} isoform. [37] Early studies, based on the possibility that most strongly positive GAD-IR interneurons are PV-IR, estimated that PV-IR cells represent 3-5% of striatal neurons in the rat. [34] This estimate is greater than the 0.7% obtained in the present stereological study. The observation that a significant proportion of CR-IR interneurons are also strongly immunoreactive for GAD, may account in part for the lower than expected proportion of striatal PV-IR neurons estimated. [37] Furthermore, since both CR and PV interneurons show heterogeneous regional distribution within the striatum [6, 17, 64, 68], previous estimates based on a small number of sections may not

be representative. The total number of neostriatal neurons counted in the present study (2.54 X 10^6) is quite similar to those previously obtained in rodents by other authors using similar stereological methods. [18, 56] Previous stereological estimates of striatal interneurons have been limited to SS-IR interneurons, which comprise 21,300 cells in each neostriatum, corresponding to approximately 0.8% of total neurons in the neostriatum. [86] PV-IR and SS-IR neurons are therefore found in comparable numbers in the neostriatum.

2.5.2 Parvalbumin-immunoreactive Interneurons of the Neostriatum as a Model for Examining Neurotransmitter Influence on Early Forebrain Development

The adult number of striatal neurons is determined by neurogenesis, successful cell migration, establishment of connections, and developmental cell death. [12, 42, 49, 63] Neurogenesis in the striatum takes place mainly from E13 to E22 (E0 corresponding to day of detection of vaginal plug). [2, 47] Whereas projection neurons become post-mitotic over a prolonged prenatal period [2, 3], the neurogenetic timetable for specific interneuron subpopulations is relatively restricted. Cholinergic interneurons are born early, with peak neurogenesis at E12-E14 [73]. The SS/NPY/NOS subtype becomes post-mitotic mainly between E15-16 [73], and CR-IR interneurons are born at E14-E17. [71] Detailed neurogenesis studies of PV-IR neurons of the neostriatum demonstrate that they also become post-mitotic over a relatively restricted time period, mainly between E14 and E17. [68]

Factors influencing proliferation, migration and survival of forebrain interneurons remain largely unexplored. [1, 20, 31, 69] We have developed an *in vivo* model for determining factors that influence morphogenesis in the prenatal striatum and cerebral cortex. [45, 69] Since GABAergic PV-IR neurons express their phenotype only

in the postnatal period [78], it is difficult to determine early developmental influences on this neuronal subclass *in vitro*. The relatively restricted period of striatal interneuron genesis allows for *in vivo* examination of microenvironmental influence on cell proliferation and post-proliferative events. In a previous study, the model was used to demonstrate that the NMDA subclass of glutamate receptors promotes proliferation of striatal PV-IR interneuron progenitors [69], suggesting a role for glutamate in early forebrain development. [8, 15, 32, 43]

2.5.3 GABA and Early Forebrain Development

In the striatum, GABA is derived mainly from intrinsic sources, including principal neurons and GABAergic interneurons. [34, 27, 28, 89] In the developing rat brain, significant GABA-IR can be detected in the ganglionic eminence as early as E12. [39] Studies using embryonic (E18) striatal cultures show that GABA promotes survival of striatal neurons during the post-proliferative period. [31] The effect is blocked by bicuculline and picrotoxin, but not 2-hydroxysaclofen, indicating a mechanism mediated via the $GABA_A$, but not $GABA_B$, receptor subtype. [31] $GABA_A$-mediated depolarizing responses are well documented in the embryonic and early postnatal CNS, and likely related to elevated intracellular chloride ion concentration in immature neurons. [5, 44, 66, 74, 85, 88, 90] In rat striatum progenitors studied *in vitro*, $GABA_A$ receptor agonists induce modest depolarization as early as E12-13, with marked increase in response by E14. [23, 52] Inhibitory responses to GABA develop progressively in the postnatal period, and are associated with developmental changes in receptor subunit composition and maturation of chloride ion homeostasis. [5, 27, 41, 62, 65, 81] $GABA_A$ receptor-mediated effects on developing cells in the mammalian CNS are regionally specific. [40] For example, $GABA_A$ receptor-mediated mechanisms increase proliferation of cerebellar granule cells [24], but decrease proliferation of

progenitors giving rise to the principal neurons of the cerebral cortex. [43] In the post-mitotic period GABA increases both motility and directed migration of immature cortical cells. [4] Different GABA concentrations differentially promote migration of distinct cortical GABAergic or non-GABAergic neuronal subpopulations. [4]

Prenatal exposure to both $GABA_A$ and NMDA receptor antagonists reduces the adult number of PV-IR striatal neurons. [69] However, $GABA_A$-mediated influence on striatal morphogenesis occurs predominantly during the post-proliferative phase, whereas NMDA receptor-mediated mechanisms promote neuroblast proliferation. Since a tendency to reduced total PV-IR neuron number was noted when $GABA_A$ receptor antagonist was given at E15-18, we cannot rule out a minor effect on cell proliferation. However, differences in neuron number were not statistically significant compared to pair-fed controls, even after doubling the dose of BMI. Furthermore, total PV-IR neuron number is not altered significantly after exposure to the $GABA_A$ receptor agonist muscimol during the proliferative phase, suggesting that ligand concentrations exceeding the physiological range do not alter proliferation or survival. Taken together, these observations suggest lack of proliferative effect of GABA on PV-IR striatal progenitors. These findings contrast with observations suggesting that $GABA_A$ receptor activation reduces neuronal proliferation in the developing cortex [43], and promotes proliferation of cerebellar granule cells. [24] Cortical interneurons, unlike projection neurons, appear to originate in the lateral ganglionic eminence. [1, 20, 80] It would therefore be of interest to determine whether PV-IR interneurons destined for the cerebral cortex, show similar behavior to their striatum counterparts with respect to proliferation or migration in response to classical neurotransmitters.

Reduction in the adult number of PV interneurons as a result of $GABA_A$ receptor blockade is in keeping with previous data demonstrating that GABA promotes striatal neuron survival *in vitro.* [31] Of note, neostriatal volume decreased

significantly following BMI treatment in the post-proliferative period, suggesting that in addition to PV-IR interneurons, the GABAergic principal neurons of the striatum likely show a reduction in cell number. However, the present model is designed to specifically distinguish proliferative effects of prenatal drug treatment from post-proliferative effects, and is tailored to the relatively restricted neurogenesis period of PV-IR interneurons. [68] Since subpopulations of principal neurons have a relatively heterogeneous and protracted time course of neurogenesis, [47, 82] we do not specifically address $GABA_A$ effects on projection neurons in the present study.

The observed reduction in PV-IR neuron survival after $GABA_A$ receptor antagonist exposure during the prenatal post-proliferative period may be related to impaired migration of post-mitotic striatal neuroblasts, reduced cell survival, or both. Gradient analysis of PV-IR neuron density suggests a possible effect on cell migration. In the adult neostriatum, PV-IR neuron density is greater in the dorsolateral, sensorimotor sector compared to the ventromedial region. [17, 35, 68] It is established that dorsolateral neostriatal PV-IR interneurons are born prior to ventromedial cells [68], suggesting an "outside-in" gradient of neurogenesis common to other ventral forebrain neurons. [3] Embryos exposed to $GABA_A$ receptor antagonist (BMI) during the post-proliferative period show loss of the dorsolateral-ventromedial gradient in PV-IR cell density, suggesting a GABAergic influence on cell migration. Failure to achieve normal final position in the striatum may also impair survival. We cannot exclude the possibility that reduced PV expression results from prenatal exposure to $GABA_A$ receptor antagonists, but this is less likely since forebrain PV is expressed mainly in the postnatal period. [78] Furthermore, altered PV-IR phenotype should occur in all sectors of the striatum, and would not account for the observed loss of density gradient. Future studies using transgenic mutant animals with altered GABA receptor expression, or models based on *in vitro* explants, may help further

establish a role for GABA in promoting neural migration in the developing ventral telencephalon.

2.6 Conclusion

Stereological analysis suggests that PV-IR GABAergic interneurons comprise approximately 1% of neostriatal neurons. The relatively restricted period of neurogenesis of striatal PV-IR cells (E14-E17) allows for an *in vivo* model for determining microenvironmental factors that influence prenatal striatal development. We provide evidence for a role for GABA in prenatal striatal morphogenesis. $GABA_A$ receptors have little influence on cell proliferation, but rather mediate migration of striatal PV-IR neurons. This *in vivo* data is consistent with *in vitro* evidence suggesting a role for GABA in survival and migration of forebrain neurons. [4, 31, 40] Recent data suggest that an as yet unidentified subpopulation of cortical GABAergic interneurons, rather than arising in the dorsal telencephalon germinal zone, may derive from the ganglionic eminence, a striatal precursor. [1, 58, 80] This finding suggests the possibility that cortical and striatal forebrain GABAergic interneurons may share homologous developmental mechanisms. Future studies will focus on comparative aspects of early neurotransmitter influence on GABAergic interneurons populating either the striatum or cerebral cortex.

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in total PV-IR cell number (B, D). (* $p < 0.01$, one way ANOVA with SNK post-hoc test)

Figure 3(A-D): Quadrant analysis to determine regional variations in PV-IR cell density after $GABA_A$ receptor modulation. Image-analysis-based map of distribution of PV-IR cells at the coronal level at which the anterior commissure forms the ventral border of the striatum (Bregma –0.26 mm, Paxinos and Watson rat atlas [61]) in progeny of saline controls (A), and offspring of dams given BMI during the post-proliferative phase (B). PV-IR cells were counted in dorsolateral, dorsomedial, ventromedial, and ventrolateral quadrants in the E18-21 drug-treated group, and cell densities were plotted and compared. The normal dorsal/ventral and medial/lateral gradients in PV-IR neuron density persist in pair-fed controls (C), but are not present after treatment with the $GABA_A$ antagonist (D). The external capsule (EC) forms the dorso-lateral border (scale bar = 100 μm), and the anterior commissure (AC) and lateral ventricle (LV) respectively form the ventral and medial borders of the neostriatum at this level (scale bar = 500 μm).

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Figure 1

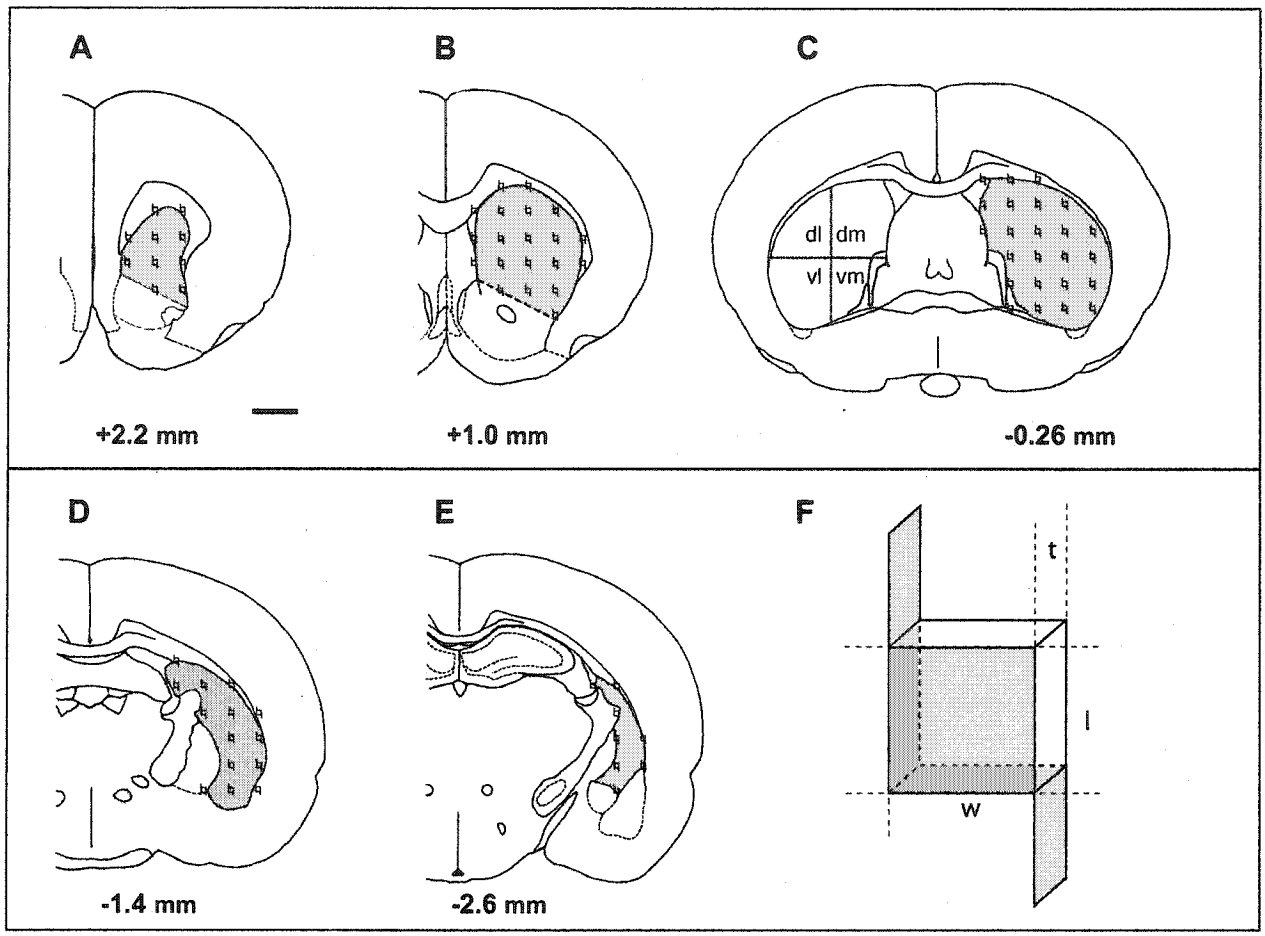


Figure 2

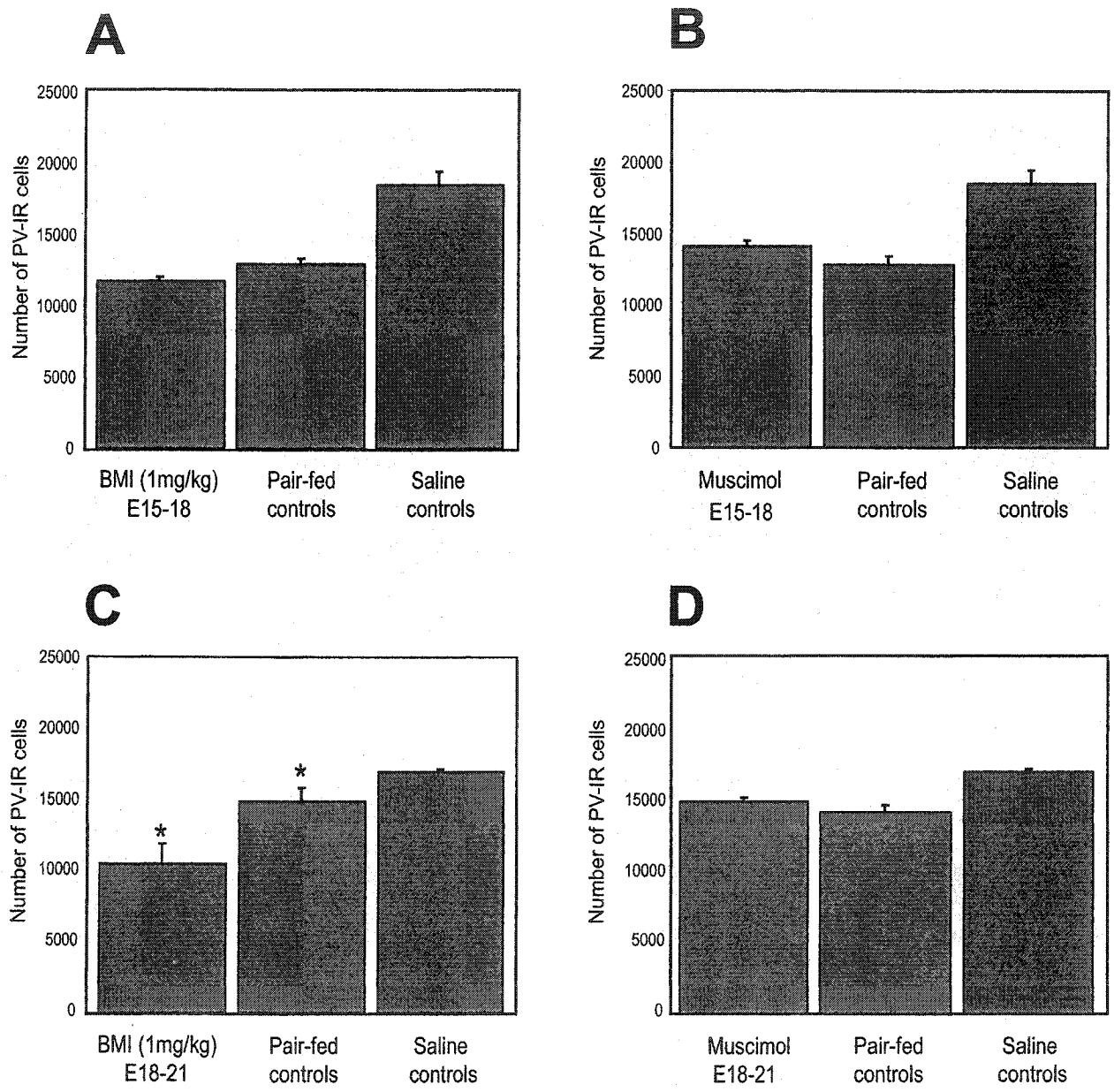
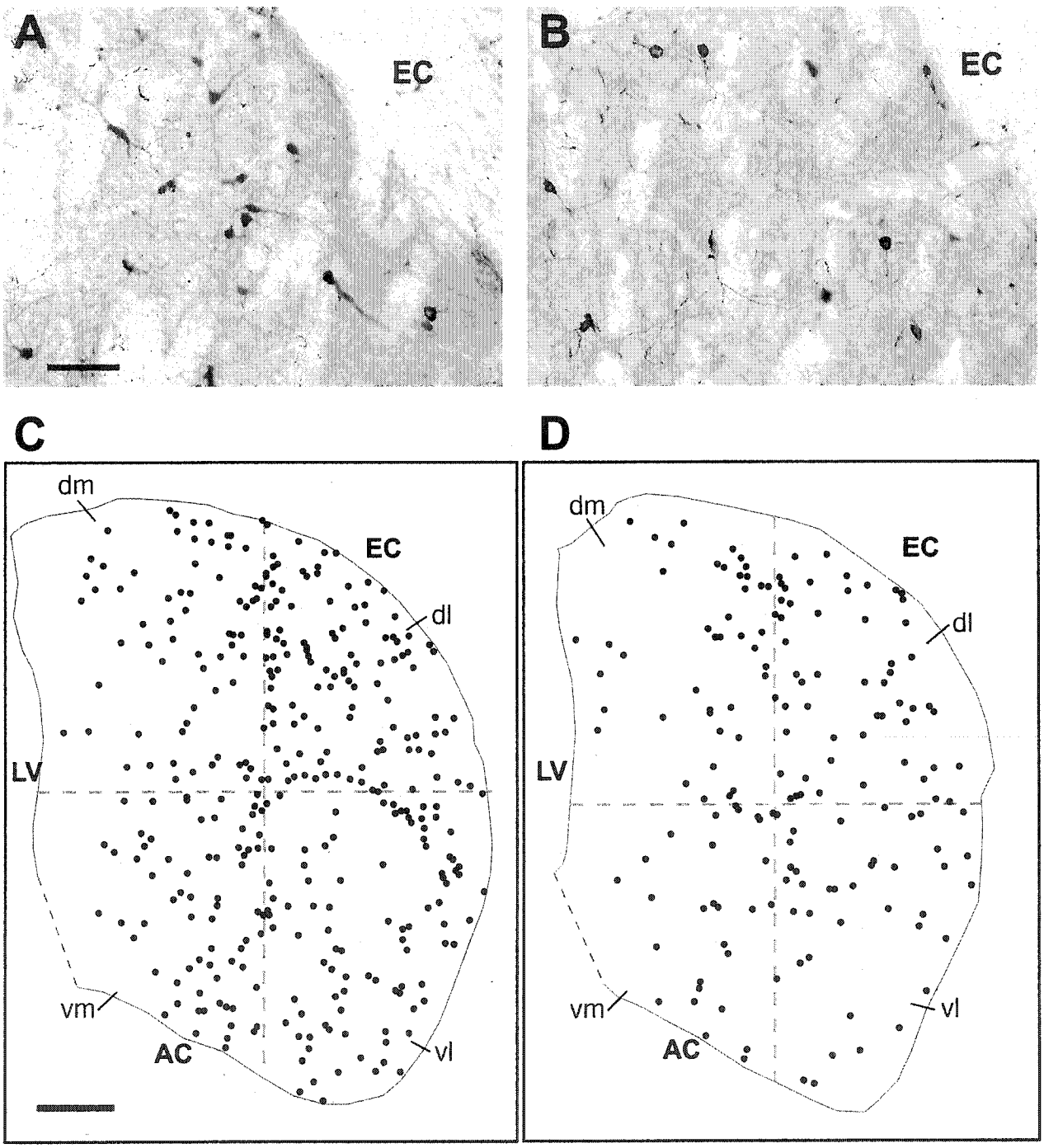

A
B
C
D
Number of PV-IR cells
25000
20000
15000
10000
5000
0
BMI (1mg/kg) E15-18
Pair-fed controls
Saline controls
Muscimol E15-18
BMI (1mg/kg) E18-21
Muscimol E18-21
*

Figure 3



Chapter 3: Glutamate promotes proliferation of striatal neuronal progenitors by an NMDA receptor-mediated mechanism

Glutamate Promotes Proliferation of Striatal Neuronal Progenitors by an NMDA Receptor-mediated Mechanism.

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Abbreviated title: NMDA and proliferation of striatal progenitors
Key Words: forebrain, striatum, neurogenesis, neurotransmitter, BrdU, stereology.

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3.1 Abstract

Increasing evidence suggests that classical neurotransmitters play important roles in the development of the mammalian central nervous system. We used *in vivo* and *in vitro* models to identify a novel role for glutamate in striatal neurogenesis mediated by N-Methyl D-Aspartate (NMDA) receptors. *In utero* exposure to NMDA receptor antagonists during striatal neurogenesis caused a dramatic reduction in the total number of adult striatal neurons. In contrast, embryos exposed to NMDA receptor antagonists immediately following the main period of neurogenesis showed no significant change in neuronal number in the adult striatum. In addition, examination of embryos shortly after NMDA receptor blockade revealed reduced proliferation in the lateral ganglionic eminence (LGE). In culture, dividing neuronal progenitors derived from the embryonic LGE showed marked reduction in 5'-bromodeoxyuridine (BrdU) uptake when exposed to NMDA receptor antagonists, indicating reduced DNA synthesis. Low concentrations of NMDA significantly increased proliferation, whereas high concentrations were toxic. α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid/kainate (AMPA/KA) receptor antagonists had no significant effect on striatal neuroblast proliferation either *in vivo* or *in vitro*. These results support the hypothesis that glutamate plays a novel role during early development of the ventral telencephalon, promoting proliferation of striatal neuronal progenitors by an NMDA receptor-dependent mechanism. In contrast, previous findings suggest that proliferation of cortical progenitors derived from the dorsal telencephalon is regulated by activation of AMPA/KA but not NMDA receptors. Heterogeneous responses to glutamate in different germinal zones of the

telencephalon may be an important mechanism contributing to generating neuronal diversity in the forebrain.

3.2 Introduction

Central nervous system (CNS) development is the result of coordinated cell proliferation, migration, differentiation, synaptogenesis, and apoptosis (reviewed by Sanes et al., 2000). Proliferation is determined by expression of distinct genetic programs and extracellular cues (Lillien, 1998; Edmund and Jessell, 1999). Differences in proliferative response to growth factors or neurotransmitters in embryonic germinal zones may be an important mechanism for achieving the appropriate number of neurons in different CNS regions (Lauder, 1993; Caviness and Takahashi, 1995; Levitt et al., 1997). Glutamate, the major excitatory neurotransmitter (Curtis et al., 1959; Watkins, 2000), is excitotoxic at high concentrations and implicated in CNS pathology (Olney, 1982; Choi, 1988). Increasing evidence suggests that glutamate plays novel roles in morphogenesis. Glutamate regulates migration, survival, differentiation, and neuritogenesis of neurons (Mattson and Kater, 1987; Simon et al., 1992; Rossi and Slater, 1993; Rakic and Komuro, 1995; Behar et al., 1996; Bhave and Hoffman, 1997; Dammerman and Kriegstein, 2000). Recent studies indicate that glutamate also plays an important modulatory role in proliferation of forebrain neuronal precursors (LoTurco et al., 1995; Cameron et al., 1995; Sadikot et al., 1998; Haydar et al., 2000; reviewed by Contestabile, 2000; Arvidsson et al, 2001).

The mammalian telencephalon is derived from dorsal germinal zones that generate glutamatergic principal neurons of the cerebral cortex, and ventral germinal zones that produce basal forebrain populations, including GABAergic principal neurons of the striatum (Holmgren, 1925; Fentress et al., 1981; Bayer, 1984;

Marchand and Lajoie, 1986, Kawaguchi et al., 1995). With the exception of cortical GABAergic interneurons, derived mainly from the ventral telencephalon (deCarlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997), most migrating neurons do not cross the corticostriatal boundary (Fishell et al., 1993). Dorsal and ventral telencephalic germinal zones express distinct transcription factors (Puelles and Rubenstein, 1993; Shimamura et al., 1995; Casarosa et al., 1999), exhibit distinct patterns of clonal heterogeneity (Halliday and Cepko, 1992; Acklin and Van der Kooy, 1993), and may show unique morphogenetic responses to extracellular factors.

Glutamate is present in the telencephalic germinal zones during embryogenesis, and exerts morphogenetic effects that vary with receptor subtype (Blanton and Kriegstein, 1991; Behar et al., 1999). Activation of AMPA/KA, but not NMDA, subclasses of ionotropic glutamate receptors alters proliferation in the cortical germinal zone (LoTurco et al., 1995; Haydar et al., 2000). Little is known about the role of glutamate in morphogenesis of the embryonic basal telencephalon. *In utero* NMDA receptor blockade markedly reduces proliferation of striatal GABAergic interneuron progenitors (Sadikot et al., 1998).

We hypothesize that proliferative responses to glutamate in dorsal and ventral telencephalic germinal zones are regionally specific. We investigate the influence of glutamate on proliferation of progenitors of striatal projection neurons, focusing on NMDA receptor activation using an *in vivo* model and proliferating primary neuronal cultures (Sadikot et al., 1998; Luk and Sadikot, 2001). We report that NMDA receptor activation is required for proliferation of striatal progenitors, whereas AMPA/KA mediated receptor mechanisms have no significant effect. These results

suggest distinct reciprocal roles for NMDA and non-NMDA receptors in proliferation of neuronal progenitors in dorsal and ventral telencephalic germinal zones. This heterogeneous response to glutamate may be an important mechanism for generating neuronal diversity in the dorsal and ventral forebrain.

3.3 Materials and Methods

Ionotropic Glutamate Receptors and Proliferation of Striatal Neuronal Progenitors In Vivo

Animals

Female Sprague-Dawley rats (Charles River, LaSalle, Quebec) were coupled with males between 3 p.m. and 5 p.m. The first 24 hours (h) after coupling was designated as embryonic day zero (E0). A second group of females was coupled 48 h later in order to provide control animals, including dams matched for food and water intake with experimental groups. All animal procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

In utero drug treatments for adult stereology

The NMDA receptor antagonists MK-801 (non-competitive; 0.2 mg/kg/day) or CGS-19755 (competitive; 5 mg/kg/day; RBI, Natick, MA), or the AMPA/KA receptor antagonist 1, 2, 3, 4-tetrahydro-6-nitro-2, 3-dioxo-benzol(f)quinoxaline-7-sulfonamide (NBQX; 10 mg/kg/day) were administered to separate groups of rats. Drugs were dissolved in sterile normal saline and administered daily by intraperitoneal (i.p.) injection over a period of 4-days from either E15-18 or E18-21.

These time intervals correspond respectively to mainly proliferative or post-proliferative periods for striatal neurons (Bayer, 1984; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987).

Each animal's food and water intake and weight was recorded daily. As controls, age-matched pregnant females were given daily i.p. injections of saline (1 ml/kg/day) over identical 4-day periods as the drug-treated dams. In addition, pair-fed control groups were given access to the amount of food and water consumed by their drug-treated counterparts. A separate control group was given saline injections (i.p.) during the period of interest and free access to food and water. After birth, 5 males were randomly chosen from each litter and sacrificed between postnatal days (P) 35-42 for histology by transcardial perfusion with 4% paraformaldehyde in phosphate buffer (PFA; 4°C, 0.1M, pH 7.4).

Stereology

Coronal sections of the entire adult striatum were cut at 50 μm on a freezing microtome. Upon identifying the most rostral extent of the striatum, section collection was started randomly between the first and sixth section, as determined by a roll of dice. Serial free-floating sections were collected in phosphate buffered saline (PBS, 0.1M, pH 7.4) as separate sets so that each set contained every sixth serial section. One set of sections from each brain was processed using 0.1% Cresyl Violet as a Nissl stain. Sections were then cleared in xylene substitute, and coverslipped with Permount (Fisher, Fair Lawn, NJ).

An unbiased stereological technique, the optical fractionator (Moller et al., 1990; West et al., 1996), was used to estimate the total number of neurons in the striatum and frontal agranular cortex as previously described (Luk and Sadikot, 2001). The apparatus used consisted of a light microscope (BX40, Olympus, Japan) coupled with a video camera (DC200, DAGE, Michigan City, IN, USA), motorized X-Y stage (BioPoint XYZ, LEP, Hawthorne, NY), Z-axis indicator (MT12 microcator, Heidenhain, Traunreut, Germany), and a computer running Stereo Investigator software (Microbrightfield Inc., Colchester, VT). The rostral and caudal limits of the reference volume were determined by the first and last coronal sections with visible caudate-putamen (neostriatum, dorsal striatum; approximately Bregma 2.20 to -2.60 mm; Paxinos and Watson, 1986). Every sixth serial section within this zone was examined, i.e. at 300 μm intervals along the rostrocaudal axis. The corpus collosum, external capsule, lateral ventricle, globus pallidus, and anterior commissure were used as boundaries (Fig. 1). In the most rostral sections, the ventral striatum was excluded from analysis by a line drawn from the ventral tip of the lateral ventricle to the dorsal border of the piriform cortex, corresponding to an angle of 20-30^{o} below the horizontal axis. In more caudal sections, the caudate-putamen borders included the external capsule, globus pallidus, bed nucleus of the stria terminalis, the substantia innominata, and the dorsal amygdala. Stereology was also performed for the motor cortex in the same sections. Areas corresponding to Fr1 and Fr2 (frontal agranular cortex) were delineated with the help of an atlas (Zilles, 1985). The granular somatosensory cortex was excluded from analysis. Surface areas of each region of interest were estimated from tracings of the neostriatum at 4X magnification using the

software. Volumes of the reference space were estimated using the Cavalieri method (Gundersen and Jensen, 1987).

Systematic random sampling of neurons in the neostriatum was performed by randomly translating a grid with 500 x 500 μm squares onto the section of interest using the software (Fig. 1). At each intersection of grid lines a 60 x 60 μm counting frame with exclusion lines was then applied (Fig. 1). All randomly assigned sample sites were then examined using a 100X objective (oil, N.A. 1.3). Neurons were distinguished using the nucleus as a unique identifier, and glial cells were excluded on the basis of morphology and by counting only profiles greater than 7 μm in diameter according to previously described criteria (Dam, 1992; Oorschot, 1996; Luk and Sadikot, 2001). Only neurons falling within the counting frame without contact with the exclusion lines were enumerated. Objects seen in the counting frame were only counted if they came into focus within a predetermined 8 μm thick optical dissector positioned 2 μm below the surface of the mounted section as indicated by the microcator. Each optical dissector therefore consisted of a 60 x 60 x 8 μm brick with three exclusion planes (Fig. 1, inset). Calculated estimates of the total number of neurons in each neostriatal and cortical reference volume were determined using the Stereo Investigator software. Statistical analysis was performed by one-way analysis of variance (ANOVA, α=0.01) with the Student-Newman-Keuls post-hoc test (SNK) for comparison between groups.

Analysis of embryos following drug treatment

The immediate effects of receptor antagonists on cell proliferation *in vivo* were examined in embryos. Separate groups of timed pregnant rats were given MK-801 (0.2 mg/kg/day), CGS-19755 (5 mg/kg/day), NBQX (10 mg/kg/day), or saline via i.p. injection on E15 and 16. On E16, drug administration was followed 1 h later by a single injection of BrdU (50mg/kg). Embryos were removed after a further 12 h by Caesarian section, decapitated, and fixed overnight in 4% PFA. Heads were then transferred to 10% formalin, dehydrated, and embedded in paraffin. Embryonic brains in paraffin blocks were sectioned at 5μm with a microtome. Antigen retrieval was achieved by heating sections in citrate buffer (0.01M, pH 6.0) for 15 min at 90°C. Sections were then exposed to 2N HCl for 1h, followed by 3 washes in PBS (5 min each), and then incubated overnight with a monoclonal antibody against BrdU (1:10, Becton Dickinson). Cells were then washed 3 times with PBS and labeled using the avidin-biotin-complex (ABC) peroxidase method by incubating for 1h with biotinylated goat anti-mouse IgG secondary antibody (1:200) followed by ABC solution (Vector, Burlinghame, CA). The final reaction was revealed by exposing cells to a solution (NiDAB) containing Tris buffer (0.05 M, pH 7.6), nickel ammonium sulfate (3.7 mg/ml), 3,3'-diaminobenzidine (DAB, 0.25 mg/ml), and 0.0006% hydrogen peroxide. Sections were then counterstained with nuclear fast red, dehydrated, and coverslipped in permount.

In order to determine the immediate effects of treatment on proliferation, BrdU immunoreactive nuclei were quantified in coronal sections at the level of the ganglionic eminence. The striatal and cortical periventricular zones were delineated by a 100 μm wide box placed approximately 100 μm from the cortical striatal angle

(Fig. 3a,b). The first 50 μm from the ventricular surface was arbitrarily designated as the ventricular zone (vz), while the area from 100 to 200 μm was considered the subventricular zone (svz). Proliferative nuclei were counted through the entire thickness of the section and the results expressed as a percentage of nuclei that were BrdU+.

Ionotropic Glutamate Receptors and Proliferation of Striatal Neuronal Progenitors In vitro

Microdissection and preparation of proliferative cultures

Cultures were prepared from E15 rat embryos using techniques similar to those previously described (Ivkovic et al., 1997; Ikeda et al., 1997; Ventimiglia et al., 1998). The lateral ganglionic eminence (LGE) that gives rise to the striatum anlage (Bayer, 1984; Deacon et al., 1994) was microdissected in cold magnesium-free Hank's Balanced Salt Solution (HBSS, Sigma, MO). The dissected tissue was then incubated in trypsin and DNAse at 37°C and centrifuged at 1200 rpm for 5 min in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS). The pellet was resuspended in Neurobasal medium supplemented with B27 (Life Technologies, Burlington, Ontario). Cells were dissociated by passing through a series of fire-polished Pasteur pipettes of decreasing caliber. Viable cells were then counted by Trypan Blue exclusion, diluted in Neurobasal/B27 medium (Bottenstein, 1985; Brewer, 1995) containing 2 mM L-glutamine, penicillin/streptomycin. Cells were then plated on 8-well chamber slides pre-coated with poly-D-lysine (Becton Dickinson, Franklin Lakes, NJ) at a density of 2×10^5

cells/cm^2 and incubated at 37°C in an air:CO_2 (20:1) mixture for 1-5 days *in vitro* (DIV).

Treatment and BrdU incorporation

24h after initial plating, cells in individual slide chambers were exposed to either MK-801 (2 μg/ml), CGS-19755 (20 μg/ml), NMDA (0.1-100 μM), NBQX (10 μM), or vehicle control. Drugs were prepared immediately prior to addition to the culture medium. In order to label cells passing through S-phase, BrdU (20 μg/ml; Sigma, St Louis, MO) was added to each chamber either 4 or 24 h after treatment. Cells were then fixed for 20 minutes with cold 4% PFA either 2 h or 12 h after addition of BrdU, and processed for immunocytochemistry.

Immunocytochemistry

BrdU uptake by proliferating cells was revealed by immunostaining (Gratzner, 1982). Briefly, cells were permeabilized in cold acetone/methanol (1:1), washed in PBS, and denatured in 2N HCl for 20 minutes. The cells were then washed with PBS, sodium borate (0.1 M), followed by another PBS wash. Anti-BrdU antibody (1:10, Becton Dickinson) was added and incubated overnight at 4°C. Cells were then washed 3 times with PBS and labeled with an Alexa 594-conjugated goat anti-mouse IgG antibody (1:500; Molecular Probes, Eugene, OR).

In order to characterize cell types, cultures were also immunostained for neuron specific βIII-microtubulin (TuJ1; 1:500; Babco, Richmond, CA) or microtubule-associated protein-2 (MAP-2; 1:1000; Sigma) as early or late neuronal markers, respectively (Lee et al., 1990; Memberg and Hall, 1995), or for glial

fibrillary acidic protein (GFAP; 1:1000; Sigma). Cells were counterstained with 4', 6-diamidino 2-phenylindole dihydrochloride (DAPI, 1 μg/ml in H_2O, 15 min, 37°C) to reveal cell nuclei.

To determine whether proliferating neuroblasts express NMDA receptor subunits, double-labeling for BrdU and NMDA receptor subunits was performed in culture. Following initial pretreatment and incubation with anti-BrdU antibody (see above), cells were incubated overnight in primary antibodies for NMDA receptor subunits. Primary antibodies were dissolved in PBS containing 0.3% Triton-X 100 and 1% normal goat serum and washed in PBS (3 x 5 min). Polyclonal antibodies for NR1 (1:500; Transduction Laboratories), NR2A (1:400, Chemicon, Temecula, CA), NR2B (1:500; Sigma), and NR2C (1:250; Chemicon) were used. Cells were then incubated with an appropriate secondary antibody conjugated to either Alexa-488 or -594 (1:500) for 1 h, then washed in PBS (5 x 5 min). For double-labeling of NR1 and NR2A, a monoclonal antibody for NR1 (1:1000; Chemicon) was used.

Analysis

Fluorescently labeled cells were visualized under a fluorescence microscope using a 40X objective and the appropriate filters. Density of cells and nuclei was determined in 40-50 random fields generated using the Stereo Investigator software. Results were compared by ANOVA as described above.

3.4 Results

Exposure to NMDA receptor antagonists reduces proliferation of neostriatal neuronal precursors in vivo

In order to determine whether ionotropic glutamate receptors mediate proliferation in the developing mammalian striatum, rat embryos were exposed *in utero* to competitive and non-competitive NMDA receptor antagonists. Drugs were administered either during proliferative (E15-18) or mainly post-proliferative (E18-21) period for striatal neurogenesis (Smart and Sturrock, 1978; Bayer, 1984; Marchand and Lajoie, 1986; Van der Kooy and Fishell, 1987). To quantify total neuron number in the striatum and motor cortex of adult offspring, we applied the optical fractionator technique to Cresyl Violet-stained brain sections. This quantitative method allowed for efficient and unbiased estimates of total neuronal number within the entire striatal or cortical reference volume. Stereology is preferred over non-stereological estimates based on profile counts, as the latter method is subject to bias resulting from changes in either the volume of the reference space or the size of the object counted (Moller et al., 1990; West et al., 1996). Changes in total neuronal count and striatal volume were compared based on analysis of sections obtained from drug-treated and control animals.

The estimated number of neurons per striatum in rats receiving only the saline vehicle during the proliferative (E15-18) or post-proliferative (E18-21) phases was 2.58 ± 0.10 million and 2.54 ± 0.12 million, respectively (Fig. 2a; all data represented as mean $\pm$ SEM). These estimates are in agreement with results from previous studies using similar stereological methods to quantify principal neurons in the rodent striatum (Dam, 1992; Oorschot, 1996). In comparison to pair-fed control animals, administration of NMDA-receptor antagonists during the maximal proliferative period for projection neurons (E15-18) resulted in a 38-54% reduction of striatal

neuronal number (Fig. 2a). One-way ANOVA revealed significantly decreased neuron numbers in rats treated with either the non-competitive antagonist MK-801 (1.61 $\pm$ 0.06 million) or the competitive antagonist CGS-19755 (1.20 $\pm$ 0.08 million) in comparison to both pair-fed (2.13 $\pm$ 0.14 million) and saline (2.58 $\pm$ 0.10 million) controls. Animals receiving either NMDA antagonist during the proliferative period also exhibited significantly reduced neostriatal volumes (MK-801: 14.8 $\pm$ 0.49 mm^3; CGS: 16.9 $\pm$ 0.32 mm^3), compared to pair-fed (19.9 $\pm$ 1.1 mm^3) and saline (20.1 $\pm$ 1.2 mm^3) control groups (Fig. 2b). These reduced volumes likely reflect reductions in striatal neuron number.

In contrast, *in utero* exposure to MK-801 during the predominantly post-proliferative period for striatal projection neurons (E18-21) did not result in statistically significant changes in striatal neuron number or striatal volume (18.95 $\pm$ 0.28 vs. 20.1 $\pm$ 0.92 mm^3; Fig. 2b) in comparison to the pair-fed control group (2.0 $\pm$ 0.17 vs 2.11 $\pm$ 0.13 million; Fig. 2a). Decreased neuron number following prenatal NMDA receptor antagonist exposure is therefore due mainly to reduced proliferation of striatal neuroblasts or precursors. To determine if proliferation of striatal neuroblasts is dependent on non-NMDA receptor-mediated glutamatergic mechanisms, as is the case in the dorsal telencephalic germinal zone (Haydar et al., 2000), dams were exposed to NBQX during the prenatal proliferative period. NBQX failed to significantly alter striatal volume or neuron number indicating that AMPA/KA receptor blockade does not influence proliferation of neuroblasts derived from the prenatal ventral telencephalon (Fig. 2a, b). Additionally, administration of MK-801 from E15-18 did not significantly alter neuron number or volume in the

frontal agranular cortex (Fig. 2a, b), suggesting that the observed effects of the NMDA receptor antagonists are regionalized to the ventral telencephalon.

Progenitors of medium spiny GABAergic projection neurons likely account for the observed NMDA-mediated proliferative effects since this population comprises 90% of rat striatal neurons (Kitai, 1981; Smith and Bolam, 1990; Kawaguchi et al., 1995). Decreases in proliferation of precursors of other minor striatal neuronal subpopulations may also account for a small proportion of observed changes though these were not distinguished using Nissl stains. For example, parvalbumin-positive GABAergic interneurons, which comprise approximately 1% of striatal neurons (Luk and Sadikot, 2001), likely contribute to the observed changes, since our previous studies indicate that proliferation of precursors of this subpopulation is also positively influenced by NMDA receptor activation (Sadikot et al., 1998).

To account for possible nutritional effects, pair-fed groups (E15-18 and E18-21) matched for food and water intake were used as controls. Striatal neuronal counts and volumes were lower in pair-fed groups compared to saline controls (Fig. 2), but the decreases did not reach statistical significance, suggesting that nutritional effects were minor in these experiments.

NMDA receptor blockade reduces proliferation in the ganglionic eminence

In order to observe the effects of NMDA receptor blockade on striatal development more directly, proliferation in the lateral ganglionic eminence was examined in embryos shortly after exposure to MK-801. The final administration of

the antagonist was followed by 12h BrdU exposure in order to label cells in S-phase. Analysis of the ventricular zones of the ganglionic eminence revealed an 18% decrease in the percentage of BrdU-labeled nuclei with respect to saline controls following MK-801 exposure (Figs. 3c-e). Overall nuclear density in the LGE was similar in both treated and untreated animals (not shown). The percentage of nuclei positive for BrdU in the subventricular zone was comparable in both MK-801 treated and control animals. Our *in vivo* data collectively indicate that NMDA, but not AMPA/KA, ionotropic glutamate receptors mediate an important proliferative effect on striatal neuroblasts or precursors. Interestingly, in the developing dorsal telencephalon AMPA/KA activation increases DNA-synthesis in cortical progenitors in the ventricular zone, whereas NMDA receptor activation has no effect on proliferation (Haydar et al., 2000).

Characterization of proliferating striatal neuroblasts in vitro

Dissociated cultures of proliferating striatal neuroblasts derived from E15 lateral ganglionic eminence (LGE) were incubated in serum free Neurobasal/B27 medium for 1-5 DIV (Ventimiglia et al., 1998; Ivkovic et al., 1997). Immunochemical markers revealed that the cultures were predominantly neuronal in composition (Fig. 4). A TuJ1 antibody that recognizes neuron–specific βIII-microtubulin, was used to label cells committed to a neuronal lineage (Geisert and Frankfurter, 1989; Moody et al., 1989). This antigen is not expressed by astrocytes or oligodendrocytes, and is an early marker of neuronal differentiation, including neuroblasts undergoing mitosis and postmitotic neurons (Lee et al., 1990; Easter et al., 1993; Memberg and Hall,

1995; Jacobs and Miller, 2000). MAP-2, a specific marker for postmitotic and differentiated neurons (Johnson and Jope, 1992), was also used. Using DAPI as a nuclear counterstain, 42% of cells in 2 DIV cultures were positive for TuJ1, and 62% of cells were positive for MAP-2 (Matus et al., 1980). At 4 DIV, the majority of cells were also NeuN positive (data not shown). GFAP staining cultures indicated that glia comprised less than 2% of the total cell population. These results are in agreement with previous reports suggesting that GABAergic medium spiny neurons and their precursors make up the majority of cells in these early cultures, with a minority of interneurons and glia (Ventimiglia et al., 1998; Ivkovic et al., 1997; Petersen et al., 2000). The results are also consistent with evidence that forebrain gliogenesis (with the exception of radial glia) occurs in the late prenatal and early postnatal periods (Schultze and Maurer, 1974).

In order to quantify cell proliferation, cultures were exposed to the thymidine analog BrdU for a period of 24 h after 1, 2, or 4 DIV. The proportion of nuclei that were positive for BrdU was maximal during the first 48 h *in vitro*, and decreased to low levels by 96 h (data not shown). This is similar to the proliferative chronology *in vivo* (Bayer, 1984; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987).

The presence of NMDA receptors *in vitro* was confirmed by staining with antibodies against subunits of the receptor heteromer. The NR1-, NR2A-, and NR2B subunits were detected in proliferative cultures by immunofluorescence (Fig. 5). However, no NR2C staining was not detected. Immunoblots of lysates prepared from 2 DIV cultures showed similar results (data not shown). In double-labeling experiments, the NR1 subunit, which is common to functional NMDA receptors

(Monyer et al, 1994), was detected in approximately 76% of BrdU+ cells after 4h of exposure (Fig. 5a,b). Smaller subsets of BrdU+ cells also expressed NR2A (Fig. 5c,d) and NR2B subunits (not shown). These results indicate that dividing neuroblasts may express a functional forms of NMDA receptor.

NMDA receptor activation promotes proliferation of striatal neuronal precursors in vitro

To identify the role of NMDA receptors in neuroblast proliferation, 1 DIV embryonic striatal cultures were exposed to NMDA antagonists (MK-801, CGS-19755), NMDA (1-100 μM), or vehicle. Similar cultures were also exposed to NBQX in order to compare NMDA and AMPA/KA receptor mediated effects. Cultures were incubated in medium containing drug or vehicle for 24 h, followed by a brief 2h exposure to BrdU prior to fixation (Fig. 6). BrdU-labeling in this preparation was therefore limited to proliferating populations that were in S-Phase at the time BrdU was added. The presence of either MK-801 or CGS-19755 reduced the percentage of BrdU+ nuclei to less than 50% of control levels (Fig. 6a). To further establish that NMDA receptors mediate the effects of glutamate on cell proliferation, cultures were exposed to varying concentrations of NMDA.

Exposure to 1 μM NMDA resulted in a 29% increase in proportion (and density, data not shown) of BrdU+ nuclei (Fig. 6a). However, progressively higher concentrations of NMDA (10, 100 μM) resulted in decreases in the proportion of both BrdU+ nuclei (Fig. 6a) and cells positive for neuronal markers (MAP-2 or TuJ-1; Fig. 6b,c), in keeping with the expected excitotoxic effects at these doses (Koroshetz et al.,

1990). Despite marked reduction in BrdU uptake, cultures exposed to NMDA antagonist for 24h showed no significant reduction in MAP2+ cell number, suggesting that maturation and survival of post-mitotic neurons was unaltered. On the other hand, the density of neurons expressing the earlier neuronal marker TuJ1+ decreased significantly after exposure to CGS-19755 or MK-801 (Fig. 6b). The contrasting results with the two markers are in keeping with the observation that MAP-2 is expressed in the processes of more mature neurons (Johnson and Jope, 1992), whereas TuJ1 expression is initiated during the final mitosis of neuronal progenitors (Memberg and Hall, 1995; Jacobs and Miller, 2000). Reduction in the number of TuJ1+ neurons may therefore reflect decreased proliferation of neuroblasts giving rise to early postmitotic TuJ1+ populations. These results collectively suggest that NMDA-mediated glutamatergic mechanisms have a marked influence on neuroblast proliferation, but not on survival of postmitotic neurons. Exposure to NBQX had no effect on the proportion of cells with BrdU+ nuclei, or TuJ1+ and MAP-2+ cells, indicating lack of an effect of AMPA/KA on proliferation of striatal progenitors, in keeping with our *in vivo* results.

To determine whether NMDA-mediated glutamatergic mechanisms influence cell proliferation after short-term exposure, 1 DIV cultures were incubated with the same agonist/antagonists for only 4h followed by a 2h pulse of BrdU prior to fixation (Fig. 7a). Given estimated cell cycle times of 10-20 h for striatal progenitors (Acklin and Van der Kooy, 1993; Bhide, 1996) this experiment has the advantage of minimizing possible fluctuations in cell number due to proliferation or apoptosis during the treatment period.

As in the previous experiment, exposure to either MK-801 or CGS-19755 resulted in a significant decrease in the percentage of BrdU+ nuclei (-35% and 33%, respectively) compared to control cultures (Fig. 7a), indicating that effects on neuroblast proliferation can be detected within 4h after initial receptor blockade. In keeping with the hypothesis that NMDA positively influences proliferation, exposure to 1 μM NMDA also increased the percentage of cells with BrdU+ nuclei, although this value did not reach strong statistical significance (Fig. 7a, $p<0.03$). The density of MAP-2+ and TuJ1+ neurons following exposure to either glutamate receptor antagonists or low concentrations of NMDA remained unchanged (Fig. 7b,c). Unaltered neuronal density is expected since the treatment period is within the cell cycle time for striatal neuroblasts. However, the addition of 100 μM NMDA to cultures reduced the density and number of both TuJ1+ and BrdU+ nuclei, respectively, indicating toxicity. As in previous experiments, there was no significant change in BrdU uptake after application of NBQX.

To determine whether the reduction in proliferation following NMDA receptor blockade is reversible, NMDA was added at various concentrations to cultures treated with the competitive receptor antagonist CGS-19755. A dose-dependent rescue was observed in which NMDA concentration was directly proportional to the number of BrdU+ nuclei (Fig. 7a). With the addition of 100 μM NMDA, BrdU uptake in CGS-19755 treated cultures recovered to 80% of control levels. However, no recovery in proliferation was observed with application of MK-801, in keeping with the non-competitive nature of this receptor antagonist (data not shown). These experiments collectively suggest that NMDA-, but not AMPA/KA-,

mediated glutamatergic mechanisms promote proliferation of striatal neuronal progenitors.

3.5 Discussion

In the present study, we identify a novel role for the classical neurotransmitter glutamate in promoting proliferation of neuronal precursors derived from the germinal zone of the ventral telencephalon. Using an *in vivo* model, we demonstrate that proliferation of progenitors of the principal neurons of the striatum is dependent on activation of NMDA glutamate receptors. We further investigated ionotropic glutamate receptor-mediated effects in proliferative cultures derived from the lateral ganglionic eminence, the main source of striatal progenitors. Our results indicate that NMDA receptor mediated glutamatergic activity promotes proliferation of striatal neuronal progenitors. Conversely, AMPA/KA mediated mechanisms have no significant effect on proliferation of striatal progenitors. In contrast, previous studies indicate that cortical neuronal progenitors proliferate in response to activation of AMPA/KA, but not NMDA receptors. We propose that the germinal epithelium of the embryonic telencephalon is spatially heterogeneous with respect to proliferative response to glutamate. Regional variation in neurotransmitter effects on proliferation in the germinal zones of the telencephalon may be an important novel mechanism for generating neuronal phenotypic diversity in the forebrain.

3.5.1 Glutamate promotes proliferation in the ventral telencephalon by an NMDA receptor mediated mechanism

Little is known about factors governing proliferation of neuronal progenitors of the striatum, the major derivative of the ventral telencephalon. Growth factors

including EGF, bFGF, and TGFα modulate proliferation in the developing ventral forebrain (Reynolds and Weiss, 1992; Temple and Qian, 1995; Cavanagh et al., 1997; Ciccolini and Svendsen, 1998). Recent evidence suggests that amino acid neurotransmitters play an important role in proliferation and survival of forebrain neurons. $GABA_A$ receptor activation, which can depolarize embryonic neurons (Cherubini et al., 1991), mediates survival, but not proliferation of striatal neuronal progenitors (Ikeda et al., 1997; Luk and Sadikot, 2001). The role of glutamate-induced depolarizing activity in early morphogenesis of the striatum remains largely unexplored.

The present results indicate that non-NMDA ionotropic receptors do not influence proliferation of striatal neuronal precursors. However, the *in vivo* and *in vitro* evidence suggests that NMDA receptors play an important role in proliferation of ventral telencephalon-derived striatal progenitors. Prenatal exposure to NMDA antagonists results in a marked decrease in neuronal proliferation. *In utero* exposure to NMDA antagonists during the post-proliferative period has no effect on neuronal survival, in keeping with previous work (Ikonomidou et al., 1999). Interestingly, postnatal exposure to NMDA antagonists dramatically increases developmental cell death, suggesting NMDA exerts a trophic influence during the major period of striatal neuronal apoptosis (Ikonomidou et al., 2000).

The GABAergic projection neurons, which comprise 90% of striatal neurons (Smith and Bolam, 1990), are a major target of NMDA-mediated proliferative effects. Previous work suggests that NMDA also promotes proliferation of other striatal populations such as parvalbumin-positive GABAergic interneurons (Sadikot et al., 1998) that originate in the medial ganglionic eminence (Lavdas et al., 1999; Marin et al., 2000). Since striatal projection neurons arise mainly from the LGE, NMDA

receptors mediate proliferation of neuronal precursors arising from both major germinal zones of the ventral telencephalon. Recent evidence suggests a large proportion of neocortical (de Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Marin et al., 2000; Parnavelas, 2000) and hippocampal GABAergic interneurons (Pleasure et al., 2000) are derived from the ventral telencephalon. NMDA-dependent proliferation may thus be a common property of all forebrain GABAergic interneuronal precursors. Whether NMDA effects are restricted to GABAergic neurons or extend to cholinergic and somatostatinergic subtypes produced in the ventral germinal zones remains to be addressed.

3.5.2 Proliferative Reponses to NMDA are regionally diverse in forebrain germinal zones

With the exception of GABAergic interneurons (Marin et al., 2000), cells generated in the germinal zones of the dorsal and ventral telencephalon remain largely separate during forebrain morphogenesis (Fishell, et al, 1993). Furthermore, the dorsal and ventral zones express distinct transcription factors, some of which are implicated in neurogenesis (Shimamura et al., 1995; Metin et al., 1997; Casarosa et al., 1999). Cells in the proliferative epithelium generating the cortex or striatum on either side of the corticostriatal boundary (Holmgren, 1925; Puelles et al., 2000; Inoue et al., 2002) may therefore show distinct responses to the extracellular environment. Regional differences in proliferative behavior of neuronal progenitors are evident in response to amino acid neurotransmitters (Cameron et al., 1998; Contestabile, 2000). In cortex, both glutamate and GABA regulate proliferation of neuronal precursors (LoTurco et al., 1995; Haydar et al., 2000). AMPA/KA receptors mediate altered DNA synthesis in cortical progenitors (LoTurco et al., 1995), but have no effect on proliferation of striatal progenitors. Such regional differences are also apparent with respect to NMDA-mediated responses. NMDA receptor activation

promotes proliferation of striatal precursors, has no effect on proliferation of neocortical progenitors (LoTurco et al., 1995), and reduces proliferation of hippocampal granule cell precursors (Cameron et al., 1995; Seki and Arai, 1995).

Recent evidence in the dorsal telencephalon suggests that proliferative responses may vary between cellular subtypes. For example, activation of AMPA/KA receptors increases proliferation of neuronal precursors in the ventricular zone, but decreases proliferation in the subventricular zone (Haydar et al., 2000), a domain that generates mainly glia (Sidman et al, 1959; Altman, 1969). Our data suggest that NMDA effects on proliferation of neuronal progenitors in the ganglionic eminence occur mainly in the ventricular zone, with no effect in the subventricular zone. It would be of interest to determine whether distinct responses also exist amongst neuronal and glial precursors derived from the ventral germinal zones.

3.5.3 Potential mechanisms mediating NMDA effects on proliferation

Calcium entry following NMDA receptor activation mediates a variety of developmental effects in the CNS (Ascher and Nowak, 1988; Yuste and Katz, 1991), including cell survival (Balazs et al., 1990; Bhave and Hoffman, 1997; Ikonomidou et al, 2000), neurite outgrowth (Mattson and Kater, 1987), and synaptic plasticity (Stevens et al., 1994). The downstream intracellular mechanisms that may couple NMDA receptor-mediated calcium entry to DNA synthesis in striatal progenitors are unknown. Extracellular regulated kinases (ERKs) have been observed to mediate proliferative responses invoked by a variety of growth factors (Finkbeiner and Greenberg, 1996; Fukunaga and Miyamoto, 1998). With respect to classical neurotransmitter effects, proliferation in cortical progenitors following muscarinic receptor activation appears to be mediated by phosphatidylinositol-3-kinase (PI3) and ERK signaling pathways (Li et al., 2001). NMDA receptor activation in striatal

neurons has been demonstrated to initiate this pathway in a PI3 dependent manner (Vincent et al, 1998; Schwarszchild et al., 1999; Perkinton et al, 2002). Components of this cascade (e.g. CaM-kinase, Ras, Mek, ERK1/2, and PI3) can be localized to glutamatergic postsynaptic densities (Husi et al, 2000). The ERK/PI3 pathway is therefore a strong candidate mechanism coupling NMDA receptor activation to DNA synthesis in striatal progenitors.

Contrasting proliferative responses to NMDA receptor activation during CNS morphogenesis may be the result of spatial and temporal differences in glutamate receptor expression (Goebel and Poosch, 1999; Kovacs et al, 2001). Both NMDA and non-NMDA ionotropic receptors are widely expressed in the prenatal rat telencephalon and exhibit developmentally regulated patterns of subunit expression that determine distinct functional effects (Misgeld and Dietzel, 1989; Monyer et al., 1994; Landwehrmeyer et al., 1995; Wullner et al., 1997; Nansen et al., 2000). *In vitro*, cells derived from the LGE exhibit NMDA responses (Vincent et al., 1998). Here, we provide evidence for NR1 and NR2 subunits in proliferating neuroblasts, suggesting a role for a functional channel in the observed events. Developmental variations in local glutamate concentration (Haydar et al., 2000), or interaction between NMDA receptors and other growth factors (Dobbertin et al., 2000; Roceri et al., 2001) may also contribute to variations in functional effects. Finally, distinct intracellular downstream mechanisms coupling NMDA receptor activation to the cell cycle machinery may also underlie variations in proliferative responses (reviewed by Platénik et al, 2000).

3.5.4 Relevance to developmental abnormalities

Disruptions in forebrain development due to pathological responses to amino acid neurotransmitters may be relevant to a wide variety of human diseases including

schizophrenia (Harrison, 1999), prenatal brain injury from trauma or ischemia (Vexler and Ferreiro, 2001), cortical dysplasias (Flint and Kriegstein, 1997), and developmental disorders following prenatal exposure to drugs of abuse (e.g. PCP, ethanol; Deutsch et al., 1998; Tabakoff et al., 1991) sedatives, anticonvulsants, and anesthetics (Jevtovic-Todorovic et al., 1998; Reich and Silvay, 1989; Morrell, 1999). The importance of NMDA-mediated glutamatergic mechanisms was recently highlighted in the pathogenesis of fetal alcohol syndrome (FAS; Ikonomidou et al., 2000). Magnetic resonance imaging in human FAS reveals markedly reduced volumes in the striatum as well as the cerebral cortex and cerebellum (Mattson et al., 1994; Archibald et al., 2001). Ethanol acts at NMDA and $GABA_A$ receptor sites (Lovinger et al., 1989; Hoffman et al., 1989). Based on previous work, alcohol induced activation of $GABA_A$ receptors would not be expected to mediate a proliferative effect on striatal neuronal precursors (Ikeda et al, 1997; Luk and Sadikot, 2001). In rodent models, exposure to MK-801 or alcohol results in a dramatic increase in postnatal apoptosis in the striatum, supporting the hypothesis that NMDA receptors mediate a trophic effect (Ikonomidou et al., 1999, 2000). Our current data suggests that alcohol and other NMDA antagonists may also impair neuroblast proliferation, and therefore contribute to abnormal striatal morphogenesis as early as the first trimester of human pregnancy (O'Rahilly and Müller 1994). In conclusion, temporal and spatial variations in morphogenic responses to glutamate play an important in normal development, and may contribute to distinct abnormalities of forebrain development in a wide variety of pathologies.

Figure Legends

Figure 1. Stereological estimation of the number of striatal and motor cortical neurons. The total number of projection neurons in the adult rat striatum and frontal agranular cortex was estimated using the optical fractionator technique. Striatal and cortical reference volumes encompassed the first and last sections containing visible caudate putamen, corresponding approximately to 2.20 to –2.60 mm Bregma (Paxinos and Watson, 1986; Zilles, 1985). Sections were cut at 50 μm with every sixth section being examined. Counting frames were arranged 500 μm apart to form a grid that was randomly superimposed over the reference area at each coronal level using the analysis software. Each counting frame measured 60 x 60 μm with a thickness of 10 μm (inset). Exclusion lines and planes (shaded) were also implemented. Cell nuclei touching or falling within the counting frame were only recorded if there was no visible contact with any of the exclusion planes.

Figure 2. Total striatal and cortical neuron number and volume after treatment with glutamate receptor antagonists. Stereological estimates of the total number of striatal and cortical neurons for all experimental groups are shown (**a**). Treatment with glutamate receptor drugs extended between either E15-18 or E18-21, corresponding to the main proliferative and post-proliferative periods, respectively, for neostriatal neurons. Striatal neuron number in rats were significantly reduced following administration of non-competitive and competitive NMDA glutamate

receptor antagonists (MK-801 and CGS 19755, respectively) when compared to saline (SAL) and pair-fed saline (PF/SAL) control groups (all data expressed as mean ± SEM; $F_{7,24}$=17.01; * $p<0.01$ vs pairfed control). Effects were statistically significant only following treatment between E15-18, but not between E18-21. Treatment with MK-801 did not significantly alter neuron number in the frontal agranular cortex, suggesting that NMDA receptor mediated effects on proliferation are restricted to the striatum in this model. Striatal volumes (**b**) also showed a significant decrease after MK-801 or CGS-19755 treatment, likely reflecting the loss of neurons ($F_{7,24}$=12.32; * $p<0.01$ vs pairfed control). Treatment with NBQX did not change neuron number or striatal volume, suggesting that the observed effect is mediated by NMDA, but not AMPA/KA receptors. Decreased striatal volume and neuron number following exposure to MK-801 was visible at the level of the anterior commissure in Cresyl Violet stained sections (**c,d**; scale bars = 500 μm).

Figure 3. Effects of MK-801 treatment on embryonic basal forebrain proliferative zones. Embryonic rats were given MK-801 (0.2mg/kg) on E15, and again on E16, followed by BrdU injection 1h later. Embryos were fixed 12h later at E16.5, sectioned, and stained using a monoclonal antibody against BrdU. (**a,b**) The periventricular striatal proliferative zones were delineated 100 μm from the cortical striatal angle (CSA). An area 100 μm wide and 50 μm deep from the ventricular surface was assigned as the ventricular zone (vz). The subventricular zone (svz) was defined as a 100 x 100 μm area located 50 μm ventral to the vz (**b**). BrdU+ nuclei in the two zones were quantified following treatment with saline (TBS) and MK-801.

(**c,d**) Photomicrographs showing BrdU uptake near the CSA region following treatment. Following treatment with the NMDA receptor antagonist, the percentage of BrdU+ nuclei was reduced in the vz but not svz. (**e**; * $p<0.01$, $n = 4$). Abbreviations: cortex (Ctx); lateral ganglionic eminence (LGE). Scale bars: a (100 μm); c,d (40 μm).

Figure 4. Embryonic striatal cultures. Cells obtained from rat ganglionic eminence at E15 were cultured in serum-free defined medium. Neurons were immunostained against TUJ1 (**a**) and MAP2 (**c**) after 2 DIV. GFAP staining did not reveal the presence of any astrocytes, suggesting a population of predominantly neuronal cells in vitro (**e**). DAPI counterstaining for Fig.4a,c,e (**b,d,f**). Scale bars = 10 μm.

Figure 5. NMDA receptor profile of embryonic striatal cultures. Embryonic neostriatal cultures were fixed at 1 DIV after 4h BrdU exposure and immunostained using antibodies against various NMDA receptor subunits. A large number of cells were positively stained for the NR1 subunit. A small subset of NR1 immunoreactive cells colocalized with BrdU, indicating that proliferative or recently post-mitotic cells express functional NMDA receptor subunits (**a,b**). Staining using polyclonal antibodies against NR2 subunits also revealed a significant number of BrdU+ cells expressing the NR2A subunit (**c,d**). Coexpression of the NR2A subunit was also detected in a subpopulation of NR1 positive cells (**e,f;** indicated by arrowheads). However, no staining for the NR2C subunit was observed. Scale bars: **a** (10 μm); **c** (10 μm); **e** (12 μm).

Figure 6. Effects of glutamate receptor antagonists and NMDA following 24h exposure. 1 DIV striatal cultures were exposed to different ionotropic glutamate receptor antagonists, NMDA or vehicle control. (**a**) Treatment duration was 24h and was followed by 2h exposure to BrdU (20 μg/ml) and fixation in PFA (diagram). Treatment with MK-801 or CGS 19755 significantly reduced the proportion of nuclei incorporating BrdU with respect to control cultures. Effect of the competitive antagonist (CGS) could be countered by addition of NMDA. Addition of 100μM NMDA to CGS treated cultures restored BrdU uptake close to normal levels. Exposure to NBQX did not alter BrdU uptake, suggesting that NMDA but not AMPA receptor blockade result in decreased proliferation. In cultures where only the agonist was added, BrdU uptake was inversely proportional to NMDA concentration. At 1μM, NMDA significantly upregulated proliferation, whereas increasing doses exhibited toxic effects. (**b**) The proportion of Tuj1 positive cells were also quantified following each treatment. Exposure to MK-801 or CGS 19755 reduced the proportion of Tuj1 cells, suggesting that production of early postmitotic neurons was reduced following receptor blockade. Tuj1 cell number also decreased following treatment with 100μM NMDA. (**c**) MAP2+ cell number did not alter significantly after treatment, with the exception of NMDA at excitotoxic concentrations, suggesting that the short-term survival of more mature neurons was not affected by the agents added. (data expressed as mean ± SEM, n=3; * $p<0.01$ vs. control)

Figure 7. Effects of glutamate receptor antagonists and NMDA following 4h exposure. 1 DIV striatal cultures were exposed to various ionotropic glutamate

receptor antagonists, NMDA or vehicle control for 4h, followed by 2h exposure to BrdU (timeline). Since treatment duration was much shorter than the expected cell cycle time for these cultures, the changes in cell population number due to drugs should be minimized. (**a**) After 4h treatment with MK-801 or CGS 19755, BrdU incorporation was significantly reduced, suggesting that NMDA receptor blockade exerts its influence on proliferation within this period. BrdU uptake in cultures treated with CGS 19755 could be restored to near control levels by adding increasing concentrations of NMDA. As in previous culture experiments, exposure to NBQX did not alter BrdU uptake. Exposure to NMDA at high concentration (100μM) resulted in decreased numbers of BrdU+ nuclei whereas at 1μM, proliferation was above control levels. (**b,c**) No significant alterations in the proportion of Tuj1+ (**b**) or MAP2+ (**c**) cells were observed following any of the treatments (data expressed as mean ± SEM, n=3; * $p<0.01$, ** $p<0.05$ vs. control). (**d-g**) Photomicrographs of BrdU labeled cells *in vitro* following exposure to NMDA and receptor antagonists. Scale bar = 8 μm.

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Figure 1

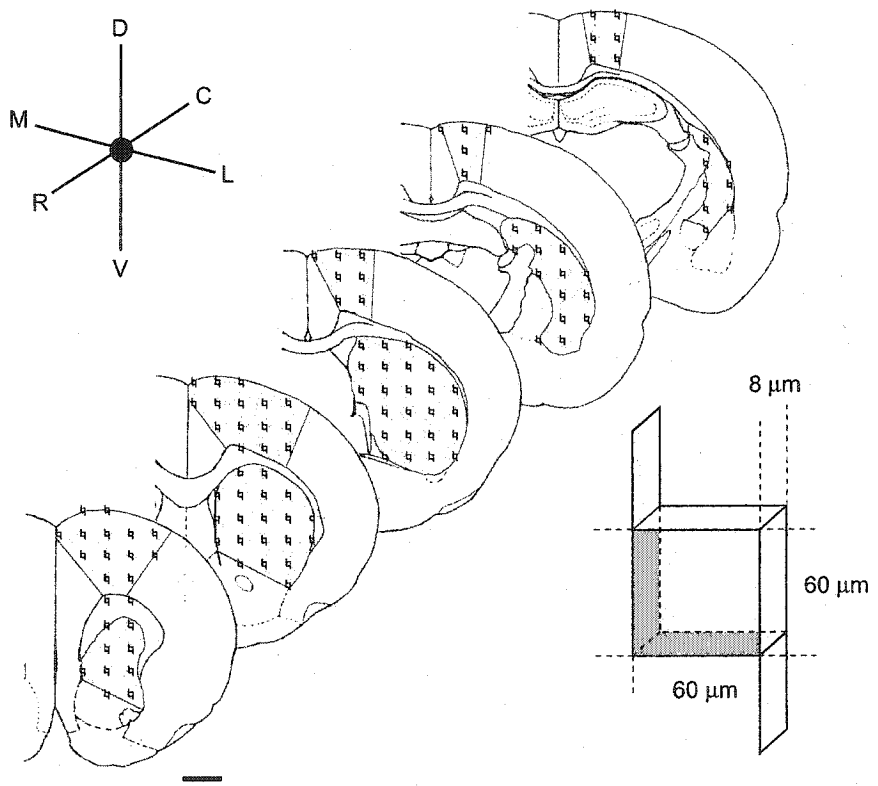


Figure 2

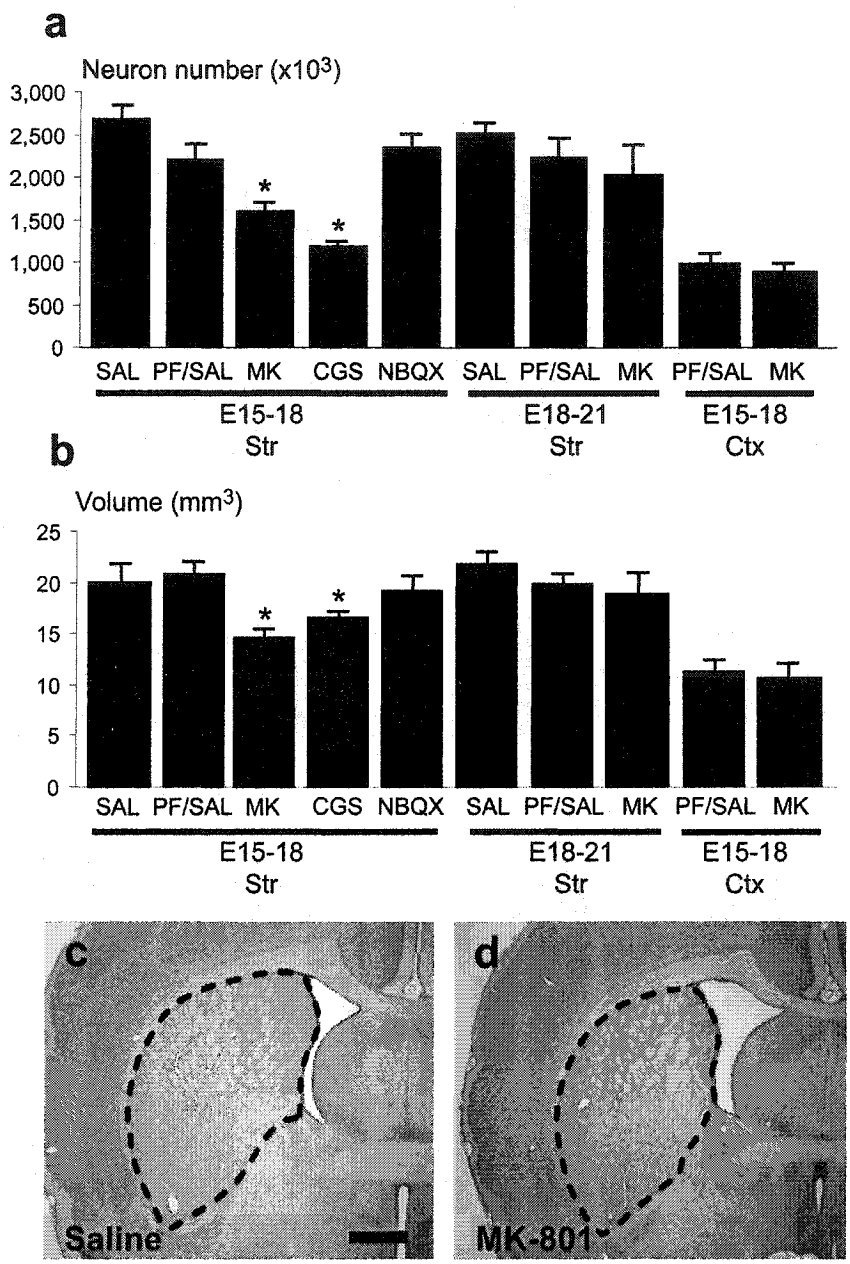

a
Neuron number (x10³)
3,000
2,500
2,000
1,500
1,000
500
0
SAL PF/SAL MK CGS NBQX SAL PF/SAL MK PF/SAL MK
E15-18 Str
E18-21 Str
E15-18 Ctx
b
Volume (mm³)
25
20
15
10
5
0
SAL PF/SAL MK CGS NBQX SAL PF/SAL MK PF/SAL MK
E15-18 Str
E18-21 Str
E15-18 Ctx
c
Saline
d
MK-801

Figure 3

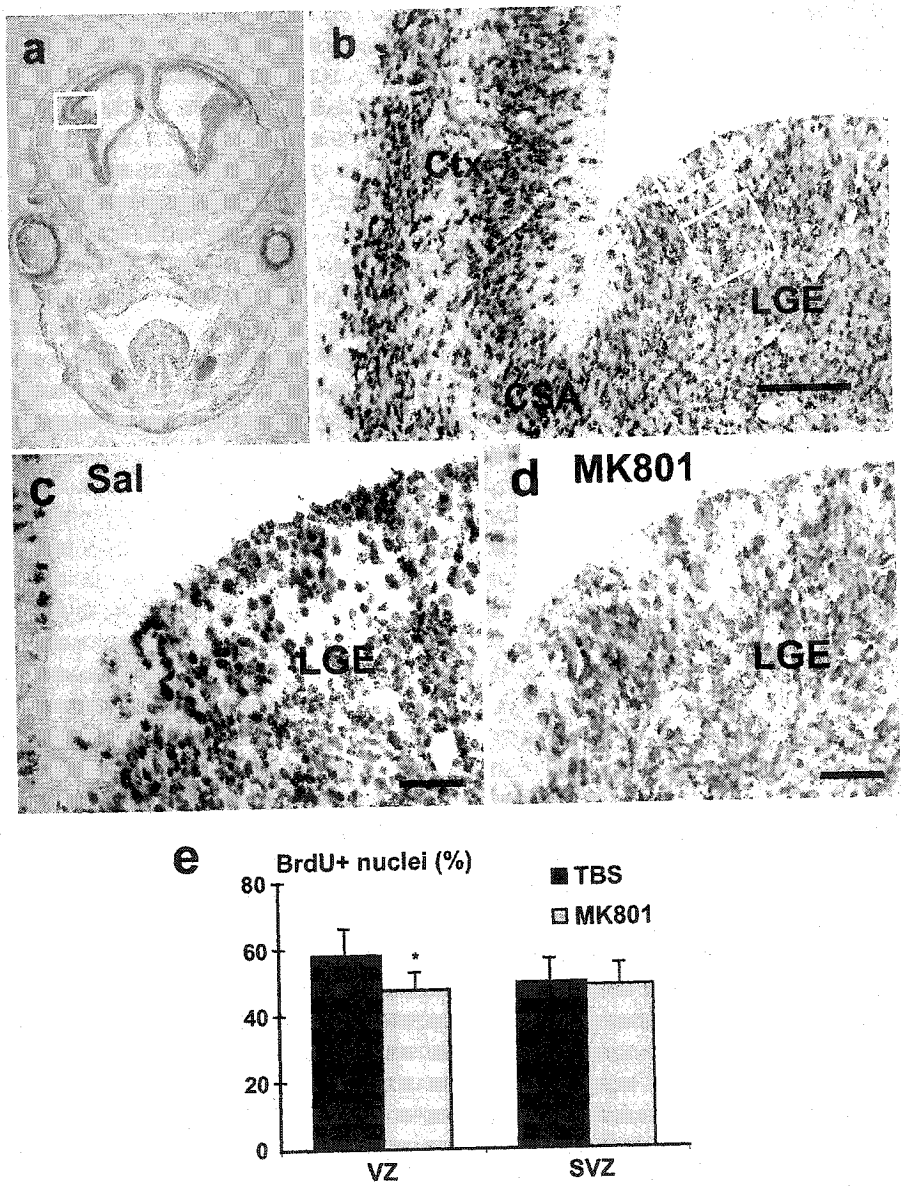

a
b
Ctx
CSA
LGE
c
Sal
LGE
d
MK801
LGE
e
BrdU+ nuclei (%)
TBS
MK801
80
60
40
20
0
*
VZ
SVZ

Figure 4

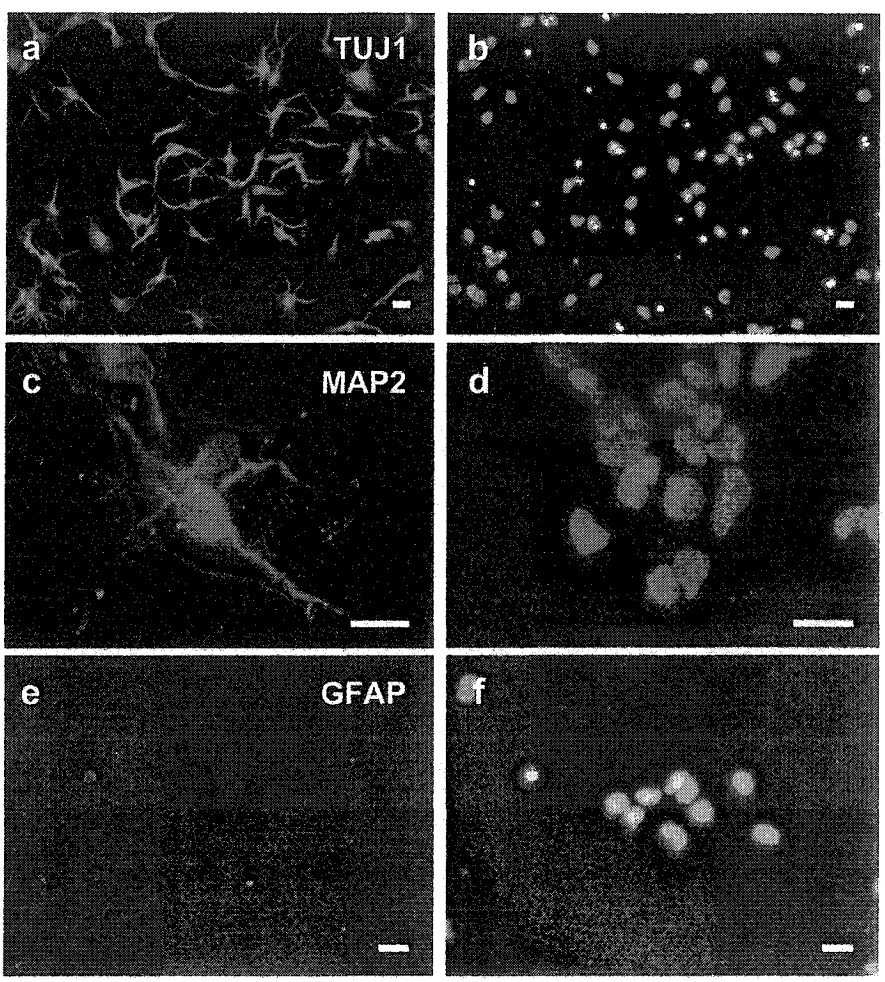


Figure 5

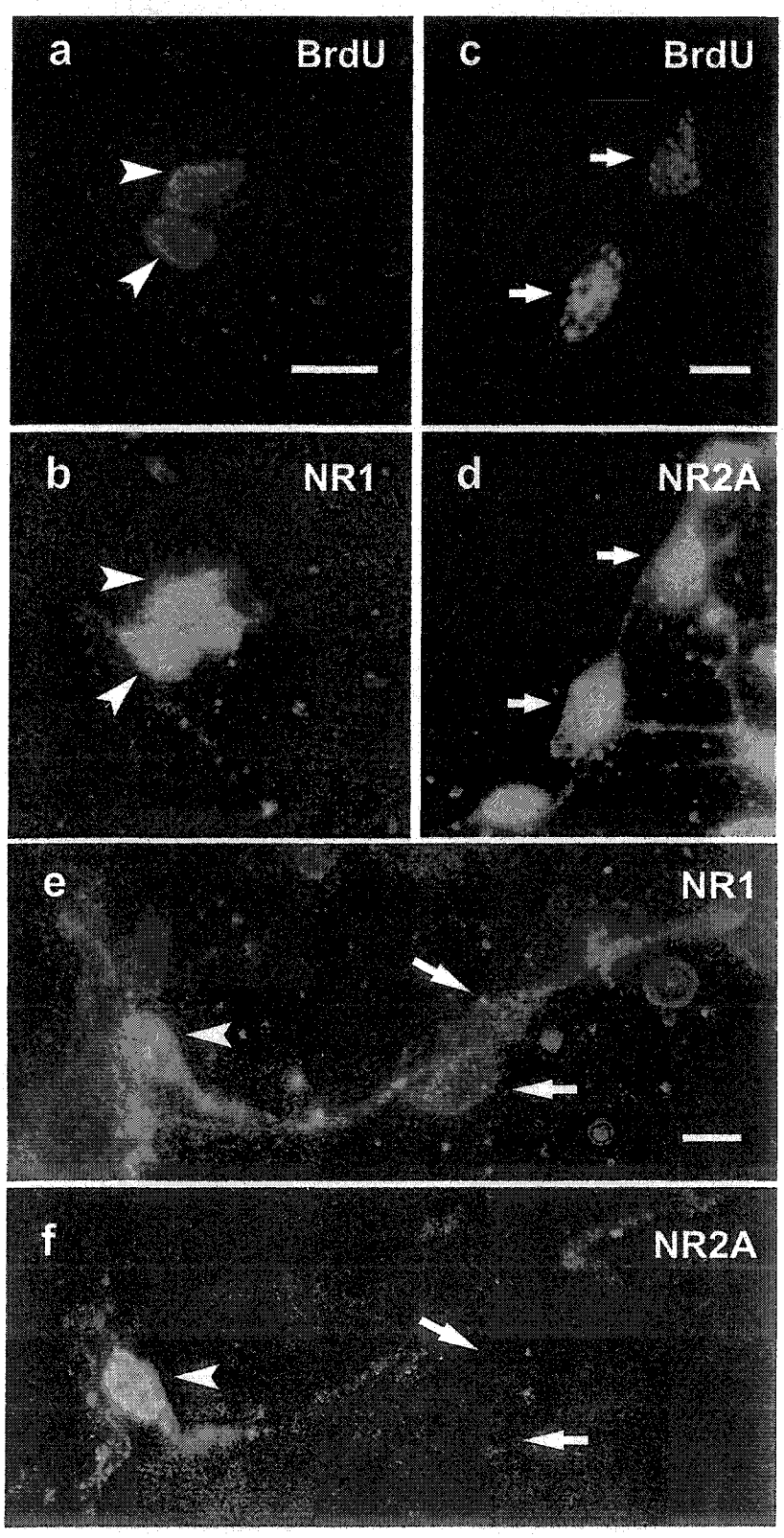


Figure 6

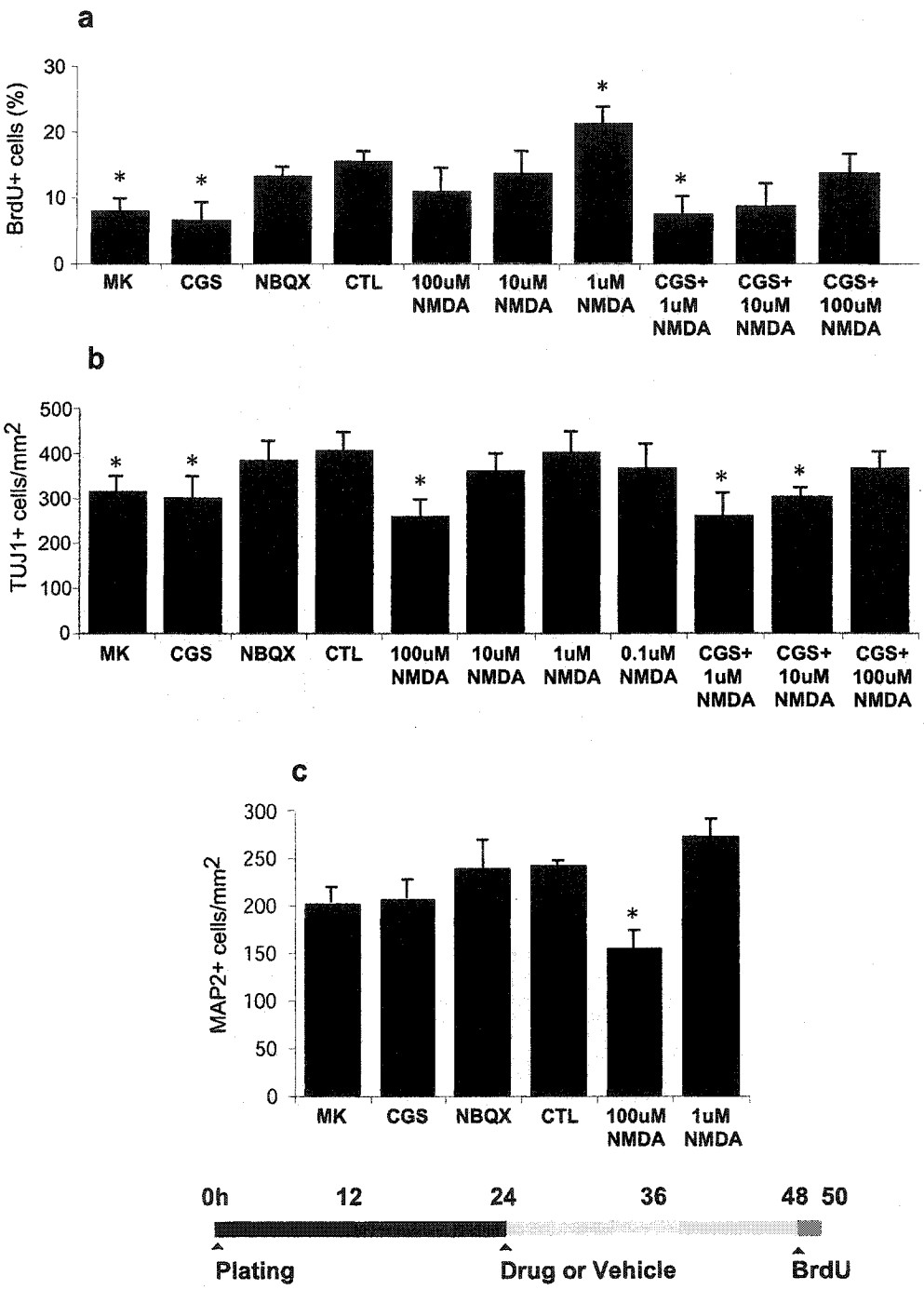
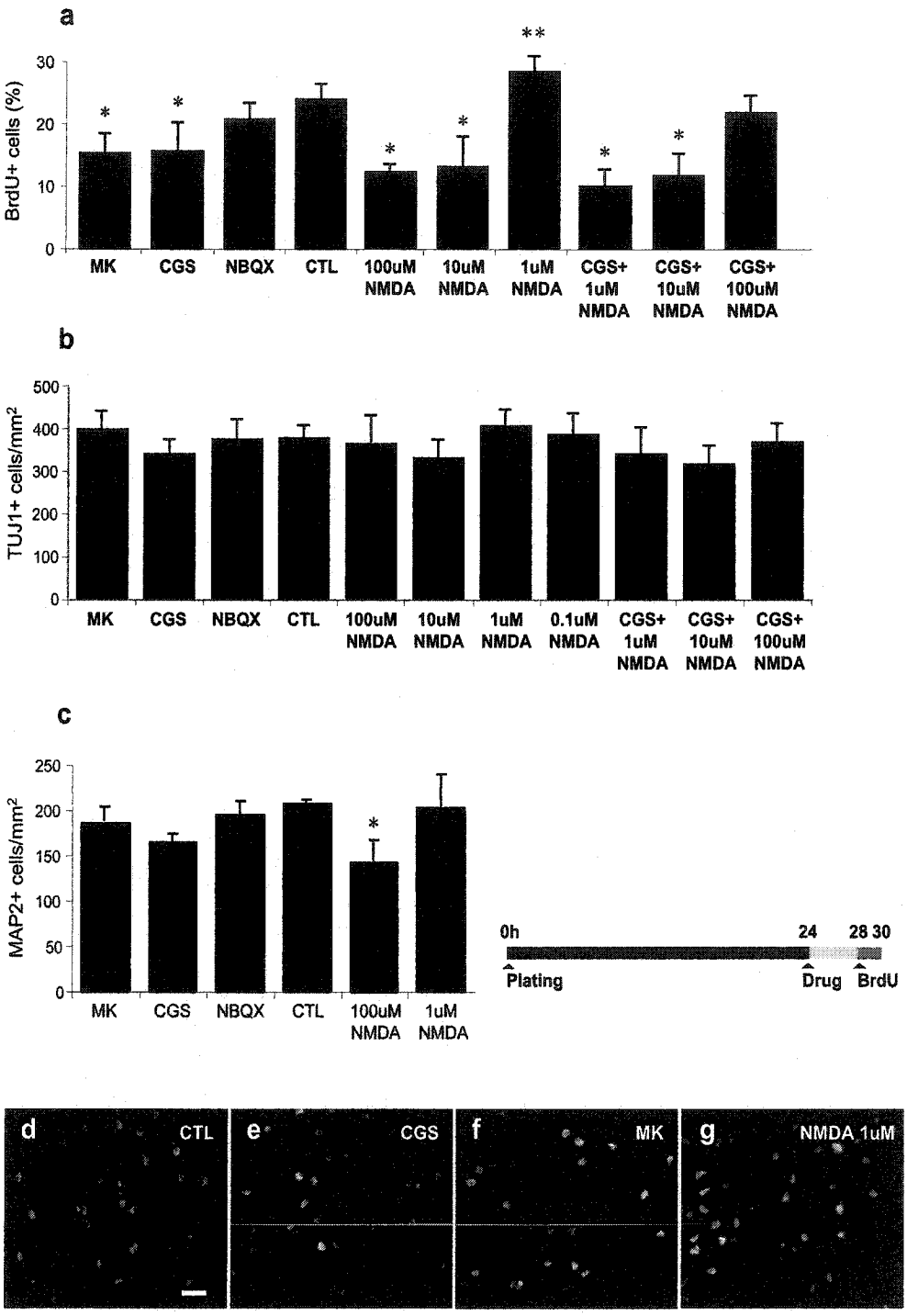

a
BrdU+ cells (%)
30
20
10
0
MK
CGS
NBQX
CTL
100uM NMDA
10uM NMDA
1uM NMDA
CGS+ 1uM NMDA
CGS+ 10uM NMDA
CGS+ 100uM NMDA
b
TUJ1+ cells/mm2
500
400
300
200
100
0
MK
CGS
NBQX
CTL
100uM NMDA
10uM NMDA
1uM NMDA
0.1uM NMDA
CGS+ 1uM NMDA
CGS+ 10uM NMDA
CGS+ 100uM NMDA
c
MAP2+ cells/mm2
300
250
200
150
100
50
0
MK
CGS
NBQX
CTL
100uM NMDA
1uM NMDA
0h
12
24
36
48
50
Plating
Drug or Vehicle
BrdU

Figure 7


a
BrdU+ cells (%)
30
20
10
0
MK
CGS
NBQX
CTL
100uM NMDA
10uM NMDA
1uM NMDA
CGS+ 1uM NMDA
CGS+ 10uM NMDA
CGS+ 100uM NMDA
b
TUJ1+ cells/mm2
500
400
300
200
100
0
MK
CGS
NBQX
CTL
100uM NMDA
10uM NMDA
1uM NMDA
0.1uM NMDA
CGS+ 1uM NMDA
CGS+ 10uM NMDA
CGS+ 100uM NMDA
c
MAP2+ cells/mm2
250
200
150
100
50
0
MK
CGS
NBQX
CTL
100uM NMDA
1uM NMDA
0h
24
28 30
Plating
Drug
BrdU
d
CTL
e
CGS
f
MK
g
NMDA 1uM

Chapter 4: Regional differences in proliferative response to glutamate in the developing telencephalon

Glutamate and regulation of proliferation in the developing mammalian telencephalon. ***Developmental Neuroscience*** (Manuscript in preparation)

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4.1 Abstract

Increasing evidence suggests that classical neurotransmitters play important roles in the development of the mammalian central nervous system. Using *in vitro* and *in vivo* models, we have previously identified a role for the N-Methyl D-Aspartate subclass of glutamate receptors in striatal progenitor proliferation. Here, we compare the developing embryonic cortex and striatum with respect to their proliferative responses to ionotropic glutamate receptor activation or inhibition. In culture, reduced neuroblast proliferation was observed following glutamate receptor antagonist treatment. However, cortical cultures were sensitive to AMPA/KA receptor blockade whereas striatal cultures were responded only to NMDA agonists and antagonists. *In vivo*, BrdU uptake in the proliferative ventricular zone was also reduced in embryos following acute administration of antagonists, with no observed differences in the subventricular zone. Similar to what was observed *in vitro*, proliferation in cortical and striatal ventricular regions were respectively affected by AMPA/KA and NMDA receptor blockade. Together, these data suggest a complementarity in the subclass of ionotropic glutamate receptors mediating proliferative response to this neurotransmitter during early development.

4.2 Introduction

In addition to their traditionally associated roles as chemical messengers, neurotransmitters are increasingly recognized as trophic and mitogenic factors (Levitt et al., 1997; Cameron et al., 1998; Nguyen et al., 2001). Within germinal zones of the developing telencephalon proliferative activity appears to be influenced by various amino acid and monoamine transmitters including GABA, glutamate, and dopamine (LoTurco et al., 1995; Sadikot et al., 1998; Haydar et al., 2000; Ohtani et al., 2003). We have previously demonstrated that proliferative responses to glutamate in the ventral telencephalic germinal zone are predominantly mediated by the NMDA subclass of receptors (Luk et al., 2003). This contrasts with the cortical germinal zone where α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or kainate (AMPA/KA) receptors have been shown to be responsible for modulating progenitor proliferation *ex vivo* (LoTurco *et al.*, 1995; Haydar *et al.*, 2000).

The mammalian telencephalon derives from dorsal and ventral germinal zones that generate the cerebral cortex and basal forebrain, respectively (Smart and Sturrock, 1979; Fentress *et al.*, 1981; Bayer, 1984; Marchand and Lajoie, 1986). Neurons within the cortex are primarily glutamatergic whereas the striatum, the main component of the basal telencephalon, consists mainly of GABAergic projection neurons (Kawaguchi *et al.*, 1995). Forebrain neurons remain close to their area of generation in the periventricular region and the majority of migrating neurons do not cross the corticostriatal boundary (Fishell *et al.*, 1993; Krushel *et al.*, 1993; Inoue *et al.*, 2001). The exception is a small population of cortical GABAergic interneurons

derived mainly from the ventral telencephalon (de Carlos *et al.*, 1996; Anderson *et al.*, 1997; Tamamaki *et al.*, 1999).

Dorsoventral differences in proliferative behavior of neuronal progenitors in response to growth factors and amino acid neurotransmitters are evident (Cameron et al., 1998; Nguyen et al., 2001; Luk et al., 2003). In cerebral cortex, both glutamate and GABA regulate proliferation of neuronal precursors (LoTurco et al., 1995; Haydar et al., 2000). AMPA/KA receptors mediate altered DNA synthesis of cortical progenitors (LoTurco et al., 1995), but have no effect on proliferation of striatal progenitors (Luk et al., 2003). On the other hand, NMDA receptor activation promotes proliferation of striatal precursors (Luk et al., 2003), reduces proliferation of hippocampal granule cell precursors (Cameron et al., 1995), and has no effect on proliferation of neocortical progenitors (LoTurco et al., 1995). Variations in the transcription factor expression between dorsal and ventral telencephalic germinal zones may underlie much of the observed heterogeneity in morphogenetic responses to extracellular factors (Puelles and Rubenstein, 1993; Shimamura et al., 1995; Casarosa et al., 1999). A number of these have been implicated in neurogenesis and the control of cell fate decisions (Shimamura et al., 1995; Casarosa et al., 1999; Torii et al., 1999; Toresson et al., 2000).

Although a number of studies have addressed the effects of glutamate on dorsal and ventral forebrain progenitors at various stages of development, the dorsal and ventral derivatives have seldom been directly compared. Furthermore, significant variation exists between the *in vitro* and *in vivo* models used in each study. We now investigate and compare glutamatergic influences on the proliferation of cortical and

striatal forebrain progenitor populations in the same model. Using proliferating primary neuroblast cultures and an *in vivo* acute exposure model (Luk et al., 2003), we report here that glutamate positively influences proliferation in both cortical and striatal progenitors. However, complementary subclasses of ionotropic glutamate receptors appear to be responsible, indicating a pattern of dorsoventral hetereogeneity in proliferative response to this neurotransmitter.

4.3 Materials and Methods

Animals

Sprague-Dawley rats were obtained from Charles River Canada (LaSalle, Quebec). Females were coupled with males between 3 p.m. and 5 p.m. The first 24 hours (h) after coupling was designated as embryonic day zero (E0). All animal procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

Primary neuroblast cultures

Primary cortical and striatal cultures were prepared from E15 rat embryos as previously described (Ivkovic *et al.*, 1997; Luk *et al.*, 2003). Briefly, the lateral ganglionic eminence (LGE) and future motor cortex were microdissected in cold magnesium-free Hank's Balanced Salt Solution (HBSS, Sigma, St. Louis, MO). The dissected tissue was then incubated in trypsin and DNAse at 37°C and centrifuged at 1200 rpm for 5 min in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS). The pellet was resuspended in Neurobasal/B27 medium supplemented with 2mM L-glutamine and

penicillin/streptomycin (Life Technologies, Burlington, Ontario). Cells were triturated through polished Pasteur pipettes. Cells were then plated on 8-well chamber slides pre-coated with poly-D-lysine (Becton Dickinson, Franklin Lakes, NJ) at a density of 2 x 10^5 cells/cm^2 and incubated at 37°C with 5% CO_2. Cells were used for experiments after 24h *in vitro*.

Drug Treatment

24h after initial plating, cells in individual slide chambers were exposed to either MK-801 (2 μg/ml), CGS-19976 (20 μg/ml), NMDA (1-100 μM), NBQX (10 μM), or vehicle control. In order to label cells passing through S-phase, BrdU (20 μg/ml; Sigma) was added to each chamber either 4 or 24 h after treatment. Cells were then fixed for 20 minutes with cold 4% PFA 2 h following addition of BrdU, and processed for immunocytochemistry.

Immunocytochemistry

BrdU uptake by proliferating cells was revealed by immunostaining as previously described (Luk et al, 2003). Briefly, cells were permeabilized in cold acetone/methanol (1:1), washed in phosphate buffered saline (PBS), and denatured in 2N HCl. The cells were then washed with sodium borate (0.1 M), followed by another wash in PBS. Anti-BrdU antibody (1:10, Becton Dickinson) was added and incubated overnight at 4°C. As a negative control, non-specific mouse IgG_1 was used as the primary serum. Cells were then washed 3 times with PBS and labeled with Alexa 594-conjugated rabbit anti-mouse IgG antibody (1:1,000; Molecular Probes, Eugene, OR). Labeled cells were visualized under a fluorescence microscope using a 40X objective and the appropriate filters. Cells and nuclei were quantified in 40-50

random fields generated with the help of the Stereo Investigator software (Microbrightfield Inc, Colchester, VT). Results were compared by one-way ANOVA.

Embryonic drug exposure

Separate groups of timed pregnant rats were given MK-801 (0.2 mg/kg/day), CGS-19755 (5 mg/kg/day), NBQX (10 mg/kg/day), or saline via intraperitoneal injection at E15 and 16. On E16, drug administration was followed 1 h later by a single injection of BrdU (50mg/kg). Embryos were removed after a further 12 h by Caesarian section, decapitated, and fixed overnight in 4% PFA. Heads were then transferred to 10% formalin, dehydrated, and embedded in paraffin. Embryonic brains were sectioned at 5μm with a microtome.

Immunohistochemistry

Antigen retrieval was achieved by heating sections in citrate buffer (0.01M, pH 6.0) for 15 min at 90°C. Sections were then exposed to 2N HCl for 1h, followed by washes in PBS (3 x 5 min each), and then incubated overnight with monoclonal anti-BrdU antibody. Sections were then washed in PBS (3 x 5 mins) and labeled using the avidin-biotin-complex (ABC) peroxidase method by incubating for 1h with biotinylated goat anti-mouse IgG secondary antibody (1:200) followed by ABC solution (Vector, Burlinghame, CA). The final reaction was revealed by exposing cells to a solution of nickel ammonium sulfate (3.7 mg/ml), 3,3'-diaminobenzidine (DAB, 0.25 mg/ml), and 0.0006% hydrogen peroxide in 0.05M Tris buffer (pH 7.6).

Sections were then counterstained with Nuclear Fast red (Vector), dehydrated, and coverslipped in permount.

BrdU immunoreactive nuclei were quantified in coronal sections at the level of the ganglionic eminences with the help of the Stereo Investigator software. The cortical and striatal periventricular zones were delineated by a 100 μm wide box placed approximately 100 μm from the cortical striatal angle (Fig. 3a). The first 50 μm from the ventricular surface was arbitrarily designated as the ventricular zone (VZ), while the area from 100 to 200 μm was considered the subventricular zone (SVZ). Proliferative nuclei were counted through the entire thickness of the section and the results expressed as a percentage of nuclei that were $BrdU^+$.

Western blotting for PCNA

Cortical and striatal neuroblasts were grown on poly-D-lysine coated 6-well plates (2 x 10^5 cells/cm^2). 24 h after plating, cells were incubated in medium containing drugs for an additional 24 h. Cells were then washed once in cold PBS, and lysed in Cell-Lytic M buffer (Sigma) containing protease inhibitors, sodium fluoride, and sodium orthovanadate, and boiled in sample buffer for 5 minutes. 10 μg of each sample was separated by electrophoresis on 4-15% gradient SDS-polyacrylamide gels (Biorad, Mississauga, Ontario) and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h with TBS/0.1% Tween 20 (TBST) containing 5% non-fat milk powder, washed, and incubated overnight with a monoclonal antibody against proliferating cell nuclear antigen (PCNA; 1:1,000, Sigma) at 4°C. Following washes in TBST (3 x 5 minutes), blots were incubated with

HRP-conjugated anti-mouse antibody raised in rabbit (1:30,000; Sigma) for 1 h at room temperature. After washing in TBST (3 x 5 minutes), blots were developed and exposed on film using a chemiluminescence kit (PerkinElmer, Boston, MA) as per manufacturer's instructions.

4.4 Results

Cortical and striatal neuroblast proliferation is influenced by different ionotropic glutamate receptor subtypes

In order to determine whether ionotropic glutamate receptors mediate proliferation in the developing mammalian forebrain, dissociated cultures of proliferating cortical and striatal neuroblasts were exposed to different glutamate receptor antagonists and agonists (Figure 1). Cultures were then exposed to BrdU for a 2 h period following either 4 h or 24 h of treatment with agonist or antagonist. Immunoblotting for PCNA was also performed using lysates from treated cultures in order to confirm the presence of proliferating cells after 24 h treatment (Figure 1c,d).

Administration of the AMPA/KA antagonist NBQX significantly reduced the proportion of BrdU+ nuclei in cortical cultures after 24 h (Figure 1a). Levels of PCNA were also reduced compared to control levels, indicating a decrease in proliferation (Figure 1c). However, the NMDA antagonists MK-801 and CGS 19755 had no significant effect, suggesting that proliferation in cortical cultures is sensitive to AMPA/KA receptor blockade but not NMDA receptor antagonists. On the other hand, striatal cultures showed a marked (>50%) reduction in BrdU+ nuclei following treatment with MK-801 and CGS 19755 (Figure 1b) along with a concomitant decrease in PCNA expression (Figure 1d). Addition of NBQX did not elicit any significant alterations in either BrdU staining or PCNA levels. These results are in agreement with previous data that indicate striatal neuroblast proliferation is sensitive to NMDA- but not AMPA/KA-receptor blockade *in vitro*.

To further determine the effects of NMDA on proliferation of telencephalic cultures, cells were exposed to NMDA at a final concentration of 1, 10, or 100 μM. NMDA at 1 μM, but not 10 μM, increased BrdU uptake and PCNA expression in striatal cultures, whereas cortical cultures did not respond. This observation further indicates a positive role for NMDA receptor activation in the proliferation of striatal but not cortical derived neuroblasts. 100 μM NMDA visibly reduced proliferation in both cortical and striatal, likely as a result of excitotoxicity (Koroshetz et al., 1990).

In order to minimize possible fluctuations in cell number due to proliferation or cell death during the treatment period, cultures were exposed to the same panel of agonist/antagonists for only 4 h before addition of BrdU. As in the previous experiment, exposure to NBQX in cortical cultures resulted in a significant decrease in the percentage of BrdU+ nuclei compared to controls (Figure 1e), indicating that effects on cortical neuroblast proliferation can be detected within 4h after initial receptor blockade. In contrast, MK-801, CGS-19755 and NMDA at different concentrations failed to elicit any significant changes in BrdU uptake (Figure 1e).

Striatal cultures exposed to NMDA antagonists for 4 h also showed significant decreases in the proportion of BrdU+ nuclei, indicating a down regulation in proliferation (Figure 1f). As in the case after 24 h exposure with the same antagonists, this effect was not present upon addition of NBQX, and is in agreement with previous observations that AMPA/KA antagonists do not alter proliferation of striatal neuroblasts (Luk et al., 2003). The addition of 100 μM NMDA reduced BrdU uptake by approximately 35% while no significant alteration was observed at 10 μM.

Although NMDA significantly increased proliferation by nearly 30% at 1 μM following 24 h exposure, this effect was less pronounced at 4 h ($p>0.03$).

Proliferation in telencephalic germinal zones is modulated by glutamate antagonists in utero

In order to directly establish whether proliferation within the developing telencephalic germinal zones is influenced by glutamate, BrdU uptake was quantified within cortical and striatal proliferative zones shortly following AMPA/KA or NMDA antagonist administration. Since the developing telencephalon contains a proliferative periventricular epithelium (ventricular zone) and secondary proliferative population (subventricular zone), $BrdU^+$ nuclei within each region was quantified in the cortex and striatum (Figure 2a). Drugs were given intraperitoneally twice (on E15 and E16) followed 1 h later by a single injection of BrdU (Figure 2b). Embryos were fixed and immunostained 12 h after BrdU injection.

In the cortical ventricular zone, administration of NBQX lead to a 31% decrease in the density of $BrdU^+$ nuclei, while MK-801 had no significant effect with respect to saline injected controls (Figure 2c-e, k). However, the density of proliferative nuclei in the subventricular zone remained relatively unchanged with either antagonist (Figure 2i). In contrast, the same embryos exposed to NBQX did not show significant changes in $BrdU^+$ nuclei density in the striatal ventricular or subventricular zones (Figure 2k). However, MK-801 treatment *in utero* led to a 21% reduction in $BrdU^+$ nuclei density in the striatal ventricular zone, suggesting that NMDA receptor inactivation results in lower proliferation in this region. Furthermore,

activation of AMPA/KA receptors does not appear to be required for normal levels of S-phase activity.

4.5 Discussion

We have previously demonstrated a role for the classical neurotransmitter glutamate in promoting proliferation of ventral telencephalic neuronal precursors (Luk *et al.*, 2003). This influence is dependent on activation of NMDA glutamate receptors and contrasts with other data indicating a role for AMPA/KA receptors in mediating proliferation following glutamatergic stimulation in the developing neocortex (LoTurco *et al.*, 1995; Haydar *et al.*, 2000). Here, we have further investigated ionotropic glutamate receptor-mediated effects in proliferative cultures derived from both the cortex and lateral ganglionic eminence. Our *in vitro* and *in vivo* results are in agreement with previous studies and indicate that glutamate influences proliferation of both populations, but via separate receptor subclasses. Thus proliferative responses in cortical and striatal progenitors are mediated by AMPA/KA and NMDA receptors, respectively, under the same culture or *in utero* conditions. These data lend direct evidence to our hypothesis that neighboring portions of the embryonic telencephalic germinal epithelium display heterogeneity in their response to glutamate. Such variation in neurotransmitter effects on proliferation in the germinal zones of the telencephalon may represent a mechanism for generating structural and phenotypic diversity in adjacent CNS domains.

The developing telencephalon is clearly distinguished by dorsal and ventral regions that give rise to cortical and basal forebrain, respectively. Cells in each part derive from adjacent periventricular zones which actively undergo cell division

during the latter half of embryogenesis (Smart and Sturrock, 1979). With the exception of tangentially migrating GABAergic interneurons arising from the ganglionic eminences, neurons remain on the side of the corticostriatal boundary from which they originated (Fishell *et al.*, 1993; de Carlos *et al.*, 1996).

Previous data suggest that responses to glutamate may vary between proliferative subpopulations in the dorsal telencephalon. For example, activation of AMPA/KA receptors increases proliferation of neuronal precursors in the ventricular zone, but decreases proliferation in the subventricular zone (Haydar *et al.*, 2000), a domain that generates mainly glia (Sidman *et al.*, 1959). In the present study, decreased proliferation was also observed in the cortical ventricular zone following AMPA/KA antagonist treatment. However, we failed to detect any significant changes in the subventricular zone as mentioned in previous reports. One possible explanation may stem from the fact that embryos used here differ in gestational age from those in previous studies and that different rostrocaudal levels were examined (LoTurco *et al.*, 1995; Haydar *et al.*, 2000). Also, the proliferative behavior of the cortical subventricular zone may differ considerably between the explant and *in utero* conditions applied. Our data suggest that NMDA effects on proliferation of neuronal progenitors in the ganglionic eminence occur mainly in the ventricular zone, with no effect in the subventricular zone.

The precise mechanisms by which this dichotomous response is mediated remain unknown. Spatial and temporal differences in glutamate receptor expression is one possibility as both AMPA/KA and NMDA receptor expression follow developmentally regulated patterns (Misgeld and Dietzel, 1989; Monyer *et al.*, 1994;

Goebel and Poosch, 1999; Nansen *et al.*, 2000). *In vitro* studies have also indicated intrinsic differences in ionotropic glutamate receptor activation between cortical and striatal neurons (Kovacs *et al.*, 2001). Variations in local glutamate concentration may also arise due to differences in regional glutamate production (Haydar *et al.*, 2000) or differential innervation from extrinsic glutamatergic populations. For example, fibres from the thalamus have been postulated to influence cell cycle parameters in the developing cortex (Dehay *et al.*, 2001). Thalamic afferents are also believed to temporarily transit the ganglionic eminences before reaching their final cortical targets, and represents a possible source of glutamate during the striatal neurogenesis period (Metin and Godement, 1996; Dehay *et al.*, 2001).

Glutamate activity may also be mediated by interaction with other mitogenic factors that modulate proliferation in the embryonic and postnatal forebrain, including epidermal growth factor (EGF), basic fibroblastic growth factor (bFGF), and transforming growth factor α/β (TGFα,β) (Reynolds and Weiss, 1992; Temple and Qian, 1995; Cavanagh et al., 1997; Ciccolini and Svendsen, 1998). In immature neurons, production of TGFβ is up regulated following ionotropic glutamate receptor activation (Dobbertin *et al.*, 2000) while bFGF production is regulated by NMDA and dopamine receptor activation (Roceri *et al.*, 2001).

Distinct intracellular downstream mechanisms coupling NMDA receptor activation to the cell cycle machinery may also underlie variations in proliferative responses (Platenik *et al.*, 2000). A wide range of developmental effects in different CNS regions such as cell survival (Balazs *et al.*, 1988; Bhave and Hoffman, 1997; Ikonomidou *et al.*, 1999), neurite outgrowth (Lipton and Kater, 1989), and synaptic

plasticity (Stevens *et al.*, 1994) have been shown to be mediated by Ca^{2+} entry following NMDA receptor activation. Although the precise downstream intracellular mechanisms that couple glutamate receptor-mediated calcium entry to DNA synthesis in forebrain progenitors are unknown, a number of studies on the effects of growth factors and other mitogenic neurotransmitters suggest possible answers.

In particular, the mitogen activated kinases (MAPK) belonging to the extracellular regulated kinase (ERK) family have been observed to mediate proliferative responses invoked by bFGF and EGF (Fukunaga and Miyamoto, 1998; Learish *et al.*, 2000). With respect to classical neurotransmitters, proliferation in expanded cortical progenitors following muscarinic receptor activation by carbachol is regulated by phosphatidylinositol-3-kinase (PI3K) activated ERK signaling (Li *et al.*, 2001). In embryonic striatal neurons, activation of the NMDA receptor has been shown to induce PI3K activity, leading to activation of MAPK, cyclic-AMP response element-binding protein (CREB) phosphorylation, and expression of genes such as c-fos (Das *et al.*, 1997; Vincent *et al.*, 1998; Schwarzschild *et al.*, 1999; Perkinton *et al.*, 2002). These processes are Ca^{2+}dependent and are attenuated with the inhibition of calcium/calmoldulin kinases (CaM-k) (Das *et al.*, 1997). In addition, components of this cascade (e.g. CaM-k, ERK1/2, and PI3k) form part of the glutamatergic postsynaptic density (PSD) complex (Husi et al, 2000) and are thus within close proximity to NMDA receptors. In cortical cells, the activation of AMPA/KA receptors also results in immediate early gene expression (Vaccarino *et al.*, 1992) but may trigger MAPK activation via a different mechanism (Wang and Durkin, 1995).

In summary, the present study demonstrates that two adjacent progenitor populations show distinct morphogenetic responses to a single neurotransmitter. Such differences between progenitor populations throughout the CNS may represent a mechanism for pattern generation in the developing brain. However, neurotransmitters do not act alone as morphogens and a number of questions remain as to their interaction with other growth factors, precise contribution and schedule of participation with respect to growth and pattern formation in the CNS.

Figure Legends

Figure 1. Proliferation in cortical and striatal neuroblasts following modulation of NMDA receptor activity. Cultured neuroblasts from embryonic cortex or striatum were exposed to either ionotropic glutamate receptor antagonists or varying concentrations of NMDA for 4 h. Proliferation in cortical neuroblasts was reduced following application of the AMPA/KA antagonist NBQX but not NMDA antagonists (MK-801 and CGS 19755) as quantified by the proportion of nuclei incorporating BrdU in the final 2 h of treatment (a). In contrast, NMDA antagonists resulted in decreased proliferation in striatal neuroblasts which were not affected by NBQX (b). Addition of 1 uM NMDA also increased proliferation in these cultures, but not in cortical cells. At 100 uM, NMDA markedly reduced BrdU uptake in both types of cultures, possibly due to excitotoxicity. Levels of PCNA expression reflected that of BrdU uptake in both treated cortical and striatal cultures (c,d). BrdU uptake in cultures following 24 h of treatment showed a similar pattern, although the proportion of proliferative nuclei was reduced in comparison to younger cultures (e,f). Photomicrographs of BrdU immunoreactivity in cortical (g,h,i) and striatal (j,k,l) cultures after 4 h treatment. (*, $p < 0.01$; **, $p < 0.05$; $n = 3\text{-}4$).

Figure 2. BrdU uptake in telencephalic proliferative zones following glutamate receptor blockade in vivo. Proliferation in assigned regions of the cortical and striatal germinal zones was assessed by BrdU immunohistochemistry in embryos exposed in utero to MK-801 or NBQX (a). Embryos were exposed to BrdU following two administrations of drug and fixed in PFA 12 h later (b). Micrographs of the

cortex (c,d,e) and lateral ganglionic eminence (LGE) (f,g,h) following treatment. Quantification revealed that NBQX treatment resulted in a significant reduction of $BrdU^+$ nuclei in the cortical ventricular zone, whereas the subventricular zone was unaffected (i). In the LGE, exposure to MK-801, but not NBQX, decreased proliferation in the ventricular zone but not subventricular zone (k). Scale bars: 100 um (a), 40 um (c-h). (*, $p > 0.01$; $n = 3$).

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Figure 1

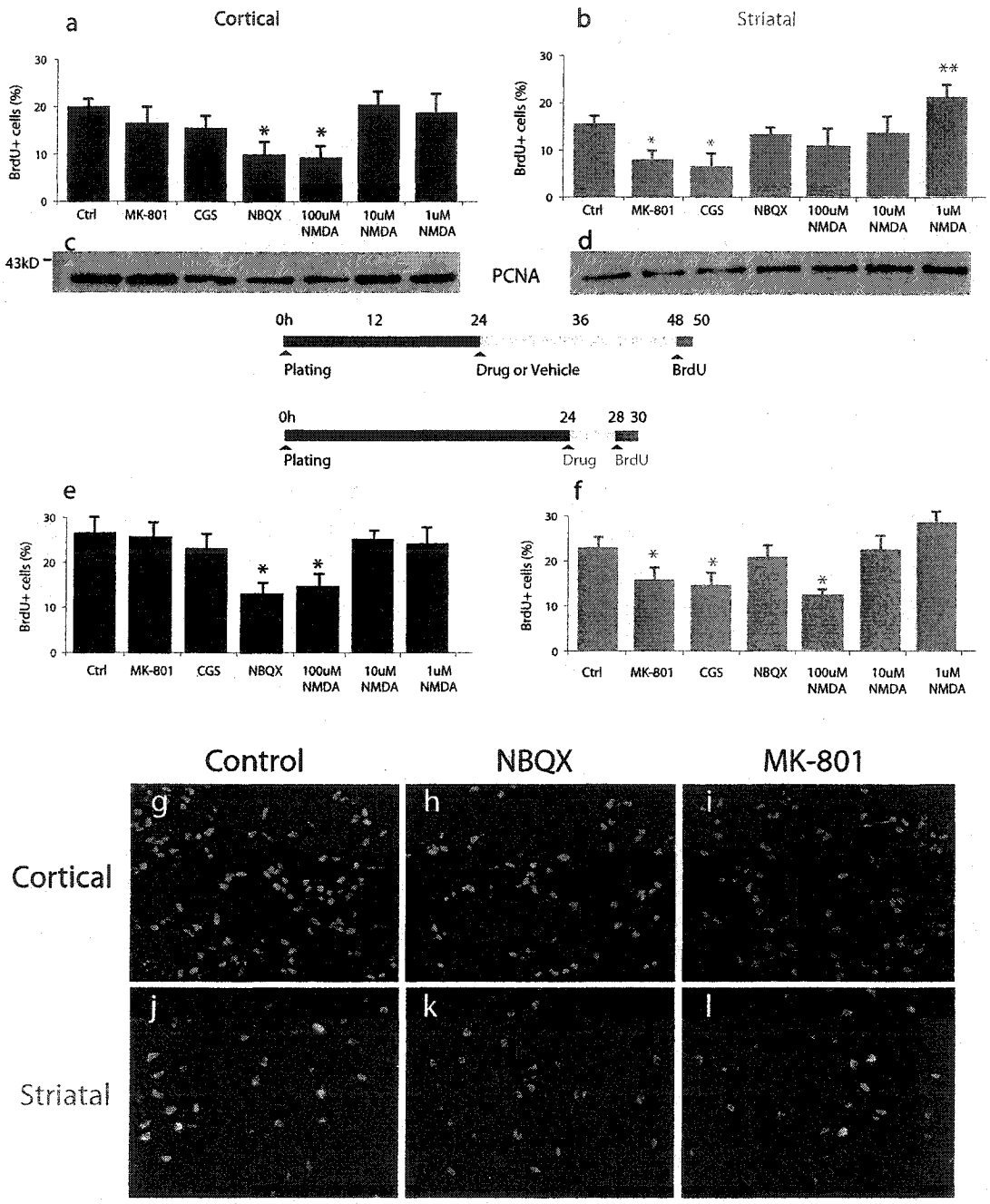
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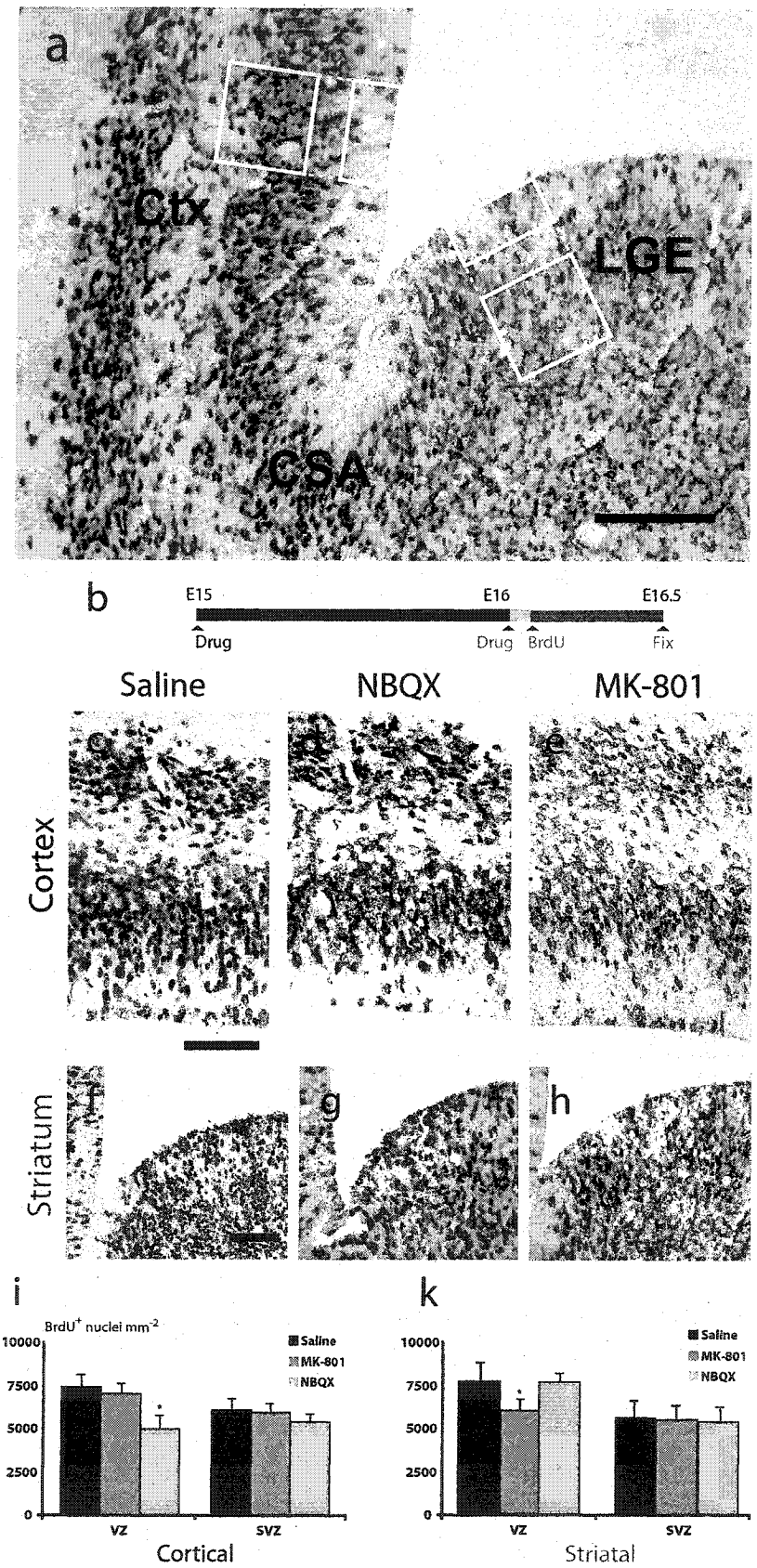
Figure 2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Chapter 5: Data from investigations in progress

5.1 Ontogeny of amino acid neurotransmitter-mediated proliferative responses in the developing telencephalon

5.1.1 Introduction

In the previous chapters, the role of the amino acid neurotransmitters glutamate and GABA were demonstrated to have important roles in the development of the mammalian central nervous system. In particular, glutamate has a positive influence on the proliferation of neuroblasts derived from basal and dorsal forebrain (telencephalic) regions. Using *in vitro* and *in vivo* models, we have previously identified a role for the N-Methyl D-Aspartate (NMDA) subclass of glutamate receptors in striatal progenitor proliferation while receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid/kainate (AMPA/KA) subclass appears to modulate proliferation of cortical progenitors. Here the ontogeny of the response to glutamate in basal telencephalic progenitors was investigated using neurospheres derived from embryonic striatum. In order to determine at what developmental stage glutamate begins to influence proliferation in precursors, cell proliferation was quantified by Ki-67 immunocytochemistry at 1, 3, or 7 days following growth factor withdrawal.

5.1.2 Methods

Embryonic cortical and striatal neurosphere cultures

Rat striatal neurospheres were obtained as described by others (Reynolds *et al.*, 1992; Ghosh and Greenberg, 1995). The lateral ganglionic eminence (LGE) was microdissected at E15 in cold magnesium-free Hank's Balanced Salt Solution (HBSS, Sigma, MO). Dissected tissue was then incubated in trypsin and DNAse I (Sigma) at

37°C and centrifuged at 1200 rpm for 5 min in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS). The pellet was resuspended in Neurobasal/B27 medium supplemented with 2 mM L-glutamine and penicillin/streptomycin (Life Technologies, Burlington, Ontario). Cells were triturated through polished Pasteur pipettes and then grown in 75 ml flasks containing Neurobasal medium with B27, Glutamax I (Life Technologies), antibiotics, and supplemented with basic fibroblastic growth factor (bFGF) and epidermal growth factor (EGF) (20ng each; Peprotech, NJ) at 37°C in an 5% CO_2. Expanded neurospheres (Figure 1a) were passaged every 4-5 days by trypsinization and gentle dissociation. Neurospheres were plated on PDL-coated 8-well chamber slides in medium lacking growth factors.

Differentiation and immunocytochemistry

At 1, 3, or 7 days after growth factor withdrawl, MK-801 (2 μg/ml), CGS-19975 (20 μg/ml), NMDA (1-100 μM), NBQX (10 μM), or vehicle control (DMEM) was added to individual wells. Cells were incubated in drug containing medium for an addition 24 h washed, and fixed with 4% paraformaldehyde. Cells were then permeabilized in 0.1% Triton-X100 in phosphate buffered saline (PBS) and incubated overnight at 4°C with a monoclonal antibody against Ki67 (MIB-5, 1:200; Dako, Glostrup, Denmark), a protein expressed exclusively in cycling cell populations (Duchrow *et al.*, 1995; Endl *et al.*, 2001). Cells were then washed with PBS (3 x 5 mins) and incubated with a biotinylated anti-mouse-IgG antibody raised in rabbit (1:200; Vector, Burlinghame, CA) for 1 h. After washing in PBS (3 x 5 mins), cells

were incubated for 30 mins with Alexa 594-conjugated streptavidin (1 μg/ml; Molecular Probes, Eugene, OR). Cells were counterstained with 4', 6-diamidino 2-phenylindole dihydrochloride (DAPI, 1 μg/ml in H_2O, 15 min, 37°C) to reveal cell nuclei. DAPI-stained nuclei and Ki67 positive cells were visualized under a fluorescence microscope using a 20X objective and appropriate filters. The number of nuclei and $Ki67^+$ cells were determined in 40-50 random fields generated with the help of Stereo Investigator software (Microbrightfield, Colchester, VT).

5.1.3 Results

Exposure to glutamate results in the up regulation of proliferation of neuroblasts derived from embryonic striatum (Li *et al.*, 1999; Haydar *et al.*, 2000; Luk *et al.*, 2003). Although the schedule of appearance of AMPA/KA and NMDA receptors in expanded cortical progenitors have been investigated (Maric *et al.*, 2000b; Maric *et al.*, 2000a), it remains unclear at what developmental stage basal telencephalic neural precursors first become responsive to glutamate. In order to address this issue, proliferation was evaluated in striatal neurospheres following treatment with NMDA agonists or antagonists. 1 day following withdrawal of growth factor and attachment to an adhesive substrate, the Ki67 labeling indices between treated neurosphere derived cultures showed no significant change (Table 1, Figure 1b-d). Similar results were observed after 3 days of differentiation, suggesting that proliferation of stem cells is not altered following exposure to NMDA or NMDA receptor antagonists. Proliferation was not assessed in 7 day cultures as neurospheres

had fully differentiated and consisted mainly of astrocytes with a small proportion of presumed neurons (Figure 1d).

5.2 bFGF release is not responsible for proliferation of striatal neuroblasts following NMDA-receptor activation *in vitro*.

5.2.1 Introduction

NMDA receptor activation leads to increased proliferation by striatal neuroblasts in culture (Luk *et al.*, 2003), but not multipotential precursors expanded *in vitro* (see previous section). However, it is unclear whether this phenomenon is the direct result of receptor activation on the target cell or indirectly mediated by other cells or factors. A possible explanation is that activity-dependent release of a mitogenic growth factor may be responsible. Basic FGF represents a likely candidate as it is a recognized mitogen for CNS precursors (Reynolds *et al.*, 1992; Vescovi *et al.*, 1993; Ghosh and Greenberg, 1995) and is released in an activity-dependent manner *in vitro* (Uchida *et al.*, 1998; Roceri *et al.*, 2001). *In vivo*, subcutaneous injection of bFGF in neonates can upregulate proliferation in cortex (Wagner *et al.*, 1999).

A number of studies have demonstrated the presence of FGF receptors (FGFRs) in the cortical and striatal ventricular zones of the mammalian telencephalon (Burrows *et al.*, 2000; Raballo *et al.*, 2000). In addition, these FGFRs exhibit a developmentally regulated pattern of expression, suggesting that this growth factor may play a significant role in the generation and maturation of this region. In order to examine whether bFGF release via depolarization is responsible for the proliferation observed in striatal neuroblasts following exposure to NMDA, the levels of bFGF in culture medium were quantified using enzyme-linked immunosorbent assay (ELISA).

5.2.2 Methods

Primary striatal cultures

Primary striatal cultures were prepared from E15 rat embryos as previously described (Luk et al, 2003). Briefly, the lateral ganglionic eminence (LGE) was microdissected in cold magnesium-free Hank's Balanced Salt Solution (HBSS, Sigma, MO). The dissected tissue was then incubated in trypsin and DNAse at 37°C and centrifuged at 1200 rpm for 5 min in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS). The pellet was resuspended in Neurobasal/B27 medium supplemented with 2 mM L-glutamine and penicillin/streptomycin (Life Technologies, Burlington, Ontario). Cells were triturated through polished Pasteur pipettes. Cells were then plated on 6-well plates pre-coated with poly-D-lysine (Costar) at a density of 2×10^5 cells/cm^2 and incubated at 37°C in an air:CO_2 (20:1) mixture for 24h.

Drugs and ELISA

Drugs were prepared immediately prior to addition to the culture medium. MK-801 (2 μg/ml), CGS-19975 (20 μg/ml), NMDA (1-100 μM), NBQX (10 μM), or vehicle control (DMEM) was added to individual wells after 24h in culture. Conditioned medium was obtained after 4h of treatment and frozen at -80°C until quantified. Concentrations of bFGF in samples were measured using a FGF-2 ELISA kit (Quantikine, RD Systems, Minneapolis, MN) as per the manufacturer's instructions. Triplicate samples were analyzed.

5.2.4 Results

In order to evaluate whether proliferation elicited by glutamate receptor activation in striatal neuroblasts is mediated by the release of a mitogenic growth factor such as bFGF, we quantified the amount of bFGF released into the surrounding medium following a variety of stimuli associated with glutamate receptor activation. We found measurable levels of this cytokine/growth factor using an ELISA method with sensitivity of <0.1 pg/ml. In concordance with previous studies, we observed increased concentrations of bFGF in the medium following exposure to NMDA (50-100 μM), but not at lower concentrations (Figure 2). However, addition of 50 μM glutamate for 4 h failed to elicit a significant increase, suggesting that higher concentrations may be necessary. Unstimulated control cultures and those treated with various glutamate receptor antagonists showed similar levels. This suggests that blockade of glutamate receptors does not reduce bFGF release in these cultures. Alternatively, under these conditions bFGF release is already at basal levels. Interestingly, despite detecting a 3-4 fold increase in free growth factor following NMDA treatment, significant bFGF concentrations were detected only at NMDA levels far exceeding that which we have previously determined to be optimal for proliferation (Luk et al, 2003). Together, this data suggests that bFGF is not responsible for the increased proliferation observed in striatal neuroblasts following NMDA receptor activation *in vitro*.

5.3 Evidence that glutamate induced proliferation in striatal neuroblasts is mediated by a MAP Kinase pathway *in vitro*.

5.3.1 Introduction

The extracellular signal-regulated kinase (ERK) family of mitogen-activated kinases (MAPK) mediate intracellular signaling following a variety of extracellular stimuli. Numerous studies have shown evidence of a link between ERK activation and proliferative activity. For example, constitutive ERK 1/2 (p42/p44) activation is responsible for maintaining melanoma cells in the cell cycle by down regulating the p27 inhibitory protein (Kortylewski *et al.*, 2001). Activation of ERK 1/2 also results in the induction of immediate early genes associated with cell cycle progression such as c-*fos* (Liu and Almazan, 1995; Boegman and Vincent, 1996) and is necessary for the activation of cyclin D (Lavoie *et al.*, 1996; Suzuki *et al.*, 2002). More importantly, activation of ERK is strongly associated with NMDA receptor activation and subsequent Ca^{2+} entry in a variety of CNS cell types (Boegman and Vincent, 1996; Vanhoutte *et al.*, 1999; Perkinton *et al.*, 2002), making this pathway an attractive candidate for mediating proliferation of striatal neuroblasts. Here, the expression of phosphorylated ERK (pERK) in neuroblasts was investigated by means of immunoblotting following exposure to NMDA agonists or antagonists. Antibodies specific to pERK and non-phosphorylated forms were used. Immunoblotting for proliferating cell nuclear antigen (PCNA) was also performed to quantify cell proliferation following blockade of ERK activity using a specific inhibitor.

5.3.2 Materials and Methods

Primary cortical and striatal neuroblasts cultures were prepared from E15 rat embryos as described above (Section 5.2.2). Cells were plated on 6-well plates pre-coated with poly-D-lysine (Costar) at a density of 2 x 10^5 cells/cm^2 and incubated at 37°C in 5% CO_2 for 24h before addition of drugs. CGS-19975 (20 μg/ml), NMDA (1, 10 μM), or vehicle control (DMEM) was added to individual wells. The MAPK inhibitor U0126 (15μM, Sigma) was added 30 minutes prior to other drugs. Cells were harvested either 15 mins or 6 h following addition of drugs by washing in cold PBS and lysis in Cell-Lytic M buffer (Sigma) containing protease inhibitors, sodium fluoride, and sodium orthovanadate. Protein content was determined using a BCA kit (Pierce). Samples were boiled in Laemmli sample buffer for 5 minutes and loaded (10 μg) into 4-15% gradient gels (Biorad, Mississauga, Ontario) for SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes, blocked for 1 h with TBS/0.1% Tween 20 (TBST) containing 5% non-fat milk powder, washed, and incubated overnight with a antibody against either PCNA (monoclonal; 1:1,000; Sigma), activated-MAPK (polyclonal; 1:3,000; Promega, Madison, WI), or pan-ERK 1/2 (polyclonal; 1:1,000; Cell Signaling Technologies, Beverly, MA) at 4°C. Following washes in TBST (3 x 5 minutes), blots were incubated with the appropriate HRP-conjugated secondary antibody raised (1:30,000; Sigma) for 1 h at room temperature. After washing in TBST (3 x 5 minutes), blots were developed and exposed on film using a chemiluminescence kit (PerkinElmer, Boston, MA) as per manufacturer's instructions.

5.3.3 Results

In order to determine whether NMDA activity results ERK 1/2 phosphorylation in striatal neuroblasts, the levels of pERK was quantified 15 mins following treatment with either NMDA or NMDA receptor antagonists (Figure 3). No changes in the expression of total ERK protein (pan-ERK) was detected (Figure 3a). Basal levels of pERK were readily detectable, and exposure to CGS-19975 neither increased nor decreased levels of pERK. However, addition of 1 or 10 μM NMDA markedly increased levels of pERK by nearly 50% and 100%, respectively (Figure 3b), indicating that NMDA receptor activation leads to ERK phosphorylation. Preincubation with U0126, a specific inhibitor of MAPK, was able to reduce pERK expression in the presence of NMDA to levels below control conditions, although this was higher than in cells without NMDA.

In order to establish whether ERK phosphorylation is necessary for proliferation of striatal neuroblasts following NMDA receptor activation, PCNA expression was examined in cultures following 6 h exposure to 1 μM NMDA in the presence or absence of U0126 (Figure 3c). As previously noted (see Section 5), NMDA upregulates proliferation as reflected by PCNA levels approximately 9% above control. In contrast, addition of U0126 alone resulted in a 22% decrease. Cells exposed to 1 μM NMDA following preincubation with U0126 also exhibited PCNA expression levels that were below control. Therefore, the ERK 1/2 pathway appears to play a role in mediating glutamate induced proliferation in striatal neuroblasts. However, inhibition of ERK activity does not completely abolish proliferation, suggesting other signaling pathways may also be involved. Alternatively, the

observed effect may be due to the short half-life of U0126 under these culture conditions.

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Figure 1

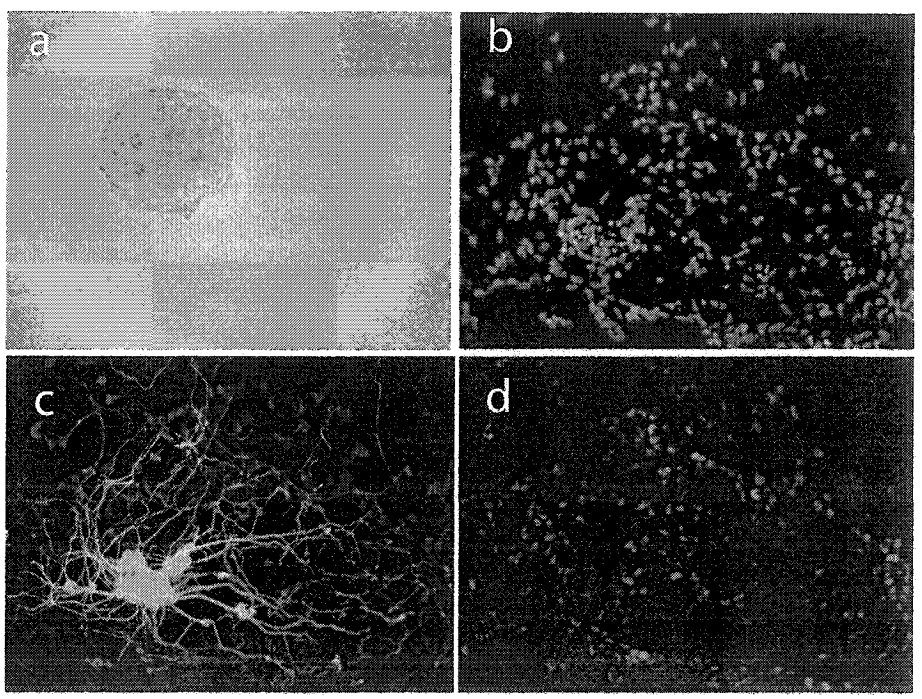


Table 1

Time *in vitro*	Treatment						
	Control	MK-801	CGS-19975	NBQX	1 μM NMDA	10 μM NMDA	100 μM NMDA
1 day	28%	30%	27%	28%	30%	28%	29%
3 days	26%	27%	24%	23%	26%	26%	25%

Figure 2

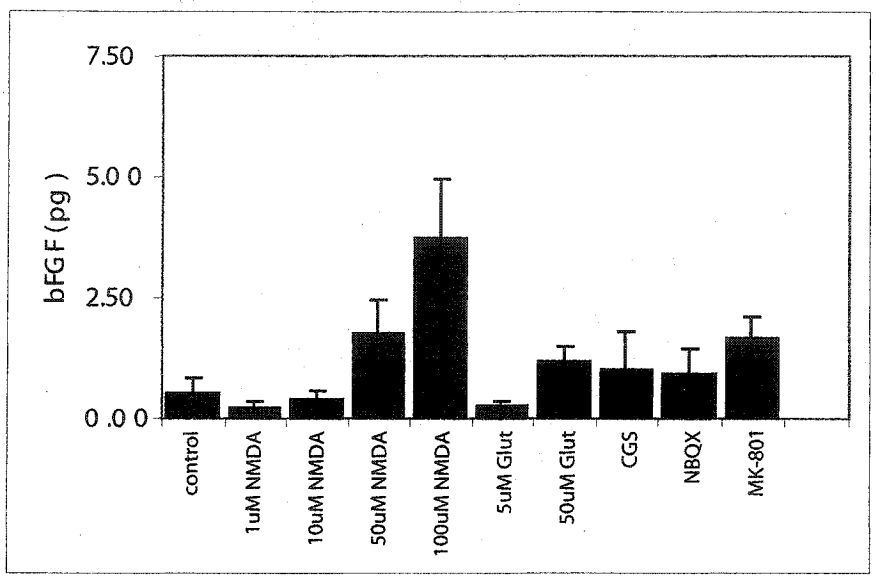
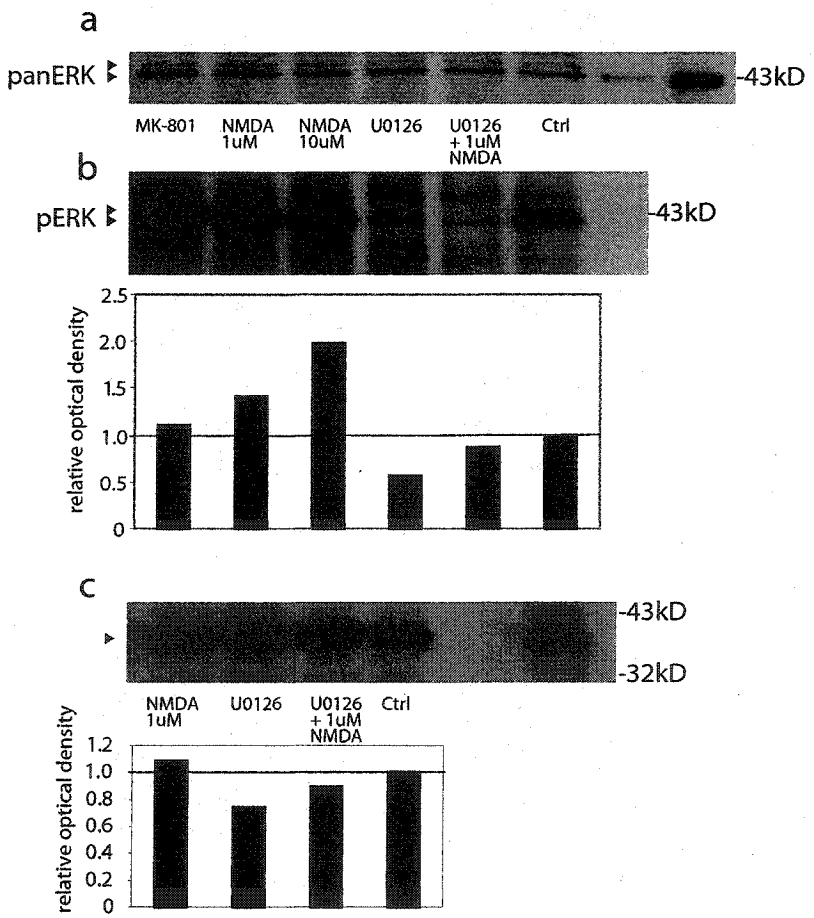


Figure 3



Chapter 6: Summary and Conclusion

6.1 Discussion

The remarkable ability of the mature CNS to complete its vast and complex range of functions is evidenced by the extensive diversity of neuronal and non-neuronal populations. In mammals a large proportion of higher motor and cognitive function is regulated through the telencephalon or forebrain. This superstructure consists of a number of smaller substructures including the septum, fornix, cerebral cortex, and basal ganglia, the latter two representing the most visible of the telencephalic subdivisions. How neuronal and structural diversity between these adjoining structures is generated has been the focus of many recent studies (see Chapter 1 and below). In this work, this question has been addressed from the perspective of possible developmental differences in cell proliferation between the dorsal and ventral telencephalon. As most neuronal populations cease to proliferate shortly after their generation, the number and phenotype of cells originally produced has long-lasting consequences.

A number of extrinsic factors, i.e. those which are secreted or part of transmembrane complexes which act in a cell-autonomous manner, have been demonstrated to influence proliferation in a variety of cell types in the CNS. Most are growth factors with known mitogenic potential in a wide range of cells. The most studied of these are basic fibroblastic growth factor (bFGF), epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1). In fact, these molecules, either alone or in combination, have been shown to generate or maintain stem-cell like precursors from embryonic and adult CNS tissues (Reynolds and Weiss, 1992; Barres and Raff, 1994; Ciccolini and Svendsen, 1998). In situ hybridization and immunohistochemical studies

have confirmed the expression of these molecules *in vivo* during development (Kornblum *et al.*, 1997; Burrows *et al.*, 2000; Maric *et al.*, 2003).

In the past decade, endogenous neurotransmitters traditionally regarded as synaptic messengers have also emerged as potential regulators of proliferation in the CNS. This list now includes acetylcholine, glutamate, GABA, serotonin, norepinephrine, and dopamine (Nguyen *et al.*, 2001). Target populations which interpret these transmitters as mitogens have been identified in various locations within and even outside the CNS (Skerry and Genever, 2001). In the CNS, these include cerebellar granule cells (Ashkenazi *et al.*, 1989; Fiszman *et al.*, 1999), hippocampal dentate gyrus cells (Cameron and McKay, 2001), and subependymal cells destined for the olfactory bulb (Mandairon *et al.*, 2003). With the exception of serotonin, these neurotransmitters have also been demonstrated to influence proliferation in the developing telencephalon. Acetylcholine increases proliferation in cortical precursors (Li *et al.*, 2001) while glutamate and GABA acts on both cortical and striatal proliferating populations (LoTurco *et al.*, 1995; Haydar *et al.*, 2000; Luk *et al.*, 2003). Meanwhile, dopamine has been found to influence striatal but, to date, not cortical progenitors (Ohtani *et al.*, 2003).

6.2 Summary of findings

The intention of the studies outlined in previous chapters was to investigate the role of amino-acid neurotransmitters in proliferation during telencephalic development. Glutamate and GABA were chosen due to a number of factors that made them likely candidates to influence cells during this period. Firstly, the levels of endogenous glutamate and GABA in the developing telencephalon are substantial and appear to be developmentally regulated (Lidow and Wang, 1995). Furthermore, receptors for these

neurotransmitters are also present during this period and show developmentally regulated patterns of expression (Lauder *et al.*, 1986; Xia and Haddad, 1992; Williams *et al.*, 1993; Monyer *et al.*, 1994). Lastly, both molecules have depolarizing properties in the embryonic brain, leading to the influx of Ca^{2+}, a known regulator of many cellular processes including proliferation (Berridge, 1995). Moreover, a limited number of studies supported this hypothesis in the cortex (LoTurco *et al.*, 1990; LoTurco *et al.*, 1995) and striatum (Sadikot *et al.*, 1998).

In Chapter 2 (Luk and Sadikot, 2001), the possible role of GABA in striatal proliferation was investigated by focusing on a subpopulation of striatal parvalbumin-immunoreactive GABAergic interneurons. Our group had previously demonstrated that proliferation of these neurons was mediated by NMDA receptor activation and that blockade of these receptors reduced their final number *in vivo* (Sadikot *et al.*, 1998). However, this effect was only observed when NMDA antagonists were administered during the known neurogenetic period for these cells (i.e. E15-18). Administration in the post-proliferative period (E18-21) did not result in any significant reduction. In the subsequent study, the effects of GABA were investigated utilizing the same *in vivo* model whereby the proliferative effect of a neurotransmitter was evaluated by *in utero* exposure to its receptor antagonists during or following its chief proliferative period. Because significant expression of parvalbumin (PV) does not commence until the postnatal period (Celio, 1990), the final number of PV interneurons was quantified in the adult offspring. The $GABA_A$ receptor antagonist BMI was used as previous studies in the literature showed little or no response to $GABA_B$ agents in the striatal neurons during this period (Ikeda *et al.*, 1997).

Our resulting data suggested that BMI had no significant effect on the final number of PV interneurons in the striatum when administered during the proliferative period. However, post-proliferative exposure resulted in a significant loss, suggesting that $GABA_A$ receptor activation may mediate survival, but not proliferation of these cells. Comparison of PV interneuron distribution in treated and control animals revealed that cell loss in the dorsolateral striatum was relatively pronounced, suggesting that migration from their ventromedial origins could have been impaired. In support of this hypothesis, previous studies in the cortex suggest that GABA may be a chemotactic factor required for migrating neurons (Behar *et al.*, 1996), and in particular, interneurons. The accompanying decrease following BMI exposure in striatal volume, as detected by stereology, also suggests that other striatal populations, namely GABAergic projection neurons, which comprise the neuronal majority, were affected to some extent. However, given that striatal volumes of E15-18 BMI treated animals were relatively unchanged, it seems unlikely that projection neuron progenitor division is affected by $GABA_A$ blockade during their proliferative period, which overlaps that of PV interneurons (Bayer, 1984; van der Kooy and Fishell, 1987; Sadikot and Sasseville, 1997).

In Chapter 3 (Luk *et al.*, 2003), the role of glutamate on striatal proliferation was investigated using a similar model that targeted specific neurogenetic periods. In contrast to GABA, we found that blockade of glutamate receptors during the proliferative period resulted in markedly decreased striatal neuron number and striatal volume in the offspring. Interestingly, the effect was mediated by NMDA receptors, but not their AMPA/KA counterparts. Analysis of the frontal agranular cortex in MK-801 treated animals by stereology also revealed no significant alterations, suggesting that the effect

of NMDA receptor blockade was restricted to the striatum. BrdU uptake in the striatum of embryos following acute exposure to NMDA antagonists was also reduced, suggesting that NMDA-receptor activation mediated proliferation *in vivo*. Treatment of proliferating neuroblasts obtained from embryonic striatum also gave similar results. *In vitro*, proliferation in cells treated with a competitive NMDA antagonist could be re-established by addition of NMDA, while application of NMDA alone significantly increased proliferation above control levels. Therefore we concluded from these data that glutamate, in contrast to GABA, plays a positive role in regulating the proliferation of striatal neurons. Interestingly, when compared to previous studies on glutamatergic effects in the cortex, our results indicated that dorsal and ventral telencephalic germinal zones respond with complementary subclasses of ionotropic receptors.

In order to investigate this implied difference, we examined the proliferative responses of the developing cortex and striatum in parallel under identical *in vitro* and *in vivo* experiments (Chapter 4). Embryonic neuroblasts from both telencephalic zones showed decreased proliferation as measured by BrdU uptake and expression of PCNA, a protein associated with cycling cells. However, cortical and striatal cultures were only sensitive to AMPA/KA (NBQX) and NMDA (MK-801) antagonists, respectively. Embryos examined immediately following administration of NBQX exhibited a sharp reduction in BrdU uptake in the cortical ventricular zone (VZ) with the striatal VZ apparently unaffected. The reverse was observed following MK-801 treatment while the subventricular zone was not significantly affected following exposure to either antagonist. These observations seem to affirm the hypothesis that dorsal and ventral progenitors show distinct responses to glutamate with respect to proliferation.

6.3 Comments

6.3.1 Possible mechanisms

The abovementioned differences existing between the developing dorsal and ventral telencephalon could result from a number of possible scenarios. These include intrinsic properties such as heterogeneous expression of glutamate receptors or variations in the cellular signaling pathways and machinery that mediate proliferation. Alternatively, extrinsic differences in the form of differential local concentrations of glutamate present in dorsal and ventral ventricular zones may be responsible. Growth factors which interact with glutamate may also vary between regions.

6.3.2 Depolarization and proliferation

The observations that a variety of neurotransmitters may induce CNS cell proliferation collectively suggest that a common pathway for proliferation may exist. For precursors, neuroblasts, or multipotential progenitors expanded *in vitro*, dependence on Ca^{2+} appears to be a common denominator in proliferation. Barker and colleagues have demonstrated in expanded cortical precursors that Ca^{2+} mediates proliferation induced by acetylcholine or the muscarinic receptor agonist carbachol (Li *et al.*, 2001). In addition, Ca^{2+} influx activates the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAPK) in a PI3k (phosphatidylinositol 3-kinase)-mediated manner. As its name implies, MAPKs are associated with cellular growth. In particular, the ERK pathway has been demonstrated to be active in a number of proliferative contexts including growth factor stimulation and cell transformation mediated by the oncogene Ras (Marshall, 1994; Ghosh and Greenberg, 1995; Temple and Qian, 1995). Both AMPA/KA and NMDA receptor activity in neurons have been associated with

MAPK activity, including activation of the ERK pathway (Fukunaga and Miyamoto, 1998; Perkinton *et al.*, 2002). Once activated, ERK 1/2 phosphorylate and activate a variety of transcription factors such as Elk1, CREB and immediate early genes such as c-fos in cortical and striatal neurons (Vanhoutte *et al.*, 1999; Davis *et al.*, 2000). Another early gene product, c-jun, is thought to play a role in controlling the expression of cyclin D, a key regulatory protein in cell cycle progression (Bakiri *et al.*, 2000). The precise molecular link that bridges ERK activation with cell cycle machinery has yet to be elucidated. However, increasing evidence indicates that phosphorylation of ERK 1/2 may regulate cyclin D activity via modulatory phosphorylation of inhibitory proteins such as Tob (Suzuki *et al.*, 2002) and p27(Kip1) (Kortylewski *et al.*, 2001).

Two parameters control the total cellular output in a developing tissue. Firstly, the number of cells generated can be influenced by changes in the proliferative rate of existing dividing cells (i.e., changes in cell cycle duration). Alternatively, the dividing population can be regulated via cell cycle exit or recruitment of quiescent progenitors. It is unclear which of these processes is enhanced in response to neurotransmitter activity although a number of observations suggest that both may be occurring simultaneously (Takahashi *et al.*, 1995). Despite the evidence that certain neurotransmitters activate mitogenic signaling pathways and cell cycle machinery, the ability of GABA to reduce the proliferating proportion in bFGF treated cortical progenitors suggests that cell cycle withdrawal occurs (Antonopoulos *et al.*, 1997). This raises an interesting question as to whether neurotransmitters may alter the specification of developing CNS cells, especially in the context of precursors and progenitors. For example, specification of multipotential precursors into a more restricted lineage may reduce or remove their capacity for dividing.

Given that the observed change in gene expression following neurotransmitter stimuli does not include any known specification genes, a direct link is unlikely. Furthermore, specifying genes appear to be stable in CNS cells that have been heterotopically transplanted (Carletti *et al.*, 2002). A more likely scenario is that progenitor fate may be affected through other growth factor-mediated mechanisms. For example, glutamate activity enhances both bFGF release while suppressing the production of TGFβ (Dobbertin *et al.*, 2000; Roceri *et al.*, 2001). In addition to its mitogenic effects, bFGF also affects the responsiveness of precursors to other growth factors such as EGF by up regulating expression of EGF receptors in a process that is antagonized by bone morphogenetic proteins (BMPs) (Ciccolini and Svendsen, 1998; Lillien and Raphael, 2000). Precursors bearing only bFGF receptor or both bFGF and EGF receptors exhibit different proliferative properties and potentiality (Cavanagh *et al.*, 1997; Ciccolini, 2001). Other factors involved in bFGF-mediated maturation of telencephalic precursors include Wnt and sonic hedgehog (Gunhaga *et al.*, 2003; Viti *et al.*, 2003).

6.3.3 Sources of neurotransmitter in the developing telencephalon

Regional fluctuations in the levels of glutamate and other neurotransmitters represent another possible mechanism by which neuronal production may be modulated in different parts of the CNS. In the mature CNS, the cerebral cortex primarily contains glutamatergic pyramidal neurons whereas the striatum is distinctly GABAergic in composition. In the developing telencephalon, glutamate is produced endogenously by the cortex in both ventricular areas and the cortical plate (Blanton and Kriegstein, 1991). Whether the embryonic striatum produces significant amounts of glutamate remains unclear. However, innervation from other brain nuclei may enrich the levels of

neurotransmitter in non-expressing areas. In this respect, glutamate from the thalamus may influence proliferation in the forebrain via thalamocortical and thalamostrial afferents (Dehay *et al.*, 2001), as may corticostriatal afferents. Interestingly, corticofugal and thalamocortical fibres passage through the ganglionic eminence transiently, and may thus correspond to a timed source of glutamate in the developing striatum (Metin and Godement, 1996). By as early as E11.5, corticofugal fibres begin traverse the cortical striatal angle into the lateral ganglionic mantle. While they do not appear to invade the striatal ventricular zone, growth cones interact with a population of calbindin expressing cells directly adjacent to the VZ. It has been reported that these projections interact with a transient population of neurons arising from the cortical preplate as well (Hamasaki *et al.*, 2001). While their role appears to be towards fasciculation of axons, they may represent another temporary source of glutamate in the striatum. In addition, projections originating from the thalamus also cross the LGE simultaneously but much closer to the VZ. Axons from both projections transit in the LGE for approximately 1-2 days. It is worth noting that the maximum number of axons passing through occurs at about E16, which falls within the peak proliferative phase of most LGE neuronal populations.

Another source of neurotransmitters in the developing telencephalon is the glial population. Astrocytes, for example, produce can produce glutamate and GABA which can affect developing neurons and neuroblasts *in vitro* (Liu *et al.*, 2000). Apart from neurotransmitter production, astrocytes also express a number of transporters for glutamate and GABA, allowing them another means of regulating extracellular concentrations of these molecules (see Schousboe, 2003 for review). Nonetheless, while microenvironmental variations in neurotransmitter levels may modulate neuronal

production, it does not effectively explain our observation that cortical and striatal neuroblasts respond via different receptor subclasses to glutamate.

6.5 Applications of research

In Chapter 2, the role of amino-acid neurotransmitters in a number of pathogenic processes was discussed. While glutamate excitotoxicity remains a major theme, evidence for glutamate as a trophic factor has advanced considerably. Our data here suggests that in addition to neuronal survival and proper synaptic establishment (Ikonomidou *et al.*, 1999), glutamate may be essential to the initial production of appropriate progenitor cell number. This warrants further consideration of the effects that exposure to substances which attenuate AMPA/KA or NMDA receptor activity including both abused and prescribed drugs may have. Clinical studies indicating that exposure to valproate (a sodium channel blocker), but not carbamazepine (a cholinergic antagonist), during pregnancy leads to an increased incidence of neural and other malformations (Mawer *et al.*, 2002) support this view. Furthermore, close anatomical examination of patients of fetal alcohol syndrome reveals significant reduction in basal grey matter and is less pronounced in the cerebral cortex (Archibald *et al.*, 2001). In the light of these findings in humans, animals exposed to NMDA or AMPA/KA antagonists during critical periods of neurogenesis represent potentially valuable models of diseases where abnormal neuron number in the telencephalon are thought to underlie pathogenesis, including alcohol and drug exposure syndromes and schizophrenia (Thune and Pakkenberg, 2000).

In conclusion, the work here has demonstrated novel roles of two amino acid neurotransmitters in the development of the brain. This is significant because humans

(and other mammals) are exposed to an extraordinary number of substances which can alter neurotransmitter function. The data presented here provides evidence that neurotransmitters influence formation and development of the CNS, in addition to mediating synaptic activity. While this had been previously postulated over a decade ago in the context of cell survival, synaptogenesis and maturation, we have shown here that GABA regulates cell survival while glutamate regulates proliferation in the ventral telencephalon. As such, the developmental timeframe during which neurotransmitters serve as relevant morphogens has been pushed back earlier.

However, our current knowledge in this area is far from complete and many questions remain to be answered before we can directly apply this knowledge as clinical solutions. Issues such as the schedule of receptor ontogeny, the cell types involved, and the underlying molecular mechanisms which tie receptor activation to cell growth and survival still need to be addressed. Furthermore, the relative contribution and importance of neurotransmitters to other parallel growth factors has yet to be evaluated. For example, do synergies between neurotransmitters exist in the context of CNS development, or are there redundancies? These are all fascinating questions and they will undoubtedly keep laboratories and graduate students occupied for many years to come.

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Experimental work involving animals was approved by the Montreal Neurological Institute's Animal Care Committee. A copy of the cover page of the approved protocol is attached.

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GABA PROMOTES SURVIVAL BUT NOT PROLIFERATION OF PARVALBUMIN-IMMUNOREACTIVE INTERNEURONS IN RODENT NEOSTRIATUM: AN *IN VIVO* STUDY WITH STEREOLOGY

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Abstract—Amino-acid neurotransmitters regulate a wide variety of developmental processes in the mammalian CNS including neurogenesis, cell migration, and apoptosis. In order to investigate the role of GABA in early development of forebrain interneurons, we determined the survival of parvalbumin-immunoreactive GABAergic interneurons in the adult rat striatum following prenatal exposure to either $GABA_A$ receptor agonist or antagonist. Unbiased stereology was used to quantify parvalbumin-immunoreactive neuron number in the neostriatum of adult rats exposed to the drugs *in utero*, and the results were compared to pair-fed or vehicle controls. Embryos were exposed to the $GABA_A$ antagonist (bicuculline) or agonist (muscimol) during previously defined proliferative or post-proliferative periods for parvalbumin-immunoreactive interneurons. Unbiased stereology using the optical fractionator was used to estimate the total number of parvalbumin-immunoreactive neurons in neostriatum of experimental and control rats. No significant alteration in parvalbumin-immunoreactive neuron number was observed in rats treated with either bicuculline (1 or 2 mg/kg/day) or muscimol (1 mg/kg/day) during the proliferative phase. Administration of bicuculline during the post-proliferative phase significantly reduced parvalbumin-immunoreactive neuron number in the neostriatum. A concomitant decrease in neostriatal volume was also observed, suggesting that the effect is not restricted to parvalbumin-immunoreactive interneurons. Positional analysis revealed loss of normal regional distribution gradients for parvalbumin-immunoreactive neurons in neostriatum of rats exposed to bicuculline in the embryonic post-proliferative phase.

This data collectively suggests that GABA promotes survival but not proliferation of parvalbumin-immunoreactive progenitors. GABA may also promote migration of subpopulations of interneurons that ultimately populate the ventral telencephalon. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: $GABA_A$ receptor, neostriatum, morphogenesis, cell survival, cell migration, calcium binding proteins.

The final number of neurons in the adult mammalian forebrain is determined by neurogenesis, cell migration to appropriate domains, and developmental cell death.[12,49] A growing body of evidence from cell culture, explant, and *in vivo* studies suggests that the microenvironment plays an important role in these processes.[4,31,40,42,43,63,69] GABA and other neurotransmitters are implicated in a variety of morphogenetic events in the mammalian and non-mammalian CNS.[40,42,58,59] Recent studies suggest that the amino acids glutamate and GABA play an important role in determining final neuron numbers in the developing telencephalon, including the cerebral cortex,[4,15,40,43] and striatum.[31,69]

GABA is the major inhibitory neurotransmitter of the CNS.[9,75] It exerts its physiological inhibitory effect by altering the chloride ion permeability of GABA receptor channels, thus hyperpolarizing the cell membrane in the majority of adult neurons. In early embryonic forebrain development, GABA possesses the ability to depolarize subpopulations of immature neurons at a time when the chloride gradient across the cell membrane is reduced.[4,5,65] GABA receptors include ionotropic $GABA_A$ and $GABA_C$ subtypes, and the metabotropic $GABA_B$ receptor.[9,50,54,71,75,77] $GABA_A$ receptors may mediate a variety of developmental CNS disorders, including some genetic epilepsies (e.g. Angelman syndrome), fetal drug and alcohol syndromes, motor disorders and psychiatric disorders.[21,36,53] $GABA_A$ receptor-mediated depolarizing responses may have an important influence on several calcium-dependant developmental processes, including cell proliferation, migration, differentiation, apoptosis, neurite outgrowth, and synapse formation.[4,25,31,43,48,55,79,90]

In cerebral cortex anlage derived from the dorsal telencephalon, varying concentrations of GABA differentially promote migration of distinct GABAergic or non-GABAergic neuronal populations.[4] Little information exists on the influence of GABA in the early morphogenesis of the striatum, a ventral telencephalon derivative

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Abbreviations: ABC, avidin–biotin–peroxidase complex; ANOVA, analysis of variance; BMI, bicuculline methoiodide; CR, calretinin; DAB, 3,3′-diaminobenzidine; DMSO, dimethyl sulfoxide; E, embryonic day; GAD, glutamic acid decarboxylase; IR, immunoreactive; NMDA, *N*-methyl-D-aspartate; NOS, nitric oxide synthase; NPY, neuropeptide-Y; P, postnatal day; PBS, phosphate-buffered saline; PV, parvalbumin; SNK, Student–Newman–Keuls; SS, somatostatin.

arising mainly from the lateral ganglionic eminence.[19] Recent evidence suggests that the ganglionic eminences not only give rise to GABAergic interneurons of the ventral telencephalon, but are also a source of a large proportion of GABAergic interneurons that ultimately populate the cerebral cortex, a dorsal telencephalon derivative.[1,20,80] Thus, factors determining proliferation and migration of GABAergic interneurons in the lateral ganglionic eminence may not only influence the final number of striatal interneurons, but may also determine GABAergic interneuron density in the dorsal telencephalon. We hypothesize that GABA influences cell proliferation or post-proliferative events of different neuronal subpopulations that ultimately comprise the adult striatum. *In vitro* studies show that activation of $GABA_A$, but not $GABA_B$, receptors results in increased number of embryonic striatal neurons.[30] We investigate the effects of GABA in prenatal striatal development by utilizing agonists and antagonists to the $GABA_A$ receptor *in vivo*.

The mammalian striatum contains a chemically heterogeneous mosaic of medium spiny GABAergic projection neurons, and subclasses of interneurons.[33,57,76] In the rodent, interneurons comprise less than 10% of striatal neurons, and include GABAergic aspiny neurons which are mainly small to medium-sized,[10,33,57,76] and a population of large aspiny cholinergic cells.[11,51,61] Distinct GABAergic interneuron subpopulations may be identified by co-localization with specific chemical markers, including the calcium binding proteins calretinin (CR) and parvalbumin (PV), and the peptide somatostatin (SS).[7,13,16,17,33,35,37,38,67,70,84] In comparison to projection neurons,[47,82] interneuron subtypes have restricted neurogenesis periods.[68,73] Their well-defined morphological chemical characteristics[33,37,57] and relatively restricted periods of proliferation make interneurons a well-suited model for determining factors that influence cell morphogenesis in the prenatal basal telencephalon.[45,69] We determine whether GABA regulates proliferation, migration, or survival of striatum PV-immunoreactive (IR) interneurons. Separate groups of embryos are exposed *in utero* to $GABA_A$ receptor agonist or antagonist during or immediately after the main proliferative period for neuroblasts giving rise to PV-IR cells.[68–70] In order to evaluate effects of $GABA_A$ receptor modulation, we employ stereological analysis[29,56,86] to provide unbiased estimates of the number of PV-IR interneurons in the striatum of control and experimental animals. Our findings suggest that GABA has little effect on cell proliferation. Rather, a $GABA_A$ receptor-mediated mechanism promotes survival of striatum PV-IR neurons during the prenatal post-proliferative phase.

EXPERIMENTAL PROCEDURES

Animals

Female Sprague–Dawley rats (Charles River, LaSalle, Quebec) were coupled with males between 15.00 and 17.00. The first 24 h after coupling was designated as embryonic day (E) zero. A second group of females was coupled 48 h later in order to provide control animals matched for food and water intake with experimental groups.

Drugs

Either the $GABA_A$ receptor agonist, muscimol, or the antagonist, bicuculline methoiodide (BMI; Research Biochemicals International, Natick, MA), was administered to separate groups of rats. Drugs were administered by i.p. injections for four-day periods from either E15–18 or E18–21, corresponding to mainly proliferative or post-proliferative periods for PV-IR neurons. Muscimol (1 mg/kg/day) was dissolved in dimethyl sulfoxide (DMSO; 1 mg/ml) and administered daily by i.p. injection during proliferative or post-proliferative periods. BMI (either 1 or 2 mg/kg/day) was dissolved in normal sterile saline (1 or 2 mg/ml) and administered daily during identical four-day periods by i.p. injection. Each animal's food and water intake and weight were recorded daily.

As controls, age-matched pregnant females were given daily i.p. injections of vehicle (saline or DMSO; 1 ml/kg/day) over identical four-day periods as drug-treated dams. Pair-fed control groups were given access to the amount of food and water consumed by their drug-treated counterparts. A separate control group was given saline injections during the period of interest, without food restrictions. After birth, five males were randomly chosen from each litter and killed between postnatal days (P) 35–42 for histology and immunohistochemistry. All animal protocols were previously approved by the McGill University Animal Care Committee. All efforts were made to minimise the number of animals used and their suffering.

Sectioning and immunohistochemistry

Rats were perfused transcardially, under deep sodium pentobarbital anesthesia (75 mg/kg, i.p.), with an initial wash of 0.9% saline (50–100 ml, 4°C) followed by 4% paraformaldehyde in phosphate buffer (300 ml, 0.1 M, pH 7.4, 4°C). Brains were immersed in the same fixative for 12 h, followed by immersion in cold 30% phosphate-buffered sucrose solution (pH 7.4) for an additional 48 h. Brains were then cut into 50-μm coronal sections encompassing the entire striatum using a freezing microtome. Upon identifying the rostral pole of the striatum, section collection was started at random, between the first and sixth section, as determined by the role of dice. Serial free-floating sections were collected in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) as separate sets so that each set contained every sixth serial section. One set of sections was immunostained for PV, and another set was processed using 0.1% Cresyl Violet as a Nissl stain.

Drug-treated and control animals were processed simultaneously for PV immunohistochemistry using a modified avidin–biotin–peroxidase complex (ABC) method as previously described.[30,68] In brief, free-floating sections were preincubated for 1 h in PBS containing 0.3% Triton X-100 and 5% bovine serum albumin. This was followed by overnight incubation with a primary monoclonal antibody against PV (1:5000 in PBS; Sigma, St. Louis, MO), followed by rinsing with PBS (3×5 min each). Next, sections were incubated in PBS containing secondary antibody (biotinylated anti-mouse IgG, 1:200; Vector, Burlinghame, CA). After rinsing again in PBS (3×5 min each) sections were incubated for 1 h in ABC (1% in PBS; Vector). The final reaction product was revealed by exposure to a solution containing 3,3′-diaminobenzidine (DAB, 0.25 mg/ml; Sigma) dissolved in Tris buffer (0.05 M, pH 7.6) containing 1% imidazole (1.0 M; Sigma) and 0.06% hydrogen peroxide. After 10–15 min exposure to DAB, sections were thoroughly washed with PBS, mounted out of distilled water, air-dried, and dehydrated using a graded series of ethanol concentrations. Sections were then cleared in xylene substitute, and coverslipped with Permount (Fisher, Fair Lawn, NJ).

Unbiased stereological estimate of total number of parvalbumin-immunoreactive neurons in the neostriatum

An unbiased stereological technique, the optical fractionator, was used to estimate the total number of PV-IR neurons in the neostriatum. The apparatus consisted of a light microscope (BX40, Olympus, Japan) coupled with a video camera (DC200, DAGE, Michigan City, IN), motorized X–Y stage (BioPoint

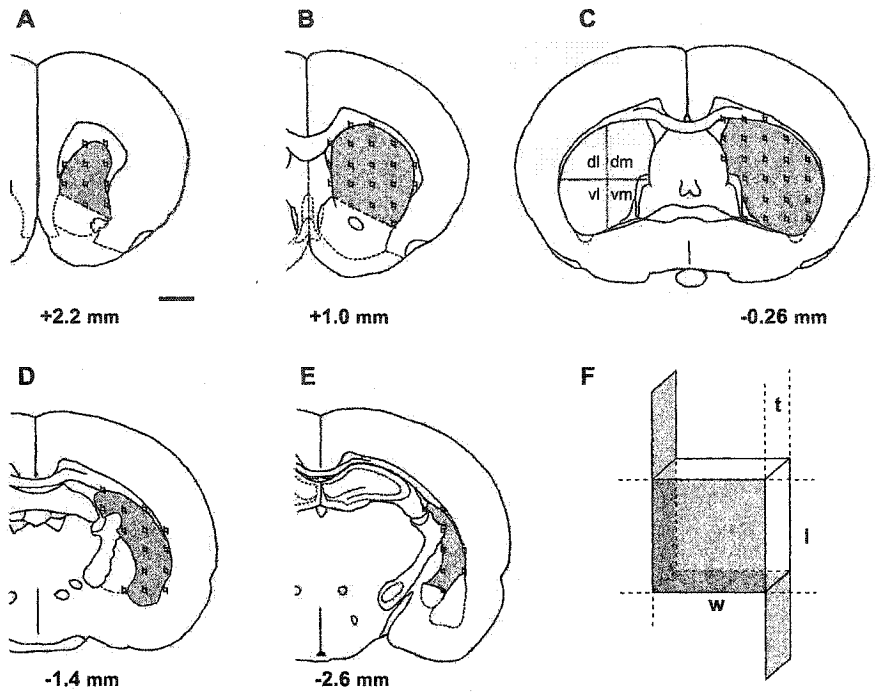

Fig. 1. Representative rostro-caudal coronal levels illustrating regions of interest used to delineate neostriatum, and the stereological scheme for estimating PV-IR cell number. Optical dissector size was $80\times80\times10$ μm for PV-IR counts, and $60\times60\times8$ μm for Nissl stained material. Dissectors with illustrated exclusion planes (shaded) were applied at intersections of a virtual square grid (500×500 μm; F). Adapted from atlas of Paxinos and Watson.[60] Scale bar = 500 μm.

XYZ, LEP, Hawthorne, NY), Z-axis indicator (MT12 microcator, Heidenhain, Traunreut, Germany), and a computer running Stereo Investigator software (Microbrightfield, Colchester, VT). The rostral and caudal limits of the reference volume were determined by the first and last coronal sections with visible caudate–putamen (approximately Bregma 2.20 to −2.60 mm, Paxinos and Watson[60] atlas). Every sixth serial section within this zone was examined, that is at 300 μm intervals through the reference volume. The corpus collosum, external capsule, lateral ventricle, globus pallidus, and anterior commissure were used as boundaries (Fig. 1A–E). In the most rostral sections, the ventral striatum was excluded from analysis by a line drawn from the ventral tip of the lateral ventricle to the dorsal border of the piriform cortex, corresponding to an angle of 20–30° below the horizontal axis. In more caudal sections, the caudate–putamen was bordered by the external capsule, globus pallidus, bed nucleus of the stria terminalis, the substantia innominata, and the dorsal amygdala. Surface areas of each region of interest were estimated from tracings of the neostriatum generated using a 4× objective and the Stereo Investigator software. All tracings derived from the same animal were used to estimate the reference volume using the Cavalieri method.[56]

Systematic random sampling of the neostriatum was performed by randomly translating a grid with 500×500 μm squares onto the section of interest using the Stereo Investigator software (Fig. 1A–E). Each intersection represented a sample site where an 80×80 μm counting frame with exclusion lines[29,86] was then applied (Fig. 1F). All randomly distributed computer-generated sample sites were then examined using a 100× objective (oil, numerical aperture 1.3). Only PV-IR cell bodies falling within the counting frame without contact with the exclusion lines were enumerated. PV-IR cell bodies were included in the count provided they had a visible dendritic process. Objects seen in the counting frame were only counted if they came into focus within a predetermined 10-μm thick optical dissector positioned 2 μm below the surface of the mounted section as indicated by the Z-axis microcator. Each optical dissector therefore consisted of an $80\times80\times10$ μm brick.[29,86] Estimates of the total number of PV-IR cells in each animal were generated using the Stereo Investigator software. Mean estimates of the total number of PV-IR cells in the neostriatum in treatment or control groups were compared using one-way analysis of variance (ANOVA, $\alpha = 0.05$) with Student–Newman–Keuls (SNK) post hoc test.

The proportion of PV neurons with respect to the total number of neostriatal neurons was also determined from Nissl-stained sections obtained from the saline control animals. Total neostriatal neuron number was estimated using the same unbiased stereological method described for PV neurons, except that the brick size (optical dissector) was 60×60 μm with an 8-μm thickness. Neurons were distinguished using the nucleus as a unique identifier, and glia were excluded on the basis of morphology[56] and by only counting profiles greater than 7 μm in diameter.

Image analysis of spatial distribution of parvalbumin-immunoreactive neurons

In order to investigate possible regional differences in PV-IR cell distribution induced by prenatal modulation of $GABA_A$ receptors, the striatum was divided into four equal quadrants at the coronal level at which the anterior commissure forms its ventral boundary (Bregma −0.26 mm in the Paxinos and Watson[60] atlas, Fig. 1C). The spatial distribution of PV-IR neurons was plotted using a light microscope (Leica Orthoplan, Wetzlar, Germany) equipped with an X–Y movement-sensitive stage and video camera coupled to a computer running image analysis software (Biocom, Les Ulis, France). Cell profile counts were obtained from the entire section thickness within the defined neostriatal area. The surface area of the region of interest was determined. Results are expressed as cell profile density per unit of striatal surface area. The mean cell profile densities for each treatment group were compared using the one-way ANOVA with the SNK post hoc test ($\alpha = 0.05$). Each mapped neostriatum section was further subdivided along dorsal/ventral and medial/lateral axes into representative quadrants. Mean PV-IR cell profile densities were obtained for each quadrant in different treatment groups and compared using the one-way ANOVA test.

RESULTS

Stereological estimate of the total number of parvalbumin-immunoreactive neurons in the neostriatum

PV-IR neurons appeared as mainly medium-sized light to dark brown DAB-stained cell bodies with multiple aspiny processes (Fig. 3A, B). In keeping with other studies,[13,16,17,35,68] PV-IR cell density is higher in the lateral aspect of the pre-commissural striatum compared to the medial part (Fig. 3C). Highest PV-IR neuron densities are noted in the dorsolateral neostriatum, and lowest densities are observed in the ventromedial sector (Fig. 3C). In the post-commissural striatum, PV-IR cell density is higher in the dorsal part, compared to the ventral part. A 300-μm interval separated coronal sections used for analysis, and a median average of 10 sections was analyzed per brain, representing 3000 μm along the rostro-caudal axis. Mean section thickness after immunohistochemical processing, mounting, and coverslipping was 16 μm, as measured by the microcator using a 100× (oil) objective.[29,86] A counting frame of 80×80 μm coupled with a 500×500 μm sampling grid at 100× (oil) magnification was applied to all sections. Our particular scheme examined 10–12 sections per neostriatum, with each section containing 10–64 sampling sites depending on its surface area. Mean coefficient of error of all PV-IR neuron number estimates was 0.12. The optical fractionator method revealed a total of 16,875 ± 203 (mean ± S.E.M., $n=4$) PV-IR neurons in the neostriatum of (E18–21) saline control animals.

A similar sampling scheme was applied to Nissl stained sections. The sections had an average thickness of 12 μm. The mean coefficient of error of all Nissl-based neostriatum neuron counts was 0.06. The optical fractionator estimated the total number of neostriatal neurons as 2.54 ± 0.13 million (mean ± S.E.M., $n=4$). Thus, approximately 0.7% of dorsal striatum neurons are PV-IR in young adult male Sprague–Dawley rats.

Stereological counts of neostriatal parvalbumin-immunoreactive neurons after prenatal exposure to $GABA_A$ receptor antagonist or agonist

Stereological analysis was performed on all experimental and control animal groups in order to obtain an unbiased estimate of the total number of PV-IR neurons in the neostriatum. Section thickness and sampling scheme (including characteristics of the sampling grid, dissector size, number of sections sampled and coefficient of error) were similar in animals derived from control and experimental groups (see previous section). Sections from a minimum of four animals were analyzed from each group treated with a $GABA_A$ receptor agonist/antagonist or vehicle.

One-way ANOVA revealed statistically significant differences in total PV-IR cell number between groups treated during the proliferative ($F_{6,21}$: 6.91; $P<0.001$) or post-proliferative phase ($F_{4,15}$: 8.62; $P<0.001$). The mean total number of PV-IR neurons in the neostriatum of animals treated with BMI (1 mg/kg/day) between E18–21 (10,547 ± 1331, $n=4$) was significantly lower than that of pair-fed (14,854 ± 967, $n=4$; $P<0.01$, SNK post hoc test) and saline control groups (16,875 ± 203, $n=4$; $P<0.01$, SNK post hoc test) (Fig. 2C). Administration of BMI (1 mg/kg/day) during the proliferative phase (E15–18) also resulted in a decrease in the total number of neostriatal PV-IR neurons (11,777 ± 300, $n=4$), but the difference was not statistically significant in comparison to pair-fed controls (12,920 ± 439, $n=4$; Fig. 2A). Even when the dose of BMI administered during the proliferative phase was doubled (2 mg/kg/day), no significant difference in the total number of PV-IR neurons was seen in comparison to the pair-fed control group (11,699 ± 1694 vs. 14,150 ± 1039, $n=4$).

Groups exposed to the $GABA_A$ agonist muscimol during the prenatal proliferative phase showed an increase in total neostriatal PV-IR neuron number compared to pair-fed controls (14,149 ± 390 vs. 12,832 ± 545, $n=4$), as did those treated during the post-proliferative phase (14,890 ± 310 vs. 14,063 ± 517, $n=4$). However, statistical analysis revealed no significant difference in PV-IR neuron number after $GABA_A$ agonist exposure during either period (Fig. 2B, D). Of note, in the case of both the E15–18 and E18–21 groups, mean PV-IR cell number in neostriatum of experimental or pair-fed animals was lower than in corresponding saline controls (E15–18 saline control: 18,517 ± 915, $n=4$; E18–21 saline control: 16,875 ± 203).

Mean neostriatal volume for each group of animals analyzed was as follows (mm^3, mean ± S.E.M.): BMI (1 mg/kg/day, E15–18) 18.37 ± 1.58; BMI pair-fed (E15–18) 19.98 ± 1.57; muscimol (E15–18) 18.06 ± 1.21; muscimol pair-fed (E15–18) 19.51 ± 0.62; saline (E15–18) 19.00 ± 0.69; BMI (E18–21) 17.62 ± 0.87; BMI pair-fed (E18–21) 20.11 ± 0.73; muscimol (E18–21) 18.65 ± 0.67; muscimol pair-fed (E18–21) 19.62 ± 0.38; saline (E18–21) 22.60 ± 1.02. Neostriatal volumes were largely comparable except for the E18–21 BMI treated group, which had a significantly lower neostriatal volume compared to both pair-fed and saline control groups (ANOVA: $F_{11,34}$: 10.28; $P<0.01$ for both SNK post hoc tests). This reduction in volume likely reflects a reduction in total number of neostriatum GABAergic projection neurons, the principal neurons of the striatum. However, quantification of this population is not performed here since the present experimental model is dependent on the relatively restricted neurogenesis period of PV-interneurons, compared to GABAergic projection neurons. Since total cell count obtained using the optical fractionator method does not vary with changes in size of the reference space, estimates of total number of PV-IR neurons in the neostriatum remain valid regardless of changes in neostriatal volume.[29,86]

Single section quadrant analysis of parvalbumin-immunoreactive cell density

In order to determine the spatial morphology of neuronal

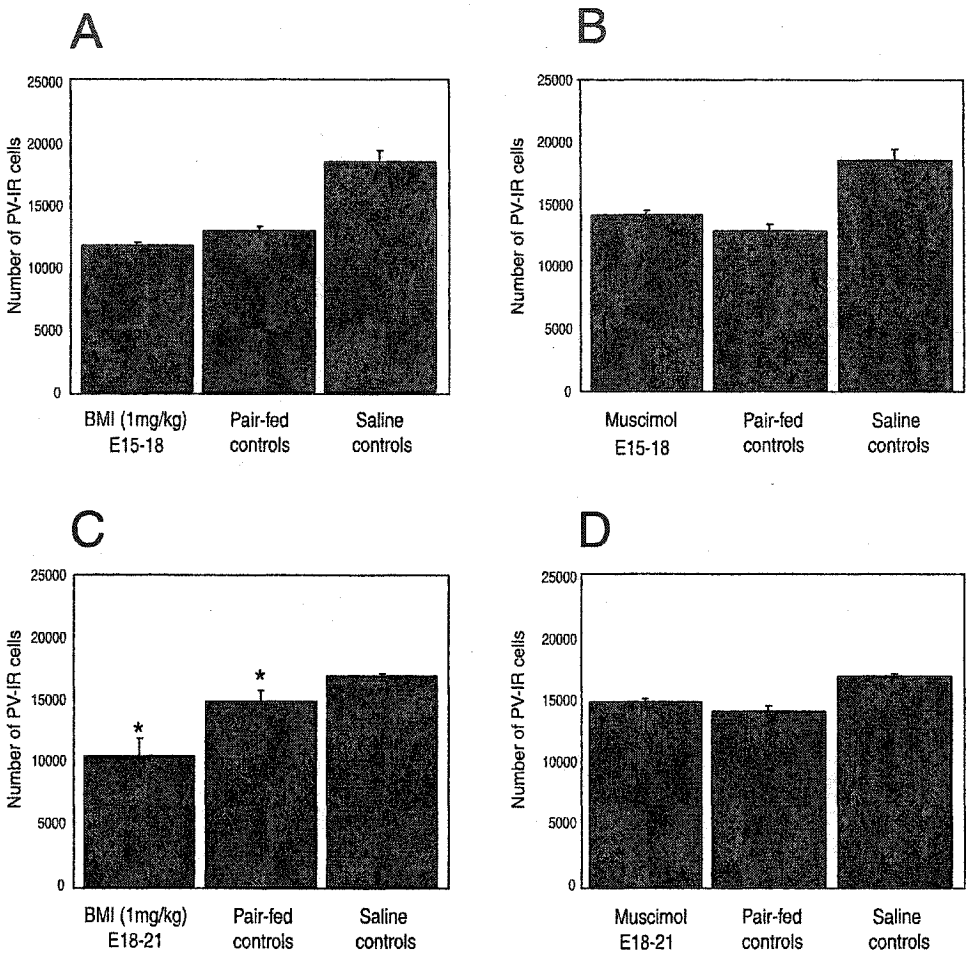

Fig. 2. Effects of prenatal $GABA_A$ receptor modulation on total PV-IR cell number in the neostriatum of young adult progeny. Administration of BMI during the embryonic proliferative phase (E15–18) for neuroblasts giving rise to PV-IR neurons did not alter total neostriatum PV-IR cell counts (mean ± S.E.M.) in comparison to pair-fed and saline controls (A). During the post-proliferative phase (E18–21), BMI (1 mg/kg/day) significantly reduced the total number of PV-IR cells in the neostriatum compared to pair-fed and saline controls (C). Animals given muscimol during either proliferative or post-proliferative phases did not show statistically significant changes in total PV-IR cell number (B, D). $*P < 0.01$, one way ANOVA with SNK post hoc test.

loss in the striatum, we examined sections corresponding to coronal level Bregma −0.26 mm, from animals treated with BMI (1 mg/kg/day) and their control groups.[60] At this level, the anterior commissure forms the ventral border of the striatum, and can be used as a reference for selecting comparable coronal sections in experimental and control groups (Fig. 3C, D). Mean PV-IR cell profile numbers and cell profile densities are shown in Table 1.

PV-IR neurons were categorized according to their position in quadrants (dorsolateral/dorsomedial/ventrolateral/ventromedial) from plots obtained from image analysis (Biocom, Les Ulis, France). BMI administered during E18–21 resulted in a significant reduction in PV-IR cell profile density compared to pair-fed controls (ANOVA: $F_{1,8}$: 11.34, $P < 0.01$, SNK post hoc test; Table 1 and Fig. 3C, D). Visible gradients in cell density were evident between quadrants in control groups. The dorsolateral region had a significantly higher PV-IR cell density than the ventromedial region in both saline (ANOVA: $F_{1,8}$: 19.04, $P < 0.01$, SNK post hoc test) and pair-fed control groups (ANOVA: $F_{1,8}$: 12.01, $P < 0.01$, SNK post hoc test; Fig. 3C). This observed gradient in PV-IR cell density is not visible in progeny of dams treated with BMI between E18–21 (1 mg/kg/day; Table 1, Fig. 3D), and statistical analysis revealed no significant difference in cell density between dorsolateral and ventromedial sectors.

DISCUSSION

The main findings of the present study are: (1) Prenatal rodents exposed to the $GABA_A$ receptor antagonist BMI during early forebrain development, show significant reduction in the total number of PV-IR GABAergic interneurons in the adult neostriatum. The effect is seen when BMI is administered during the prenatal post-proliferative phase, but not during the proliferative period for PV-IR neurons. These *in vivo* findings are compatible with prior *in vitro* evidence suggesting that GABA increases the

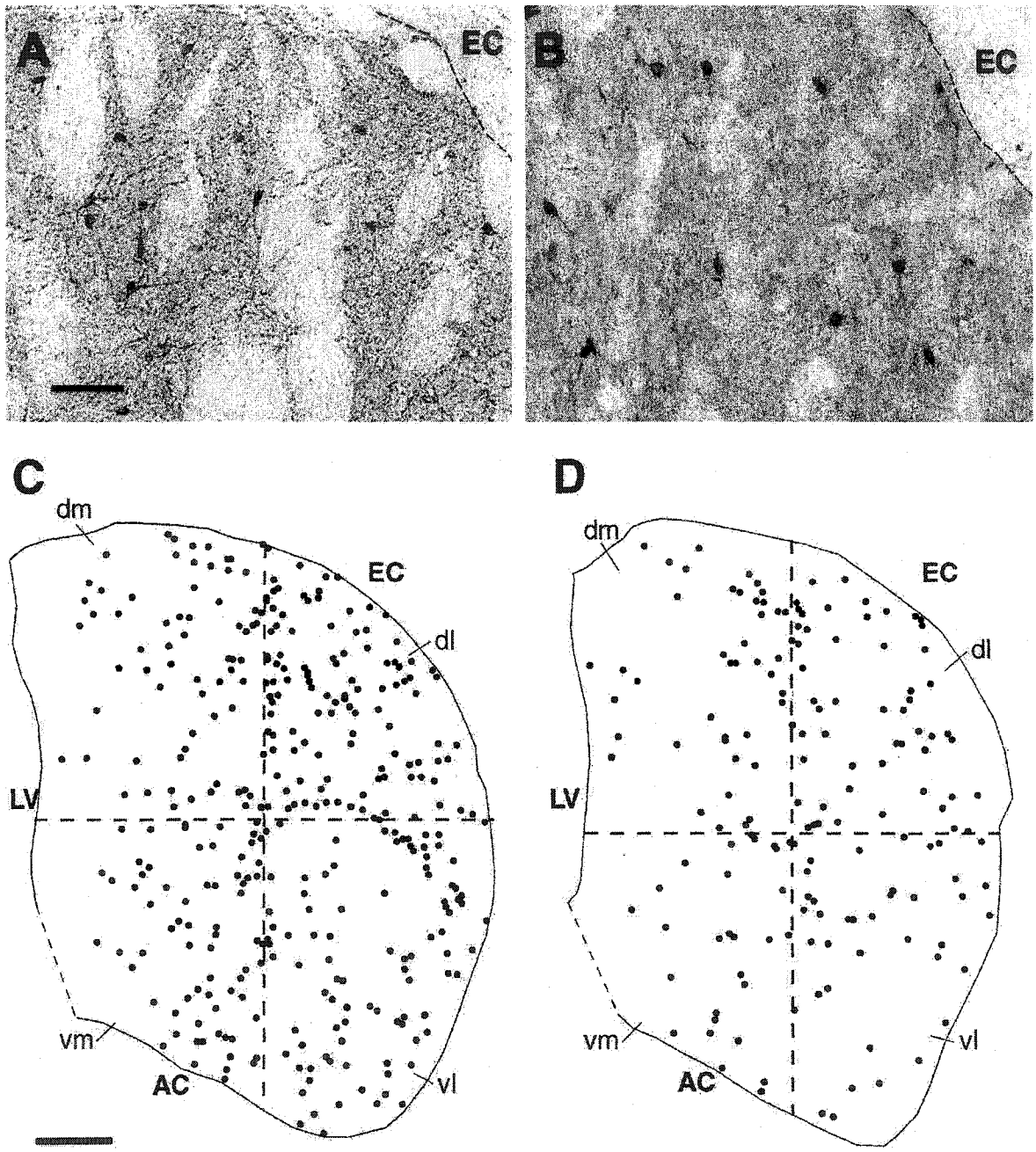

Fig. 3. (A, B) Photomicrographs of PV-IR cells in the dorsolateral neostratium at the coronal level at which the anterior commissure forms the ventral border of the striatum (Bregma −0.26 mm, Paxinos and Watson.)[60] PV-IR cells in progeny of saline controls (A) and offspring of dams given BMI during the post-proliferative (E18–21) phase (B) are shown. The number of neurons in the dorsolateral quadrant was significantly reduced in E18–21 $GABA_A$ receptor modulation. PV-IR cells were counted in dorsolateral, dorsomedial, ventromedial, and ventrolateral quadrants in the E18–21 drug-treated group, and cell densities were plotted and compared. Image-analysis-based maps of distribution of PV-IR cells in the neostratium of offspring of pair-fed control dams (C), and $GABA_A$ antagonist E18–21 treated animals (D) are illustrated. The normal dorsal/ventral and medial/lateral gradients in PV-IR neuron density persist in pair-fed controls (C), but are not present after treatment with the $GABA_A$ antagonist (D). The external capsule (EC) forms the dorso-lateral border and the anterior commissure (AC) and lateral ventricle (LV) respectively form the ventral and medial borders of the neostriatum at this coronal level. Scale bars = 100 μm (A, B); 500 μm (C, D).

number of striatal neurons by a $GABA_A$ receptor-mediated mechanism.[31] Our study extends these observations to a subclass of striatal interneurons with a well-defined period of neurogenesis.[68] The data suggests that GABA influences survival, but not proliferation of neuronal progenitors giving rise to PV-IR striatal neurons. (2) Prenatal treatment with BMI is also associated with loss of normal medial/lateral and dorsal/ventral distribution gradients of PV-IR interneurons in the neostriatum. This observation suggests that GABA

Table 1. Regional PV-IR cell density in the striatum after treatment with $GABA_A$ receptor antagonist

BMI (1 mg/kg) E18–21		
	Medial	Lateral
Dorsal	14.0 ± 1.7	22.9 ± 3.8
Ventral	15.0 ± 2.6	14.76 ± 1.5
Pair-fed Control E18–21		
	Medial	Lateral
Dorsal	17.3 ± 1.2	27.0 ± 1.1*
Ventral	15.6 ± 3.1*	17.4 ± 1.6
Saline Control E18–21		
	Medial	Lateral
Dorsal	18.38 ± 1.5	34.1 ± 1.7*,#
Ventral	23.5 ± 1.7*	24.1 ± 1.2#

PV-IR cell profile densities were obtained for each quadrant of the neostriatum at the coronal level at which the anterior commissure forms its ventral border (Bregma −0.26 mm, Paxinos and Watson[61] rat atlas). In normal controls, dorsolateral regions contained significantly higher PV-IR cell densities than ventromedial regions (*, #: $P < 0.01$, one way ANOVA with SNK post hoc test). No significant difference was observed for animals treated with BMI in the post-proliferative period, indicating a loss of normal density gradient. Densities are expressed as cells/mm^2 (mean ± S.E.M.).

may influence migration of PV-IR interneurons. (3) Finally, we provide a stereological estimate of the total number of PV-IR interneurons in the neostriatum.

Unbiased estimate of the total number of parvalbumin-immunoreactive neurons in the neostriatum

The number of PV-IR neurons in the striatum has not been previously determined. The use of stereology allowed unbiased estimates of total PV-IR cell number in the neostriatum from control and experimental groups. Optical fractionator-based estimates of cell count within the entire volume of interest are preferred over non-stereological estimates based on profile counts, since the latter method is subject to bias resulting from changes in volume of either the reference space or the object counted.[29,86]

Non-cholinergic aspiny interneurons include at least three groups of largely distinct GABAergic cells.[10,33] The calcium binding proteins CR and PV identify two subclasses.[6,7,13,16,17,35,37,38,64,67] A third class of aspiny neurons contains nitric oxide synthase (NOS), and co-localizes the peptides SS and neuropeptide Y (NPY).[14,22,83] In the absence of colchicine pre-treatment, antibodies to GABA or its synthesizing enzyme glutamic acid decarboxylase (GAD) fail to immunostain most NOS-IR cells, reveal weak to intense staining in CR-IR neurons, and show strong staining in PV-IR neurons.[37] Following colchicine pre-treatment, all three subclasses of interneurons immunostain intensely for the GAD_{67} isoform.[37] Early studies, based on the possibility that most strongly positive GAD-IR interneurons are PV-IR, estimated that PV-IR cells represent 3–5% of striatal neurons in the rat.[34] This estimate is greater than the 0.7% obtained in the present stereological study. The observation that a significant proportion of CR-IR interneurons are also strongly immunoreactive for GAD, may account in part for the lower than expected proportion of striatal PV-IR neurons estimated.[37] Furthermore, since both CR and PV interneurons show heterogeneous regional distribution within the striatum,[6,17,64,68] previous estimates based on a small number of sections may not be representative. The total number of neostriatal neurons counted in the present study (2.54×10^6) is quite similar to those previously obtained in rodents by other authors using similar stereological methods.[18,56] Previous stereological estimates of striatal interneurons have been limited to SS-IR interneurons, which comprise 21,300 cells in each neostriatum, corresponding to approximately 0.8% of total neurons in the rodent neostriatum.[86] PV-IR and SS-IR neurons are therefore found in comparable numbers in the neostriatum.

Parvalbumin-immunoreactive interneurons of the neostriatum as a model for examining neurotransmitter influence on early forebrain development

The adult number of striatal neurons is determined by neurogenesis, successful cell migration, establishment of connections, and developmental cell death.[12,42,49,63] Neurogenesis in the striatum takes place mainly from E13 to E22 (E0 corresponding to day of detection of vaginal plug).[2,47] Whereas projection neurons become post-mitotic over a prolonged prenatal period,[2,3] the neurogenetic timetable for specific interneuron subpopulations is relatively restricted. Cholinergic interneurons are born early, with peak neurogenesis at E12–E14.[72,73] The SS/NPY/NOS subtype becomes post-mitotic mainly between E15–16,[73] and CR-IR interneurons are born at E14–E17.[70] Detailed neurogenesis studies of PV-IR neurons of the neostriatum demonstrate that they also become post-mitotic over a relatively restricted time period, mainly between E14 and E17.[68]

Factors influencing proliferation, migration and survival of forebrain interneurons remain largely unexplored.[1,20,31,69] We have developed an *in vivo* model for determining factors that influence morphogenesis in the prenatal striatum and cerebral cortex.[45,69] Since GABAergic PV-IR neurons express their phenotype only in the postnatal period,[78] it is difficult to determine early developmental influences on this neuronal subclass *in vitro*. The relatively restricted period of striatal interneuron genesis allows for *in vivo* examination of microenvironmental influence on cell proliferation and post-proliferative events. In a previous study, the model was used to demonstrate that the *N*-methyl-D-aspartate (NMDA) subclass of glutamate receptors promotes proliferation of striatal PV-IR interneuron progenitors,[69] suggesting a role for glutamate in early forebrain development.[8,15,32,43]

GABA and early forebrain development

In the striatum, GABA is derived mainly from intrinsic sources, including principal neurons and GABAergic interneurons.[34,27,28,89] In the developing rat brain, significant GABA-IR can be detected in the ganglionic eminence as early as E12.[39] Studies using embryonic

(E18) striatal cultures show that GABA promotes survival of striatal neurons during the post-proliferative period.[31] The effect is blocked by bicuculline and picrotoxin, but not 2-hydroxysaclofen, indicating a mechanism mediated via the $GABA_A$, but not $GABA_B$, receptor subtype.[31] $GABA_A$-mediated depolarizing responses are well documented in the embryonic and early postnatal CNS, and likely related to elevated intracellular chloride ion concentration in immature neurons.[5,44,46,66,74,85,88,90] In rat striatum progenitors studied *in vitro*, $GABA_A$ receptor agonists induce modest depolarization as early as E12–13, with marked increase in response by E14.[23,52] Inhibitory responses to GABA develop progressively in the postnatal period, and are associated with developmental changes in receptor subunit composition and maturation of chloride ion homeostasis.[5,26,27,41,62,65,81,87,91] $GABA_A$ receptor-mediated effects on developing cells in the mammalian CNS are regionally specific.[40] For example, $GABA_A$ receptor-mediated mechanisms increase proliferation of cerebellar granule cells,[24] but decrease proliferation of progenitors giving rise to the principal neurons of the cerebral cortex.[43] In the post-mitotic period GABA increases both motility and directed migration of immature cortical cells.[4] Different GABA concentrations differentially promote migration of distinct cortical GABAergic or non-GABAergic neuronal subpopulations.[4]

Prenatal exposure to both $GABA_A$ and NMDA receptor antagonists reduces the adult number of PV-IR striatal neurons.[69] However, $GABA_A$-mediated influence on striatal morphogenesis occurs predominantly during the post-proliferative phase, whereas NMDA receptor-mediated mechanisms promote neuroblast proliferation. Since a tendency to reduced total PV-IR neuron number was noted when $GABA_A$ receptor antagonist was given at E15–18, we cannot rule out a minor effect on cell proliferation. However, differences in neuron number were not statistically significant compared to pair-fed controls, even after doubling the dose of BMI. Furthermore, total PV-IR neuron number is not altered significantly after exposure to the $GABA_A$ receptor agonist muscimol during the proliferative phase, suggesting that ligand concentrations exceeding the physiological range do not alter proliferation or survival. Taken together, these observations suggest lack of proliferative effect of GABA on PV-IR striatal progenitors. These findings contrast with observations suggesting that $GABA_A$ receptor activation reduces neuronal proliferation in the developing cortex,[43] and promotes proliferation of cerebellar granule cells.[24] Cortical interneurons, unlike projection neurons, appear to originate in the ganglionic eminences.[1,20,80] It would therefore be of interest to determine whether PV-IR interneurons destined for the cerebral cortex, show similar behavior to their striatum counterparts with respect to proliferation or migration in response to classical neurotransmitters.

Reduction in the adult number of PV interneurons as a result of $GABA_A$ receptor blockade is in keeping with previous data demonstrating that GABA promotes striatal neuron survival *in vitro*.[31] Of note, neostriatal volume decreased significantly following BMI treatment in the post-proliferative period, suggesting that in addition to PV-IR interneurons, the GABAergic principal neurons of the striatum likely show a reduction in cell number. However, the present model is designed to specifically distinguish proliferative effects of prenatal drug treatment from post-proliferative effects, and is tailored to the relatively restricted neurogenesis period of PV-IR interneurons.[68] Since subpopulations of principal neurons have a relatively heterogeneous and protracted time-course of neurogenesis,[47,82] we do not specifically address $GABA_A$ effects on projection neurons in the present study.

The observed reduction in PV-IR neuron survival after $GABA_A$ receptor antagonist exposure during the prenatal post-proliferative period may be related to impaired migration of post-mitotic striatal neuroblasts, reduced cell survival, or both. Gradient analysis of PV-IR neuron density suggests a possible effect on cell migration. In the adult neostriatum, PV-IR neuron density is greater in the dorsolateral, sensorimotor sector compared to the ventromedial region.[17,35,68] It is established that dorsolateral neostriatal PV-IR interneurons are born prior to ventromedial cells,[68] suggesting an "outside–in" gradient of neurogenesis common to other ventral forebrain neurons.[3] Embryos exposed to $GABA_A$ receptor antagonist (BMI) during the post-proliferative period show loss of the dorsolateral-ventromedial gradient in PV-IR cell density, suggesting a GABAergic influence on cell migration. Failure to achieve normal final position in the striatum may also impair survival. We cannot exclude the possibility that reduced PV expression results from prenatal exposure to $GABA_A$ receptor antagonists, but this is less likely since forebrain PV is expressed mainly in the postnatal period.[78] Furthermore, altered PV-IR phenotype should occur in all sectors of the striatum, and would not account for the observed loss of density gradient. Future studies using transgenic mutant animals with altered GABA receptor expression, or models based on *in vitro* explants, may help further establish a role for GABA in promoting neural migration in the developing ventral telencephalon.

CONCLUSIONS

Stereological analysis suggests that PV-IR GABAergic interneurons comprise approximately 1% of neostriatal neurons. The relatively restricted period of neurogenesis of striatal PV-IR cells (E14–E17) allows for an *in vivo* model for determining microenvironmental factors that influence prenatal striatal development. We provide evidence for a role for GABA in prenatal striatal morphogenesis. $GABA_A$ receptors have little influence on cell proliferation, but rather mediate migration of striatal PV-IR neurons. This *in vivo* data is consistent with *in vitro* evidence suggesting a role for GABA in survival and migration of forebrain neurons.[4,31,40] Recent data suggest that an as yet unidentified subpopulation of cortical GABAergic interneurons, rather than arising in the dorsal telencephalon germinal zone, may derive from the ganglionic eminence, a striatal precursor.[1,58,80] This

finding suggests the possibility that cortical and striatal forebrain GABAergic interneurons may share homologous developmental mechanisms. Future studies will focus on comparative aspects of early neurotransmitter influence on GABAergic interneurons populating either the striatum or cerebral cortex.

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Glutamate Promotes Proliferation of Striatal Neuronal Progenitors by an NMDA Receptor-Mediated Mechanism

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Increasing evidence suggests that classical neurotransmitters play important roles in the development of the mammalian CNS. We used *in vivo* and *in vitro* models to identify a novel role for glutamate in striatal neurogenesis mediated by NMDA receptors. *In utero* exposure to NMDA receptor antagonists during striatal neurogenesis caused a dramatic reduction in the total number of adult striatal neurons. In contrast, embryos exposed to NMDA receptor antagonists immediately after the main period of neurogenesis showed no significant change in neuronal number in the adult striatum. In addition, examination of embryos shortly after NMDA receptor blockade revealed reduced proliferation in the lateral ganglionic eminence (LGE). In culture, dividing neuronal progenitors derived from the embryonic LGE showed marked reduction in 5′-bromodeoxyuridine (BrdU) uptake when exposed to NMDA receptor antagonists, indicating reduced DNA synthesis. Low concentrations of NMDA significantly increased proliferation, whereas high concentrations were toxic. AMPA–KA receptor antagonists had no significant effect on striatal neuroblast proliferation either *in vivo* or *in vitro*. These results support the hypothesis that glutamate plays a novel role during early development of the ventral telencephalon, promoting proliferation of striatal neuronal progenitors by an NMDA receptor-dependent mechanism. In contrast, previous findings suggest that proliferation of cortical progenitors derived from the dorsal telencephalon is regulated by activation of AMPA–KA but not NMDA receptors. Heterogeneous responses to glutamate in different germinal zones of the telencephalon may be an important mechanism contributing to generating neuronal diversity in the forebrain.

Key words: forebrain; striatum; neurogenesis; neurotransmitter; BrdU; stereology

Introduction

CNS development is the result of coordinated cell proliferation, migration, differentiation, synaptogenesis, and apoptosis (for review, see Sanes et al., 2000). Proliferation is determined by expression of distinct genetic programs and extracellular cues (Lillien, 1998; Edmund and Jessell, 1999). Differences in proliferative response to growth factors or neurotransmitters in embryonic germinal zones may be an important mechanism for achieving the appropriate number of neurons in different CNS regions (Lauder, 1993; Caviness and Takahashi, 1995; Levitt et al., 1997). Glutamate, the major excitatory neurotransmitter (Curtis et al., 1959; Watkins, 2000), is excitotoxic at high concentrations and implicated in CNS pathology (Olney, 1982; Choi, 1988). Increasing evidence suggests that glutamate plays novel roles in morphogenesis. Glutamate regulates migration, survival, differentiation, and neuritogenesis of neurons (Mattson and Kater, 1987; Simon et al., 1992; Rossi and Slater, 1993; Rakic and Komuro, 1995; Behar et al., 1996; Bhave and Hoffman, 1997; Dammerman and Kriegstein, 2000). Recent studies indicate that glutamate also plays an important modulatory role in proliferation of forebrain neuronal precursors (Cameron et al., 1995; LoTurco et al., 1995; Sadikot et al., 1998; Haydar et al., 2000) (for review, see Contestabile, 2000; Arvidsson et al., 2001).

The mammalian telencephalon is derived from dorsal germinal zones that generate glutamatergic principal neurons of the cerebral cortex and ventral germinal zones that produce basal forebrain populations, including GABAergic principal neurons of the striatum (Holmgren, 1925; Fentress et al., 1981; Bayer, 1984; Marchand and Lajoie, 1986; Kawaguchi et al., 1995). With the exception of cortical GABAergic interneurons, derived mainly from the ventral telencephalon (de Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997), most migrating neurons do not cross the corticostriatal boundary (Fishell et al., 1993). Dorsal and ventral telencephalic germinal zones express distinct transcription factors (Puelles and Rubenstein, 1993; Shimamura et al., 1995; Casarosa et al., 1999), exhibit distinct patterns of clonal heterogeneity (Halliday and Cepko, 1992; Acklin and van der Kooy, 1993), and may show unique morphogenetic responses to extracellular factors.

Glutamate is present in the telencephalic germinal zones during embryogenesis, and it exerts morphogenetic effects that vary with receptor subtype (Blanton and Kriegstein, 1991; Behar et al., 1999). Activation of AMPA–KA, but not NMDA, subclasses of ionotropic glutamate receptors alters proliferation in the cortical germinal zone (LoTurco et al., 1995; Haydar et al., 2000). Little is known about the role of glutamate in morphogenesis of the embryonic basal telencephalon. *In utero* NMDA receptor blockade markedly reduces proliferation of striatal GABAergic interneuron progenitors (Sadikot et al., 1998).

We hypothesize that proliferative responses to glutamate in

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dorsal and ventral telencephalic germinal zones are regionally specific. We investigate the influence of glutamate on proliferation of progenitors of striatal projection neurons, focusing on NMDA receptor activation using an *in vivo* model and proliferating primary neuronal cultures (Sadikot et al., 1998; Luk and Sadikot, 2001). We report that NMDA receptor activation is required for proliferation of striatal progenitors, whereas AMPA–KA-mediated receptor mechanisms have no significant effect. These results suggest distinct reciprocal roles for NMDA and non-NMDA receptors in proliferation of neuronal progenitors in dorsal and ventral telencephalic germinal zones. This heterogeneous response to glutamate may be an important mechanism for generating neuronal diversity in the dorsal and ventral forebrain.

Materials and Methods

Ionotropic glutamate receptors and proliferation of striatal neuronal progenitors in vivo

Animals. Female Sprague Dawley rats (Charles River, LaSalle, Quebec, Canada) were coupled with males between 3:00 P.M. and 5:00 P.M. The first 24 hr after coupling was designated as embryonic day zero (E0). A second group of females was coupled 48 hr later to provide control animals, including dams matched for food and water intake with experimental groups. All animal procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

In utero *drug treatments for adult stereology.* The NMDA receptor antagonists MK-801 (noncompetitive; 0.2 mg · kg^{-1} · d^{-1}) or CGS-19755 (competitive; 5 mg · kg^{-1} · d^{-1}; RBI, Natick, MA), or the AMPA–KA receptor antagonist 1, 2, 3, 4-tetrahydro-6-nitro-2, 3-dioxo-benzol(f)-quinoxaline-7-sulfonamide (NBQX; 10 mg · kg^{-1} · d^{-1}) were administered to separate groups of rats. Drugs were dissolved in sterile normal saline and administered daily by intraperitoneal injection over a period of 4 d from either E15 to E18 or from E18 to E21. These time intervals correspond respectively to mainly proliferative or postproliferative periods for striatal neurons (Bayer, 1984; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987).

Food and water intake and weight for each animal was recorded daily. As controls, age-matched pregnant females were given daily intraperitoneal injections of saline (1 ml · kg^{-1} · d^{-1}) over identical 4 d periods as the drug-treated dams. In addition, pair-fed control groups were given access to the amount of food and water consumed by their drug-treated counterparts. A separate control group was given intraperitoneal saline injections during the period of interest and *ad libitum* access to food and water. After birth, five males were randomly chosen from each litter and killed between postnatal days 35 and 42 for histology by transcardial perfusion with 4% paraformaldehyde in phosphate buffer (PFA; 4°C, 0.1 M, pH 7.4).

Stereology. Coronal sections of the entire adult striatum were cut at 50 μm on a freezing microtome. After identifying the most rostral extent of the striatum, section collection was started randomly between the first and sixth section, as determined by a roll of dice. Serial free-floating sections were collected in PBS (0.1 M, pH 7.4) as separate sets so that each set contained every sixth serial section. One set of sections from each brain was processed using 0.1% cresyl violet as a Nissl stain. Sections were then cleared in xylene substitute and coverslipped with Permount (Fisher, Fair Lawn, NJ).

An unbiased stereological technique, the optical fractionator (Moller et al., 1990; West et al., 1996), was used to estimate the total number of neurons in the striatum and frontal agranular cortex as previously described (Luk and Sadikot, 2001). The apparatus used consisted of a light microscope (BX40; Olympus, Tokyo, Japan) coupled with a video camera (DC200; Dage, Michigan City, IN), motorized X-Y stage (BioPoint XYZ; LEP, Hawthorne, NY), *z*-axis indicator (MT12 microcator; Heidenhain, Traunreut, Germany), and a computer running Stereo Investigator software (Microbrightfield, Inc., Colchester, VT). The rostral and caudal limits of the reference volume were determined by the first and last coronal sections with visible caudate–putamen (neostriatum, dorsal striatum; approximately bregma 2.20 to −2.60 mm; Paxinos and Watson, 1986). Every sixth serial section within this zone was examined, i.e., at 300 μm intervals along the rostrocaudal axis. The corpus callosum, external capsule, lateral ventricle, globus pallidus, and anterior commissure were used as boundaries (Fig. 1). In the most rostral sections, the ventral striatum was excluded from analysis by a line drawn from the ventral tip of the lateral ventricle to the dorsal border of the piriform cortex, corresponding to an angle of 20–30° below the horizontal axis. In more caudal sections, the caudate–putamen borders included the external capsule, globus pallidus, bed nucleus of the stria terminalis, the substantia innominata, and the dorsal amygdala. Stereology was also performed for the motor cortex in the same sections. Areas corresponding to Fr1 and Fr2 (frontal agranular cortex) were delineated with the help of an atlas (Zilles, 1985). The granular somatosensory cortex was excluded from analysis. Surface areas of each region of interest were estimated from tracings of the neostriatum at 4× magnification using the software. Volumes of the reference space were estimated using the Cavalieri method (Gundersen and Jensen, 1987).

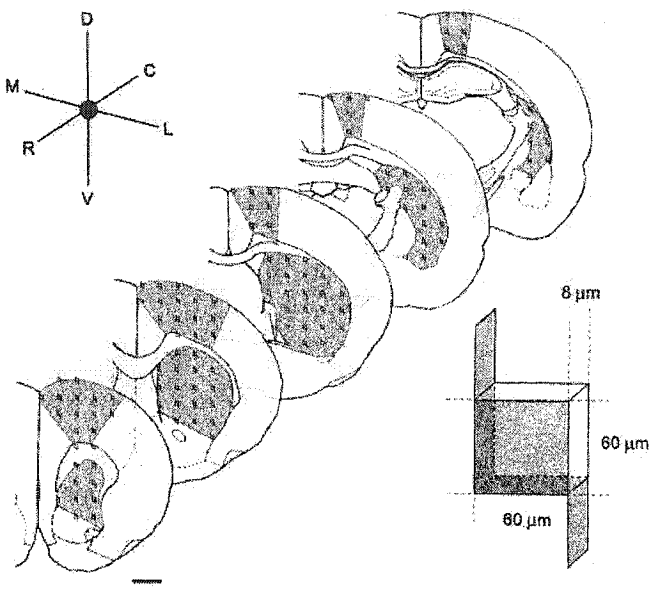

Figure 1. Stereological estimation of the number of striatal and motor cortical neurons. The total number of projection neurons in the adult rat striatum and frontal agranular cortex was estimated using the optical fractionator technique. Striatal and cortical reference volumes encompassed the first and last sections containing visible caudate–putamen, corresponding approximately to 2.20 to −2.60 mm bregma (Zilles, 1985; Paxinos and Watson, 1986). Sections were cut at 50 μm with every sixth section being examined. Counting frames were arranged 500 μm apart to form a grid that was randomly superimposed over the reference area at each coronal level using the analysis software. Each counting frame measured 60 × 60 μm with a thickness of 8 μm (*inset*). Exclusion lines and planes (*shaded*) were also implemented. Cell nuclei touching or falling within the counting frame were only recorded if there was no visible contact with any of the exclusion planes. Scale bar, 500 μm.

Systematic random sampling of neurons in the neostriatum was performed by randomly translating a grid with 500 × 500 μm squares onto the section of interest using the software (Fig. 1). At each intersection of grid lines a 60 × 60 μm counting frame with exclusion lines was then applied (Fig. 1). All randomly assigned sample sites were then examined using a 100× objective (oil; numerical aperture, 1.3). Neurons were distinguished using the nucleus as a unique identifier, and glial cells were excluded on the basis of morphology and by counting only profiles >7 μm in diameter according to previously described criteria (Dam, 1992; Oorschot, 1996; Luk and Sadikot, 2001). Only neurons falling within the counting frame without contact with the exclusion lines were enumerated. Objects seen in the counting frame were only counted if they came into focus within a predetermined 8-μm-thick optical dissector positioned 2 μm below the surface of the mounted section as indicated by the microcator. Each optical dissector therefore consisted of a 60 × 60 × 8 μm brick with three exclusion planes (Fig. 1, *inset*). Calculated estimates

of the total number of neurons in each neostriatal and cortical reference volume were determined using the Stereo Investigator software. Statistical analysis was performed by one-way ANOVA ($\alpha = 0.01$) with the Student–Newman–Keuls *post hoc* test (SNK) for comparison between groups.

Analysis of embryos after drug treatment. The immediate effects of receptor antagonists on cell proliferation *in vivo* were examined in embryos. Separate groups of timed pregnant rats were given MK-801 ($0.2\ mg \cdot kg^{-1} \cdot d^{-1}$), CGS-19755 ($5\ mg \cdot kg^{-1} \cdot d^{-1}$), NBQX ($10\ mg \cdot kg^{-1} \cdot d^{-1}$), or saline via intraperitoneal injection on E15 and E16. On E16, drug administration was followed 1 hr later by a single injection of BrdU (50 mg/kg). Embryos were removed after a further 12 hr by Cesarean section, decapitated, and fixed overnight in 4% PFA. Heads were then transferred to 10% formalin, dehydrated, and embedded in paraffin. Embryonic brains in paraffin blocks were sectioned at 5 μm with a microtome. Antigen retrieval was achieved by heating sections in citrate buffer (0.01 M, pH 6.0) for 15 min at 90°C. Sections were then exposed to 2N HCl for 1 hr, followed by three washes in PBS (5 min each), and then incubated overnight with a monoclonal antibody against BrdU (1:10, Becton Dickinson, Franklin Lakes, NJ). Cells were then washed three times with PBS and labeled using the avidin–biotin–complex (ABC) peroxidase method by incubating for 1 hr with biotinylated goat anti-mouse IgG secondary antibody (1:200) followed by ABC solution (Vector Laboratories, Burlingame, CA). The final reaction was revealed by exposing cells to a solution (NiDAB) containing Tris buffer (0.05 M, pH 7.6), nickel ammonium sulfate (3.7 mg/ml), 3,3′-diaminobenzidine (DAB; 0.25 mg/ml), and 0.0006% hydrogen peroxide. Sections were then counterstained with nuclear fast red, dehydrated, and coverslipped in Permount.

To determine the immediate effects of treatment on proliferation, BrdU-immunoreactive nuclei were quantified in coronal sections at the level of the ganglionic eminence. The striatal and cortical periventricular zones were delineated by a 100 μm wide box placed ~100 μm from the cortical striatal angle (see Fig. 3*a*,*b*). The first 50 μm from the ventricular surface was arbitrarily designated as the ventricular zone (VZ), whereas the area from 100 to 200 μm was considered the subventricular zone (SVZ). Proliferative nuclei were counted through the entire thickness of the section, and the results were expressed as a percentage of nuclei that were BrdU+.

Ionotropic glutamate receptors and proliferation of striatal neuronal progenitors in vitro

Microdissection and preparation of proliferative striatal cultures. Cultures were prepared from E15 rat embryos using techniques similar to those previously described (Ikeda et al., 1997; Ivkovic et al., 1997; Ventimiglia and Lindsay, 1998). The lateral ganglionic eminence (LGE) that gives rise to the striatum anlage (Bayer, 1984; Deacon et al., 1994) was microdissected in cold magnesium-free HBSS (Sigma, St. Louis, MO). The dissected tissue was then incubated in trypsin and DNase at 37°C and centrifuged at 1200 rpm for 5 min in DMEM (Sigma) containing 10% fetal bovine serum. The pellet was resuspended in Neurobasal medium supplemented with B27 (Life Technologies, Burlington, Ontario, Canada). Cells were dissociated by passing through a series of fire-polished Pasteur pipettes of decreasing caliber. Viable cells were then counted by Trypan Blue exclusion, diluted in Neurobasal/B27 medium (Bottenstein, 1985; Brewer, 1995) containing 2 mM L-glutamine and penicillin–streptomycin. Cells were then plated on 8-well chamber slides precoated with poly-D-lysine (Becton Dickinson) at a density of 2×10^5 cells/cm^2 and incubated at 37°C in an air:CO_2 (20:1) mixture for 1–5 d *in vitro* (DIV).

Treatment and BrdU incorporation. Twenty-four hours after initial plating, cells in individual slide chambers were exposed to either MK-801 (2 μg/ml), CGS-19755 (20 μg/ml), NMDA (0.1–100 μM), NBQX (10 μM), or vehicle control. Drugs were prepared immediately before addition to the culture medium. To label cells passing through S-phase, BrdU (20 μg/ml; Sigma) was added to each chamber either 4 or 24 hr after treatment. Cells were then fixed for 20 min with cold 4% PFA either 2 hr or 12 hr after addition of BrdU, and processed for immunocytochemistry.

Immunocytochemistry. BrdU uptake by proliferating cells was revealed by immunostaining (Gratzner, 1982). Briefly, cells were permeabilized in cold acetone–methanol (1:1), washed in PBS, and denatured in 2N HCl for 20 min. The cells were then washed with PBS, sodium borate (0.1 M), followed by another PBS wash. Anti-BrdU antibody (1:10; Becton Dickinson) was added and incubated overnight at 4°C. Cells were then washed three times with PBS and labeled with an Alexa 594-conjugated goat anti-mouse IgG antibody (1:500; Molecular Probes, Eugene, OR).

To characterize cell types, cultures were also immunostained for neuron-specific βIII-microtubulin (TuJ1; 1:500; Babco, Richmond, CA) or microtubule-associated protein-2 (MAP-2; 1:1000; Sigma) as early or late neuronal markers, respectively (Lee et al., 1990; Memberg and Hall, 1995), or for glial fibrillary acidic protein (GFAP; 1:1000; Sigma). Cells were counterstained with 4′, 6-diamidino 2-phenylindole dihydrochloride (DAPI; 1 μg/ml in H_2O, 15 min, 37°C) to reveal cell nuclei.

To determine whether proliferating neuroblasts express NMDA receptor subunits, double-labeling for BrdU and NMDA receptor subunits was performed in culture. After initial pretreatment and incubation with anti-BrdU antibody (see above), cells were incubated overnight in primary antibodies for NMDA receptor subunits. Primary antibodies were dissolved in PBS containing 0.3% Triton X-100 and 1% normal goat serum and washed in PBS (3 × 5 min). Polyclonal antibodies for NR1 (1:500; Transduction Laboratories, Lexington, KY), NR2A (1:400; Chemicon, Temecula, CA), NR2B (1:500; Sigma), and NR2C (1:250; Chemicon) were used. Cells were then incubated with an appropriate secondary antibody conjugated to either Alexa-488 or -594 (1:500) for 1 hr, then washed in PBS (5 × 5 min). For double-labeling of NR1 and NR2A, a monoclonal antibody for NR1 (1:1000; Chemicon) was used.

Analysis. Fluorescently labeled cells were visualized under a fluorescence microscope using a 40× objective and the appropriate filters. Density of cells and nuclei was determined in 40–50 random fields generated using the Stereo Investigator software. Results were compared by ANOVA as described above.

Results

Exposure to NMDA receptor antagonists reduces proliferation of neostriatal neuronal precursors *in vivo*

To determine whether ionotropic glutamate receptors mediate proliferation in the developing mammalian striatum, rat embryos were exposed *in utero* to competitive and noncompetitive NMDA receptor antagonists. Drugs were administered either during proliferative (E15–E18) or mainly postproliferative (E18–E21) period for striatal neurogenesis (Smart and Sturrock, 1978; Bayer, 1984; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987). To quantify total neuron number in the striatum and motor cortex of adult offspring, we applied the optical fractionator technique to cresyl violet-stained brain sections. This quantitative method allowed for efficient and unbiased estimates of total neuronal number within the entire striatal or cortical reference volume. Stereology is preferred over nonstereological estimates based on profile counts, because the latter method is subject to bias resulting from changes in either the volume of the reference space or the size of the object counted (Moller et al., 1990; West et al., 1996). Changes in total neuronal count and striatal volume were compared based on analysis of sections obtained from drug-treated and control animals.

The estimated number of neurons per striatum in rats receiving only the saline vehicle during the proliferative (E15–E18) or postproliferative (E18–E21) phases was 2.58 ± 0.10 million and 2.54 ± 0.12 million, respectively (Fig. 2*a*) (all data represented as mean ± SEM). These estimates are in agreement with results from previous studies using similar stereological methods to quantify principal neurons in the rodent striatum (Dam, 1992; Oorschot, 1996). In comparison with pair-fed control animals, administration of NMDA receptor antagonists during the maximal proliferative period for projection neurons (E15–E18) resulted in a 38–54% reduction of striatal neuronal number (Fig. 2*a*). One-way ANOVA revealed significantly decreased neuron

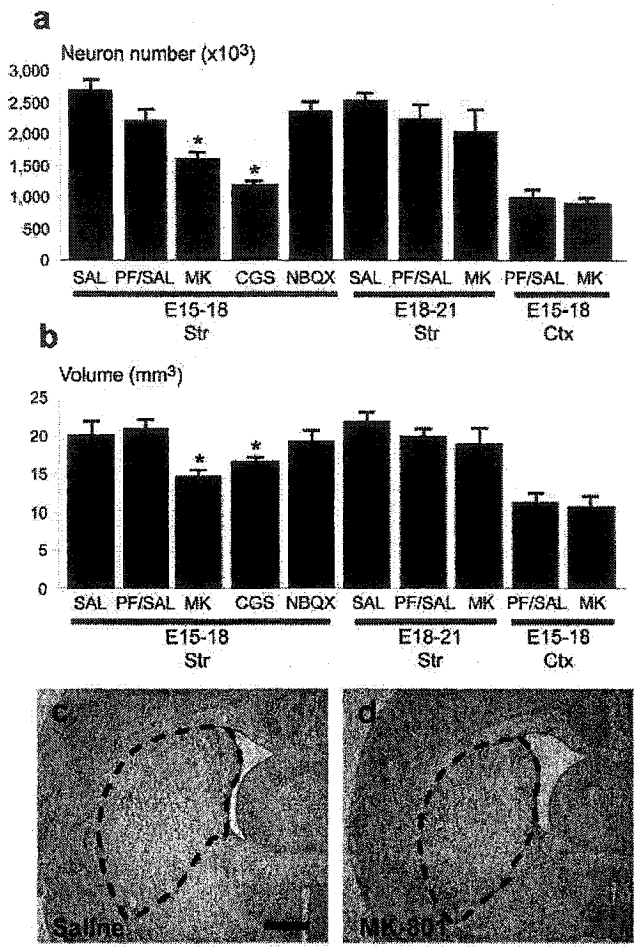

Figure 2. Total striatal and cortical neuron number and volume after treatment with glutamate receptor antagonists. Stereological estimates of the total number of striatal and cortical neurons for all experimental groups are shown (*a*). Treatment with glutamate receptor drugs extended between either E15–E18 or E18–E21, corresponding to the main proliferative and postproliferative periods, respectively, for neostriatal neurons. Striatal neuron number in rats was significantly reduced after administration of noncompetitive and competitive NMDA glutamate receptor antagonists (MK-801 and CGS 19755, respectively) when compared with saline (*SAL*) and pair-fed saline (*PF/SAL*) control groups (all data expressed as mean ± SEM; $F_{(7,24)} = 17.01$; $^*p < 0.01$ vs pair-fed control). Effects were statistically significant only after treatment between E15 and E18, but not between E18 and E21. Treatment with MK-801 did not significantly alter neuron number in the frontal agranular cortex, suggesting that NMDA receptor-mediated effects on proliferation are restricted to the striatum in this model. Striatal volumes (*b*) also showed a significant decrease after MK-801 or CGS-19755 treatment, likely reflecting the loss of neurons ($F_{(7,24)} = 12.32$; $^*p < 0.01$ vs pair-fed control). Treatment with NBQX did not change neuron number or striatal volume, suggesting that the observed effect is mediated by NMDA, but not AMPA–KA receptors. Decreased striatal volume and neuron number after exposure to MK-801 was visible at the level of the anterior commissure in cresyl violet-stained sections (*c*,*d*). Scale bars, 500 μm.

numbers in rats treated with either the noncompetitive antagonist MK-801 (1.61 ± 0.06 million) or the competitive antagonist CGS-19755 (1.20 ± 0.08 million) in comparison with both pair-fed (2.13 ± 0.14 million) and saline (2.58 ± 0.10 million) controls. Animals receiving either NMDA antagonist during the proliferative period also exhibited significantly reduced neostriatal volumes (MK-801: 14.8 ± 0.49 mm^3; CGS: 16.9 ± 0.32 mm^3), compared with pair-fed (19.9 ± 1.1 mm^3) and saline (20.1 ± 1.2 mm^3) control groups (Fig. 2*b*). These reduced volumes likely reflect reductions in striatal neuron number.

In contrast, *in utero* exposure to MK-801 during the predominantly postproliferative period for striatal projection neurons (E18–E21) did not result in statistically significant changes in striatal neuron number (2.0 ± 0.17 vs 2.11 ± 0.13 million) (Fig. 2*a*) or striatal volume (18.95 ± 0.28 vs 20.1 ± 0.92 mm^3) (Fig. 2*b*) in comparison with the pair-fed control group. Decreased neuron number after prenatal NMDA receptor antagonist exposure is therefore attributable mainly to reduced proliferation of striatal neuroblasts or precursors. To determine if proliferation of striatal neuroblasts is dependent on non-NMDA receptor-mediated glutamatergic mechanisms, as is the case in the dorsal telencephalic germinal zone (Haydar et al., 2000), dams were exposed to NBQX during the prenatal proliferative period. NBQX failed to significantly alter striatal volume or neuron number indicating that AMPA–KA receptor blockade does not influence proliferation of neuroblasts derived from the prenatal ventral telencephalon (Fig. 2*a*,*b*). Additionally, administration of MK-801 from E15 to E18 did not significantly alter neuron number or volume in the frontal agranular cortex (Fig. 2*a*,*b*), suggesting that the observed effects of the NMDA receptor antagonists are regionalized to the ventral telencephalon.

Progenitors of medium spiny GABAergic projection neurons likely account for the observed NMDA-mediated proliferative effects because this population comprises 90% of rat striatal neurons (Kitai, 1981; Smith and Bolam, 1990; Kawaguchi et al., 1995). Decreases in proliferation of precursors of other minor striatal neuronal subpopulations may also account for a small proportion of observed changes, although these were not distinguished using Nissl stains. For example, parvalbumin-positive GABAergic interneurons, which comprise ~1% of striatal neurons (Luk and Sadikot, 2001), likely contribute to the observed changes, because our previous studies indicate that proliferation of precursors of this subpopulation is also positively influenced by NMDA receptor activation (Sadikot et al., 1998).

To account for possible nutritional effects, pair-fed groups (E15–E18 and E18–E21) matched for food and water intake were used as controls. Striatal neuronal counts and volumes were lower in pair-fed groups compared with saline controls (Fig. 2), but the decreases did not reach statistical significance, suggesting that nutritional effects were minor in these experiments.

NMDA receptor blockade reduces proliferation in the ganglionic eminence

To observe the effects of NMDA receptor blockade on striatal development more directly, proliferation in the lateral ganglionic eminence was examined in embryos shortly after exposure to MK-801. The final administration of the antagonist was followed by 12 hr BrdU exposure to label cells in S-phase. Analysis of the ventricular zones of the ganglionic eminence revealed an 18% decrease in the percentage of BrdU-labeled nuclei with respect to saline controls after MK-801 exposure (Fig. 3*c–e*). Overall nuclear density in the LGE was similar in both treated and untreated animals (data not shown). The percentage of nuclei positive for BrdU in the subventricular zone was comparable in both MK-801-treated and control animals. Our *in vivo* data collectively indicate that NMDA, but not AMPA–KA, ionotropic glutamate receptors mediate an important proliferative effect on striatal neuroblasts or precursors. Interestingly, in the developing dorsal telencephalon, AMPA–KA activation increases DNA synthesis in cortical progenitors in the ventricular zone, whereas NMDA receptor activation has no effect on proliferation (Haydar et al., 2000).

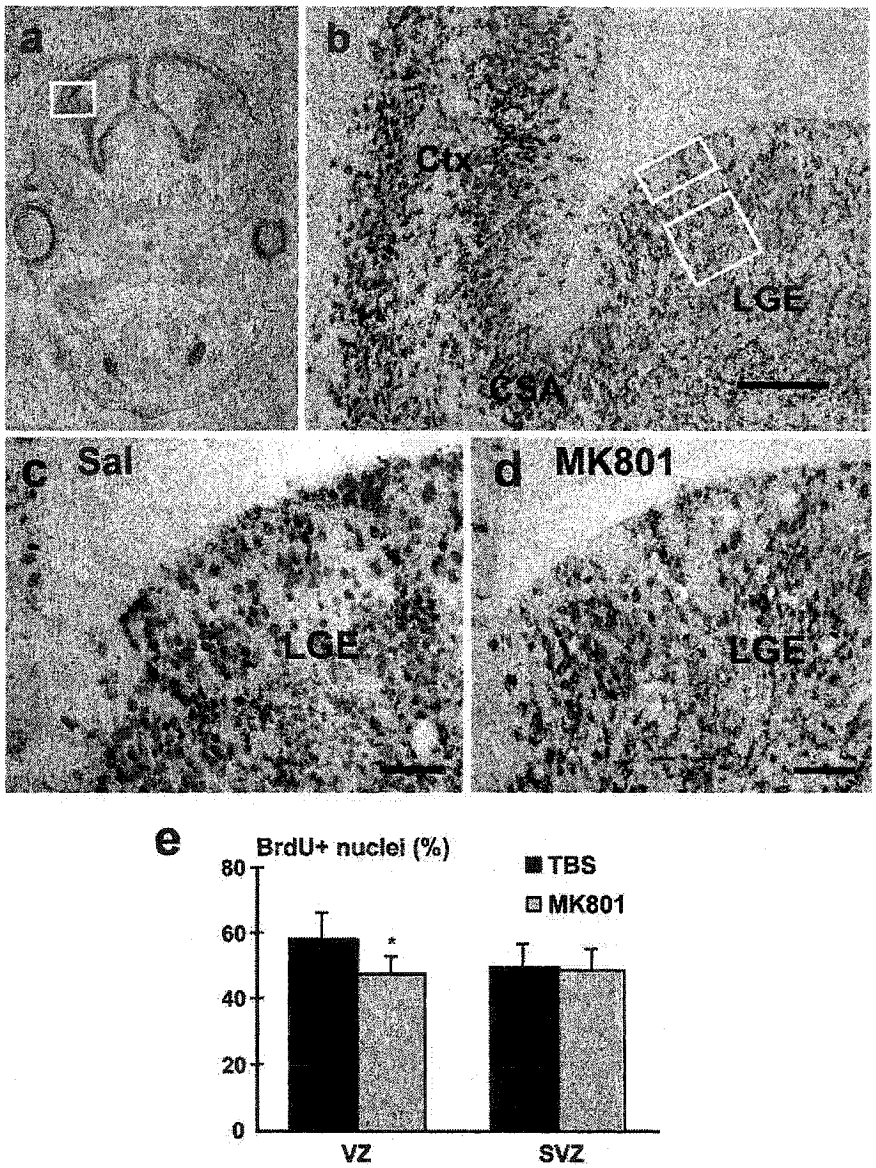

Figure 3. Effects of MK-801 treatment on embryonic basal forebrain proliferative zones. Embryonic rats were given MK-801 (0.2 mg/kg) on E15 and again on E16, followed by BrdU injection 1 hr later. Embryos were fixed 12 hr later at E16.5, sectioned, and stained using a monoclonal antibody against BrdU. *a, b,* The periventricular striatal proliferative zones were delineated 100 μm from the cortical striatal angle (*CSA*). An area 100-μm-wide and 50-μm-deep from the ventricular surface was assigned as the ventricular zone (*VZ*). The subventricular zone (*SVZ*) was defined as a 100 × 100 μm area located 50 μm ventral to the VZ (*b*). BrdU+ nuclei in the two zones were quantified after treatment with saline (*TBS*) and MK-801. *c, d,* Photomicrographs showing BrdU uptake near the CSA region after treatment. After treatment with the NMDA receptor antagonist, the percentage of BrdU+ nuclei was reduced in the VZ but not SVZ. (*e*; $^*p < 0.01$; $n = 4$). *Ctx,* Cortex; *LGE,* lateral ganglionic eminence. Scale bars: *b,* 100 μm; *c, d,* 40 μm.

Characterization of proliferating striatal neuroblasts *in vitro*

Dissociated cultures of proliferating striatal neuroblasts derived from E15 LGE were incubated in serum-free Neurobasal/B27 medium for 1–5 DIV (Ivkovic et al., 1997; Ventimiglia and Lindsay, 1998). Immunochemical markers revealed that the cultures were predominantly neuronal in composition (Fig. 4). A TuJ1 antibody that recognizes neuron-specific βIII-microtubulin, was used to label cells committed to a neuronal lineage (Geisert and Frankfurter, 1989; Moody et al., 1989). This antigen is not expressed by astrocytes or oligodendrocytes and is an early marker of neuronal differentiation, including neuroblasts undergoing mitosis and postmitotic neurons (Lee et al., 1990; Easter et al., 1993; Memberg and Hall, 1995; Jacobs and Miller, 2000). MAP-2, a specific marker for postmitotic and differentiated neurons (Johnson and Jope, 1992), was also used. Using DAPI as a nuclear counterstain, 42% of cells in 2 DIV cultures were positive for TuJ1, and 62% of cells were positive for MAP-2 (Matus et al., 1980). At 4 DIV, the majority of cells were also NeuN-positive (data not shown). GFAP staining cultures indicated that glia comprised <2% of the total cell population. These results are in agreement with previous reports suggesting that GABAergic medium spiny neurons and their precursors make up the majority of cells in these early cultures, with a minority of interneurons and glia (Ivkovic et al., 1997; Ventimiglia and Lindsay, 1998; Petersen et al., 2000). The results are also consistent with evidence that forebrain gliogenesis (with the exception of radial glia) occurs in the late prenatal and early postnatal periods (Schultze et al., 1974).

To quantify cell proliferation, cultures were exposed to the thymidine analog BrdU for a period of 24 hr after 1, 2, or 4 DIV. The proportion of nuclei that were positive for BrdU was maximal during the first 48 hr *in vitro* and decreased to low levels by 96 hr (data not shown). This is similar to the proliferative chronology *in vivo* (Bayer, 1984; Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987).

The presence of NMDA receptors *in vitro* was confirmed by staining with antibodies against subunits of the receptor heteromer. The NR1, NR2A, and NR2B subunits were detected in proliferative cultures by immunofluorescence (Fig. 5); however, no NR2C staining was detected. Immunoblots of lysates prepared from 2 DIV cultures showed similar results (data not shown). In double-labeling experiments, the NR1 subunit, which is common to functional NMDA receptors (Monyer et al., 1994), was detected in ~76% of BrdU+ cells after 4 hr of exposure (Fig. 5*a,b*). Smaller subsets of BrdU+ cells also expressed NR2A (Fig. 5*c,d*) and NR2B subunits (data not shown). These results indicate that dividing neuroblasts may express a functional form of NMDA receptor.

NMDA receptor activation promotes proliferation of striatal neuronal precursors *in vitro*

To identify the role of NMDA receptors in neuroblast proliferation, 1 DIV embryonic striatal cultures were exposed to NMDA antagonists (MK-801, CGS-19755), NMDA (1–100 μM), or vehicle. Similar cultures were also exposed to NBQX to compare NMDA and AMPA–KA receptor-mediated effects. Cultures were incubated in medium containing drug or vehicle for 24 hr, followed by a brief 2 hr exposure to BrdU before fixation (Fig. 6).

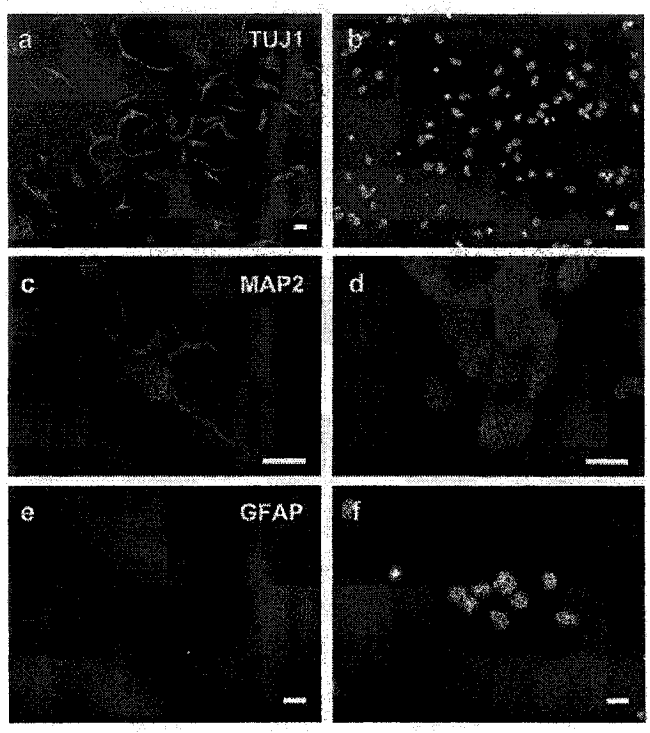

Figure 4. Embryonic striatal cultures. Cells obtained from rat ganglionic eminence at E15 were cultured in serum-free defined medium. Neurons were immunostained against TUJ1 (*a*) and MAP2 (*c*) after 2 DIV. GFAP staining did not reveal the presence of any astrocytes, suggesting a population of predominantly neuronal cells *in vitro* (*e*). DAPI counterstaining for *a*, *c*, and *e* is found in *b*, *d*, and *f*, respectively. Scale bars, 10 μm.

BrdU labeling in this preparation was therefore limited to proliferating populations that were in S-Phase at the time BrdU was added. The presence of either MK-801 or CGS-19755 reduced the percentage of BrdU+ nuclei to <50% of control levels (Fig. 6*a*). To further establish that NMDA receptors mediate the effects of glutamate on cell proliferation, cultures were exposed to varying concentrations of NMDA.

Exposure to 1 μM NMDA resulted in a 29% increase in proportion (and density, data not shown) of BrdU+ nuclei (Fig. 6*a*). However, progressively higher concentrations of NMDA (10, 100 μM) resulted in decreases in the proportion of both BrdU+ nuclei (Fig. 6*a*) and cells positive for neuronal markers (MAP-2 or TuJ-1) (Fig. 6*b*,*c*), in keeping with the expected excitotoxic effects at these doses (Koroshetz et al., 1990). Despite marked reduction in BrdU uptake, cultures exposed to NMDA antagonist for 24 hr showed no significant reduction in MAP2+ cell number, suggesting that maturation and survival of postmitotic neurons was unaltered. On the other hand, the density of neurons expressing the earlier neuronal marker TuJ1+ decreased significantly after exposure to CGS-19755 or MK-801 (Fig. 6*b*). The contrasting results with the two markers are in keeping with the observation that MAP-2 is expressed in the processes of more mature neurons (Johnson and Jope, 1992), whereas TuJ1 expression is initiated during the final mitosis of neuronal progenitors (Memberg and Hall, 1995; Jacobs and Miller, 2000). Reduction in the number of TuJ1+ neurons may therefore reflect decreased proliferation of neuroblasts giving rise to early postmitotic TuJ1+ populations. These results collectively suggest that NMDA-mediated glutamatergic mechanisms have a marked influence on neuroblast proliferation, but not on survival of postmitotic neurons. Exposure to NBQX had no effect on the proportion of cells with BrdU+ nuclei, or TuJ1+ and MAP-2+ cells, indicating lack of an effect of AMPA–KA on proliferation of striatal progenitors, in keeping with our *in vivo* results.

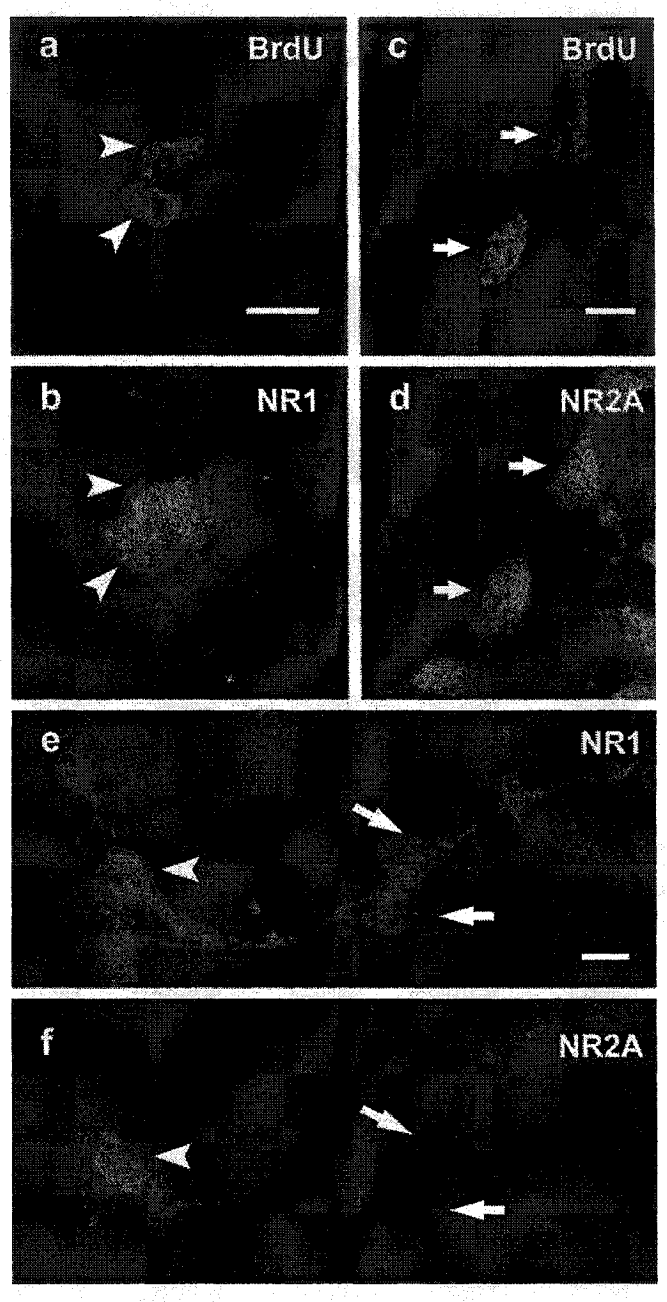

Figure 5. NMDA receptor profile of embryonic striatal cultures. Embryonic neostriatal cultures were fixed at 1 DIV after 4 hr BrdU exposure and immunostained using antibodies against various NMDA receptor subunits. A large number of cells were positively stained for the NR1 subunit. A small subset of NR1-immunoreactive cells colocalized with BrdU, indicating that proliferative or recently postmitotic cells express functional NMDA receptor subunits (*a*,*b*). Staining using polyclonal antibodies against NR2 subunits also revealed a significant number of BrdU+ cells expressing the NR2A subunit (*c*,*d*). Coexpression of the NR2A subunit was also detected in a subpopulation of NR1-positive cells (*e*,*f*; indicated by *arrowheads*). However, no staining for the NR2C subunit was observed. Scale bars: *a*, *c*, 10 μm; *e*, 12 μm.

To determine whether NMDA-mediated glutamatergic mechanisms influence cell proliferation after short-term exposure, 1 DIV cultures were incubated with the same agonist/antagonists for only 4 hr followed by a 2 hr pulse of BrdU before fixation (Fig. 7*a*). Given estimated cell cycle times of 10–20 hr for

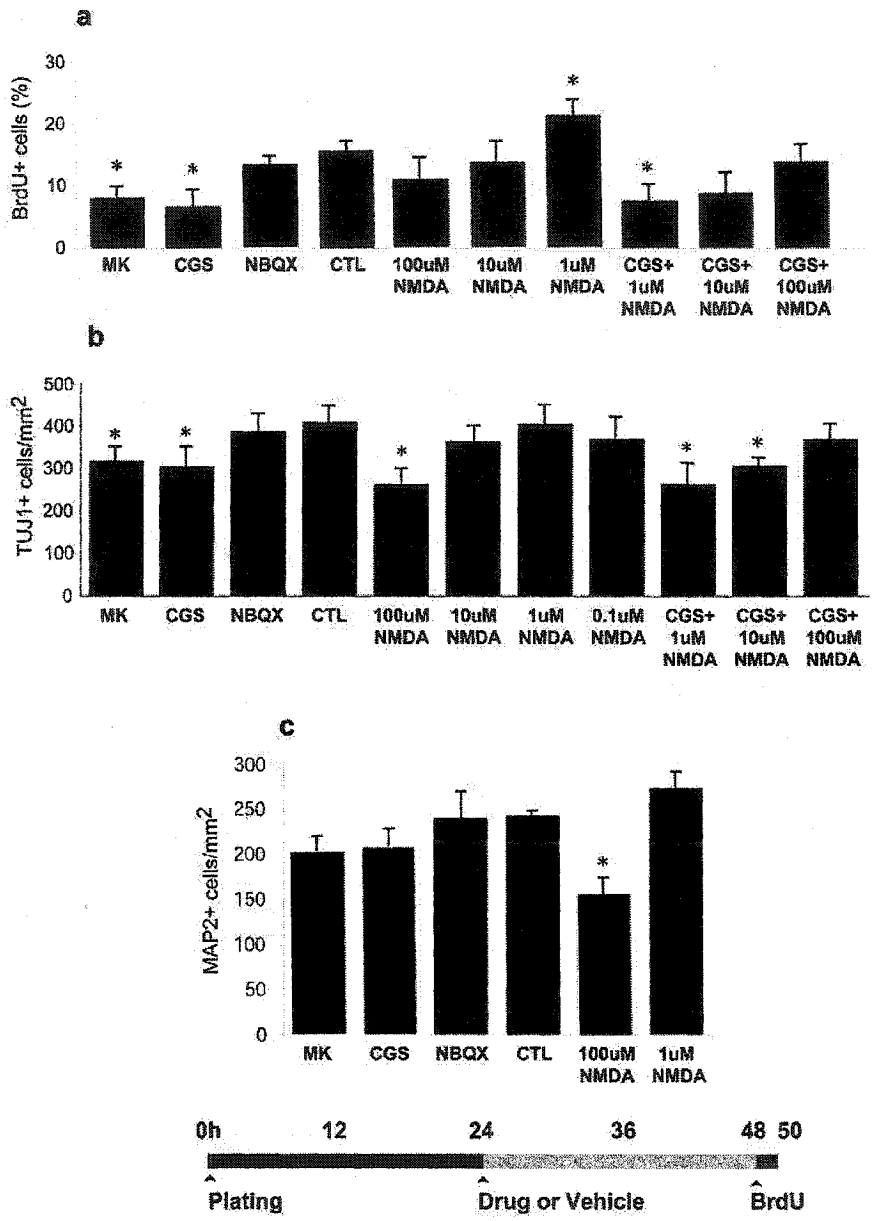

Figure 6. Effects of glutamate receptor antagonists and NMDA after 24 hr exposure. Striatal cultures of 1 DIV were exposed to different ionotropic glutamate receptor antagonists, NMDA, or vehicle control. *a*, Treatment duration was 24 hr and was followed by 2 hr exposure to BrdU (20 μg/ml) and fixation in PFA (*diagram*). Treatment with MK-801 or CGS 19755 significantly reduced the proportion of nuclei incorporating BrdU with respect to control cultures. Effect of the competitive antagonist (*CGS*) could be countered by addition of NMDA. Addition of 100 μM NMDA to CGS-treated cultures restored BrdU uptake close to normal levels. Exposure to NBQX did not alter BrdU uptake, suggesting that NMDA but not AMPA receptor blockade results in decreased proliferation. In cultures in which only the agonist was added, BrdU uptake was inversely proportional to NMDA concentration. At 1 μM, NMDA significantly upregulated proliferation, whereas increasing doses exhibited toxic effects. *B*, The proportion of Tuj1-positive cells were also quantified after each treatment. Exposure to MK-801 or CGS 19755 reduced the proportion of TUJ1 cells, suggesting that production of early postmitotic neurons was reduced after receptor blockade. Tuj1 cell number also decreased after treatment with 100 μM NMDA. *c*, MAP2+ cell number did not alter significantly after treatment, with the exception of NMDA at excitotoxic concentrations, suggesting that the short-term survival of more mature neurons was not affected by the agents added. Data expressed as mean ± SEM ($n = 3$; $^*p < 0.01$ vs control).

striatal progenitors (Acklin and van der Kooy, 1993; Bhide, 1996) this experiment has the advantage of minimizing possible fluctuations in cell number caused by proliferation or apoptosis during the treatment period.

As in the previous experiment, exposure to either MK-801 or CGS-19755 resulted in a significant decrease in the percentage of BrdU+ nuclei (−35 and 33%, respectively) compared with control cultures (Fig. 7*a*), indicating that effects on neuroblast proliferation can be detected within 4 hr after initial receptor blockade. In keeping with the hypothesis that NMDA positively influences proliferation, exposure to 1 μM NMDA also increased the percentage of cells with BrdU+ nuclei, although this value did not reach strong statistical significance (Fig. 7*a*) ($p < 0.03$). The density of MAP-2+ and TuJ1+ neurons after exposure to either glutamate receptor antagonists or low concentrations of NMDA remained unchanged (Fig. 7*b*,*c*). Unaltered neuronal density is expected because the treatment period is within the cell cycle time for striatal neuroblasts. However, the addition of 100 μM NMDA to cultures reduced the density and number of both TuJ1+ and BrdU+ nuclei, respectively, indicating toxicity. As in previous experiments, there was no significant change in BrdU uptake after application of NBQX.

To determine whether the reduction in proliferation after NMDA receptor blockade is reversible, NMDA was added at various concentrations to cultures treated with the competitive receptor antagonist CGS-19755. A dose-dependent rescue was observed in which NMDA concentration was directly proportional to the number of BrdU+ nuclei (Fig. 7*a*). With the addition of 100 μM NMDA, BrdU uptake in CGS-19755-treated cultures recovered to 80% of control levels. However, no recovery in proliferation was observed with application of MK-801, in keeping with the noncompetitive nature of this receptor antagonist (data not shown). These experiments collectively suggest that NMDA-mediated, but not AMPA–KA-mediated, glutamatergic mechanisms promote proliferation of striatal neuronal progenitors.

Discussion

In the present study, we identify a novel role for the classical neurotransmitter glutamate in promoting proliferation of neuronal precursors derived from the germinal zone of the ventral telencephalon. Using an *in vivo* model, we demonstrate that proliferation of progenitors of the principal neurons of the striatum is dependent on activation of NMDA glutamate receptors. We further investigated ionotropic glutamate receptor-mediated effects in proliferative cultures derived from the lateral ganglionic eminence, the main source of striatal progenitors. Our results indicate that NMDA receptor-mediated glutamatergic activity promotes proliferation of striatal neuronal progenitors. Conversely, AMPA–KA-mediated mechanisms have no significant

effect on proliferation of striatal progenitors. In contrast, previous studies indicate that cortical neuronal progenitors proliferate in response to activation of AMPA–KA, but not NMDA receptors. We propose that the germinal epithelium of the embryonic telencephalon is spatially heterogeneous with respect to proliferative response to glutamate. Regional variation in neurotransmitter effects on proliferation in the germinal zones of the telencephalon may be an important novel mechanism for generating neuronal phenotypic diversity in the forebrain.

Glutamate promotes proliferation in the ventral telencephalon by an NMDA receptor-mediated mechanism

Little is known about factors governing proliferation of neuronal progenitors of the striatum, the major derivative of the ventral telencephalon. Growth factors including epidermal growth factor, basic fibroblast growth factor, and transforming growth factor β modulate proliferation in the developing ventral forebrain (Reynolds and Weiss, 1992; Temple and Qian, 1995; Cavanagh et al., 1997; Ciccolini and Svendsen, 1998). Recent evidence suggests that amino acid neurotransmitters play an important role in proliferation and survival of forebrain neurons. $GABA_A$ receptor activation, which can depolarize embryonic neurons (Cherubini et al., 1991), mediates survival, but not proliferation of striatal neuronal progenitors (Ikeda et al., 1997; Luk and Sadikot, 2001). The role of glutamate-induced depolarizing activity in early morphogenesis of the striatum remains primarily unexplored.

The present results indicate that non-NMDA ionotropic receptors do not influence proliferation of striatal neuronal precursors. However, the *in vivo* and *in vitro* evidence suggests that NMDA receptors play an important role in proliferation of ventral telencephalon-derived striatal progenitors. Prenatal exposure to NMDA antagonists results in a marked decrease in neuronal proliferation. *In utero* exposure to NMDA antagonists during the post-proliferative period has no effect on neuronal survival, in keeping with previous work (Ikonomidou et al., 1999). Interestingly, postnatal exposure to NMDA antagonists dramatically increases developmental cell death, suggesting NMDA exerts a trophic influence during the major period of striatal neuronal apoptosis (Ikonomidou et al., 2000).

The GABAergic projection neurons, which comprise 90% of striatal neurons (Smith and Bolam, 1990), are a major target of NMDA-mediated proliferative effects. Previous work suggests that NMDA also promotes proliferation of other striatal populations such as parvalbumin-positive GABAergic interneurons (Sadikot et al., 1998) that originate in the medial ganglionic eminence (Lavdas et al., 1999; Marin et al., 2000). Because striatal projection neurons arise mainly from the LGE, NMDA receptors mediate proliferation of neuronal precursors arising from both major germinal zones of the ventral telencephalon. Recent evidence suggests a large proportion of neocortical (de Carlos et al.,

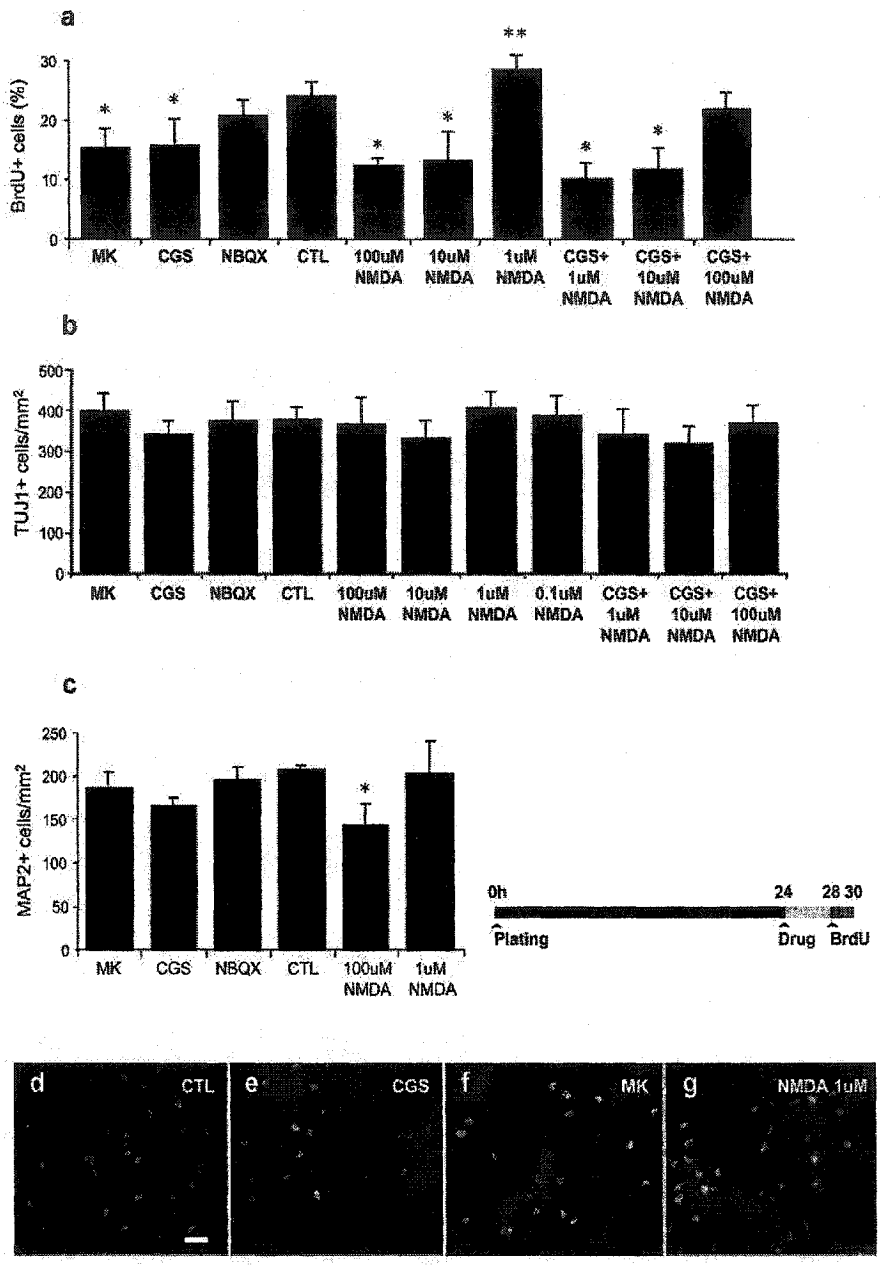

Figure 7. Effects of glutamate receptor antagonists and NMDA after 4 hr exposure. Striatal cultures of 1 DIV were exposed to various ionotropic glutamate receptor antagonists, NMDA, or vehicle control for 4 hr, followed by 2 hr exposure to BrdU (*timeline*). Because treatment duration was much shorter than the expected cell cycle time for these cultures, the changes in cell population number caused by drugs should be minimized. *a*, After 4 hr treatment with MK-801 or CGS 19755, BrdU incorporation was significantly reduced, suggesting that NMDA receptor blockade exerts its influence on proliferation within this period. BrdU uptake in cultures treated with CGS 19755 could be restored to near control levels by adding increasing concentrations of NMDA. As in previous culture experiments, exposure to NBQX did not alter BrdU uptake. Exposure to NMDA at high concentration (100 μM) resulted in decreased numbers of BrdU+ nuclei, whereas at 1 μM, proliferation was above control levels. *b*, *c*, No significant alterations in the proportion of Tuj1+ (*b*) or MAP2+ (*c*) cells were observed after any of the treatments (data expressed as mean ± SEM, $n = 3$; $^*p < 0.01$; $^{**}p < 0.05$ vs control). *d–g*, Photomicrographs of BrdU-labeled cells *in vitro* after exposure to NMDA and receptor antagonists. Scale bar, 8 μm.

1996; Anderson et al., 1997; Tamamaki et al., 1997; Marin et al., 2000; Parnavelas, 2000) and hippocampal GABAergic interneurons (Pleasure et al., 2000) are derived from the ventral telencephalon. NMDA-dependent proliferation may thus be a common property of all forebrain GABAergic interneuronal precursors. Whether NMDA effects are restricted to GABAergic neurons or extend to cholinergic and somatostatinergic subtypes produced in the ventral germinal zones remains to be addressed.

Proliferative responses to NMDA are regionally diverse in forebrain germinal zones

With the exception of GABAergic interneurons (Marin et al., 2000), cells generated in the germinal zones of the dorsal and ventral telencephalon remain primarily separate during forebrain morphogenesis (Fishell et al., 1993). Furthermore, the dorsal and ventral zones express distinct transcription factors, some of which are implicated in neurogenesis (Shimamura et al., 1995; Metin et al., 1997; Casarosa et al., 1999). Cells in the proliferative epithelium generating the cortex or striatum on either side of the corticostriatal boundary (Holmgren, 1925; Puelles et al., 2000; Inoue et al., 2001) may therefore show distinct responses to the extracellular environment. Regional differences in proliferative behavior of neuronal progenitors are evident in response to amino acid neurotransmitters (Cameron et al., 1998; Contestabile, 2000). In cortex, both glutamate and GABA regulate proliferation of neuronal precursors (LoTurco et al., 1995; Haydar et al., 2000). AMPA–KA receptors mediate altered DNA synthesis in cortical progenitors (LoTurco et al., 1995), but have no effect on proliferation of striatal progenitors. Such regional differences are also apparent with respect to NMDA-mediated responses. NMDA receptor activation promotes proliferation of striatal precursors, has no effect on proliferation of neocortical progenitors (LoTurco et al., 1995), and reduces proliferation of hippocampal granule cell precursors (Cameron et al., 1995; Seki and Arai, 1995).

Recent evidence in the dorsal telencephalon suggests that proliferative responses may vary between cellular subtypes. For example, activation of AMPA–KA receptors increases proliferation of neuronal precursors in the ventricular zone, but decreases proliferation in the subventricular zone (Haydar et al., 2000), a domain that generates mainly glia (Sidman et al., 1959; Altman, 1969). Our data suggest that NMDA effects on proliferation of neuronal progenitors in the ganglionic eminence occur mainly in the ventricular zone, with no effect in the subventricular zone. It would be of interest to determine whether distinct responses also exist among neuronal and glial precursors derived from the ventral germinal zones.

Potential mechanisms mediating NMDA effects on proliferation

Calcium entry after NMDA receptor activation mediates a variety of developmental effects in the CNS (Ascher and Nowak, 1988; Yuste and Katz, 1991), including cell survival (Balazs et al., 1988; Bhave and Hoffman, 1997; Ikonomidou et al., 2000), neurite outgrowth (Mattson and Kater, 1987), and synaptic plasticity (Stevens et al., 1994). The downstream intracellular mechanisms that may couple NMDA receptor-mediated calcium entry to DNA synthesis in striatal progenitors are unknown. Extracellular regulated kinases (ERKs) have been observed to mediate proliferative responses invoked by a variety of growth factors (Finkbeiner and Greenberg, 1996; Fukunaga and Miyamoto, 1998). With respect to classical neurotransmitter effects, proliferation in cortical progenitors after muscarinic receptor activation appears to be mediated by phosphatidylinositol-3-kinase (PI3) and ERK signaling pathways (Li et al., 2001). NMDA receptor activation in striatal neurons has been demonstrated to initiate this pathway in a PI3-dependent manner (Vincent et al., 1998; Schwarszschild et al., 1999; Perkinton et al., 2002). Components of this cascade (e.g., CaM-kinase, Ras, Mek, ERK1/2, and PI3) can be localized to glutamatergic postsynaptic densities (Husi et al., 2000). The ERK–PI3 pathway is therefore a strong candidate mechanism coupling NMDA receptor activation to DNA synthesis in striatal progenitors.

Contrasting proliferative responses to NMDA receptor activation during CNS morphogenesis may be the result of spatial and temporal differences in glutamate receptor expression (Goebel and Poosch, 1999; Kovacs et al., 2001). Both NMDA and non-NMDA ionotropic receptors are widely expressed in the prenatal rat telencephalon and exhibit developmentally regulated patterns of subunit expression that determine distinct functional effects (Misgeld and Dietzel, 1989; Monyer et al., 1994; Landwehrmeyer et al., 1995; Wullner et al., 1997; Nansen et al., 2000). *In vitro*, cells derived from the LGE exhibit NMDA responses (Vincent et al., 1998). Here, we provide evidence for NR1 and NR2 subunits in proliferating neuroblasts, suggesting a role for a functional channel in the observed events. Developmental variations in local glutamate concentration (Haydar et al., 2000), or interaction between NMDA receptors and other growth factors (Dobbertin et al., 2000; Roceri et al., 2001) may also contribute to variations in functional effects. Finally, distinct intracellular downstream mechanisms coupling NMDA receptor activation to the cell cycle machinery may also underlie variations in proliferative responses (for review, see Platénik et al., 2000).

Relevance to developmental abnormalities

Disruptions in forebrain development because of pathological responses to amino acid neurotransmitters may be relevant to a wide variety of human diseases, including schizophrenia (Harrison, 1999), prenatal brain injury from trauma or ischemia (Vexler and Ferriero, 2001), cortical dysplasias (Flint and Kriegstein, 1997), and developmental disorders after prenatal exposure to drugs of abuse (e.g., PCP, ethanol; Deutsch et al., 1998; Tabakoff et al., 1991), sedatives, anticonvulsants, and anesthetics (Reich and Silvay, 1989; Jevtovic-Todorovic et al., 1998; Morrell, 1999). The importance of NMDA-mediated glutamatergic mechanisms was recently highlighted in the pathogenesis of fetal alcohol syndrome (FAS; Ikonomidou et al., 2000). Magnetic resonance imaging in human FAS reveals markedly reduced volumes in the striatum as well as the cerebral cortex and cerebellum (Mattson et al., 1994; Archibald et al., 2001). Ethanol acts at NMDA and $GABA_A$ receptor sites (Lovinger et al., 1989; Hoffman et al., 1989; Mihic, 1999). Based on previous work, alcohol-induced activation of $GABA_A$ receptors would not be expected to mediate a proliferative effect on striatal neuronal precursors (Ikeda et al., 1997; Luk and Sadikot, 2001). In rodent models, exposure to MK-801 or alcohol results in a dramatic increase in postnatal apoptosis in the striatum, supporting the hypothesis that NMDA receptors mediate a trophic effect (Ikonomidou et al., 1999, 2000). Our current data suggests that alcohol and other NMDA antagonists may also impair neuroblast proliferation, and therefore contribute to abnormal striatal morphogenesis as early as the first trimester of human pregnancy (O'Rahilly and Müller, 1994). In conclusion, temporal and spatial variations in morphogenic responses to glutamate play an important role in normal development and may contribute to distinct abnormalities of forebrain development in a wide variety of pathologies.

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