"Development of Compartment Phenotypes in the Mammalian Striatum"

by

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Abstract

This thesis focuses on the development of the major morphological and functional units of the striatum: the striosomes and the matrix. During nervous system development, different immature phases, highly influenced by the afferent inputs, occur before the final structural and functional organization is established.

Major inputs to the striatum come from the substantia nigra, the cortex, the raphe nuclei and the thalamus. The afferents from the substantia nigra are not easily accessible to external manipulation in virtue of being hidden in the midbrain and close to neural centers crucial to survival.

To study the nigrostriatal interactions of the dopamine pathway we turned to an *in vivo* non-invasive pharmacological approach. Dopamine acts through two classes of receptors, the D1-class and the D2-class. D1-class dopamine receptors activate the enzyme adenylyl cyclase, increasing the level of the second messenger cAMP, whereas D2-class dopamine receptors have an inhibitory influence. Fos, Fra and NGFI-A, transcription factors of the immediate-early gene (IEG) family, expressions are sensitive to modifications caused by changes in cAMP level. Using the expression of Fos, Fra and NGFI-A to monitor changes at the single and population levels, we tested striatal functional responsiveness to dopaminergic stimulation during development. Dopaminergic indirect agonists, such as cocaine and amphetamine, have as their final effect the increase in the level of dopamine at the nigrostriatal synapses. We found that pharmacological dopaminergic stimulation leads to increases in IEG expression in individual developing striatal neurons. Moreover, the population profile of activated neurons changes during development. We found that, from the time of birth, the increase in IEG level of expression is mediated by the D1 pathway since pretreatments with the D1-selective antagonist, SCH23390, completely block IEG induction. On the contrary, the D2-selective antagonist, eticlopride, induces IEG expression. Moreover, we determined that D2 functional responsiveness appears to be confined to the striosomal compartment for a prolonged period of development despite the expression of D2 ligand binding site and mRNA in the matrix. We further tested the hypothesized modulation of glutamatergic and dopaminergic signals. Our results indicate that the cortical glutamatergic modulation of the dopaminergic input starts from the first postnatal week. From the evidence collected, we propose the presence of two distinct modes of regulation for Fos, Fra and NGFI-A proteins: a constitutive and an inducible. Constitutive expression of Fra and NGFI-A proteins is present even after dopamine or glutamate receptors blockade and the spatial and temporal patterns of expression are unaffected during postnatal development. This suggests that intrinsic programs of development may proceed in parallel with dopaminergic and glutamatergic activity-dependent modeling of striatal function.

The inducible and constitutive Fos, Fra and NGFI-A patterns of expression observed clearly define two prenatal and postnatal phases of striatal development. In

addition, an intermediate phase which overlaps both pre and postnatal periods has also been observed. During prenatal development IEG expression is confined to the developing striosomal compartment. This prenatal period could be divided according to IEG expression and striosomal neurons localization, into two phases. In the first phase, which goes from embryonal day 14 (E14) to E15, IEG expression and striosomal neurons are located in the VENTROLATERAL area, especially in the caudal striatum. This is followed by a DISPERSED phase (from E16-E17) in which the IEG pattern of expression and striosomal neurons are dispersed throughout the striatum. In the STRIOSOMAL (intermediate) phase, which spans a long time interval, from E18 to postnatal day P7, IEG expression and striosomal neurons cluster into the well-known STRIOSOMES. This phase also extends into the first postnatal week, by the end of which, inducible and basal IEG expressions start to spread beyond the striosomal compartment. The postnatal phases begin with the second postnatal week defining a TRANSITORY PERIOD characterized by a transient phase of vivid inducible IEG expression in the globus pallidus and in the substantia nigra in contrast with weak IEG protein product inductions in the striatum. By the third postnatal week, IEG expression enters the MATURE phase characterized by patterns typical of adulthood. We suggest that IEG developmental modifications in the patterns of expression manifest an underlying maturation of the circuits.

To study striatal development in a system accessible to external manipulation and to test the possibility of cell migration and compartment formation in a system deprived of most long range information, we developed an *in vitro* embryonal organotypic system. We labeled matrix cells with the thymidine analog 5-bromo-2-deoxyuridine (BrdU) and tracked the migration in slice cultures for increasing time periods. Indeed, we found that matrix cells progressively migrate from the germinal zone deeply into the striatum. We further investigated whether compartment formation occurs without intact nigrostriatal afferents by tracking labeled striosomal neurons in organotypic slices. We found clusters of BrdU-labeled cells only in some cultures. Even in cultures cut to preserve the substantia nigra, the formation of BrdU-positive clusters by striosomal neurons was not consistent. However, using the immunodetectable expression of the transcription factor Fra as putative marker for striosomal neurons, we detected Fra-positive patches in the medial and rostral striatum. To check whether the slice system affected the survival and differentiation of the striatal cells, we stained slices with the neurodifferentiation marker, microtubule associated protein-2 (MAP-2). At all time intervals characterized, healthy slices showed MAP-2 expression with a pattern of expression similar to the one we detected in vivo. We propose that the nigrostriatal innervation is not necessary for striatal cell migration. Whether it is possible to consistently obtain compartment formation in cultures is still unclear; however, we found that the organotypic slice system does not disrupt the already aggregated prenatal Fra-positive striosomes at E18, providing a valuable tool for analyzing the molecular mechanisms of prenatal striosome/matrix phenotypic development.

Thesis Supervisor: Dr. Ann M. Graybiel, Walter A. Rosenblith Professor

With all my heart,

To all the people that have supported me
from a smile to a scientific, technical or academic advice

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CHAPTER 1

Introduction

Mammalian Corpus Striatum

The striatum is a forebrain region included in a set of structures collectively called the basal ganglia. The basal ganglia participate in the high level functional processing related to motor control. However, increasing evidence from human patients indicate that the basal ganglia influence also cognitive and affective functions of the brain (review in Graybiel, 1995).

The basal ganglia are formed by the caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra. The globus pallidus has a diencephalic origin and it is subdivided into internal and external segments. The substantia nigra is in the midbrain and is composed by two regions: the pars compacta and the pars reticulata. The caudate nucleus and the putamen are of telencephalic origin and in rodents are collectively called the striatum. In contrast to the motor cortex, which innervates directly interneurons and motor neurons in the spinal cord and brainstem, the basal ganglia do not act directly on motor neurons but instead influence the cortex and brainstem regions such as the superior colliculus. Primary input to the basal ganglia is from the cortex and target outputs are directed through the ventrolateral (VL), the ventral anterior (VA) and the mediodorsal (MD) nuclei of the thalamus back to the motor, premotor and prefrontal cortices.

Connections of the Striatum

The striatum is the input structure of the cortico-basal ganglia functional loop. It receives its major afferents from the neocortex, the amygdala, the intralaminar nuclei of the thalamus (centromedian, CM and parafascicular nuclei, PF), the dopamine containing

substantia nigra pars compacta and the serotonin-containing raphe nuclei, and the striatum projects to the globus pallidus and the substantia nigra (review in Graybiel, 1990) (Fig.1).

The striatal inputs from the substantia nigra pars compacta and the cortical projections have been well characterized both anatomically and, to a lesser extent, physiologically. The cortical projection is glutamatergic while the nigra afferents are dopaminergic (Fig.1). Each corticostriatal or nigrostriatal fiber contacts many striatal neurons spread over a relatively large region of the striatum. For the corticostriatal fibers, it is estimated that 10-15,000 fibers converge on a single striatal projection neuron. At the synaptic level, glutamatergic and dopaminergic afferents can both terminate in relation to single spines of the striatal projection neurons, the corticostriatal afferent on the head and the nigrostriatal afferent on the neck of the same dendritic spine (Fig.2). It is claimed that the axon terminals of dopaminergic and glutamatergic fibers in the striatum possess receptors for the other neurotransmitter: presynaptic glutamate receptors on the nigrostriatal terminals (Wang, 1991; Desce et al., 1992) and dopamine receptors on the corticostriatal terminals (Maura et al., 1989; Yamamoto and Davy, 1992) (Fig.2). These arrangements show the potential for interactions between the dopaminergic and glutamatergic inputs. Indeed, it has been shown that local striatal administration of glutamate, or glutamate increment caused by cortical stimulation can increase the release of dopamine in the striatum (Wang, 1991; Desce et al., 1992). Conversely, increased extracellular levels of dopamine can lead to a decrease in glutamate release (Rowlands and Roberts, 1980; Maura et al., 1989; Yamamoto and Davy, 1992).

Striatal outputs are GABAergic and have inhibitory actions on the globus pallidus and the substantia nigra (Fig.1). The striatal projection is divided into two major pathways. Striatal neurons that give rise to the "direct pathway" (from striatal matrix to SNpr and GPi) express the neuropeptides dynorphin and substance P, whereas those that give rise to the indirect pathway (from the matrix to GPe) predominately express the neuropeptide

enkephalin (Fig.1). The direct pathway has as a final effect the activation of movement, whereas the indirect pathway has an inhibitory effect on movement.

Structure of the Striatum

Cytoarchitectonically, there is a striking contrast between the striatum and the neocortex. In conventional cell stains, the striatum appears uniform. It has neither a clear laminar organization such as that found in the cortex, nor a large morphological variety of neurons. More than 90% of the neurons are of the same type, namely, medium spiny projection neurons. Despite this apparent uniformity, much research has shown that the striatum has two major neurochemicai subsystems or compartments: the striosomes or patches and the matrix. In the context of the mammalian striatum, the term compartment is used to denote groups of contiguous cells with a common phenotype and does not necessarily denote a developmental compartment which defines a contiguous group of clonally related cells. The compartments are recognizable at the level of their different birthdates, their different connections and the cellular biochemistry of their neurons (review in Graybiel and Ragsdale, 1978; Graybiel and Ragsdale, 1983; Graybiel, 1990; Gerfen, 1992). The striosomes are made up of early-born striatal neurons and are innervated predominately by the limbic cortices, whereas the matrix is innervated by the sensory-motor and association cortex (Ragsdale and Graybiel, 1990). Matrix neurons, mostly generated later, project to both the globus pallidus and the substantia nigra pars reticulata, whereas striosomal neurons project to the dopamine-containing substantia nigra pars compacta (Graybiel, 1990; Gerfen, 1992). In rodents it has been shown that the striosomal and matrix neurons receive inputs preferentially from deep and superficial cortical layers, respectively (Gerfen, 1989). A relative selectivity of inputs to each compartment is suggested by the restriction to a single compartment of the dendritic tree of most medium spiny neurons (Walker et al., 1993). The axonal fields of many spiny neuron collaterals are also restricted mostly into a compartment (Penny et al., 1988). Moreover, both

compartments receive dopaminergic input from the substantia nigra pars compacta but only the striosomal neurons project back to it. Phenotypically, the striosome and matrix pattern is evident in the differential expression of nearly every neuroactive substance found so far in the striatum (review in Graybiel and Ragsdale, 1983; Graybiel, 1990). Some molecules, such at the neuropeptide dynorphin, substance P and the μ-class of opioid receptors have a predominant expression in striosomes (Herkenham and Pert, 1981; Moon-Edley and Herkenham, 1984; Ni and Jonakait, 1988). Other molecules, such as the neuropeptide enkephalin, are concentrated in the matrix compartment (Beckstead and Kersey, 1985; Graybiel and Chesselet, 1984). The compartmental expression of these phenotypic markers shows some species variation. For example, enkephalin, considered a marker for the matrix compartment in the monkey and mouse striatum, appears more evenly expressed in the rat (Graybiel and Chesselet, 1984).

Development of the Striatum

Development of the structural organization of the striosome/matrix pattern

How does the striosome-matrix pattern emerge during development? During development of the central nervous system, neuroblast cells divide in the proliferative layers of the ventricular zone (VZ) and subventricular zone (SVZ). When cells stop dividing, they migrate towards their final locations where they continue to differentiate, extending processes and making synaptic contacts (review in McConnell, 1995). The embryonic age at which a certain type of neuron was at the final or near final DNA synthesis phase can be identified using a radioactive DNA base such as [^{3}H] thymidine, or a DNA base analog such as BrdU. With this approach it has been shown that striosomal neurons are among the first to become postmitotic and migrate into the striatum (van der Kooy and Fishell, 1987; Graybiel, 1984; Graybiel and Hickey, 1982). In the mouse, we found that patch neurons start to be born at embryonic day 11 (E11), whereas in the rat, striosomal neurons start to be born at E13 (Marchand and Lajoie, 1986; van der Kooy and

Fishell, 1987). Subsequently, the matrix neurons become postmitotic and migrate into the striatum (van der Kooy and Fishell, 1987; Graybiel and Hickey, 1982; Song and Harlan, 1994a; see also Angenive and Connell, 1974; Fentress et al., 1981; Bayer, 1984). In the rat, the majority of matrix neurons becomes postmitotic between E17 and E20 (Marchand and Lajoie, 1986; van der Kooy and Fishell, 1987). In the mouse, we observed that most of the matrix neurons become postmitotic between E14 to E17. There is a rough topography in this process, so that the earliest born cells are eventually positioned mostly in the lateral and caudal striatum and the later born cells are medial and rostral. The striosomal and matrix cells appear initially to intermingle with each other (Krushel et al., 1995). Subsequently striosomal cells aggregate, forming the cell clusters called striosomes (or patches). A rough topography is maintained. The striosomes are first identifiable laterally and caudally, with subsequent appearance medially and rostrally. The clusters of striosomal neurons are separated from the surronding matrix, at least in early postnatal rats, by glycoconjugate-rich boundaries (Steindler et al., 1988; O'Brien et al., 1992).

During prenatal development, one of the earliest signs of striosome-matrix compartmentalization is the selective high concentration of dopamine-containing terminals in the developing striosomes. Dopaminergic afferents from the substantia nigra innervate the developing striatum early, before compartments are formed (Voorn et al., 1988). The dopaminergic innervation appears to follow the process and topography of compartmentalization, first being diffuse and then becoming patchy (Voorn et al., 1988; Olson et al.,1972). At the time that the early-born neurons are in the caudal and ventral striatum, it is possible to detect dopaminergic terminals in the same region. Later on, when the striosomal and matrix neurons are intermixed in the striatum, the dopaminergic terminals are evenly diffused throughout the striatum. Finally, when the striosomal neurons start to aggregate, thus forming the patches, the dopaminergic terminals become highly concentrated in the same patches forming the so called "dopamine islands" (Fishell and van der Kooy, 1987; Voorn et al., 1988; Graybiel et al., 1981b). These events are

coincident with the emergence of the striosomes (Graybiel, 1984). At the same time, D1-class dopamine receptors appear predominantly in striosomes (Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994). D2-class dopamine receptors are also expressed, but do not have a compartmental pattern of expression (Murrin et al., 1985; Chen and Weiss, 1991; Weiss et al., 1992; Guennoun and Bloch, 1991).

This difference suggests the hypothesis that dopaminergic afferents, acting through D1-class dopamine receptors, could be involved in the process of striosomal aggregation. However, the issue of whether the dopaminergic fibers play an instructive role is still open. The striatum is eventually innervated by several brain regions other than the substantia nigra, as is noted above, but very little is known about their influences on striatal development.

Development of the neurochemical phenotypes of striosomes and matrix

How does the phenotypic striosome/matrix architecture emerge during development? The development of the different neurochemical phenotypes of striosomes and matrix involves a series of diverse changes. As an example, acetylcholinesterase activity is first concentrated in the striosomes in the developing striatum, whereas after birth, it switches to having a higher expression in the matrix (Butcher and Hodge, 1976; Graybiel et al., 1981a). The dopamine and cyclic adenosine 3':5'-monophosphate-regulated phosphoprotein 32 (DARPP-32) also initially has a striosome-specific expression, but it is, later on, evenly expressed by both compartments (Foster et al., 1987). D1-class dopamine receptors have the same type of developmental pattern. In rodents, D1 receptors first predominate in the striosomal compartment but later are more evenly expressed by both subsystems (Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994). The "dopamine islands" themselves, whose appearance is one of the earliest signs of the formation of striosomes, become more and more difficult to detect with the maturation of

the striatum, as the dopaminergic innervation spreads throughout the striatum (Graybiel, 1984).

The mechanisms orchestrating the temporal and spatial expression of these phenotypic markers of the striosome/matrix compartmentalization are still unknown but much work has focused on examining the role of the dopamine-containing afferents because they innervate the striatum so early . Dopaminergic afferents appear to have a crucial role for striatal neuron survival as early postnatal lesions of the substantia nigra produce massive shrinkage of the striatum (van der Kooy, 1996). However, whether and how dopamine regulates striatal development and pattern formation is unclear. It has been shown that, the removal of dopaminergic afferents, just before the beginning of the striosomal aggregation, by mechanical transection of the axons, affected the normal distribution in patches of the μ-class of opioid receptors, but it did not affect the formation of striosome/matrix compartmentalization as detected with tritiated thymidine [H^3]T (van der Kooy and Fishell, 1992). However, the mechanical transection of dopaminergic fibers was performed several days after the fibers had innervated the striatum, so that an early inductive role of the dopaminergic afferents could not be ruled out. Chemical depletion of the dopaminergic afferents, obtained with the injection of 6-hydroxydopamine (6-OHDA), a toxin selective for dopamine-containing neurons, during the prenatal and postnatal development of the striatal compartments, is reported not to affect either striosome-matrix pattern formation or the expression of several phenotypic markers of the compartments (Snyder-Keller, 1991). However, the elimination of the dopaminergic fibers by toxin injection was not complete in these experiments.

Recently, the analysis of engineered mice lacking different components of the dopaminergic pathway has brought new insights into the possible role of dopamine. Mice lacking D1-class dopamine receptors have a decreased expression of the neuropeptides dynorphin and substance P in the striatum, and they lack dynorphin-positive striosomes (Drago et al., 1994; Xu et al.,1994). The general anatomy of the striatum, by contrast,

appears fairly normal and other markers, including enkephalin and calbindin, show that striosomes exist, even if their appearance is not completely normal (Xu et al., 1994). In D2-knockout mice, enkephalin expression is increased, but no clear information has been reported regarding the striosome-matrix pattern (Baik et al., 1995). Mice genetically engineered to lack tyrosine hydroxylase (TH), a crucial enzyme in dopamine synthesis, in nigrostriatal afferents, have generally normal striatum but the striatal expression of substance P and dynorphin is decreased (Zhou and Palmiter, 1995). Again no clear information has been reported about the striosome-matrix pattern. Moreover, in the dopamine transporter-deficient mouse drastic changes have occurred. D1- and D2-class dopamine receptors are down regulated, as is TH. Enkephalin expression is decreased while dynorphin expression is increased. Despite the marked changes in the biochemical parameters, the general anatomy of the striatum seems normal (Giros et al., 1996). Once again, no clear information has been reported on striosome-matrix patterning.

These results point to the possibility that dopaminergic afferents may not be required for the formation of striosomes, but may be essential to the expression of the striosome-matrix phenotype as represented by neuroactive substances such as dynorphin. Such phenotypic alterations could be causally linked to the behavioral alterations observed in these mice.

Dopamine, dopaminergic fiber systems and dopaminergic receptors

Dopaminergic fiber systems innervating the forebrain nearly all originate in the midbrain, but they innervate several different telencephalic structures in addition to the striatum. They are divided into three major systems: the mesostriatal, the mesolimbic and the mesocortical. The mesostriatal system is composed by the substantia nigra (area A9) the ventral tegmentum (A10) and the retrorubral nucleus (A8). A9 projects primarily to the striatum, with some additional projections to the globus pallidus and the subthalamic

nucleus; A10 projects to the nucleus accumbens, the olfactory tubercle, nuclei stria terminalis and the neocortex in particular to the prefrontal cortex; A8 projects to the ventral striatum. A10 and partially A9 form the mesolimbic and mesocortical dopaminergic systems which innervate limbic and cortical areas including the prefrontal cortex, the cingulate cortex, the habenula, the septum, the amygdala, the locus coeruleus, the pyriform and entorhinal cortices. Other dopaminergic cell groups in the nervous system, not considered here, lie in the retina, olfactory system and hypothalamus.

Dopamine acts through at least six types of dopamine receptors. All dopamine receptors can be divided into two major subtypes, the D1- and the D2-class. They are characterized by seven membrane-spanning domains and they are all coupled to their effector functions via guanine nucleotide regulatory (G) proteins. D1-class of dopamine receptors activate the enzyme adenylyl cyclase, increasing the intracellular level of the second messenger cAMP, whereas D2-class of dopamine receptors exert an inhibitory influence on the cAMP level (Andersen et al., 1990). The D2-class of dopamine receptors has an additional subtype which is linked through G proteins to phosphatidylinositol turnover (Vallar and Meldolesi, 1989). The D1-class of dopamine receptors has two members, the D1a or D1 and the D1b or D5. D1a is highly expressed in the striatum, nucleus accumbens, olfactory tubercule and at lower concentrations in the cerebral cortex, limbic system, hypothalamus and thalamus, whereas D5 is mostly expressed in the limbic system, hippocampus, hypothalamus and also in the frontal and temporal cortices. Only very low levels of D5 are found in the striatum. Within the striatum, the D1a receptors are highly expressed by the dynorphin positive spiny neurons which form the direct pathway (Gerfen et al., 1990). The D2-class of dopamine receptors includes at least four members: D2 short (D2s) and D2 long (D2l) forms, D3 and D4. D2s and D2l are highly expressed in the striatum, nucleus accumbens and olfactory tubercle. Messenger RNA (mRNA) for D2 receptors has been found in the dopaminergic cells of the substantia nigra pars compacta and ventral tegmental areas, suggesting an additional role as autoreceptor. Within

the striatum, D2 receptor is expressed mostly by enkephalin-positive medium spiny neurons which form the indirect pathway (Gerfen et al., 1990). Pharmacologically it is not possible to distinguish between the two isoforms of the D2 receptor. D3 and D4 receptors have a low expression in the striatum. D3 is predominately expressed in the nucleus accumbens, islands of Calleja, bed nucleus of the stria terminalis, olfactory tubercle, hippocampus and hypothalamus. The highest level of D4 has been detected in the frontal cortex and amygdala.

In this thesis, the terminology D1- class and D2-class of dopamine receptors refers to D1a and D2s/l receptors, respectively. D1a and D2s/l are, in fact, the dopaminergic receptors with the highest expression in the striatum.

In contrast with neurotransmitters such as glutamate, which acting through ion channel postsynaptic receptors affects the resting potential of the postsynaptic neuron, dopamine, applied alone, does not influence the potential of the postsynaptic cell (Cepeda et al., 1993). Dopamine, through its G coupled postsynaptic receptors, modulates the activation of second messengers in the postsynaptic cell. As a consequence, to determine which neurons have been influenced by dopamine, indicators of activation of intracellular second messenger cascades are suitable tools. In our experiments, we have used the induction of immediate-early genes for this purpose.

Immediate-Early Gene Induction as a Cellular Assay of Dopaminergic Activation

Studying the influence of afferents to the striatum on the development of their target is not an easy task. Whereas afferents to the sensory cortex are easily accessible to external manipulations such as the eyes for the visual cortex and the vibrissa for the somato-sensory cortex, this is not so for the striatum. Dopaminergic nigrostriatal afferents are hidden deep in the brainstem, and the substantia nigra pars compacta , which gives rise to these afferents, is reciprocally connected to the striatum. The corticostriatal projection is

accessible to external manipulations, but it is also part of a loop system, receiving indirect feedback from the striatum via the thalamus. Studies of hemidecortication in early postnatal life have shown that the removal of cortical input does not affect the striosome and matrix formation (Kolb et al., 1992). However, the surgical removal of the cortex was performed in the postnatal period, at a stage when the striosome and matrix pattern had already been formed. Therefore, this result may suggest that cortical input is not essential for the maintenance of compartments, but it does not give any information on their role in the establishment of the striosome-matrix organization.

The classical experiments performed to study the development of the visual cortex manipulating the afferent input by, for example, blocking the signal transmission with intraocular injections of the neurotoxin tetrodotoxin (TTX) (Shatz and Stryker, 1988; Galli and Maffei 1988; Stryker, 1991) are impractical for studying the influence of the nigrostriatal system on the development of the striatum. While intraocular TTX injections do not affect the activity of any neuronal center crucial to the animal's survival, TTX injections in the midbrain could not only affect the activity of the substantia nigra neurons but also of centers crucial for the survival of the animal such as the reticular formation. Systemic injections of 6-hydroxydopamine (6-OHDA), a selective catecholamine neurotoxin, have not been a very useful tool for removing the developing nigrostriatal system, because the injections result in high mortality in the fetuses and mothers, and in the survivors the removal of the dopaminergic afferents is not complete (Snyder-Keller, 1991).

To analyze changes in neuronal responsiveness at the population level after afferent stimulation, an approach increasingly used is to characterize the resulting immediate-early gene (IEG) induction (Hughes and Dragunow, 1995). We have monitored induction of immediate-early genes by dopamine receptor agonists in the striatum as a cellular assay to test the functional responsiveness of developing striatal neurons. The order in time and space, the type of behavior and the onset of responsiveness of striatal neurons to dopaminergic stimulation is unclear. For example, it is known that D1-class dopamine

receptors are enriched in striosomes of the immature striatum (Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994), but it has not been determined whether these are functional. Our experiments permitted testing of whether D1-class and D2-class receptors are functionally coupled to the nucleus of striatal neurons so to elicit synthesis of an immediate-early gene response.

Immediate-Early Genes: Overview

Immediate-early genes (IEGs) are members of a class of molecules so called because their rapid and transient induction is among the first changes in gene activity observed in response to extracellular stimulation. IEGs have been associated with several physiological and pathological processes in the nervous system (review in Sheng and Greenberg, 1990; Morgan, 1991; Hughes and Dragunow, 1995). We focused our attention on the transcription factor subgroup of the IEG class of molecules. The well-characterized IEG transcription factor Fos has been found to be induced by seizures (Morgan et al., 1987), kindling (Dragunow and Robertson, 1987) and psychomotor-stimulant drugs (Graybiel et al.,1990); to reflect circadian rhythms and sleep cycles (Kornhauser et al., 1990; Sherin et al., 1996); to be correlated with neuropeptide regulation (Soonenberg et al., 1989), and to be expressed in several regions during development of the CNS (Alcantara and Greenough, 1993; Caubet, 1989; Gonzales-Martin et al., 1991). NGFI-A has been found present during critical activity-dependent processes (Wisden et al., 1990; Zhang et al., 1995). Substantial research effort has been direct towards elucidating the molecular mechanisms leading to IEG induction. Several different second messenger pathways are involved. A well documented path is via an increase of intracellular calcium or cAMP that can activate, respectively, the calcium-calmodulin dependent kinase and protein kinase A. The protein kinases can phosphorylate the calcium/cAMP response element binding protein (CREB) bound to the CREB response element (CRE), which can induce the transcription of the *c-fos* gene, among others. Other stimuli can lead to intracellular

cascades inducing the cleavage by phospholipase C of the phosphatidylinositol biphosphate (PIP_2)molecule into 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG can stimulate the action of protein kinase C, leading to the activation of the serum response factor (SRF). The binding of SRF to the serum response element (SRE) in the promoter region of IEGs such as *c-fos* can regulate the transcription of these genes.

The protein products of the IEGs studied in this thesis are transcription factors that can activate or inactivate the transcription of other genes, the so called "late-response genes" in addition to regulating other IEGs (review in Sheng and Greenberg, 1990). Therefore, selective IEG induction observed after extracellular stimulation is one means of linking external signals with cellular events so as to store the information by long-lasting molecular changes. The possibility that IEGs can be functionally involved during development is also emerging. In PC12 cell lines, IEGs are expressed in response to differentiating agents (Milbrandt, 1986 and 1987). In the CNS, IEG expression has been detected during late embryonic and postnatal development in several regions (Caubet, 1989; Gonzales-Martin et al., 1991; Alcantara and Greenough, 1993; Herms et al., 1994; Labandeira-Garcia et al., 1994; Kosofsky et al., 1995a). It is reasonable to suppose that they may be involved in gene regulation necessary for normal development. Clearly, the same classes of transcription factors could be the targets of several signal transduction pathways activated both by differentiating extracellular signals during development and by "plastic" modifications later on.

Using Immediate-Early Gene Expression to study dopaminergic actions in the Adult Striatum

With the aid of IEG induction methodology, it has been possible to uncover several modalities of influence of the afferents on the postsynaptic striatal cells. It has been shown that different sets of striatal neurons are responsive after cortical and dopaminergic stimulation (Berretta et al., 1992; Berretta et al., 1997; Parthasarathy et al., 1996).

Pharmacological dopaminergic stimulation with the indirect dopamine agonist cocaine induces Fos expression predominantly in dynorphin-positive neurons (Berretta et al., 1992). In contrast, pharmacological or electrical cortical stimulation induces Fos expression mostly in enkephalin-positive neurons (Berretta et al., 1997). Even though the physiological implications of IEG induction in these striatal neurons are still unclear, it is remarkable that dopaminergic and cortical stimulation affect mainly different populations of striatal neurons. Dynorphin and enkephalin are expressed in the two major output pathways of the striatum. The dynorphin-positive neurons form the so-called direct pathway, whereas enkephalin-positive neurons the indirect pathway (reviewed in Graybiel, 1986, 1990; Albin et al., 1989).

Using IEG induction as a cellular assay, several groups have further shown that direct and indirect dopamine agonists can selectively target striosomes, matrix or both compartments (Graybiel et al., 1990; Wirtshafter and Asin 1994; Jaber et al., 1995; Moratalla et al., 1996; Liu et al., 1995).

This approach has also been used to analyze the functional interactions of the different classes of dopamine receptors. In the adult striatum, IEG induction appears following agonist activation of D1-class of dopamine receptors (Young et al., 1991; Berretta et al., 1992) and antagonist blockade of the D2-class of dopamine receptors (Dragunow et al., 1990; Marshall et al., 1993). However, synergism has been observed between the D1 and D2 classes of dopamine receptors (Paul et al., 1992; LaHoste and Marshall, 1993; Witshafter and Asin, 1994; Ruskin and Marshall, 1994).

IEG induction has also been used to monitor the effects of the glutamatergic input on the dopaminergic signals (Torres and Rivier, 1993; Wang et al., 1994; Konradi et al., 1996).

Using IEG induction as a cellular assay to monitor striatal responsiveness to dopaminergic or other stimulations is a very efficient approach but it has drawbacks as well. The pharmacological approach commonly used to increase dopamine at

dopaminergic terminals is the systemic administration of indirect catecholamine agonists like cocaine and amphetamine. The systemic administration of the drugs may affect other brain regions, some of which project to the striatum. For example, the prefrontal cortex receives dopaminergic afferents and projects extensively to the striatum. In addition, the spectrum of action of these drugs is not limited to dopaminergic terminals. Cocaine blocks the dopamine reuptake transporter (DAT), a member of the Na^+/Cl^--dependent twelve transmembrane domain transporter family. Amphetamine's most significant effect is to cause reverse transport of dopamine via the DAT. Cocaine and amphetamine also affect the serotoninergic and noradrenergic terminals, however. It has been found that the blockade of serotonin receptors reduces IEG induction by cocaine in the striatum (Bhat et al., 1992 and 1993). Therefore, striatal IEG induction provoked by these drugs could be, at least in part, due to an indirect effects.

To control for the contribution of extrastriatal regions on IEG induction in the striatum, intrastriatal infusion of pharmacological agents has been performed in our and other laboratories (Berretta et al., 1992; Keefe and Gerfen, 1992; Robertson et al., 1992). It has been shown that the dopaminergic agents administrated locally regulate IEG expression in the striatum in ways similar to that observed after systemic injection of the same substance, suggesting that IEG regulation is mostly driven by intrastriatal mechanisms (Berretta et al., 1992; Keefe and Gerfen, 1992; Robertson et al., 1992). Taken into account the benefits and problems with this technique, we felt confident in using this approach to analyze the responsiveness of developing striatal neurons to dopamine, given the extensive controls that we have performed. We emphasize that this method of remote stimulation is particularly suitable for accessing dopaminergic responsiveness in the developing striatum.

Conclusion

In this chapter we have reviewed the organization of the striatum, its subdivision into striosomes and matrix, and its connections, biochemical specialization and developmental history. We have pointed out that the striatum is innervated by the dopamine-containing substantia nigra pars compacta, which is not easily accessible to external manipulation, being hidden in the midbrain. As consequence, we have indicated that to study the influence of the nigrostriatal dopaminergic afferents on the development of striatal compartments it is appropriate to use a remote pharmacological stimulation approach. We have explained that dopamine is a neuromodulator which, through G-protein-coupled receptors, influences the intracellular activity of the postsynaptic cells, activating second messenger pathways. We have introduced the study of immediate-early gene expression as a way to monitor changes in the intracellular activity of the postsynaptic cells being a target of second messenger pathway activation. We have discussed the potential of this technique and reviewed important results achieved for the adult striatum. We have also discussed potential drawback of this methodology. We have briefly introduced the motivations and the approach used for the experiments undertaken for this thesis: our goal was to develop and to exploit a method for detecting functional activation of striatal neurons by dopamine. In the following chapters, we will examine the results obtained.

Figure 1. Schematic diagram of major basal ganglia connections.

The striatum is the input structure of the basal ganglia circuit loops. Almost all the cortical areas project to the striatum. The limbic structures amygdala, prefrontal and insular cortices project preferentially to the striosomal compartment (S), whereas the sensory-motor cortices and the cingulate girus preferentially innervate the matrix compartment (M). The centromedian (CM) and parafascicular (PF) nuclei of the thalamus project to the striatum. The dopaminergic neurons from the substantia nigra pars compacta (SNpc) innervate both the striosomes and the matrix but only the striosomal neurons project back. The matrix neurons project to the substantia nigra pars reticulata (SNpr) and to the globus pallidus (GP). The projection is subdivided into two main pathways. The "direct" pathway, characterized by the neuropeptide dynorphyn (Dyn), innervates the subastantia nigra pars reticulata and the internal segment of the globus pallidus (GPi) which then they both project to the ventrolateral (VL), ventral anterior (VA) and mediodorsal (MD) nuclei of the thalamus and from here back to the cortex. The "indirect" pathway, characterized by the neuropeptide enkephalin (Enk) projects to the external segment of the globus pallidus (GPe), which projects to the subthalamic nuclei (STN). The STN projects to the GPi and SNpr.

Excitatory glutamatergic or aspartatergic connections are represented in red. Inhibitory GABAergic connections are represented in green. The neuromodulatory dopaminergic innervation is represented in yellow.

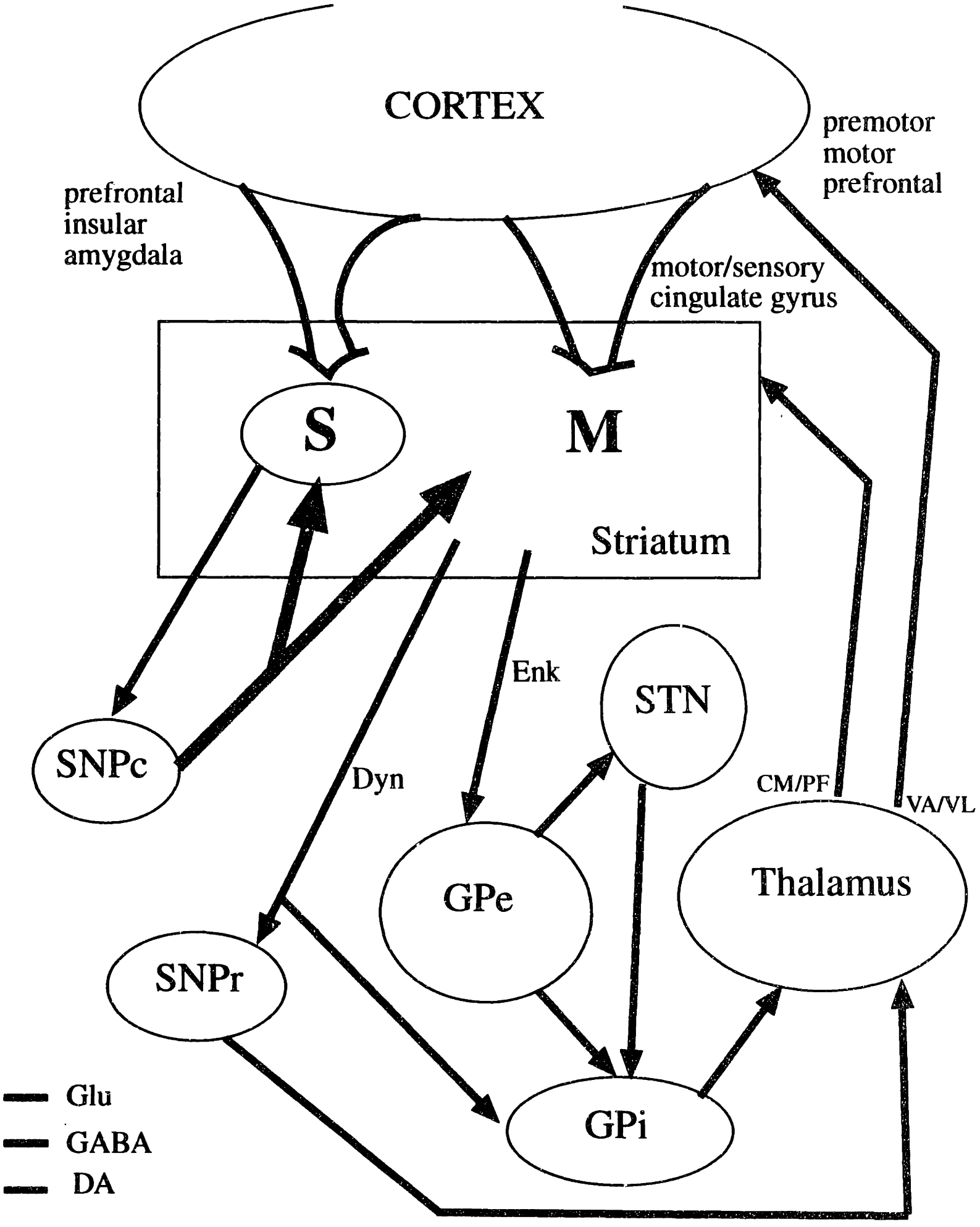
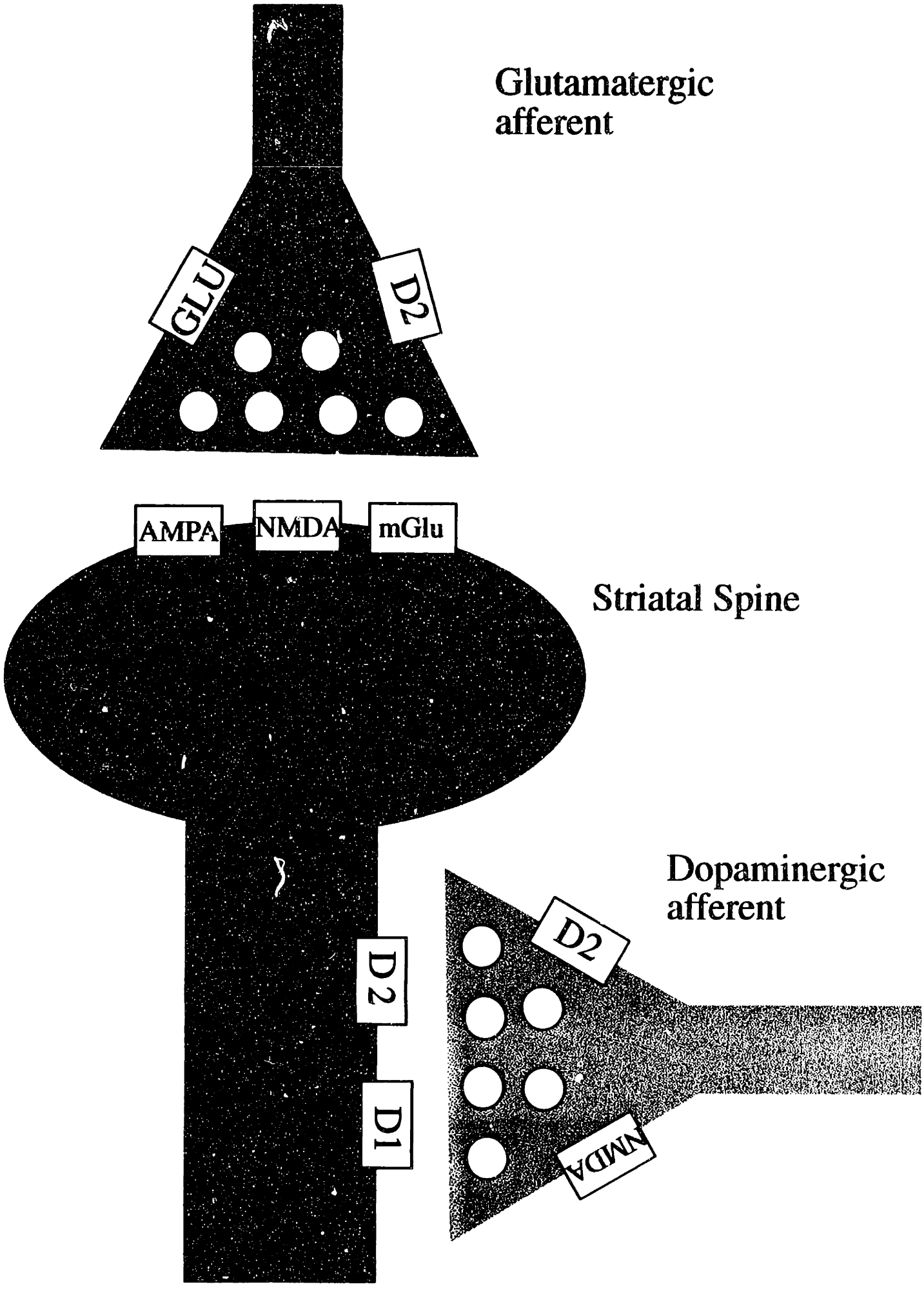

CORTEX
premotor
motor
prefrontal
prefrontal
insular
amygdala
motor/sensory
cingulate gyrus
S
M
Striatum
Enk
STN
SNPc
Dyn
CM/PF
VA/VL
GPe
Thalamus
SNPr
GPi
Glu
GABA
DA

Figure 2. Schematic representation of striatal synaptic circuit.

The corticostriatal terminal is in red, the striatal dendritic spine is in blue, the nigrostriatal terminal is in yellow. The corticostriatal afferents terminate on the head of the dendritic spine whereas the dopaminergic terminals innervate the neck of the spine. Dopaminergic terminals have D2-class dopamine receptors as autoreceptor and NMDA glutamatergic receptor as presynaptic heteroreceptors. Glutamatergic terminals have glutamatergic autoreceptors and D2 receptors as presynaptic heteroreceptors. Striatal spines display postsynaptic dopaminergic (D1, D2) and glutamatergic receptors (NMDA, AMPA/kainate and metabotropic).


Glutamatergic afferent
GLU
D2
AMPA
NMDA
mGlu
Striatal Spine
Dopaminergic afferent
D2
D2
D1
NMDA

CHAPTER 2

Developmental network-like changes in distribution and afferent control of the level of expression of Fos, Fra and NGFI-A proteins in rat striatum

Abstract

During nervous system development, different immature phases, highly influenced by the afferent inputs, occur before the final structural and functional organization is established. The development of some systems like the visual cortex can be tested by direct manipulation of these afferents. Unfortunately, the basal ganglia system does not have afferent pathways easily accessible to such direct external manipulation. Therefore, in order to study the influence of the dopaminergic nigrostriatal afferents on the functional maturation of striatal circuits, in vivo pharmacological manipulations become necessary. Using the expression of transcription factors of the immediate-early gene family Fos, Fra and NGFI-A as sensitive displays of changes at network-like level, we define three fundamental phases in the postnatal developing rat striatum. We further show that pharmacological stimulation of the dopaminergic system not only leads to increase of IEG expression in individual striatal neurons, but also to network changes in which ensembles of striatal neurons express these proteins. Moreover, we indicate that the cortical glutamatergic modulation of the dopaminergic input observed in the adult, starts to act since the first postnatal week.

Introduction

Activity-dependent regulation of cell function plays a major part in establishing the ultimate structural and functional organization of neural systems. Much of the evidence for the role of activity comes from studies of the mammalian visual system, where direct manipulations of afferent input can be made both prenatally and postnatally (Shatz and Stryker, 1988; Galli and Maffei, 1988; Stryker, 1991). The basal ganglia do not have afferent paths easily accessible to such direct intervention. However, indirect evidence suggests that the dopamine-containing afferents arising in the midbrain substantia nigra may strongly influence pattern formation and phenotypic maturation of target neurons in the striatum. The dopamine-containing afferents arrive early in striatal development, before its characteristic striosome-matrix compartments are formed (Voorn et al., 1988); some of these fibers even enter the germinal epithelium (Voorn et al., 1988). Dopamine receptors and mRNA coding for different dopamine receptor subtypes also are expressed early, before compartmentalization has occurred (Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994; Murrin et al., 1995; Chen and Weiss, 1991; Weiss et al., 1992; Guennoun and Bloch, 1991).

For directly testing the influence of dopaminergic afferents on striatal development, classical ablation/lesion approaches encounter major problems. The substantia nigra lies deep in the midbrain near brain regions essential for life, and even neurotoxin-based prenatal ablative attempts for example, with 6-hydroxydopamine have faced serious obstacles due to high rates of mortality (Snyder-Keller, 1991). Transgenic mouse technology, however, has provided evidence that dopamine receptor function is necessary for the presence of a fully normal striatum at adulthood. Deletion of D1 dopamine receptors produces a selection loss of dynorphin and substance P in the mature striatum and its efferent axons (Xu et al., 1994; Drago et al., 1994), supporting the view that D1-class receptors in the normal striatum are strongly expressed in striatal projection neurons co-expressing these neuropeptides. Deletion

of D2 dopamine receptors produces a selective loss of enkephalin in striatal neurons at maturity, fitting evidence for predominant expression of some D2-class receptors on enkephalin-positive projection neurons (Baik et al., 1995). Thus, the presence of dopamine receptors, and presumably their activation by dopaminergic afferents, is essential for the normal neurochemical phenotypes of striatal neurons, at least at adulthood.

In the experiments described here, we attempt to test directly for functional effects of stimulating dopamine receptors during the postnatal development of the striatum. This is a period of dramatically changing expression of neuroactive substances as they take on their eventual striosome-enriched, matrix-enriched or, rarely, non-compartmental distributions within the striatum (Graybiel and Ragsdale, 1983; Graybiel, 1990). We took advantage of the fact that remote, non-invasive stimulation of the dopamine receptors by dopamine receptor agonists and antagonists induces, in the adult striatum, rapid, transient induction of several groups of proto-oncogenes immediate-early genes, (IEGs) in striatal neurons. The induction occurs in cell-types specific, compartment-specific patterns, through intracellular cascades coupled to D1-class and D2-class receptors (Graybiel et al., 1990; Young et al., 1991; Berretta et al., 1992; Moratalla et al., 1996). The use of this IEG assay allowed us to test for neuronal responsiveness to dopaminergic stimulation not only in individual striatal neurons but also in populations of neurons in the developing striosome and matrix compartments. In a smaller set of experiments we combined the dopamine agonist treatments with glutamate NMDA receptor antagonist pretreatment to determine whether interacting between dopamine and glutamate, a cardinal feature of the nature striatum (review in Starr, 1995; Wang, 1991; Desce et al., 1992; Maura et al., 1989; Yamamoto and Davy, 1992), occur also during postnatal development.

Our findings demonstrate that dopaminergic stimulation induces intense activation of Fos/Fra and NGFI-A family IEGs from the time of birth, but that the population profiles of activated neurons does not reach the mature pattern either in the striatum or in its target structures until near adulthood. Throughout postnatal development, glutamate NMDA

receptors appear to contribute to these responses. Finally, we show that in addition to inducible Fra and NGFI-A proteins there are constitutively expressed Fra and NGFI-A proteins that do display sensitivity to blockade of dopamine and NMDA glutamate receptors, but that do undergo patterning during postnatal development blockade. We suggest that dopamine-containing and glutamate-containing afferents, can act in activity-dependent modeling of striatal function, but that endogenous programs of striatal development proceed in parallel.

Materials and Methods

Experimental procedure

Experiments were carried out on Sprague-Dewley rats (Taconic) of ages groups P1-P2, P6-P7, P14-P15, P21-P22 and P30. The indirect monoamine agonists cocaine (SIGMA, St. Louis, MO; 25mg/kg; s.c. in saline solution) and D-amphetamine sulfate (SIGMA, 5mg/kg; s.c. in saline solution) were administrated to pups of each age groups. The selective D1-class dopamine receptor antagonist SCH23390 (Research Biochemicals; 0.2mg/kg, i.p. in tartaric acid solution) and the selective D2-class dopamine antagonist eticlopride hydrochloride (Research Biochemicals; 0.5 mg/kg, s.c. in saline solution) were administered to siblings of the cocaine- and amphetamine-treated pups. In addition, pups of the same litters were pretreated with SCH23390 or eticlopride hydrochloride 10-20-30 mins before cocaine or amphetamine administration (SCH23390+cocaine; SCH23390+amphetamine; eticlopride+cocaine; eticlopride+amphetamine). The selective non competitive NMDA receptor antagonist MK-801 (Research Biochemicals; 5mg/kg, s.c. in saline solution) was also administrated alone or as a pretreatment followed, after 25-30 mins, by indirect monoamine agonist injection in some age groups. For each offspring analyzed there was at least one solution control (saline s.c.; tartaric acid solution) animal and often a control animal without treatment. All animals were euthanized 2hr after the last injection. More than one animal has been used for each age. More than 160 rat brains has been analyzed.

Tissue preparation

For fixation, rats were deeply anesthetized by inhalation of Halothane and were perfused transcardially with 4% paraformaldehyde in 0.1M sodium phosphate buffer/0.9% NaCl (PBS). The brains were postfixed in the same fixative for 2hrs and then were

cryoprotected at 4°C for at least 24 hrs in 20% glycerol in PBS. Serial coronal sections were cut on a freezing microtome at 20μm (sections for dual antigens immunohistochemistry) or at 40μm (sections for single antigen immunohistochemistry) and were collected in 0.1M phosphate buffer containing 0.1% Na azide.

Antisera

The primary antisera and the concentrations used were as follows: rabbit polyclonal anti-NGFI-A, 1:2,000 (generously provided by Dr. J. Milbrandt); rabbit polyclonal anti-Fos, 1:200 (Oncogene Science, Manhasset, NY); sheep polyclonal anti-Fos, 1:2,000 (Cambridge Research Biochemicals, Cambridge, UK); rabbit polyclonal anti-Fra, 1:2,000 and 1:5,000 (generously provided by Dr. M.J. Iadarola); mouse monoclonal anti-dopamine-and-adenosine 3':5'mono-phosphate regulated phosphoprotein-32000, 1:20,000 (DARPP-32; generously provided by Drs. P. Greengard and E.L. Gustafson); and mouse monoclonal anti-tyrosine hydroxylase, 1:1,000 (SIGMA). All results refer specifically to the particular antisera and antibodies listed.

Single antiserum immunohistochemistry

Free-floating sections were pretreated with 3% H_2O_2 in 0.01M PBS and 0.2% Triton X-100 (PBST) for 10 min and were then incubated in 5% normal serum from the same species in which the secondary antibody was raised. Sections were incubated in primary antisera diluted in PBST for 48-72hr at 4°C. For avidin-biotin-peroxidase complex immunocytochemistry, sections were rinsed in PBST, incubated in a 1:500 biotin-conjugated secondary antiserum (Vector Laboratories, Burlingame, CA) for 1hr, rinsed in PBST, and then incubated in avidin-biotin complex (ABC, Vectastain, Vector Laboratories; 6μl/ml) in PBST for 1hr before being reacted with diaminobenzidine (DAB, SIGMA). Some sections were subjected to nickel enhancement with DAB. Sections were mounted

on gelatin-coated slides, air dried, dehydrated, coverslipped with Eukitt mounting medium, and were analyzed by light microscopy.

Dual antiserum immunohistochemistry

The simultaneous detection of two antigens was carried out on 20μm thick free-floating sections. To demonstrate colocalization of a nuclear and a cellular antigen, such as a transcription factor and DARPP-32, we followed a procedure of two avidin-biotin peroxidase complex labeling. The first staining enhanced with nickel to produce a black, dot-like signal, and the second was stained with only DAB to produce a light brown signal. After the nickel-DAB staining, sections were washed overnight in 0.1M PO_4, then were incubated for 45 min in 0.6% H_2O_2 to eliminate the residual peroxidase activity and given blocking treatments in avidin and then in biotin before the start of the incubation for the second antigen, which was performed as described in the single antiserum immunohistochemistry protocol.

Results

Constitutive expression of immunodetectable Fos, Fra and NGFI-A undergo marked changes in expression patterns during postnatal development

To characterize the constitutive expression of Fos, Fra and NGFI-A and their patterns of distribution during striatal development, we analyzed the brains of normal and saline-treated rat pups from P1-P30. Striking compartmental changes in pattern expression occurred within the striatum and the patterns were gene specific. Immunoreactivities for all three antigens, Fos , Fra and NGFI-A, were expressed mainly in patchy aggregates of striatal neurons at P1-P2 (Fig.3 A, A', A"). To determine whether IEG distributions were coincident with the striosomal compartment, we double labeled sections for each protein class and the striosomal markers DARPP-32 and TH. There was a strict correspondence between the IEG-positive patches and the striosomal markers positive patches (Fig.5 A, B).

Despite this early coincidence of basal expression patterns the expression of Fos, Fra and NGFI-A sharply diverged after the first postnatal week. Fos expression rapidly declined to undetectable levels. Fra expression remained strong and predominately striosomal until P7 (Alcantara and Greenough, 1993), but then was downregulated along a clear lateral to medial gradient from P2 (Fig.3 A', B'). At P21, detectable Fra proteins were still present mostly confined to the medial edge of the caudatoputamen and the basal striatum (Fig.3 B'). In sharp contrast, the expression of NGFI-A, although originally largely confined to striosomes spread to the matrix compartment by the end of the first postnatal week (Fig.3 A, B). In the ventral striatum, Fra antiserum intensely characterized the nucleus accumbens (N.A.). The temporal pattern of Fra protein intensity in the N.A. mirrors the downregulation observed in the dorsal striatum.

These results demonstrate that both Fos and Fra have early striosome-specific expression and then are down-regulated, whereas NGFI-A expression with time extends from the striosomes to acquire an homogeneous distribution in both striosome and matrix compartments (Fig. 3). Even though, it is still unclear what function Fos, Fra and NGFI-A regulate in striatal development, our results suggest that the constitutive expression of Fos and Fra may be linked to early compartment-specific differentiation of the striatum, such as the striosomal-predominant expression of neuroactive substances, whereas NGFI-A proteins may be involved in general striatal development. proceeding from the early-developing striosome compartment to a generalized bi-compartment distribution.

Fos, Fra and NGFI-A were also distributed in other circumscribed forebrain regions. All three immunoreactivities were strongly expressed in the olfactory pathway. During the first postnatal week, Fra-like proteins crisply demarcated the deep cortical layers and the hyppocampal formation. With increasing postnatal age, the Fra immunostaining spread across the entire cortical width. Remarkably, neurons of the deep early-born cortical layers, some of which preferentially innervate the striosomal compartment, expressed Fra immunoreactivity with the same temporal pattern of the striosomes. NGFI-A expression in the cortex and hippocampus started more than a week later than that of Fra proteins, but followed the same anatomical pattern in the neocortex, spreading from the deep layers to the more superficial ones. The fourth cortical layer was the least immunoreactive for both Fra and NGFI-A antisera. The postnatal pattern of cortical expression Fos and Fra is generally similar, but not identical, to those reported by Alcantara and Greenough in 1993.

Cocaine and amphetamine induce a similar striosomal-specific immunodetectable IEG pattern of expression in the postnatal developing striatum

To determine whether transcription factors of the Fos/Fra and NGFI-A families were sensitive to dopaminergic stimulation at birth, and to analyze the populations of

neurons expressing inducible proteins of these classes, we treated rats of the different postnatal age groups with the indirect catecholaminergic agonists cocaine and amphetamine. In the adult rat it is known that cocaine and amphetamine both induce expression of these proteins, but do so in different patterns. Cocaine induces IEG expression in broadly distributed sets of striatal projection neurons, whereas amphetamine, has a more selective effect, inducing IEG proteins in a striosome-predominant pattern in a large anterior part of the caudatoputamen (Graybiel et al., 1990; Moratalla et al., 1996). For the developing striatum we found that a single systemic injection of cocaine (25mg/kg) or amphetamine (5mg/kg), also induced a rapid increase in expression of Fos, Fra and NGFI-A immunoreactivities above control levels at all postnatal ages studied. In sharp contrast to the induction patterns seen in adults, however, the patterns of Fos, Fra and NGFI-A induced by the acute cocaine and amphetamine treatments, at birth, were virtually identical and with a patchy distribution (Fig.4).

To reveal whether the IEG induction is circumscribed to the striosomal compartment or not, we have double labeled the IEG expression with markers, at this age striosomal-specific, such as DARPP-32 and TH. An extensive colocalization is observed both in the cocaine- and in the amphetamine-treated striatum (Fig.5 C, D).

This striosome-predominant pattern persisted for both treatments through the first postnatal week and beyond (Fig.4 A, B, D, E). Only after the third week did the IEG induction elicited by cocaine fully spread to the matrix, leading to the sharp contrast in IEG induction patterns observed in the adult (Fig.4 C, F). Our result for Fos proteins induction after cocaine treatment is in general agreement with results in literature (Kosofsky et al., 1995a and 1995b). In contrast with already reported, we found an intense induction of Fos-like proteins after amphetamine stimulation at P0 and P7 (Labandeira-Garcia et al., 1994). The discrepancy in the results does not depend on drug dosage or on the protocol of the treatment; most likely the difference is due to a slight different spectrum of Fos/Fra members recognized by the antibodies used.

Inducible but not constitutive Fra, Fos and NGFI-A proteins are sensitive to D1 receptor pharmacological block

To determine whether the striatal induction of IEGs by cocaine and amphetamine is dependent on D1-class of dopamine receptors during postnatal development, as it is at adulthood, we pretreated rat pups with the selective anatagonist SCH23390 (0.2mg/kg) before administrating the psychomotor stimulant. We found that the D1-class dopamine receptor blockade sharply reduced the Fos induction in the striatum at all ages investigated (see summary table). Since the first postnatal week, striosomal Fos constitutive and inducible expressions are completely blocked by D1 antagonist pretreatment (Fig.6 A', B'). The effect of D1 receptor blockade become visible for Fra anf NGFI-A at the end of the first postnatal week. Both NGFI-A and Fra have considerable constitutive expression that likely cover the effect of SCH23390. After D1 receptor blockade the levels of expression of these proteins were at roughly constitutive levels (Fig.6 A, B; see summary table). These results suggest that D1 dopamine receptors, even at birth, when they are primarily expressed in the striosomes, are functionally coupled to intracellular cascade capable of responding to stimulation with induction of protein synthesis. It was of special interest that some NGFI-A and Fra expression remained in the striatum in the stimulant-treated animals pretreated with SCH23390 (Fig.6 A, B). This raised the possibility that the pool of proteins of the basal expression might not be sensitive to D1 receptor blockade unlike the inducible pool of proteins. We therefore administered the D1-selective agonist SCH23390 to otherwise untreated rat pups of each age group. Neither the NGFI-A nor the Fra expression in the striatum appeared to be affected, at least, strongly by the D1 antagonist treatment (Fig.6 C and summary table). Interestingly, it has also been found in organotypic cultures of P0 rat striatum that basal Fra expression is not detectable affected

by pharmacological blockade of D1 class dopamine receptors (Liu et al., 1995). These results suggest that the tonic levels of expression of NGFI-A and Fra proteins may be, at least in part, independent of D1-class dopamine receptors. It is possible that basal NGFI-A and Fra expressions are driven by the activity of other afferents or, alternatively, that their expression may be not afferent-dependent. These results would favor the hypothesis of an afferent-independent constitutive expression for at least Fra-like and NGFI-A-like proteins. A particularly interesting possibility is that there are different pools of the proteins, only some of which are sensitive to D1 receptor stimulation.

Striosomal-predominant Fos, Fra and NGFI-A expression induced by D2-like receptor antagonist treatment alone or in combination with dopaminergic indirect agonist treatments

In the adult striatum, it has been shown that D1- and D2-classes of dopamine receptors can operate synergistically or can interact to induce IEG proteins (Dragunow et al., 1990; Marshall et al., 1993; Paul et al., 1992; LaHoste and Marhall, 1993).
During development, D1-class dopamine receptors have a dominantly striosome-specific expression (Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994), whereas D2-class dopamine receptors do not have an apparent compartmental-specific expression but instead are expressed in both striosomes and matrix (Murrin et al., 1985; Chen and Weiss, 1991; Weiss et al., 1992; Guennoun and Bloch, 1991). To determine whether D2-class of dopamine receptors are functionally coupled to IEG induction since the first postnatal week and, if so, whether the spatial patterns of IEG expression follow the non-compartmental distribution of D2 receptors, we pharmacologically blocked the D2-class of dopamine receptors with the D2-selective antagonist eticlopride (0.5 mg/kg). Eticlopride induced robust expression in the striatum of P2 and P6 age pups (see summary table).

However, there is a sharp contrast to the pattern seen at adulthood, the induction was mainly confined to striosomes for all three classes of IEG protein analyzed (Fig.7 D, E, F; see summary table). Moreover, when we gave at P6 the D2 antagonist as a pretreatment before amphetamine and cocaine, the eticlopride clearly increased the patchy induction of IEGs by both psychomotor stimulants (Fig.7 B, C, E, F; and summary table). These findings imply that not only D1-class dopamine receptors but also D2-class dopamine receptors function in determining the pattern of IEG induction elicited by dopamine agonist treatment from the early postnatal period on. Moreover, our results suggest differential actions of D2 dopamine receptors in the striosomal compartment over a prolonged postnatal period despite strong expression of D2 ligand binding site and mRNAs in the matrix (Murrin et al., 1985; Chen and Weiss, 1991; Weiss et al., 1992; Guennoun and Bloch, 1991). Thus the functional actions of the dopamine receptors cannot simply be deduced from their distributions as indicated by ligand binding or in situ hybridization.

NMDA pharmacological blockade by MK801 suppresses inducible Fos, Fra and NGFI-A expressions whereas constitutive IEG expression is still present after MK801 treatment

Glutamatergic corticostriatal afferents represent the largest single input to the striatum. Thalamic inputs numerically may constitute the second largest input, and these are also thought to be glutamatergic. It has been hypothesized that dopamine modulates the efficacy of the glutamatergic cortical inputs (review in Starr, 1995; Wang, 1991; Desce et al., 1992; Maura et al., 1989; Yamamoto and Davy, 1992; Cepeda et al., 1993). In the adult striatum, NMDA receptors blockade suppresses Fos induction provoked by cocaine (Torres and Rivier, 1993; Wang et al., 1993; Wang et al., 1995). Recent evidence has shown that glutamate receptor subtypes have a striosome-predominant expression in the developing striatum (Snyder-Keller and Costantini, 1996). To determine whether the

glutamatergic modulation of dopamine function is already operative during the first postnatal week, and whether it is under compartmental constraints, we pretreated P6 rats with the NMDA antagonist MK801 (5mg/kg) and followed this pretreatment with cocaine injection less than half an hour later. The normally observed increase in striosomal expression above baseline for all three protein immunoreactivities by cocaine treatment was completely blocked by MK801 pretreatment (Fig.8 C, D; and summary table). By contrast to the blockade of inducible, however, constitutive Fra and NGFI-A expression were still present and were not strongly reduced (Fig.8 A, D; and summary table). We further tested whether the constitutive Fra and NGFI-A expressions were regulated by the glutamatergic input, by treating P6 rats with the NMDA antagonist alone. Fra and NGFI-A baseline expressions were still present, and were at most only mildly reduced in intensity and were not changed in distribution (Fig.8 A, B; and summary table). These results suggest that glutamatergic afferents can influence dopamine function strongly during early postnatal development. Moreover, our results strengthen the hypothesis of an afferent-independent constitutive expression of Fra and NGFI-A in the developing striatum.

Transient induction of Fos, Fra and NGFI-A proteins in nonstriatal nuclei of the basal ganglia defines a crucial period of basal ganglia development

In the adult rat, amphetamine and cocaine treatments do not induce detectable expression of Fos/Fra or NGFI-A in other nuclei of the basal ganglia. However, in the course of analyzing the developmental series, we found that there is a transient period during the second postnatal week in which dopaminergic stimulation by the psychomotor stimulants induces intense expression of Fos and Fra and weak expression of NGFI-A in the globus pallidus, and in the substantia nigra, main output targets of the striatum. By contrast, in the striatum, the IEG induction appeared weaker than at earlier or later time points (Fig.10 A; Fig.11 A; and summary table). The IEG response in the pallidum and

substantia nigra was first detectable at very low level at the end of the first postnatal week, and it had almost disappeared by the third week.

Remarkably, in contrast to our results for the striatum, we found that the Fos and Fra expression induced in the pallidum and substantia nigra was not blocked by pretreatment with the D1 selective antagonist, SCH23390 (Fig.9 B; and summary table). To determine whether D2-class of the dopamine receptors might be involved in generating the IEG induction in the pallidum and substantia nigra, we pretreated P15 rats with the D2 selective antagonist, eticlopride, prior to their treatment with cocaine or amphetamine. We found that eticlopride pretreatment fully blocked the IEG induction in the pallidum and in the substantia nigra elicited by either drug (Fig.9 C; and summary table). This result again stands in contrast to our results for the striatum, where eticlopride induces IEG expression alone and IEG proteins are still widely present when eticlopride is given as pretreatment before psychomotor stimulant treatments (Fig.10 B, C; and summary table). With ligand binding methods Greenamyre et al., (1987) observed a strong transient expression of glutamate receptor binding sites in the globus pallidus. We therefore pretreated P15 rats with the non competitive NMDA antagonist, MK801 and followed the pretreatment with cocaine. We found that Fos, Fra and NGFI-A induction in the pallidum and in the substantia nigra was completely blocked by the MK801 pretreatment (Fig.9 D; and summary table). In the striatum, MK801 pretreatment also downregulated the characteristic IEG protein increases induced by cocaine, leaving very few IEG-positive neurons in tiny clusters (Fig.10 D; and summary table). We also carried out control experiments to test the effects of blockade of NMDA receptors (by MK801), D1 receptors (by SCH23390) or D2 receptors (by eticlopride) alone and not in combination with the dopaminergic stimulation. None of the drugs altered the status of IEG expression in the pallidum and in the substantia nigra (see summary table). Our results suggest that during a transient period of postnatal development, stimulation with indirect dopaminergic agonists excites second messenger pathway and gene induction in striatal output structures and that that this excitation is

mediated through D2-class dopaminergic receptors and is under the control of NMDA glutamate receptors. This transient phase could be crucial in postnatal activity-activity modeling of the basal ganglia pathways. This transitory period remarkably parallels in time the transient peaks observed for the glutamatergic and dopaminergic families of receptors in these structures (Pellegrini-Giampietro et al., 1991; Shigemoto et al., 1992; Miller et al., 1990; Chen and Weiss, 1991; Weiss et al., 1992; Creese et al., 1992; Teicher et al., 1991).

Discussion

Our findings suggest that distinct phases of postnatal development characterize the functional maturation of striatal circuits as displayed by network-level changes in distribution of both constitutive and inducible proteins of the Fos/Fra and NGFI-A families. Major shifts occur in both the constitutive and inducible expression of these transcription factors in the striosome and matrix compartments of the developing striatum. For the inducible expression patterns the developmental changes are roughly parallel for the three classes of proteins we studied. However, these shifts in inducible expression are not parallel to the shifts in constitutive expression that we found, which themselves are different for each class. In addition to these changes in gene expression in the striatum, we found equally striking developmental changes in the inducibility of all three classes of transcription factor in basal ganglia nuclei receiving inputs from the striatum. We suggest that Fos, Fra and NGFI-A proteins may play at least two roles in the developmental modeling of striatal networks. Selective constitutive expression of these proteins in the developing striosome and matrix compartments may be part of the molecular mechanisms driving compartment-specific phenotypic pattern formation in the striatum. The inducible expression of these proteins may reflect activity-related modeling of striatal compartments and the basal ganglia circuits differentially related to them.

Developmental phases in dopamine's influence on the striatum

Dopamine and dopaminergic nigrostriatal afferents are crucially important for proper function of the basal ganglia and cortico-basal ganglia systems. Degeneration of the dopaminergic nigral system results in severe motor and cognitive impairments manifested in dopamine degenerating disorders such as Parkinson's disease. A role for the nigrostriatal system in sensorimotor-reward based learning has also been established in experimental work in primates (review in Graybiel, 1995)and on the basis of

neuropsychological observations (review in Graybiel, 1995). At molecular level, dopamine' s actions are mediated by receptors coupled to G proteins. As a consequence, a methodology currently applied to study functional interactions between the dopaminergic afferents and their postsynaptic targets is to monitor molecular changes caused by the activation of these second messenger pathways, including the induction of transcription factors of the immediate-early gene group. Through the aid of this methodology, the molecular mechanisms of dopamine action on striatal neurons has been extensively studied in vivo and in vitro, and it has been possible to identify individual and synergistic contributions of D1- and D2- class and their close interactions with the glutamatergic afferents (Dragunow et al., 1990; Marshall et al., 1993; Paul et al., 1992; LaHoste and Marhall, 1993; Liu et al., 1995; Moratalla et al., 1996; Cole et al., 1995). Downstream effects of the regulation of IEG expression have not been identified, but the selectivity of the IEG induction for subsets of striatal neurons suggests that the regulation is specific and tightly constrained. Especially interesting are experiments in which the IEG induction has been used to uncover molecular mechanisms at the basis of the long-lasting changes in behavior induced by repeated exposure to drugs of addiction (Hope et al., 1994; Moratalla et al., 1996)

Our results suggest that postnatal striatal development can be divided into three functional phases based on patterns of constitutive and inducible IEG expression: a striosome-dominated period (P0-P7); a transitory period (P14-P15); and a late phase of refinement (by P21). The early or *striosome-dominated* phase, which spans the first postnatal week in the rat, is characterized by a compartment-selective constitutive and induced expression of Fra, Fos and NGFI-A proteins in striosomes. The dopamine agonist induced expression, but not the constitutive expression requires D1-class dopamine receptors but D2-class dopamine receptors also have striosome-predominant effects, even though they are widely distributed in both the striosomes and the matrix. Our results suggest that during this early phase, there is differential coupling of dopamine through both

D1 and D2 receptors to functional activation of striatal neurons of the striosomal compartment. Remarkably, the basal expression of these three classes of proteins in the striatum is mainly confined to striosomes, and we could find little evidence that this expression could be changed by dopamine receptor blockade. The second or *transitory period* phase, which occurs during the second postnatal week, is characterized by striatal constitutive and induced expression that gradually changes from the tight compartmental constraint to striosomes, and by a transient period in which dopaminergic agonists induce IEG expression in basal ganglia output structures receiving inputs from the striatum, the globus pallidum and the substantia nigra. The striatal induction of IEGs by the psychomotor stimulants is blocked by D1 antagonists whereas the IEG induction in the pallidum and substantia nigra is blocked by D2 antagonists, but not by D1 antagonists. During this time, striosomes no longer have most of the constitutive expression of the IEGs. Constitutive Fos has become undetectable, constitutive Fra have declined and constitutive NGFI-A proteins have appeared in the matrix. They still appear to be independent of D1 receptors, in sharp contrast to the inducible Fras. By the third postnatal week, striatal patterns of IEG induction by psychomotor stimulants begin to stabilize and to acquire their adult form: amphetamine induces striosome-predominant pattern rostrally, whereas cocaine induces a more homogeneous pattern of striatal activation, especially strong centromedially. Constitutive expression of Fos is almost nil, Fra appear mainly medially and ventrally, but NGFI-A is broadly distributed. This findings suggest that during normal development, most of the early effects of dopaminergic activation of the striatum are focused on the striosomal system, but that the effects of dopamine are then widely extended to both striatal compartments during a transitory period when activity in dopaminergic systems may tune basal ganglia circuits. It is during this transitory period that many of the phenotypes of the striosomes and matrix undergo sharp transitional changes before acquiring their mature form. The alterations in patterns of IEG expression could represent activity-dependent molecular changes that become translated into shifting

expression patterns of these neuroactive substances. Our results also emphasize, however, that apparently independent of dopaminergic stimulation, there is a steady series of shifts in constitutive expression of the same classes of IEG proteins. An interesting possibility raised by our findings is that the constitutive and inducible proteins include different species of the proteins with different functional effects.

Developmental IEG expression patterns may reflect distinct maturational programs for the striosomes and matrix compartments of the striatum

During the first postnatal week, the dopamine-containing innervation of the striatum is densest in striosomes, but by the third postnatal week this innervation is widely distributed throughout the striatum. The developmental pattern of IEG induction we have described is also first striosome-specific and then more broad, showing drug-specific patterns. At first glance, these results suggest that the IEG pattern we observed simply reflects the intensity of the dopaminergic innervation of the two compartments. However, other findings suggest that this simple idea does not account for our results. First, it has been shown that organotypic slices of P0 rat striatum exposed to uniform concentrations of dopamine D1-class receptor agonists show the striosomal-specific pattern for induction of Fos proteins, but that Fra proteins are induced in both striosomes and matrix in response to the same D1 agonists (Liu et al., 1995). During the first postnatal week, D1 receptors are enriched in striosomal neurons. We found that the D1-class dopamine receptors predominately mediate the IEG induction. This result suggests that the richness of functional D1 receptors in the developing striosomes could be the causal link to the compartment-specific IEG induction. However, not only the high expression of D1-class dopamine receptors in the striosomes but differential intracellular coupling may contribute strongly to the compartment-specific IEG induction observed in the striosome-dominant period. In organotypic slices, not only are some Fra proteins activated by D1 agonist

treatment, but also, such stimulation leads to the rapid phosphorylation of CREB protein in both striosomes and matrix (Liu and Graybiel, 1996a). However, the matrix CREB phosphorylation phase is very brief and does not lead to subsequent Fos induction while the striosomal CREB phosphorylation phase is sustained and does lead to Fos induction (Liu and Graybiel, 1996a). Striatal phosphatases have been singled out as controlling the duration of the CREB phosphorylation and subsequent expression of Fos and of at least one potential downstream target of IEG regulation, dynorphin (Liu and Graybiel, 1996b). Therefore it appears that the compartment-constrained early developmental pattern of IEG induction by dopaminergic stimulation is driven, at least in part, by a different functional intracellular coupling of the dopaminergic receptors as well as by distinct maturational programs reflected in the constitutive IEG expression we observed.

Interactions between glutamatergic and dopaminergic receptor function manifested by Fos, Fra and NGFI-A expression patterns during development

A range of experimental evidence suggests that interactions between the dopaminergic and the glutamatergic afferents underline normal function in basal ganglia circuits. At circuit level, glutamatergic and dopaminergic afferents can terminate in close proximity to each other and the terminal axons of the dopaminergic and glutamatergic projections to the striatum are thought to possess receptors for the other neurotransmitter: presynaptic glutamate receptors on the nigra axons and dopamine receptors on the cortical terminals (review in Starr, 1995; Wang, 1991; Desce et al., 1992; Maura et al., 1989; Yamamoto and Davy, 1992). It has been shown that local striatal administration of glutamate, or glutamate increment caused by cortical stimulation increase the release of dopamine (Wang, 1991; Desce et al., 1992). At the same time, increased extracellular levels of dopamine by amphetamine or dopamine increment due to nigrostriatal stimulation lead to a decrease of glutamate release (Rowlands and Roberts, 1980; Maura et al., 1989;

Yamamoto and Davy, 1992). Interactions between the dopaminergic and glutamatergic systems also appear possible at the level of intracellular activity (Konradi et al., 1996). In adult animals these interactions are evident in IEG induction assays: blockade of NMDA receptors blocks Fos/Fra and NGFI-A induction by psychomotor stimulants (Torres and River, 1993; Wang et al., 1995) and dopamine receptor blockade affects glutamatergic corticostriatal transmission (Berretta et al., 1997). Our results suggest that NMDA receptors can block intracellular changes triggered by dopaminergic stimulation as early as P6, and that they can affect not only members of the leucine-zipper Fos/Fra family but also members of the zinc-finger family such as NGFI-A. In contrast to this blockade of the inducible expression, constitutive expression of Fra and NGFI-A is still present and occurs in a striosome-specific pattern after NMDA blockade. During this early postnatal period, different subtypes of AMPA receptors as well as NMDA receptors are expressed with a striosome-predominant pattern (Snyder-Keller and Costantini, 1996). We did not test whether the constitutive Fra and NGFI-A expression is dependent on the AMPA glutamatergic receptors, or is glutamate receptor independent. P0 and prenatal striatal organotypic slices, however, maintained basal Fra expression, resembling that seen in vivo even though the slice procedure isolates the striatum from afferent input (Liu et al., 1995; see Chapter 4). This result suggests an afferent independent expression at least for the Fra proteins (see chapter 4).

Figure 3. Developmental changes of NGFI-A, Fra and Fos patterns of constitutive expression in the postnatal striatum.

At P2 the immunodetectable NGFI-A (A), Fra (A') and Fos (A") proteins have all a patchy pattern of expression characterized by a different level of expression. Basal Fos expression is very weak. By P21, NGFI-A (B) proteins are detected in both compartments with a more intense expression near the ventricular area; Fra (B') proteins are downregulated and remain detectable only medially near the ventricular area. Fos (B") proteins are completly downregulated and undetectable. Scale bar shown in A indicates 500 μm for A' and A". AC, anterior commissure; CP, caudate putamen; Ctx, cortex; V, ventricle. Scale bar in B indicates 500 μm for B' and B".

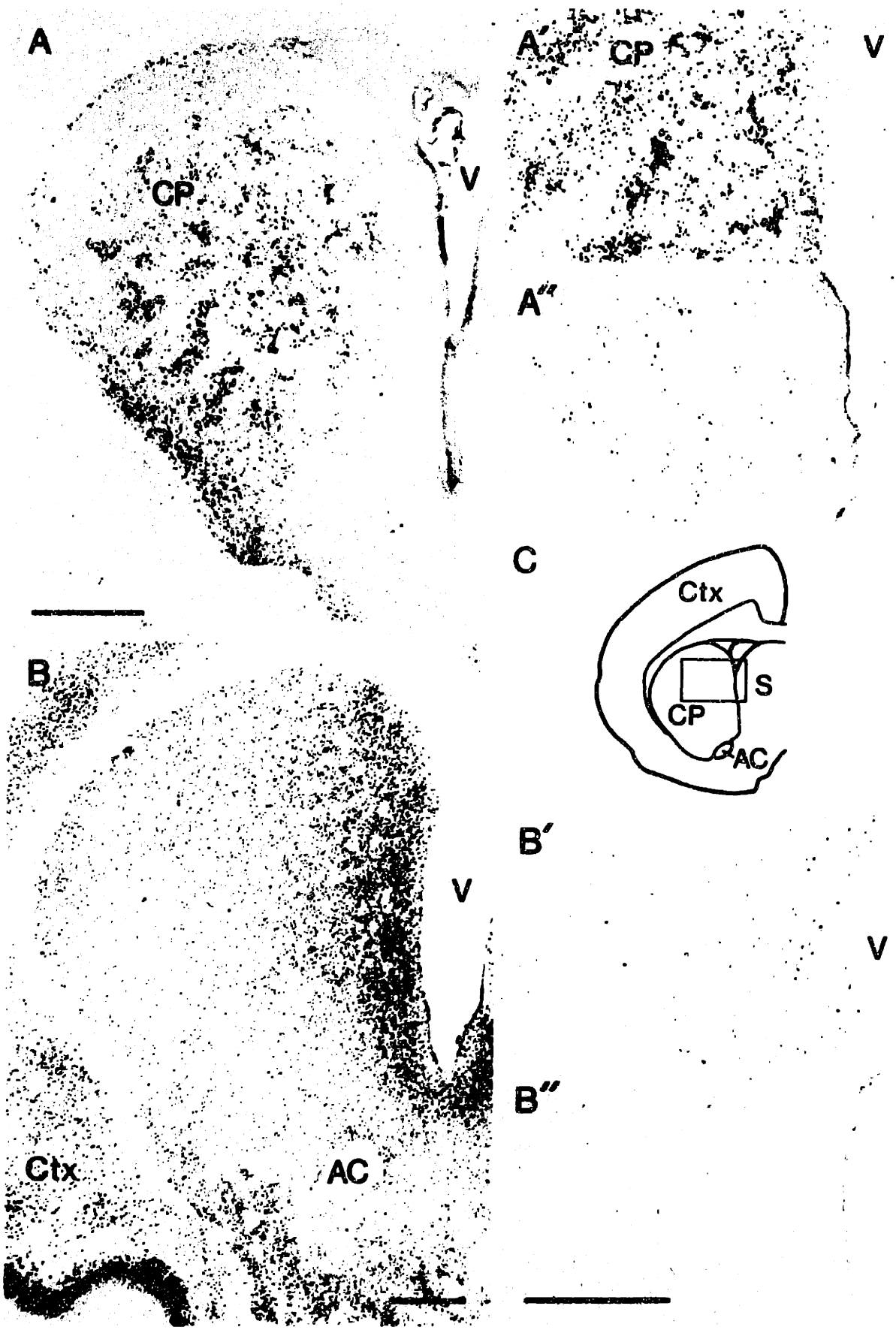

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Figure 4. Both cocaine and amphetamine induce a striosomal-specific immunodetectable pattern of IEG expression in the postnatal developing striatum.

During all the first postnatal week (A, D: P1-P2; B, E: P6-P7), cocaine (A and B) and amphetamine (D and E) induce similar patchy aggregates of Fos proteins. Only around the third postnatal week (C, F: P21), the drug-specific patterns characteristic of adulthood are detected. C, Cocaine induces Fos proteins with a dispersed pattern whereas (F) Fos pattern of expression induced by amphetamine remains mostly patchy. AC, anterior commissure; CP, caudatoputamen; V, ventricle. All scale bars indicate 500 μm.

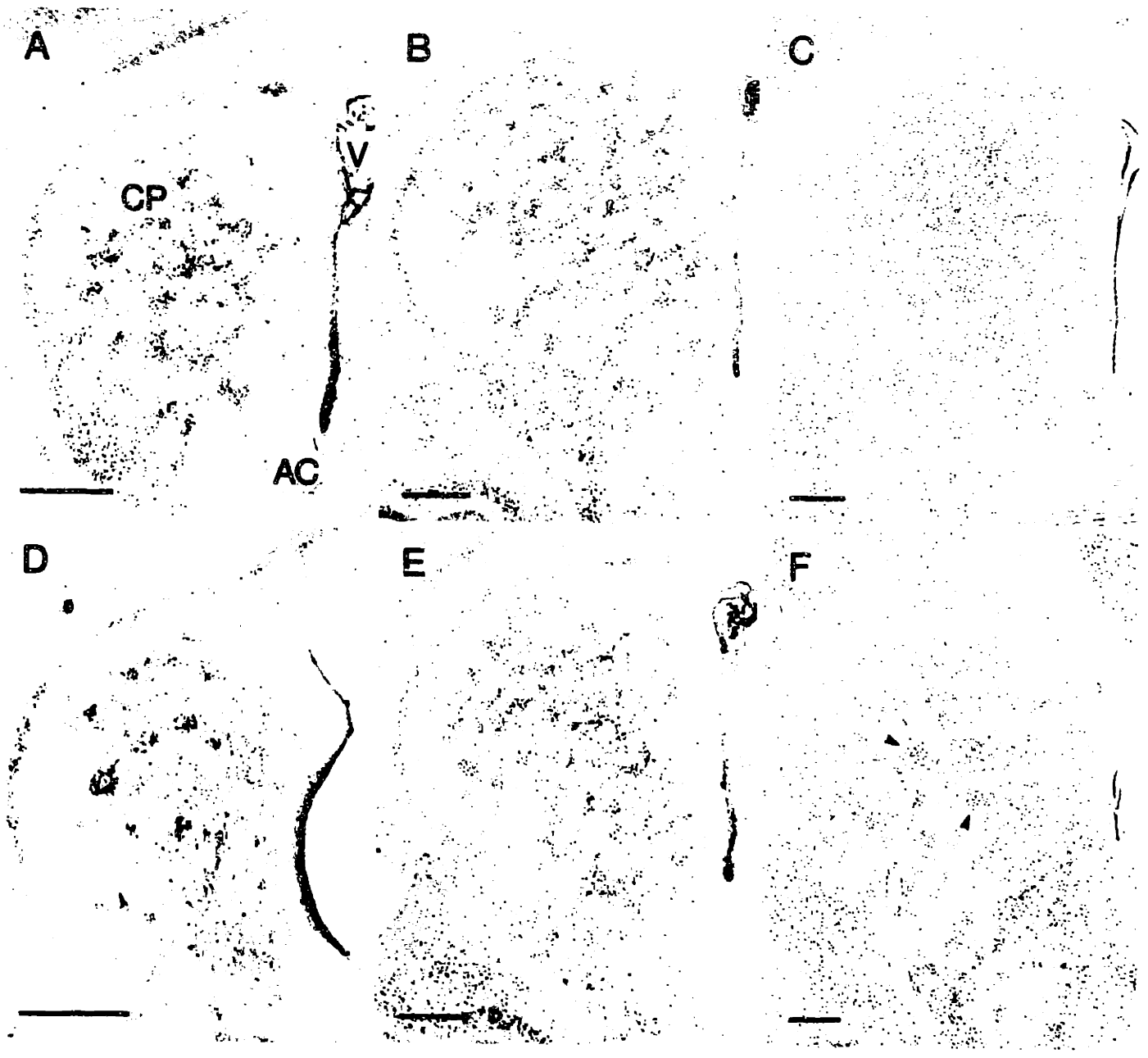
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Figure 5. NGFI-A and Fra constitutive proteins and Fos inducible proteins are expressed in the striosomal compartment during the first postnatal week.

A, A cluster NGFI-A positive colocalizes with a cluster of TH- positive fibers. B, A cluster NGFI-A positive colocalizes with a striosome manifested by the expression of the striosomal phenotypic marker DARPP-32. C, Two clusters of Fos proteins induced by amphetamine colocalize with two clusters of high nigrostriatal innervation as shown by TH immunoreactivity. D, A patch of Fos proteins induced by cocaine colocalizes with a striosome manifested by the expression of DARPP-32. Scale bar shown in C indicates 500 μm for A, B, and D.

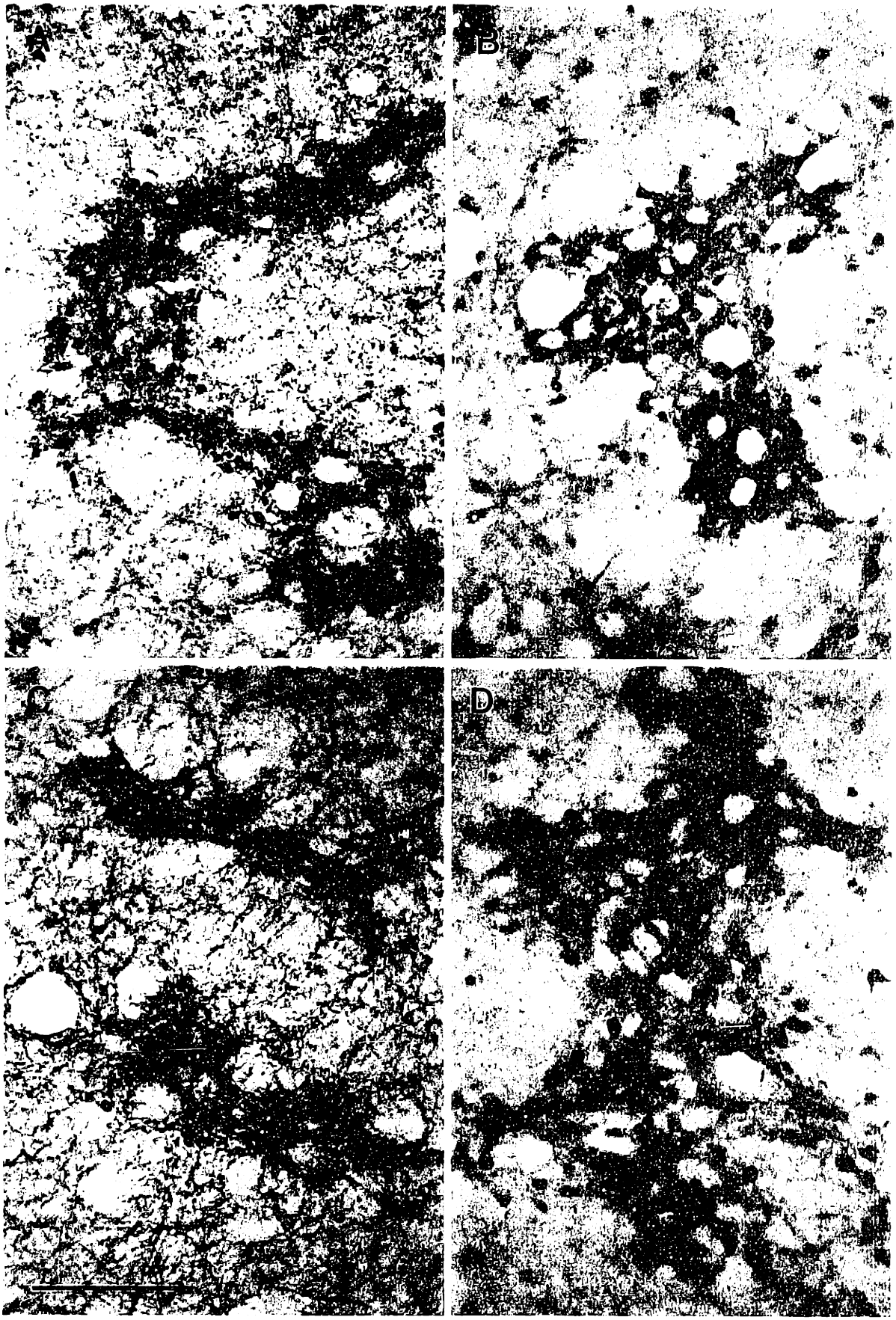
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Figure 6. Fra and NGFI-A constitutive proteins are still present after D1 receptor pharmacological blockade, whereas both Fos constitutive and inducible proteins are completly downregulated.

A, Photomicrograph of Fra immunostaining detected in P2 developing striatum treated, before dopaminergic stimulation by cocaine, with the D1 receptor selective antagonist, SCH23390. Fra proteins are present and expressed with a striosome-predominant pattern in the striatum. In the nucleus accumbens Fra constitutive proteins are detectable and the level of expression is not drastically affected. A' Photomicrography of a near section of the same brain analyzed in A here immunostained with the Fos antiserum. The inducible pool of Fos proteins is completly downregulated as the already weak pool of Fos constitutive proteins. This result is not due to immunoreactivity problems as shown by the detection of Fos-positive nuclei in the septum (as indicated by the arrowhead). B, Photomicrography of Fra immunostaining detected in the developing striatum of a P2 rat treated before amphetamine administration with SCH23390. Fra proteins are present and display a patchy pattern. B', Fos inducible and constitutive proteins are completly downregulated by the same treatment of B. C, Fra constitutive expression is still widely present in the dorsal and ventral striatum after administration of SCH23390. C' By contrast, Fos constitutive expression is completly downregulated by the same treatment of C. AC, anterior commissure; CP, caudatoputamen; NA, nucleus accumbens; V ventricle. Scale bar shown in A indicates 500 μm for B and C. Scale bar in A' indicates 500 μm for B' and C'.

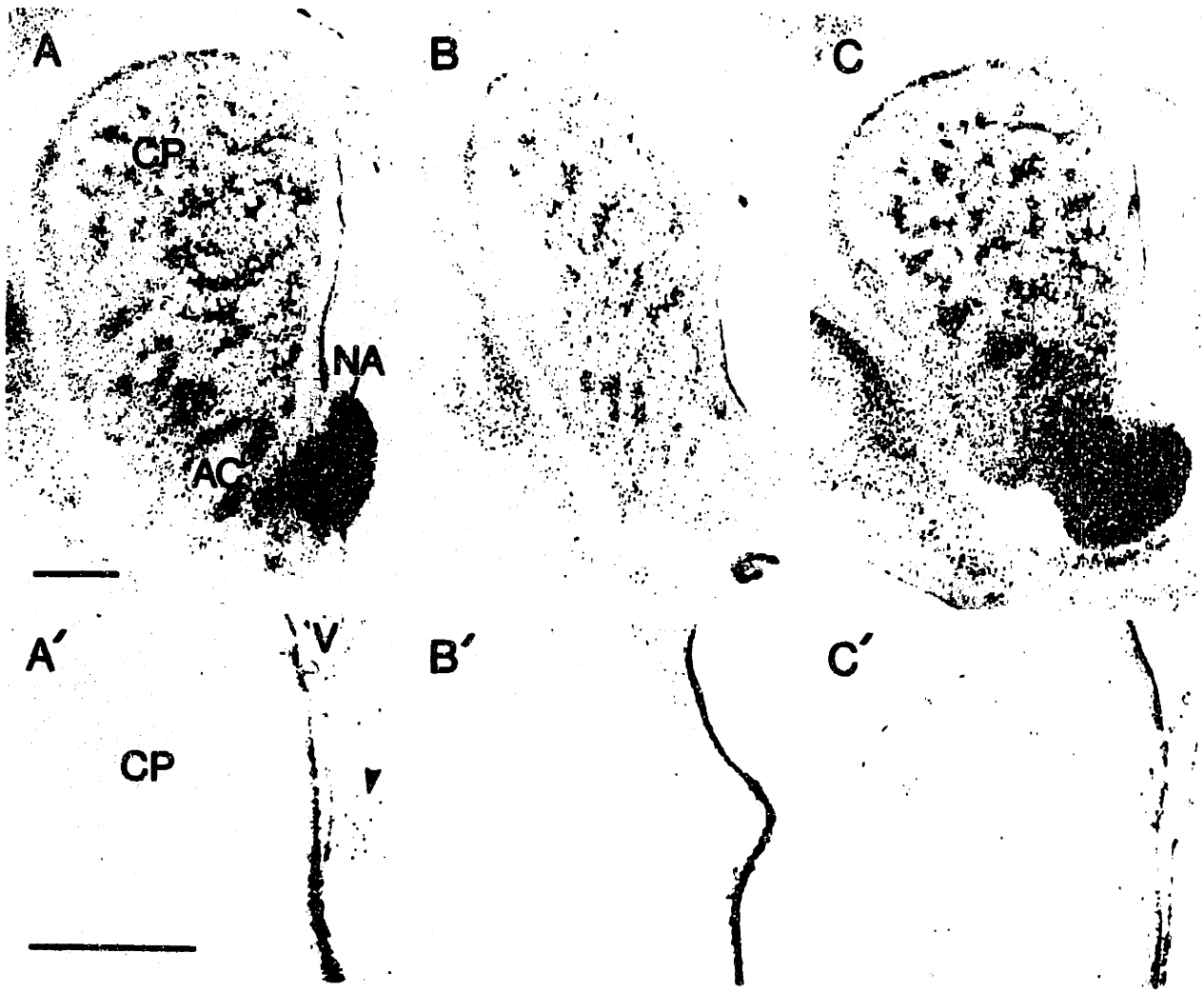
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Figure 7. D2 receptor antagonist treatments with eticlopride administred alone or before dopaminergic stimulation by cocaine or amphetamine induce NGFI-A, Fra and Fos proteins in a striosome-predominant pattern during the first postnatal week.

A, Photomicrograph of the basal level of NGFI-A striosome-predominant expression detected in P6 saline-treated striatum. B and C, NGFI-A inducible proteins are expressed after dopaminergic stimulation with respectively cocaine and amphetamine. D, D2 receptor blockade by eticlopride induce NGFI-A expression above baseline levels and with a striosome-predominant pattern. E and F, D2 receptors blockade by eticlopride before administration of respectively cocaine and amphetamine increases the striosome-predominant induction of NGFI-A expression obtained by the administration of the pyschomotorstimulants alone. AC, anterior commissure; CP, caudatoputamen; V, ventricle Scale bar shown in A indicates 500 μm for B, C, D, E, F.

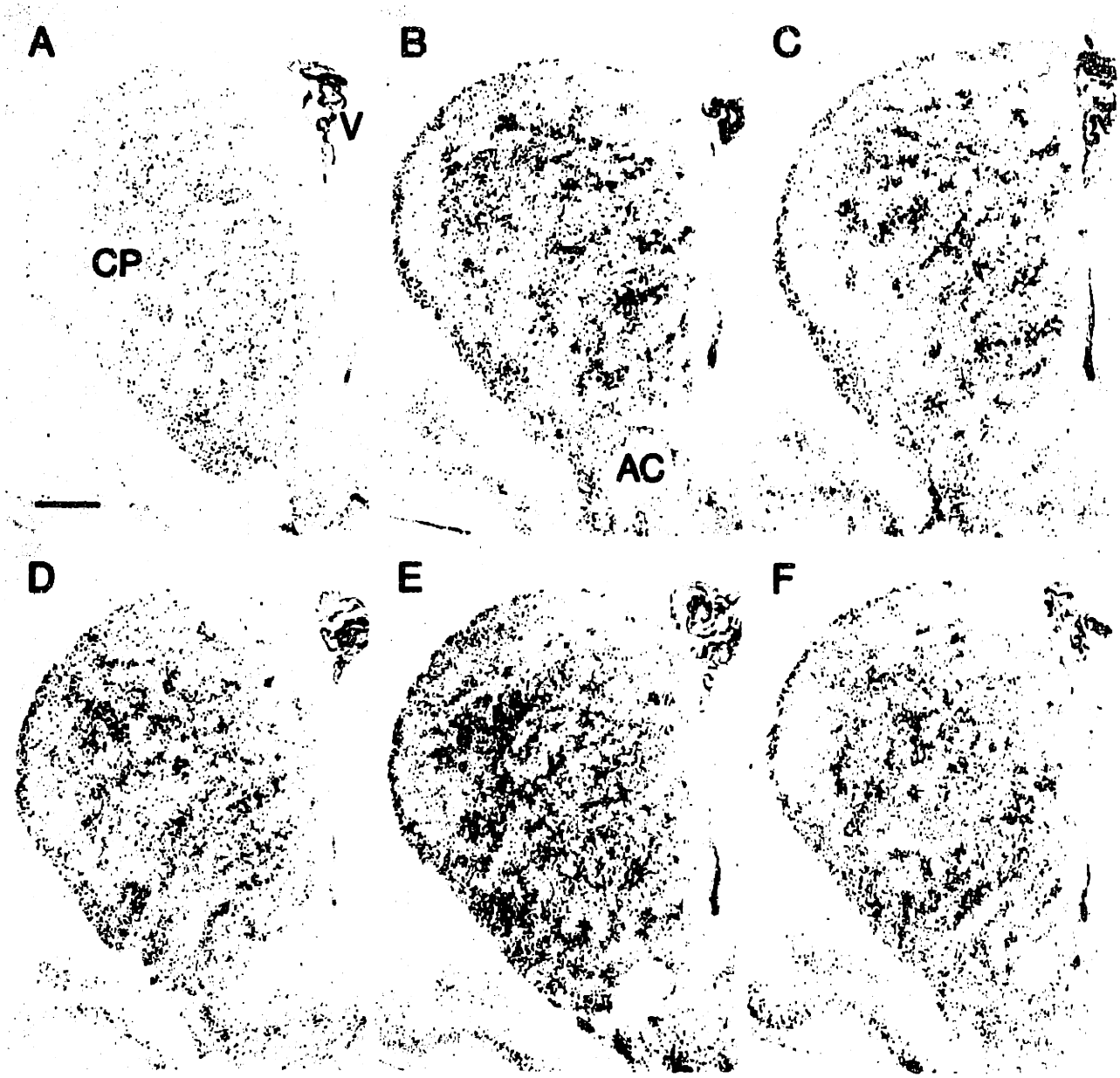
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Figure 8. NMDA receptor antagonist treatments with MK801 administred alone or before dopaminergic stimulation by cocaine completly downregulate the inducible pool of Fos, Fra and NGFI-A proteins whereas only partially affect the constitutive pool of Fra and NGFI-A proteins.

A, Photomicrograph of the striosome-predominant Fra immunostaining detected in P6 saline-treated striatum. B, NMDA receptors pharmacological blockade by MK801 only partially downregulates the constitutive pool of Fra proteins. C, Dopaminergic stimulation by the indirect dopamine agonist, cocaine, induces striosome-predominat expression of Fra proteins above baseline level. D, MK801 treatment before dopaminergic stimulation by cocaine completly blocks the expression of the inducible pool of Fra proteins and partially downregulates the basal pool. CP, caudatoputamen; V, ventricle. Scale bar shown in D indicates 500 μm for B, C, D.


A
CP
B
V
C
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Figure 9. Transient induction of IEG proteins in nonstriatal nuclei by pychomotorstimulants defines a crucial period of basal ganglia development.

A, Photomicrograph of Fra immunostaining detected in the globus pallidus (GP) after dopaminergic stimulation by cocaine. B, The induction of Fra proteins in the GP by cocaine is not sensitive to D1 receptors blockade by SCH23390. C, Fra induced proteins by cocaine in the GP is sensitive to D2 receptors blockade by eticlopride. Eticlopride pretreatment completly downregulates Fra inducible proteins by psychomotorstimulants. D, MK801 pretreatment completly downregulates the induction of Fra proteins by cocaine. The arcs deline the border between CP and GP. CP, caudatoputamen; GP, globus pallidus. Scale bar in C indicates 500 μm for A, B and D.

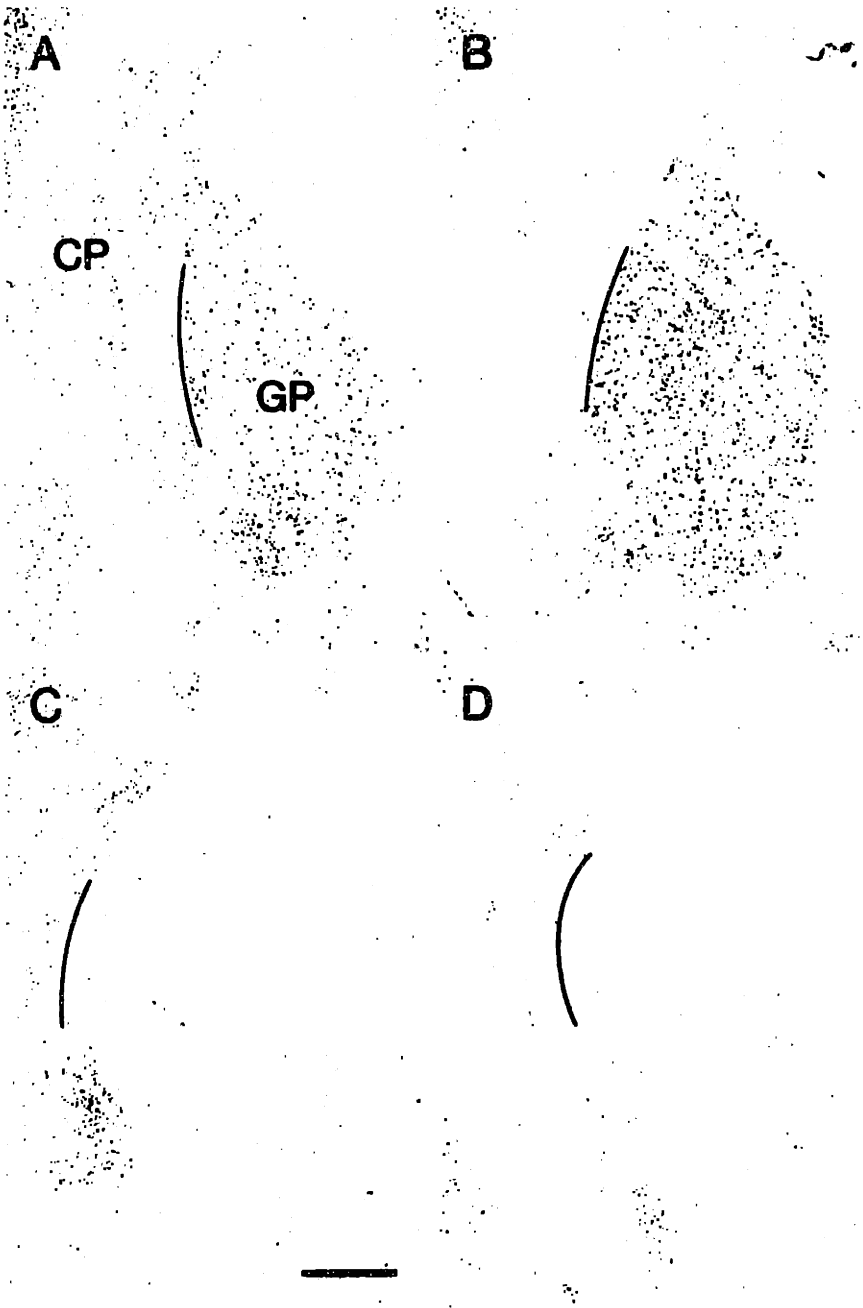
A
B
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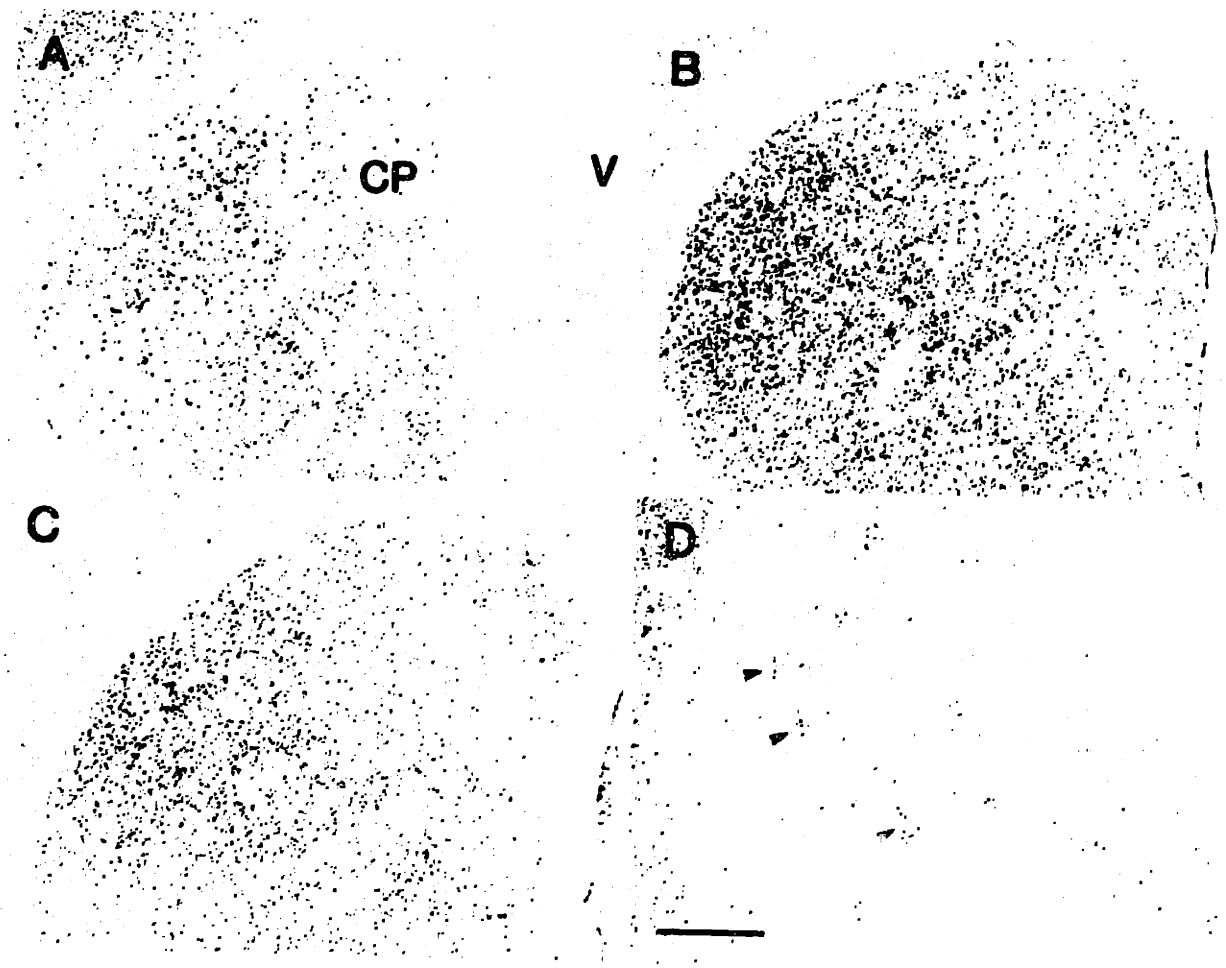
Figure 10. Dopaminergic and glutamatergic regulation of IEG expression in the striatum during the transitory phase.

A, Photomicrograph of Fra immunostaining detected in the striatum after dopaminergic stimulation by cocaine. The distribution of inducible Fra proteins is no longer confined in the striosomal compartment but it is detected in both compartments. B, Pharmacological D2 receptors blockade by eticlopride induces Fra proteins in the caudatoputamen with a distribution typical of adulthood. C, In contrast to the total downregulation of the inducible pool of Fra proteins in the globus pallidus, in the striatum eticlopride as pretreament before cocaine administration induces Fra expression above baseline. D, Pharmacological NMDA receptors blockade by MK801 before dopaminergic stimulation by cocaine causes a general downregulation of the inducible Fra proteins. Only few cells in tiny clusters are spared. CP, caudatoputamen; V, ventricle. Scale bar in D indicates 500 μm for A, B and C.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Summary Table. Data show the complete serie of experiments performed. The intensity of the immunostaining for each antiserum was rated comparing all treatments inside each age group to the saline or no treatment case. For the age group P1-P2, we could not rate the differences between treatments for Fra and NGFI-A antisera likely due to the high basal level of expression characteristic of this age. We used the term present to indicate that Fra or NGFI-A proteins were detectable.

Summary Table

AGE & PATTERN	no treatment	saline	SCH23390	cocaine	SCH23390 & cocaine	amphetamine	SCH23390 & amphetamine	Eticlopride	Eticlopride & cocaine	Eticlopride & amphetamine	MK801	MK801 & cocaine
Striatum P1-P2 Striosomal	present present	present present	present present	present present	present present	present present	present present	present present				
Striatum P6-P7 Striosomal	— ●● ●●	— ●● ●●	— ● ●●	●●● ●●● ●●●●	— ● ●●	●●● ●●● ●●●●	— ● ●●	●● ●●● ●●●●	●●●● ●●●● ●●●●●	●●●● ●●●● ●●●●●	— ● ●●	— ● ●
Striatum P14-P15 critical period	— ● ●●	— ● ●●	— ● ●	●● ●●● ●●●	— ● ●●	●● ●● ●●●	— ● ●●	●●●● ●●●● ●●●	●●● ●●● ●●●	●●● ●●● ●●●	— ● ●	— ● ●●
Globus pallidus & Substantia nigra P14-P15 critical period	— — —	— — —	— — —	●●●● ●●●● ●●	●●●● ●●●● ●●	●●●● ●●●● ●●	●●●● ●●●● ●●	— — —	— — —	— — —	— — —	— — —
Striatum P21-P22 P30 adult-like	— ● ●	— ● ●	— ● ●	●●● ●●●● ●●	— ● ●	●●● ●●●● ●●	— ● ●					

● FOS

● FRA

● NGFI-A

CHAPTER 3

Development of compartment-specific phenotypes in the mammalian striatum and influence of the dopaminergic afferents on the process

Abstract

Selective expression of regulatory genes, such as those coding transcription factors, is critically involved in the pattern formation events that lead groups of neurons to form morphological and functional units.

In the experiments described here, we found that transcription factors of two immediate-early gene (IEG) classes Fos/Fra and NGFI-A, have selective temporal and spatial patterns of expression that could define the developmental phases of compartment formation in the striatum. We traced the onset and developmental expression of Fos, Fra and NGFI-A proteins in the striatum of mouse embryos. We found that all three proteins show a strict and selective expression in parallel with the developing striosomal compartment. First there was a ventrolateral phase (E14-E16) in which Fos-, Fra- and NGFI-A-positive nuclei and future striosomal neurons, were localized at the ventrolateral edge of the striatum; following there was a dispersed phase (E16-E17), in which the IEGs were expressed in neurons dispersed throughout the striatum. IEG expression in the last prenatal days was increasingly defining clusters of neurons, corresponding to striosomes. This clustering phase extended through the first postnatal week, by the end of which IEG expression lost compartment constraints and diversified in gene-specific patterns. Fos and Fra proteins became downregulated with different timetable whereas NGFI-A expression increased by spreading to include the matrix compartment.

The early onset and precisely patterned expression of Fos, Fra and NGFI-A in the striatum suggest that they may be involved in the development of the striatal compartments. The target genes regulated by Fos, Fra and NGFI-A are mostly not known. Interestingly, however, we found a tight correlation between the temporal and spatial patterns of Fra expression and the microtubule associated protein-2, a molecule important in differentiation events in neurons.

The ingrowth of dopaminergic afferents to the striatum originating in the substantia nigra is known to parallel striosome formation, and the expression of D1-class of dopamine receptors, in particular, has a striking specificity for striosomes. In the adult striatum, stimulation of dopamine receptors has been found to regulate Fos/Fra and NGFI-A expression. Thus to determine whether such early arriving dopamine afferents are active in modulating striatal neuron activity, we exposed embryos to an indirect dopamine agonist and examined levels of Fos expression.

A single exposure, through the maternal circulation, to the catecholamine re-uptake blocker, cocaine (25mg/kg; 50mg/kg), was sufficient to increase dramatically Fos-like expression in the developing striosomes at ages as early as E16. This suggest that the early-arriving monoamine afferents are capable of activating striatal neurons, but that their effects are limited to the striosomal compartment.

To test directly whether dopamine acting through the D1 receptors regulates striosome formation, and the expression of IEGs, we characterized the early postnatal morphology and expression phenotypes of the striatum of knockout mice lacking the D1 receptors. Our results indicate that the striosomal compartment, as manifested by the expression of Fra and NGFI-A and the expression of several striosome-phenotypic markers, is indistinguishable from that of the controls. In addition, we found that the pattern of early postnatal dopaminergic innervation is not affected by the absence of the D1 receptors. However we found that there was a selective loss of at least one key neuropeptide, dynorphin. Combined with previous observations that this neuropeptide is

deficient in striosomes of adult D1 deficient mice, we suggest that the D1 dopamine receptor does not play an instructive role in striosome compartmentalization but, instead, selectively affects specific phenotypic markers of this compartment.

Introduction

The mammalian striatum has a striking mosaic structure. It is divided into two major compartments: the striosomes and the matrix. The striatal compartments are recognizable at the level of their connections and cellular biochemistry and developmental histories (Graybiel and Ragsdale, 1978, 1983; Graybiel, 1990). The inputs and outputs of the striatum are also compartment specific. The striosomes are innervated predominantly by the limbic cortices and deep cortical layers, whereas the matrix is innervated by the sensory-motor and association cortex (Graybiel, 1990; Gerfen, 1992). Both compartments receive dopamine-containing afferents from the substantia nigra pars compacta, but only the striosomal neurons project back to the region of the pars compacta. Phenotypically, the striosome and the matrix compartmentation is evident in the differential expression of nearly every neuroactive substance found in the striatum (review in Graybiel and Ragsdale, 1983; Graybiel, 1990). The molecular mechanisms that drive the aggregation of striosomes and matrix compartments during development are mostly unknown. It has been shown that the striosomal compartment is mostly formed by the early born neurons of the striatum, whereas the matrix compartment is composed mainly of the late-born striatal neurons (Graybiel and Hickey, 1932; van der Kooy and Fishell, 1987). The final striosome-matrix architecture apparently emerges from an earlier developmental period in which there is intermingling of future striosomal and matrix neurons (van der Kooy and Fishell, 1987). The ontogenic expression of several striatal phenotypic markers have been tracked through this developmental period, but comprehensive understanding of this compartmentation - the striatal equivalent of layer cortex formation - is still elusive. The molecules showing such developmental regulation range from enzyme such as acetylcholinesterase (Butcher and Hodge, 1976; Graybiel et al., 1981a) to regulators of phosphorylation such as dopamine and cyclic adenosine 3':5'- monosphosphate-regulated phosphoprotein 32 (DARPP-32) (Foster et al., 1987) to neuropeptides such as dynorphin

(Brana et al., 1995) to receptors such as the D1-class of dopamine receptors (Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994). After this early period of striosome predominance, however, there is great diversification in the expression of these and other molecules, some retain their enhanced expression in striosomes (Brana et al., 1995), other start to express in both compartments (Foster et al., 1987; Caille et al., 1995; Murrin and Zeng, 1989; Schambra et al., 1994). and yet others apparently switch compartmental patterns (Butcher and Hodge, 1976; Graybiel et al., 1981a). The mechanisms orchestrating the temporal and spatial expression of these phenotypic markers of the striosome/matrix compartments are still unknown.

The selective temporal and spatial expression of regulatory genes, such as those coding for transcription factors, is known to be among the molecular mechanisms determining the development of the nervous system (review in He and Rosenfeld, 1991; Kessel and Gruss, 1990; Struhl, 1991; Rubenstein et al., 1994). Several transcription factors have been found expressed from early embryonic stage in the developing striatum but yet no transcription factor has been selectively attributed to a specific striatal compartment at early prenatal age (Price et al., 1991; Porteus at al., 1991; Le Moine et al., 1992; Simeone et al., 1994).

We demonstrate here that, during murine embryogenesis, the immunodetectable transcription factors of the immediate-early gene (IEG) group including NGFI-A, Fra and Fos are selective markers for the forming striosomal compartment of the striatum from early prenatal ages. We suggest that genes of these families could contribute to the development of striosome/matrix phenotypes.

To test whether the dopaminergic afferents acting through the G-protein coupled striatal dopaminergic receptors could influence the expression of these transcription factors, we pharmacologically stimulated the nigrostriatal system at E15-E16, E16-E17, E18-E19 with an indirect dopamine agonist, cocaine, and we checked the levels and patterns of expression of the IEGs studied. The levels of constitutive expression of Fra and NGFI-A

were so high that we could not detect effects of the dopaminergic stimulation but for Fos, which have lower constitutive levels, we found high inducibility from as early as E15-E16. The pattern of inducible expression had the same distributions followed and the same developmental phases delineated with the constitutive expressions of the IEGs. Thus at least some of the early expressed transcription factors are influenced by dopaminergic afferents, and, more generally, dopaminergic stimulation can activate second messenger pathways in striatal neurons of the future striosomal compartment from as early as E15-E16, well before the striosomal clusters have emerged.

To further determine how D1 dopamine receptors influence the development of the striosome/matrix pattern, we characterized the neurochemical architecture of the striatum of mutant mouse pups lacking the D1 receptor. Our results show that D1 receptors are necessary for the early establishment of a phenotypic marker of the striosome/matrix pattern, as shown by the lack of dynorphin in the striosomes. However, our results demonstrate that compartmental aggregation of striatal neurons is not radically affected by the absence of D1 receptors, because we found clear striosome-predominant expression of other phenotypic markers. Remarkably, we found that the constitutive expressions of Fra and NGFI-A were not affected, suggesting that the basal expression of Fra and NGFI-A are D1-independent, if not dopamine-independent.

Materials and Methods

Experimental procedure

Observations were made on the brains of mouse fetuses and pups of CD-1 time pregnant mice (Charles River) of the following stages: embryonic days 12 (E12), E13, E14, E15, E16, E17, E18, E19, postnatal day 3 (P3), P9, P18, P24. To minimize variability on fetal age, only 4hr mating periods (8.00am-noon) was allowed (Charles River).

Some litters were exposed to the indirect monoamine agonist, cocaine, administered through maternal circulation fetuses of the age groups comprised from E15 to E19 (SIGMA, St. Louis, MO; 50mg/kg, 25mg/kg; s.c. in saline solution). Litters were of the following ages: E15, E16, E17, E19. Parallel control experiments were carried out by administering saline to time-pregnant mice at matched stages of fetal development.

Observations were also made on the brains of D1 knock-out mice generously provided by Prof. Tonegawa and generated by Xu et al. (1994) at P6-P7 and P11.

Tissue preparation

Time pregnant mice were deeply anesthetized with an overdose of Nembutal (150 mg/kg), P3 pups were anesthetized on ice and older pups were anaesthetized inhaling Metofane. Fetuses and pups were perfused transcardially with 4% paraformaldehyde in 0.1M sodium phosphate buffer/0.9% NaCl (PBS) or in 0.1M cacodylate buffer (pH 7.4). The brains were postfixed in the same fixative for 2hrs, and then were cryoprotected at 4°C for at least 24 hrs in 20% glycerol in PBS or cacodylate buffer. Serial coronal sections were cut on a freezing microtome at 20μm and were collected in 0.1M cacodylate buffer, 0.1%

Na azide (sections for double immunohistochemistry) or at 40μm and collected in 0.1M phosphate buffer, 0.1% Na azide (sections for single immunohistochemistry).

Immunostaining was carried out with the following antisera: 1) rabbit polyclonal antiserum against NGFI-A, 1:2,000 (generously donated by Dr. J. Milbrandt); 2) rabbit polyclonal antiserum against c-Fos, 1:200 (Oncogene Science, Inc., Uniondale, NY lot #392051); 3) rabbit polyclonal antiserum against the conserved M-peptide sequence in c-Fos and Fos-related antigens (Fras), 1:2,000 and 1:5,000 (generously donated by Dr. M.J. Iadarola); 4) mouse monoclonal antibody against dopamine-and-adenosine 3':5'monophosphate regulated phosphoprotein-32000, 1:20,000 (DARPP-32; kind gift of Drs. P. Greengard, H. Hemmings, and E.L. Gustafson); 5) mouse monoclonal antibody against microtubule-associated protein 2 (MAP-2), 1:400 (SIGMA, St. Louis, MO); 6) mouse monoclonal antibody against tyrosine hydroxylase (TH), 1:1,000 (SIGMA; St. Louis, MO); 7) rabbit polyclonal antiserum against rat dynorphin B (1-13), 1:10,000 (generously provided by Dr. S Watson). The immunostaining results described refer specifically to the pattern of immunoreactivity detected with the antisera and antibodies listed above.

Single antiserum immunohistochemistry

Free-floating sections were pretreated with 3% H_2O_2 in 0.01M PBS and 0.2% Triton X-100 (PBST) for 10 min and then were incubated in 5% normal serum from the same species in which the secondary antibody was raised. Sections were incubated in primary antiserum at 4°C for 48-72hr, with the antisera diluted in PBST. For avidin-biotin-peroxidase complex immunocytochemistry, sections were rinsed in PBST, incubated in a 1:500 biotin-conjugated secondary antiserum (Vector Laboratories) for 1hr, rinsed in PBST, and incubated in avidin-biotin complex (ABC, Vectastain, Vector Laboratories, 6μl/ml) in PBST for 1hr before being reacted with diaminobenzidine (DAB) and

sometimes enhanced with nickel ammonium sulfate. Sections were mounted on gelatin-coated slides, air dried, dehydrated, coverslipped with Eukitt mounting medium, and analyzed by light microscopy.

Dual antiserum immunohistochemistry

Dual staining in single cells was carried out by combining either nickel-intensified DAB and DAB or silver intensified immunogold and DAB as chromogens. The nickel/DAB and the silver intensified immunogold produce gray-black colors whereas DAB alone produces a brown staining (Batchelor et al., 1989).

To stain for two nuclear antigens, such as Fra and NGFI-A, we used a procedure combining immunogold-silver and avidin-biotin peroxidase complex labeling was used (Berretta et al., 1992). Sections 20μm thick were pretreated with 0.5M NH_4Cl for 10 min and then with 2% BSA in 0.1M tris(hydroxymethyl)aminomethane (TRIS)-buffered saline (TBS) plus Triton X-100 (TBST) for 30 min. The sections were incubated with Fra antiserum diluted 1:2,000 in 1% BSA TBST at 4°C for 24-72hr. After several washes in TBST, the sections were incubated overnight with gold conjugated secondary antisera (Auro-Probe LM, Amersham, Arlington Heights, IL; 1:400), washed for 1hr in 0.02M TBS and carried through to silver enhancement (IntenSE M kit, Amersham; 10 drops for each component) for 40 min to achieve a high-contrast, black dot-like signal. Sections were then washed overnight in 0.1M PO_4 buffer and carried through a standard avidin-biotin peroxidase immunohistochemical protocol.

To co-stain for a nuclear antigen and a cytoplasmatic antigen, such as staining for Fra, Fos, or NGFI-A in combination with DARPP-32, TH or MAP-2, we used a procedure of two avidin-biotin peroxidase complex labeling (Batchelor et al., 1989). The first staining was enhanced with nickel and so produced a black dot-like signal, whereas the second staining produced a light brown signal. After the nickel-DAB staining, sections

were washed overnight in 0.1M PO_4 and then were incubated for 45 min in 0.6% H_2O_2 to eliminate the residual peroxidase activity and treated with blocking passages in avidin and in biotin before the beginning of the primary incubation for the second antigen, performed as described in the single antiserum immunohistochemistry protocol.

Result

Early forebrain expression of Fos/Fra and NGFI-A proteins

High basal expression of transcription factors of the Fos/Fra and NGFI-A families were found in the embryonic forebrain. By E14, the earliest embryonic stage analyzed Fra protein were already strongly expressed. NGFI-A proteins were first detected at E16 and Fos protein at E17. From the earliest times they were detectable, these transcription factors showed a striking selectivity for a small subset of forebrain regions: striosomes within the striatum, and limbic-olfactory regions of the cortex and basal forebrain (Fig.11). With time, the expression patterns of each class of protein developed broader expression patterns, which were similar to one another but not identical. Fra and NGFI-A immunoreactivities were very intense, but Fos immunoreactivity, although present, was weak.

Selective expression of Fos, Fra and NGFI-A proteins in the developing striosomal compartment

Remarkably, the temporal and spatial patterns of Fra, Fos and NGFI-A expression all resembled the temporal and spatial patterns known to characterize the striosomal compartment of the striatum (Graybiel and Hickey, 1982, Graybiel, 1984; Fishell and van der Kooy, 1987). Striosomal neurons have a "dispersed" pattern in which they appear broadly distributed through the developing striatum. They then begin to aggregate into clusters, and finally they form definitive striosomal patches (Fishell and van der Kooy, 1987). We found that immunoreactivities for Fos, Fra and NGFI-A proteins all follow these developmental phases characteristics of striosome formation as shown in Fig. 11 A, B, C, using Fra expression as archetype. Thus at E14 Fra- and E16 Fra- and NGFI-A-

positive nuclei were largely at the ventrolateral edge of the striatum then at E16-E17, Fos, Fra and NGFI-A were all expressed in nuclei dispersed diffusely throughout the striatum (Fig. 11 A, B). By E18 starting caudally on late E17, aggregations of immunopositivenuclei were clearly detectable, following the progressive caudal to rostral gradient known for the aggregation of striosomal neurons (Fig.11 C).

To determine whether the expression of these transcription factors was, in fact, coincident with that of the developing striosomal system, we tested for colocalization of the IEG proteins with striosomal markers. To identify the developing striosomes we used DARPP-32 and TH (Foster et al., 1987; Voorn et al., 1988). At early stages of striatal development, DARPP-32 is specifically expressed in the striosomal compartment (Foster et al., 1987). In the same time frame, the dopamine-containing axons, marked with the tyrosine hydroxylase enzyme TH, change from a diffuse innervation pattern to a striosome-predominant pattern of innervation (Graybiel 1984; Voorn et al., 1988). We found that the IEG-positive clusters of neurons were coincident with patches of high DARPP-32 and TH staining (Fig.12).

In contrast to the tight linkage between the expression patterns of the IEGs prenatally, their expression pattern diverged postnatally. Fos expression rapidly declined and by P3 was undetectable. Fra expression remained strong and predominately striosomal until P7, but then decreased sharply with a lateral to medial gradient (Fig.13 A, B, C). NGFI-A expression remained detectable primarily in the striosomal compartment but then spread also to the matrix, finally becoming expressed in both compartment (Fig.13 D, E, F).

The onset of IEG expressions start during the differentiation process of the striosomal neurons, when the cells are already arrived in the target structure. Given that the earliest born cells are eventually positioned mostly in the lateral and caudal striatum and the later born cells are medial and rostral. IEG expressions follow the same pattern and as a

wave they spread from the lateral and caudal part to the medial and rostral part of the striatum.

NGFI-A-positive neurons define a subgroup of the Fra-positive cells before and after striosomal aggregation

We found that Fra and NGFI-A patterns of expression are very similar, but that Fra immunoreactivity was consistently expressed in a higher number of cells, thus indicating an intriguing heterogeneity inside the striosomal population. Using IEG expression as marker for the striosomal neurons, it could be possible to divide the population into different groups Fra-positive neurons and NGFI-A-positive neurons. To determine whether Fra and NGFI-A were labeling the same striosomal neurons or two different striosomal subpopulations, we double labeled with the two antisera at E17, E18 and E19. We used as an internal control Fra-positive neurons in the cortex, where NGFI-A-positive neurons are not detected at the ages studied (Fig.12 E''). We found that NGFI-A is expressed by a subpopulation of the Fra-positive neuron both before (E17) and after striosome aggregation (E18) (Fig.12 E, E').

These results suggest that, at a time crucial for compartment aggregation, there are at least two distinct subgroups in the striosomal population, Fra-positive and Fra/NGFI-A-positive neurons.

Developmental pattern of expression of Fos, Fra and NGFI-A in the mouse forebrain

In contrast to the similarity in the spatial and temporal patterns of expression of the IEGs we tracked in the striatum, the expression of these transcription factors was not tight linked in the cortex. Fra proteins were detectable in the developing neocortex and in the hippocampal formation from E14 and were strongly expressed in the claustrum by E16.

By contrast NGFI-A and Fos expression were detected in the cortex only postnatally. The patterns of expression resemble very close the one already described in chapter 2 for the rat cerebral cortex. In the cortex, the expression of Fra developed from a localized regional expression in the cingulate cortex at E14 to a more general layered expression by E18 (Fig.11 A, B, C).

High striosome-predominant expression of the microtubule-association protein 2 (MAP-2) during striosome aggregation

In an effort to investigate whether the striosomal and matrix neurons were in a similar or different stage of neurodifferentiation we characterized the expression of the neurodifferentiation marker microtubule-associated protein 2 (MAP-2) during striatal development. MAP-2 is a regulatory protein for cytoskeletal stabilization that is selectively expressed in neuronal cells and specifically localized in dendritic arborizations (review in Johnson and Jope, 1992). We found that MAP-2 is expressed at low levels in both matrix and striosomal neurons after they leave the ventricular zone (VZ) and the subventricular zone (SVZ). At E17, when the striosomal neurons start to aggregate and IEG-positive patches start to be detected, patches of high MAP-2 staining start to appear (Fig.14 A). The appearance of strongly stained MAP-2 patches followed the same spatial and temporal gradient observed for striosomes aggregation by IEG expression (Fig.14 B). To determine whether or not the MAP-2 positive patches were striosomes, we double-labeled with the Fra and NGFI-A antisera. We found that MAP-2 positive patches colocalized with Fra and NGFI-A positive patches (Fig.12 C).

Postnatally, patches of high MAP-2-positive staining were detectable until P3 (Fig.14 C). In contrast to the cortex, where MAP-2 staining remains constant during development, MAP-2 in the striatum was strongly downregulated with a gradient similar to the downregulation gradient detected for Fra expression. By P18, MAP-2 positive

patches were no longer detectable, and a general MAP-2 downregulation occurred throughout the region (Fig.14 D). MAP-2 staining was still present in the very medial striatal region, at the edge with the ventricle and in the fundus striatum in the same pattern as that for Fra expression (Fig.14 D). MAP-2 high basal expression as Fra constitutive expression were prerogative of the striosomal compartment and were never detected in the matrix compartment. These results suggest a tight correlation between Fra expression and MAP-2 expression in the developing striatum.

Prenatal responsiveness of striosomal neurons to dopamine: effects on Fos, Fra and NGFI-A expression

To test the neuronal responsiveness of striatal neurons to dopaminergic stimulation during prenatal development, we injected the dopamine indirect agonist cocaine into pregnant mice and analyzed Fos, Fra and NGFI-A expression. Remarkably, the expression of Fos, Fra and NGFI-A was largely confined to neurons of striosomes in the striatum and to a small number of neurons in limbic-related cortex. Striking was the induction of Fos proteins above baseline (Fig.15) The spatial and temporal patterns of induction of Fos-like immunoreactivity followed the spatial and temporal patterns known to characterize the early striosomal system (Fig.15, A, B, C). Fos-positive nuclei were, at E16, at the ventrolateral edge of the striatum (Fig.15 A). By E16-E17, they appeared diffusely distributed throughout the striatum (Fig.15 B, B'). By E18, patches of Fos-positive nuclei started to be clearly detectable (Fig.15 C, C'). We did not detect an appreciable induction of Fra and NGFI-A proteins, likely due to the high level of constitutive expression. They did not show, however, detectable increases in expression in the striatal matrix or in cortical regions other than limbic regions.

Our results suggest that neurons of the striosome compartment are sensitive to dopaminergic stimulation, even before any compartment organization is established.

Therefore, dopamine could influence early compartment formation during striatal development.

Striosomes but not dynorphin-positive patches are present in neonatal D1 knockout mice

The pharmacological approach of determining whether dopaminergic stimulation activates IEGs has the limitation that it tests the influence of dopamine receptors only in a transient assay. To determine the role of D1-class dopamine receptors during development of the striosome-matrix pattern, we characterized the early postnatal striatum in D1-deficient mice (Xu et al., 1994). We found that NGFI-A and Fra expression were present in the striatum in newborn D1-deficient mice and were confined to striosomes, as in normal mice (Fig.16). These findings, indicating that the striosomal compartment exists in the absence of D1 receptors, strongly support our evidence based on pharmacological treatments that the basal expression of Fra and NGFI-A survives blockade of D1-class dopamine receptors (see chapter 2). We further found that DARPP-32 striosome-predominant expression in the D1 knock-out mice was indistinguishable from the controls. To determine whether or not there is a link between the developmental phase of striosome-enriched D1-class dopamine receptors and the striosome-enriched dopaminergic innervation, we immunostained the striatum of P6 D1-deficient mice for TH. We found that the striosome-enriched dopaminergic innervation is present despite the absence of D1-class dopamine receptors (Fig.16). To test whether D1-class dopamine receptors are necessary for the development and/or maintenance of the compartment-specific phenotype of striosomal neurons, we immunostained for the neuropeptide dynorphin. Dynorphin staining was virtually absent (Fig.18). We conclude that D1 receptors are necessary for the proper striosome-predominant expression of dynorphin from the early postnatal period.

Discussion

The molecular mechanisms that lead to striosome/matrix pattern formation in the developing striatum could rely on an intrinsic genetic program and on a parallel afferent-dependent program. Our results suggest that the transcription factors Fos/Fra and NGFI-A of the immediate-early genes family have a basal expression that is not even affected by the absence of postsynaptic dopaminergic receptors, such as D1. Furthermore, the onset of Fos/Fra and NGFI-A constitutive expressions is very early and defines crucial steps of striatum development. Even though is not clear which aspect of the differentiation process Fos, Fra, and NGFI-A may regulate these results suggest that the constitutive IEG expression, could be part of the gene regulation necessary for the development of the striatum. In addition, Fos and Fra constitutive expressions are confined to the striosomal compartment thus suggesting a specific action in striosomes development. By contrast, constitutive NGFI-A expression with time looses compartment constraints and NGFI-A proteins are detected in all the striatum. Thus suggesting that NGFI-A proteins may be part of molecular processes of general striatal development. Remarkably, during the same time, the same class of IEG has another pool of proteins that show afferent-dependent regulation. Prenatal pharmacological dopaminergic stimulation by administration through the maternal circulation of the indirect agonist cocaine induce Fos proteins above baseline levels. In addition the pool of inducible proteins follow similar developmental phases defined by the constitutive expression. Thus suggesting that Fos, Fra and NGFI-A have characteristics peculiar of the intrinsic genetic program and of the parallel activity-dependent mechanisms.

Dopaminergic afferents through the D1 receptors affect the striosome/matrix phenotypic development and not the structural compartmental organization

Dopamine-containing afferents innervate the striatum from the substantia nigra very early in development. The pattern of innervation follow the developmental phases observed for striosomal neurons. At the same time striosomal neurons have a predominant expression of the D1-class of dopamine receptors. The tight link between striosomal-predominant dopaminergic innervation and striosomal-predominant detection of the D1-class of postsynaptic receptors at times crucial for striosomal pattern formation raised the possibility that dopamine through D1 receptors may influence the process. Our results challenge this hypothesis. In the D1 receptors knock-out pups, striosomes are present as manifested by the expression of Fra, NGFI-A and another phenotypic marker such as DARPP-32 with modalities indistinguishable from the controls. Furthermore the dopaminergic innervation maintains the striosome-predominant innervation even in absence of the D1 receptors. Thus suggesting that if there is a link between the dopaminergic innervation and striosomal pattern formation it does not require D1 receptors to function. Our results indicate that dopamine through D1 receptors appears to selectively regulate the striosome/matrix pattern of expression of neuroactive substances from early in development. In the adult striatum, it has been shown that both D1 and D2 receptor gene deletions strongly affect neuropeptide expression by striatal neurons. Dynorphin and substance P expression is low in D1 mutants (Xu et al., 1994; Drago et al., 1994) and enkephalin expression is increased in D2 mutants (Baik et al., 1995). We found that D1 receptors are required for the proper striosome-predominant expression of dynorphin from the early postnatal period.

Despite the early and selective expression of NGFI-A proteins, their absence in engineered mice does not prevent normal development

NGFI-A proteins are present in the striatum from very early age throughout adulthood. Furthermore NGFI-A proteins have been found correlated with fundamental activity-dependent processes (Wisden et al., 1990; Zhang et al., 1995; Moratalla et al., 1992). However, the characterization of the adult NGFI-A deficient mice (generously provided by Prof. Milbrandt) did not found any evident defect. Likely this negative result could be attributed to the redundant number of molecules that may regulate the same function.

Selective dendritic stabilization as mechanism of pattern formation

The striosomes and matrix compartments emerge from a phase where striosomal and matrix neurons are intermixed with each other (van der Kooy and Fishell, 1987). The mechanisms which lead to the final striosome/matrix pattern are still mostly unclear. It has been hypothesized that one mechanism at the basis of striosomal pattern formation is selective adhesion among striosomal neurons (Krushel et al., 1995). Our results indicate that during the period of striosome pattern formation, high expression of the microtubule associated protein-2 is selectively detected in the aggregating striosomes. Whether high MAP-2 staining in developing striosomes is due to the aggregation of striosomal neurons already expressing high levels of the protein, or whether the aggregating process induces the expression of high MAP-2 levels, is not known. We propose that when striosomal neurons are aggregating, they may need a highly stabilized dendritic arborization to counteract the desegregating force exerted by the matrix neurons. In favor of this hypothesis is the evidence that high MAP-2 expression is not a general requirement for dendritic maturation because it was not observed during the maturation of matrix neuron dendritic arborization.

Figure 11. Prenatal phases of striosomal development defined by IEG expression.

A, Photomicrograph of Fra immunostaining detected at embryonal day 14 (E14). Ventrolateral phase: striosomal neurons are localized in the ventrolateral striatum. Remarkably, Fra staining shows positive nuclei detected in the ventrolateral part. Fra-positive nuclei are also detected in the cingulate cortex, in the hyppocampal formation and in the basal forebrain. B, Dispersed phase: at E17, when striosomal neurons are dispersed throughout the striatum, Fra-positive neurons apeear also dispersed throughout the striatum. Fra-positive nuclei are also detected in the cingulate cortex, olfactory tubercule, claustrum and hyppocampal formation. C, Striosomal phase: by E19, well-defined striosomes appear, at the same time, Fra positive clusters are clearly detectable in the striatum. In the cortex, Fra expression is now evident through all the extension rostro-caudal of the deep cortical area; in the claustrum and in the olfactory tubercule. CP, caudatoputamen; Cg, cingulate cortex; Cl, claustrum; Ctx, cortex; GZ, germinal zone; S, septum; V, ventricle. Each scale bar indicates 500 μm.

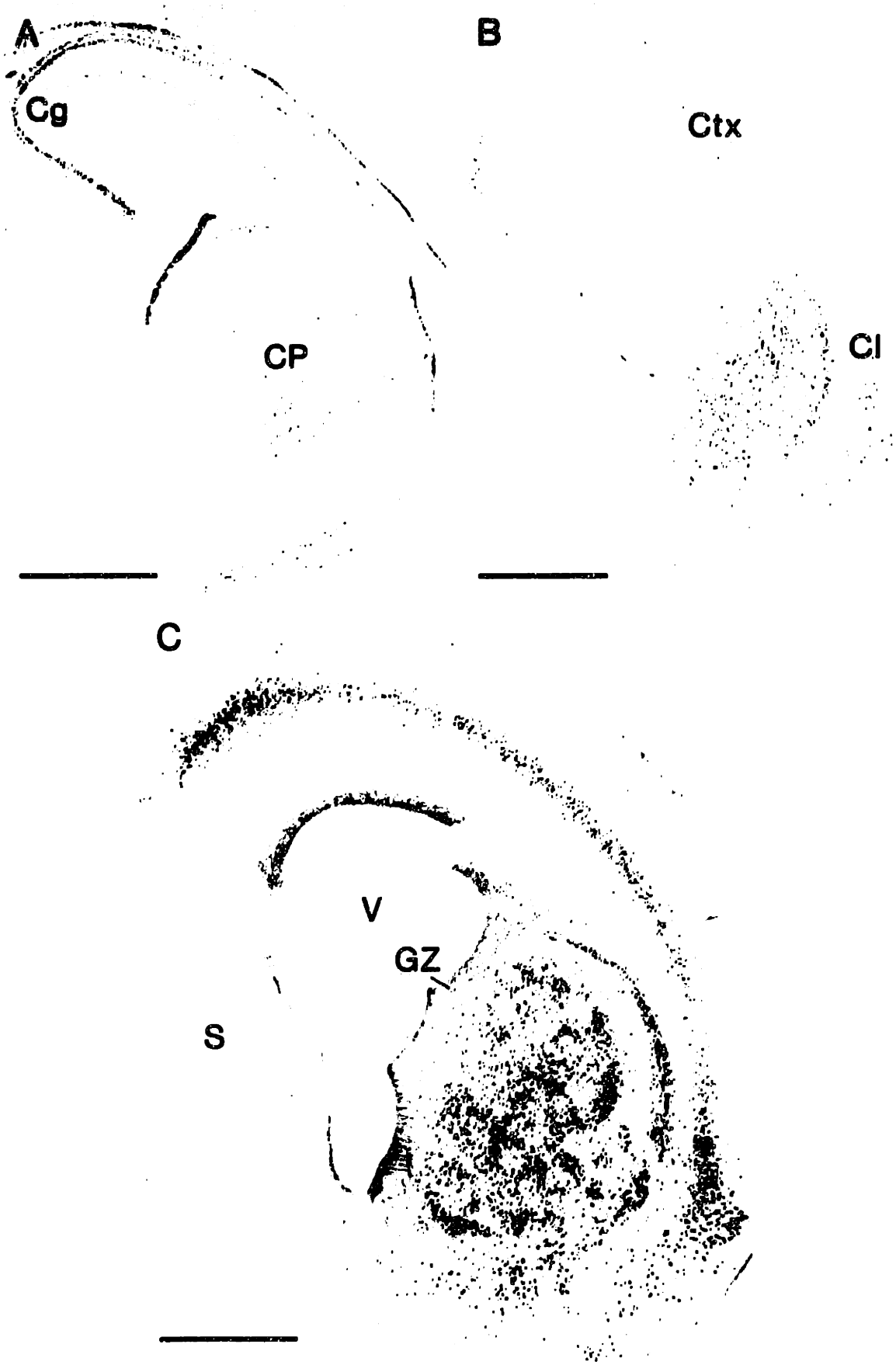
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Figure 12. Fra and NGFI-A constitutive proteins and Fos inducible proteins are expressed in the developing striosomes. NGFI-A proteins are expressed by a subpopulation of Fra-positive cells.

A, Photomicrography of a Fra-positive cluster (black) which colocalizes with a DARPP-32-positive cluster (brown). B, A cluster NGFI-A-positive (black) colocalizes with a cluster of high nigrostriatal innervation as shown by TH immunoreactivity (brown). C, A Fra-positive cluster colocalizes with a striosome delineated by the expression of the novel marker microtubule-associated protein-2 (MAP-2). D, A patch of Fos proteins (black) induced by cocaine colocalizes with a striosome, manifested by the expression of the striosomal marker DARPP-32 (brown). E, A patch stained for both NGFI-A and Fra proteins. E', Higher magnification of the same patch shows that NGFI-A-positive (brown) nuclei colocalize with Fra-positive (black grains) nuclei. There are several nuclei only Fra-positive. E", Control for the double-labeling between Fra and NGFI-A antisera. Fra-positive nuclei in the cortex, where only Fra staining is detected at this age, do not show any brown staining under the black grains. Scale bar shown in C indicates 500 μm for A, B, and D.Scale bar in B indicates 50 μm for A, C, D. Scale bar in E indicates 25 μm. Scale bar in E" indicates 10 μm for E'.

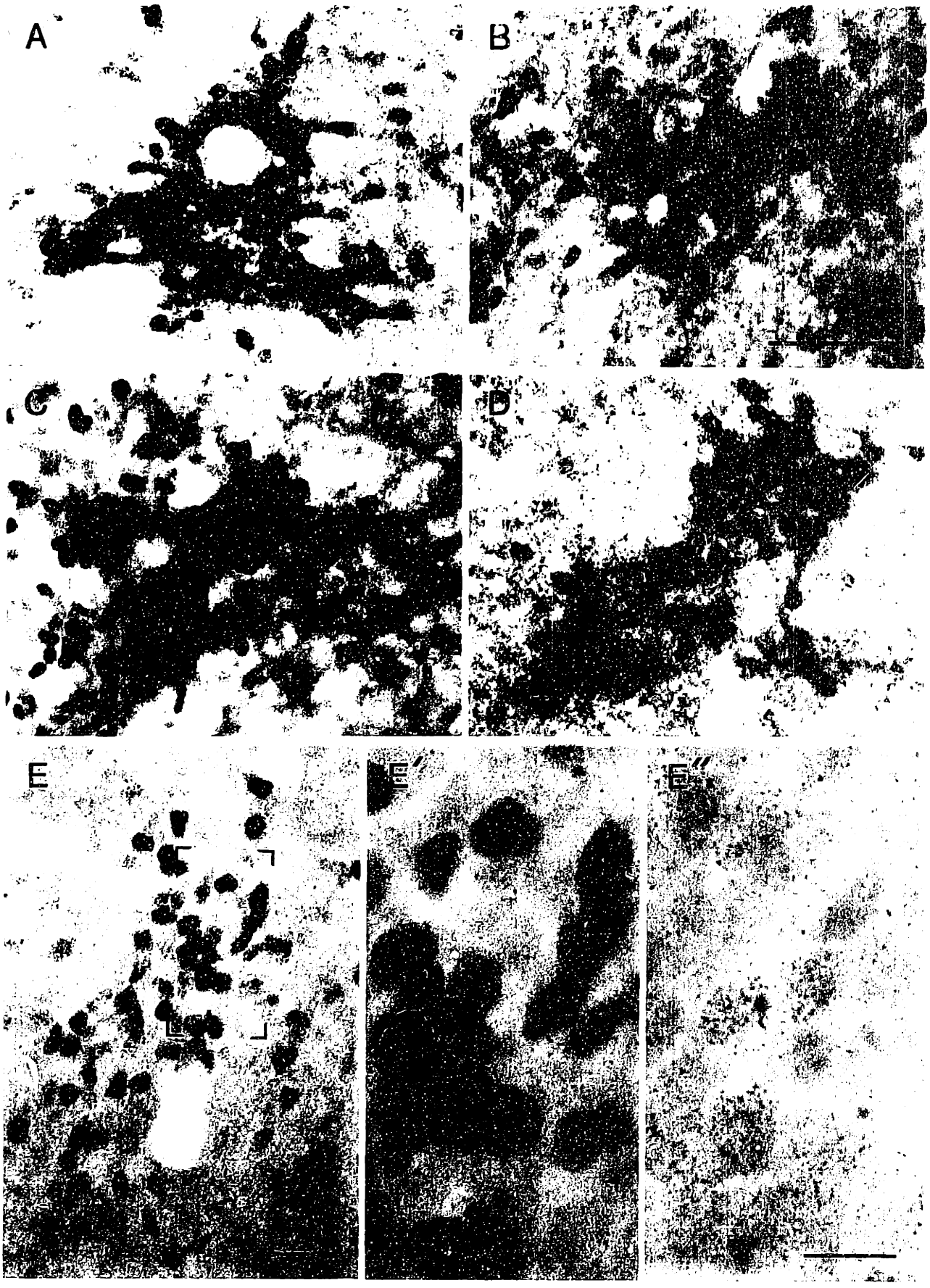
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Figure 13. Postnatal IEG expression in the developing striatum.

A, Photomicrograph of Fra imunostaining detected in the postnatal day 3 (P3) striatum. Fra-positive patches are still present but the process of Fra proteins downregulation is started ventrolaterally. B, At P9, Fra proteins are extensive downregulated in the striatum, only few Fra-positive patches remain detectable in the central and medial striatum. C, By P18, the downregulation of the constitutive pool of Fra proteins is almost complete. Only few scattered Fra-positive nuclei are detected in the very medial zone, at the edge with the ventricle and in the fundus striatum. D, In contrast with Fra immunoreactivity, NGFI-A expression is not subjected to such drastic downregulation. At P3, crispy NGFI-A-positive patches are detected in the striatum. E, By P9, NGFI-A immunoreactivity atrat to be detected also in the matrix and NGFI-A positive patches become hard to recognize. F, By P18, NGFI-A expression appear with the same intensity in both striatal compartments. CP, caudatoputamen; Ctx, cortex; V, ventricle. Scale bar in C indicates 500 μm for A, B, D, E, F.

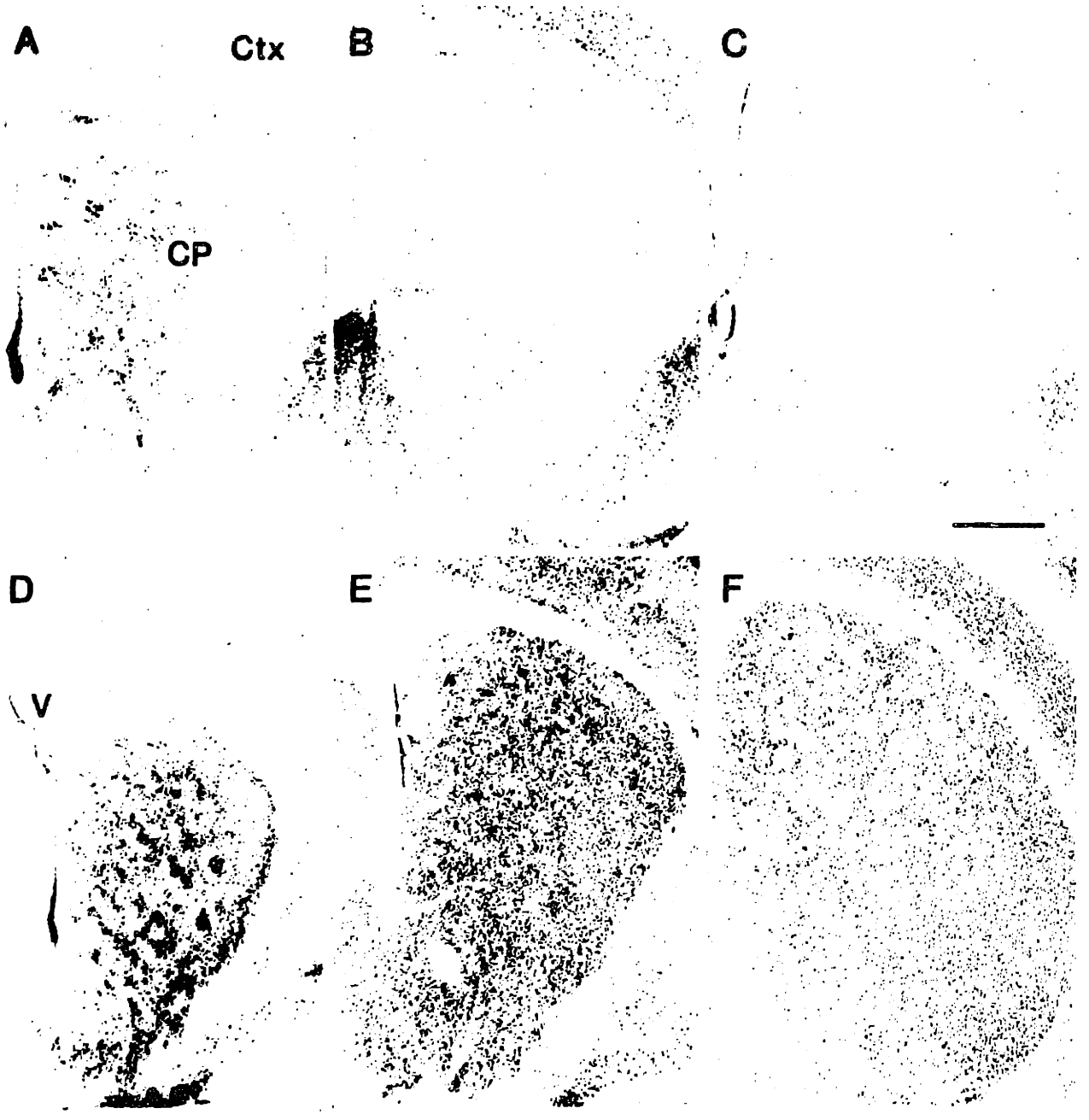
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Figure 14. Developmental pattern of expression of the novel striosomal marker, microtubule associated protein 2 (MAP-2).

A, Photomicrography of MAP-2 staining detected at E17. MAP-2 is expressed at low level in both compartments. At the time that striosomes start to aggregate, patches of high MAP-2 start to appear as indicated by the arrow heads. B, By E18, several patches high MAP-2-positive are detected throughout the striatum. C, Postnatally MAP-2 expression starts to downregulate specifically in the striatum. At P3, few MAP-2 patches are still detectable. D, By P18, MAP-2 expression is completly downregulated in the striatum. MAP-2 expression is still detectable in the very medial striatum, almost at the edge with the ventricle and in the fundus striatum. By contrast, in the cortex the level of MAP-2 expression appear unaffected during development. CP, caudatoputamen; Ctx, cortex; V, ventricle. Each scale bar indicates 500 μm.

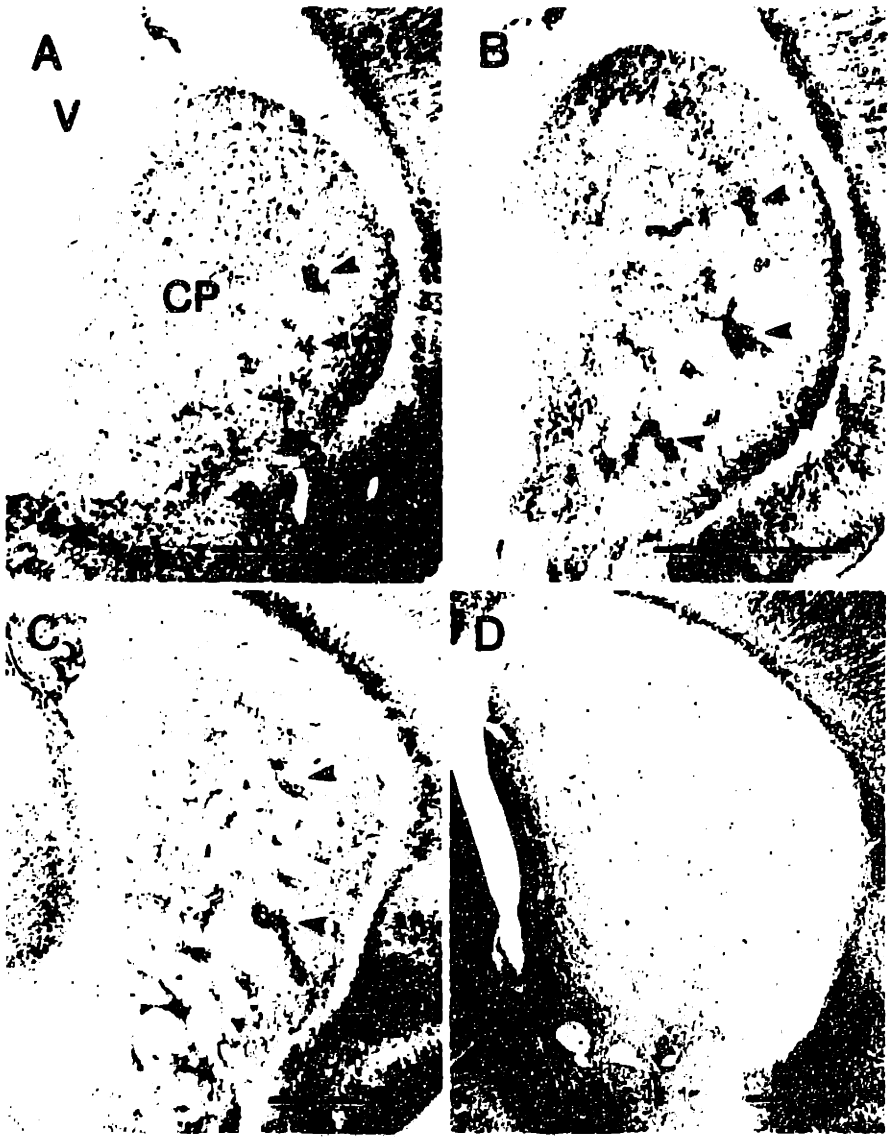
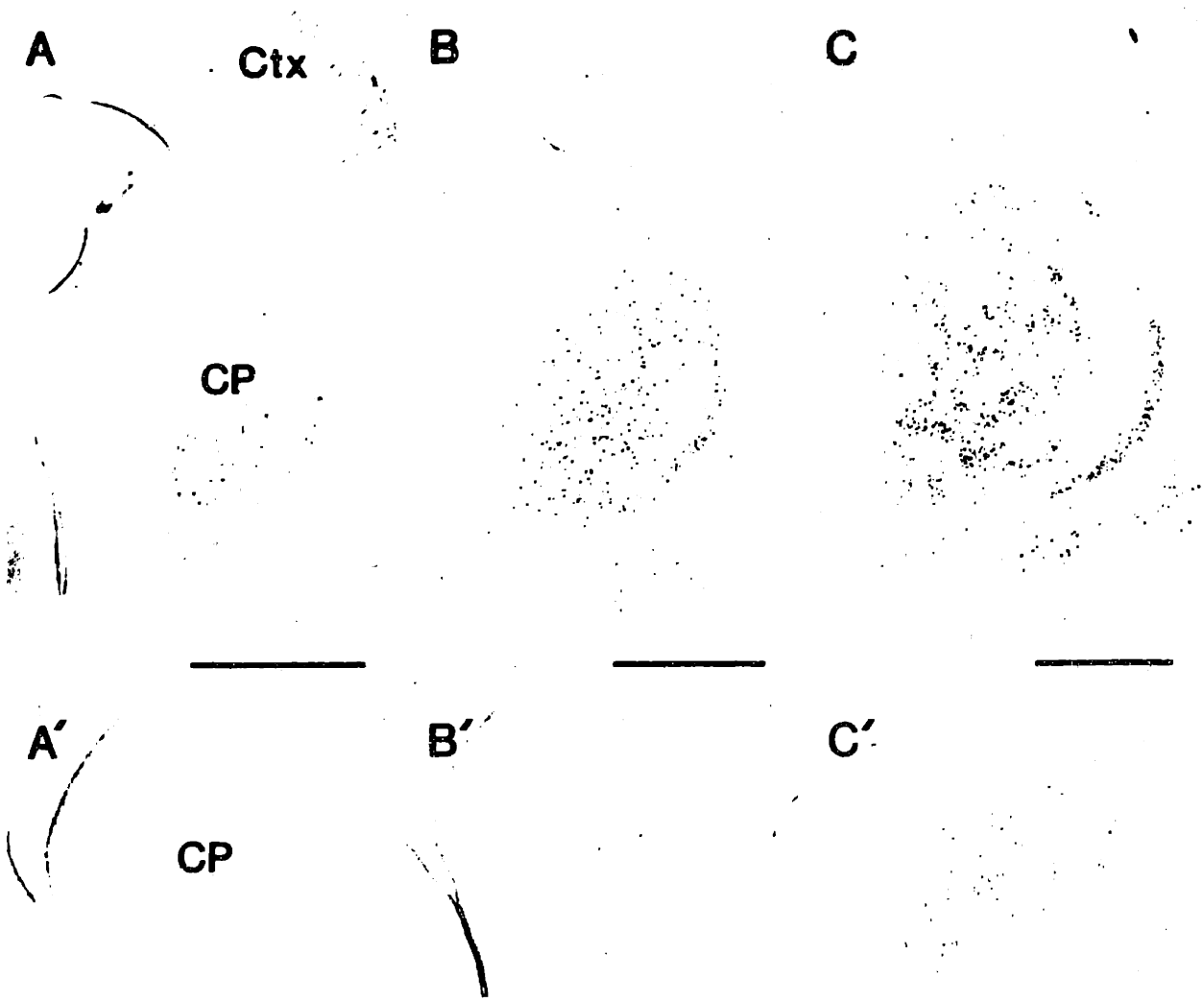

A
V
CP
B
C
D

Figure 15. Fos inducible expression indicates a prenatal responsiveness of striosomal neurons to dopamine.

A, Photomicrography of Fos staining detected at E16 after dopaminergic stimulation by cocaine administration through the maternal circulation. Fos-positive nuclei are detected in the ventrolateral striatum. At the same age striosomal neurons are in the ventrolateral striatum. A' In the control saline-treated no Fos-positive nuclei is detectable. B, At E17, when striosomal neurons are localized dispersed throughout the striatum, the dopaminergic stimulation induces an increased number of Fos-positive striatal neurons dispersed throughout the striatum. B', In the control saline-treated very few Fos-positive nuclei are detected. C, At E19 when striosomes are aggregated, the dopaminergic stimulation induces Fos-positive nuclei in clusters. C', In the control saline-treated, only weak and tiny Fos-positive patches are detected. CP, caudatoputamen; Ctx, cortex. Scale bar in A indicates 500 μm for A'. Scale bar in B indicates 500 μm for B'. Scale bar in C indicates 500 μm for C'.


A
Ctx
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CP
A′
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CP

Figure 16. NGFI-A-positive and TH-enriched patches are detected in neonatal D1 knockout mice.

A, Tyrosine hydroxylase positive patches manifest the striosome-predominant innervation of the dopaminergic afferents in the developing striatum. B, Tyrosine hydroxylase positive patches are similarly present in the D1 mutant mice. This indicates that the striosome-predominant innervation of the dopaminergic afferents is not dependent on D1 receptor activation. C, Striosomal cell clusters during the first postnatal week are manifested by the compartment specific expression of NGFI-A proteins. D, Clusters of NGFI-A positive cells are present in the D1 mutant mice, similarly to the controls. This suggests that striosomes aggregation is not dependent on D1 receptor activation. AC, anterior commissure; CP, caudatoputamen; Ctx, cortex; S, septum. Scale bar in C indicates 500 μm for A, B, D.

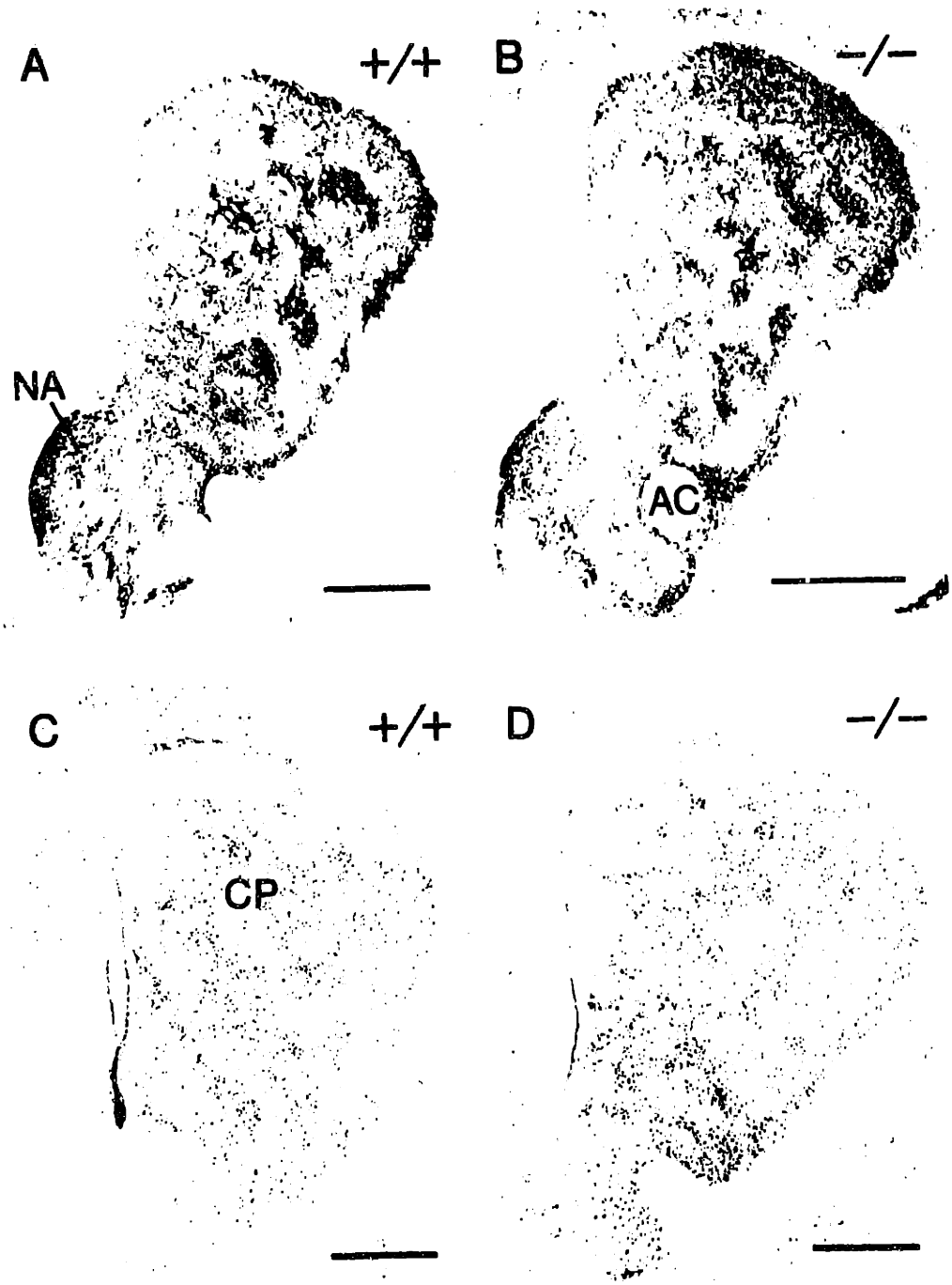
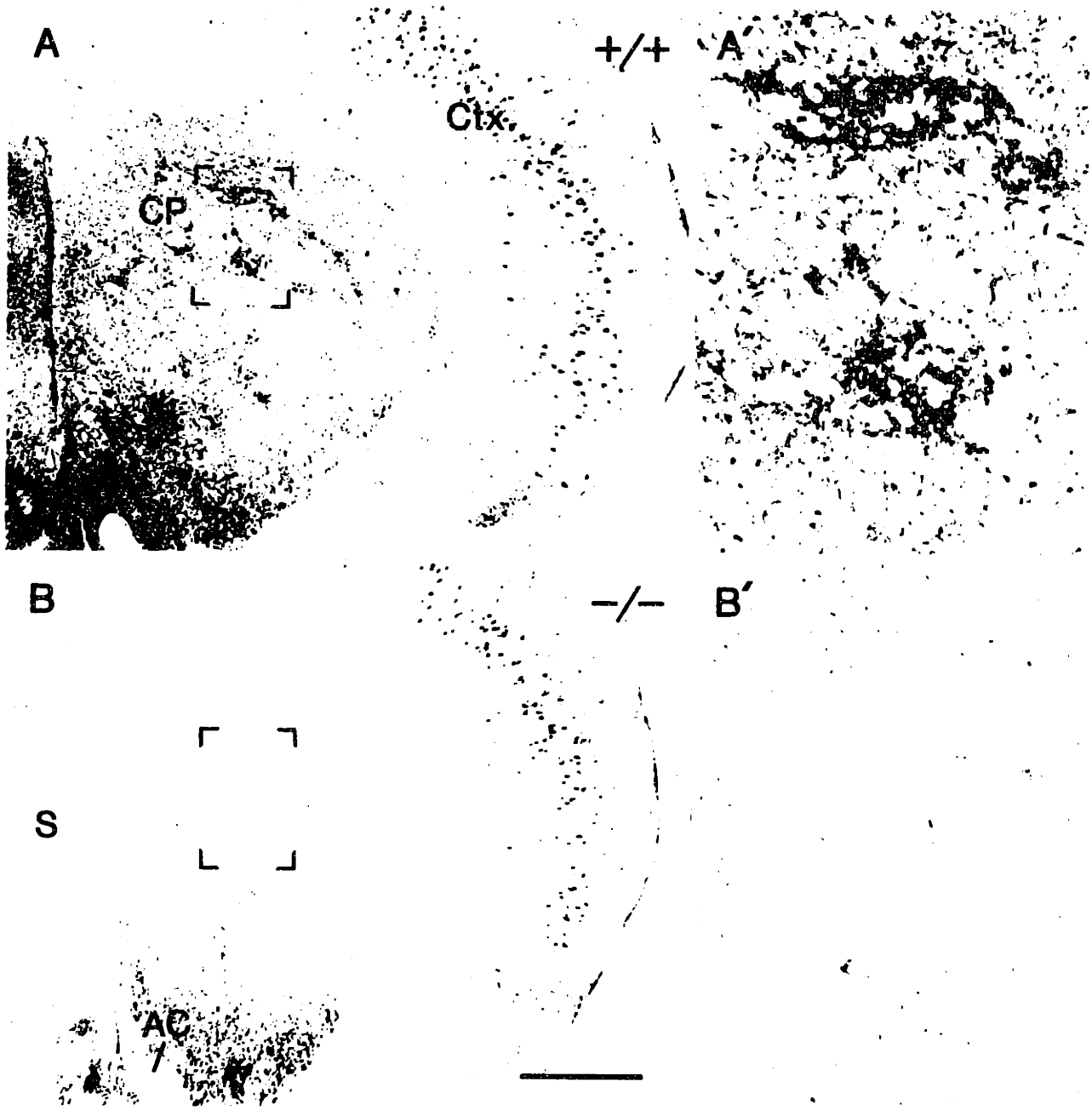

A
+/+
NA
B
−/−
AC
C
+/+
CP
D
−/−

Figure 17 Dynorphin-positive patches are barely detectable in neonatal D1 knockout mice

A, Dynorphin has a predominant expression in the striosomal compartment. A' Higher magnification of dynorphin-positive patches. B, Although dynorphin expression in the cortex of the D1 mutant mice is very close to the levels detected in the normal, there is a striking difference in the striatum. Dynorphin-positive patches are virtually absent in the postnatal D1 mutant pups. B' At higher magnification only few weakly stained dynorphin-positive cells are detected. AC, anterior commissure; CP, caudatoputamen; Ctx, cortex; S, septum. Scale bar in B indicates 500 μm for A.


A
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CHAPTER 4

Striatal cell migration and phenotypic markers characterization in prenatal organotypic slices

Abstract

The striatum of the mammalian forebrain is divided into two compartments: the striosomes and the matrix. In order to investigate striatal cell migration and compartmentalization in a system accessible to external manipulation, we have developed an in vitro embryonic organotypic system. To determine whether striatal cell migration occurs in a system deprived of most long range information, we have labeled matrix cells with the thymidine analog 5-bromo-2-deoxyuridine (BrdU) and tracked the migration in organotypic slices cultures for increasing time. Indeed, we found that matrix cells progressively migrate from the germinal zone deeply into the striatum. We excluded that this result is due to the flattening of the slice as a subpopulation of the matrix cells which is located near the germinal zone remained located in the same area even after several days of culturing.

We investigated whether compartment formation occurs independently of most of the afferents by tracking labeled striosomal neurons in organotypic slices. Only in some cultures, we found clusters of BrdU-labeled cells. Even in cultures cut to preserve at least partially the nigrostriatal afferents, the formation of BrdU-positive clusters by striosomal neurons was not consistent. However, using the immunodetectable expression of the transcription factor Fra as putative marker for striosomal neurons, we detected Fra-positive patches in the medial and rostral striatum.

To check whether the slice system affected the survival and differentiation process of the striatal cells, we stained slices with the neurodifferentiation marker, microtubule associated protein-2 (MAP-2). In all times intervals characterized, healthy slices showed MAP-2 expression with a pattern of expression similar to the one detected in vivo.

We propose that the nigrostriatal innervation is not necessary for striatal cell migration from the germinal zone in the target structure. Whether is possible to consistently obtain compartment formation in cultures is still unclear, however we found that organotypic slices system does not disrupt the already aggregated prenatal striosomes providing a valuable tool for analyzing the molecular mechanisms of prenatal striosome/matrix phenotypic development.

Introduction

The mammalian striatum is composed of two compartments the striosomes and the matrix. In the context of the mammalian striatum the term compartment is used to identify groups of contiguous cells with a common phenotype, without necessarily denote clonally related cells. The striosomes appear as clusters of contiguous cells with similar biochemical properties embedded into the matrix compartments. Neurons constituting these compartments become postmitotic in the germinal layer at different ages. In all mammals studied striosomal neurons are born first, whereas the matrix neurons become postmitotic at later times (Graybiel and Hickey, 1982; van der Kooy and Fishell, 1987). Cell migration from the germinal layer to the striatum respects the same time shift. Striosomal neurons invade the striatum first whereas matrix neurons reach the striatum in a second wave (Fishell and van der Kooy, 1987). The final structural organization of striosomes and matrix compartments emerges from a phase where striosomal and matrix neurons are intermixed with one another (van der Kooy and Fishell, 1987). The cellular mechanisms underlying striatal compartmentalization could rely on afferent-dependent processes or on intrinsic potentialities of striatal cells. To test the possibility of cell migration and compartment formation in a system deprived of most long range information, we turned to an *in vitro* approach. We developed an *in vitro* embryonic organotypic system. We chose this culture system because in short-term organotypic slice cultures, it is possible to maintain most of the cytoarchitettonic three-dimensional structure necessary for short range cell-cell interactions allowing conditions close to the physiological environment more than cell line cultures or dissociated primary striatal cells (Liu et al., 1995). We examined striatal cell migration from the germinal layer into the striatum. To track the movements of a consistent number of neurons with similar initial conditions, we chose to mark the matrix neurons, which are more numerous than the striosomal neurons. We identified matrix cells by two maternal injections of the DNA base

analog bromodeoxyuridine (BrdU) performed at the peak period of their production, embryonal day 15, E15. We found that matrix neurons do migrate from the germinal layer deeply into the striatum in less that a week of culturing. To control for the contribution of passive movement in our results, we labeled a subpopulation of the matrix neuron which, as observed in vivo, does not migrate deep into the striatum. Even in slices cultured for almost a week this subpopulation of the matrix cells remained confined near the germinal layer, thus excluding the possibility that the cell migration observed was an artifact of the culture system.

In order to assess the intrinsic potential of the isolated embryonic striatum to develop its striosome and matrix pattern organization, we have labeled striosomal cells with BrdU and tracked the compartment formation. We have been encouraged in testing the possibility of obtaining striatal compartment formation *in vitro* by the facts that embryonic striata transplanted into the anterior eye chamber of adult hosts showed heterogeneous distribution of compartment specific markers (Johnston et al., 1987) and that embryonal striatal neurons dissociated and reaggregated in suspension cultures appeared to segregate forming clumps of early-born neurons surrounded by late-born neurons (Krushel et al., 1995). Despite the encouraging results in literature, only in some cultures, clusters of BrdU-labeled cells were present. Even in slices cut to preserve part of the long range afferents the results have not been consistent. To test whether the difficulties encountered could depend on the paucity of the striosomal neurons BrdU-labeled, we decided to use a striosomal phenotypic marker. We found that the immunodetectable transcription factor Fra, shown to mark striosomal neurons in vivo, (see chapter 3) maintains the selectivity in expression in organotypic slices. Indeed, we found clearly detectable Fra-positive clusters emerging from a dispersed pattern after 5 days in culture in the medial rostral striatum. However we could not observe detectable clusters in the ventro-lateral striatum.

We propose that striatal cell migration occurs in organotypic slices even in absence of most long range interactions. Whether it is possible to obtain consistent compartment formation in these in vitro conditions is still unclear. However, the prenatal organotypic culture system does not disrupt already formed striosomes thus providing an easy accessible system for analyzing, even before birth, pharmacologically the molecular mechanisms underlying the phenotypic striosome/matrix pattern.

Materials and Methods

Experimental procedure

To minimize the time variability among the different timed pregnant CD-1 mice (Charles River), female and male mice were kept together for only 4hrs (8.00am-noon). To mark the embryonic age at which the striosomal or matrix neuronal populations were at the final or near final DNA synthesis phase, injections of the DNA analog 5-bromo-3'-deoxyuridine (BrdU) have been performed. Striosomal neurons are the first to become postmitotic mainly in the time frame from embryonic day 11 (E11) to E13; subsequently, the matrix neurons become postmitotic between E13-P0 (Angenive and Connell, 1974; Graybiel and Hickey, 1982 ; van der Kooy and Fishell, 1987; Song and Harlan, 1994a). To mark the embryonic neurons that are going to form the striosomal compartment BrdU administration through the maternal circulation has been carried out (100 mg/kg in saline; s.c.) at E11.5. To mark the matrix population 2 injections of 50mg/kg each s.c. have been performed at E15 (10.00am and 10.00pm). To mark a subgroup of the matrix neurons BrdU injection has been performed at E17 2hrs prior slicing, and the slices have been incubated with BrdU (10μM) for 3hrs after slicing.

To minimize variability only embryos of the same litter, thus exposed at the same time to the same concentration of BrdU were used to study the cell migration issue. More than one litter has been used for each experiment.

Preparation of organotypic slice cultures

The organotypic slice culture method used is an adaptation of the procedure developed by Liu et al. (1995). Fetuses were taken from deeply anaesthetized time pregnant CD-1 mothers (Charles River). Fetal brains were quickly removed and washed in cold

Hanks' balanced salt solution (GIBCO-Bethesda Research Labs, Grand Island, NY). The brain tissue was, then embedded in 37°C, 4% low melting temperature agarose (FMC Bio-Products, Rockland, ME) dissolved in Hanks'solution supplemented with 5.5 mg/ml glucose. The embedded fetal brains were cooled down on ice till the agar hardened and then cut on a sterilized vibratome into 300μm thick coronal slices. Slices were cultured in a 0.4μm microporous transparent biopore membranes in 30mm culture plate inserts (Millicell-CM, Millipore, Bedford, MA). The inserts were placed in six-well culture dishes. Brain slices were washed for almost 1hr in cold Hanks' solution then the buffer was removed and 1 ml of SF21 serum-free medium (Segal et al., 1992) was added in each well. All experimental procedure required very attentive sterile conditions. The cultures were then placed in a sterilized humidified incubator at 33 °C with 5% CO_2 , 95% air.

Immunostaining

After incubation, slices were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, 0.1M PO_4, for at least 1hr followed by several rinses in 0.1M phosphate buffer, 0.1% Na azide and then in 0.01M phosphate-buffered saline (PBS). Slices were then pretreated with 3% H_2O_2 in PBS with the addition of 0.2% Triton X-100 (PBST) for 15 min and then incubated in PBS with 5% normal serum from the same species in which the secondary antibody was raised for at least 1hr and then rinsed with PBS only.

Slices were incubated in primary antisera at 4°C for 48-72hr, with the antisera diluted in PBST or in PBS. For avidin-biotin-peroxidase complex immunocytochemistry, sections were rinsed in PBS, incubated in a 1:500 biotin-conjugated secondary antiserum (Vector Laboratories, Burlingame, CA) for 2hrs, rinsed in PBS, and incubated in avidin-biotin complex (ABC, Vectastain, Vector Laboratories; 6μl/ml) in PBS for 2hr, before being reacted with diaminobenzidine (DAB, SIGMA) sometimes enhanced by nickel.

Sections were mounted on gelatin-coated slides, air dried, dehydrated, coverslipped with Eukitt mounting medium, and analyzed with light microscopy.

The immunostaining for BrdU required modifications on the general procedure. A 1 hr step at r.t. in 2N HCl neutralized by the following rinses in Na-Borate (Borax) pH 8.6 were necessary before continuing with the commonly applied protocol.

To demonstrate colocalization between a nuclear and a cellular antigen, such as a FRA and MAP-2, we carried out the two avidin-biotin peroxidase complex labeling procedure (Batchelor et al., 1989). The first staining was intensified with nickel to produce a nuclear black dot-like signal whereas the second staining not enhanced with nickel produced a cellular brown signal. After the DAB/nickel staining, the sections were washed overnight in 0.1M PO_4. To eliminate the residual peroxidase activity, slices were then incubated for 45 min in 0.6% H_2O_2. To block the excessive biotin and avidin binding sites, before the beginning of the incubation for the second antigen, passages of 30 mins each, in avidin (Vectastain, Vector Laboratories; 6µl/ml) and in biotin (Vectastain, Vector Laboratories; 6µl/ml) were performed. Slices were then treated following the general protocol adopted in the first staining protocol with the exception that the final passage was using only DAB as chromogen.

Primary incubations were carried out with the following antisera and concentrations: 1:2,000 and 1:5,000 rabbit polyclonal anti-Fos/Fra (generously provided by Dr. M.J. Iadarola of the National Institute of Dental Research, National Institutes of Health, Bethesda, MD; batch F2 prep 1); 1:400 mouse monoclonal anti-microtubule-associated protein 2 (MAP2) (Boehringer Mannheim Biochemica, Indianapolis, IN); 1:1,000 for mouse monoclonal anti-tyrosine hydroxylase (SIGMA, St.Louis, MO); 1:100 mouse monoclonal 5-Bromo-deoxyuridine (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Result

Matrix cell migration in prenatal organotypic slices

To investigate whether the nigrostriatal afferents are necessary for the process of striatal cell migration from the germinal zone into the striatum, we marked the striatal cells and we tracked cell movements in prenatal organotypic slices. Brain slices were cut in a coronal plane to transect the dopaminergic innervation coming from the substantia nigra. To reliable assess the migration of striatal cells we needed to track the movements of a consistent number of cells labeled in a short time window. We checked the postmitotic rate of both the matrix cells and striosomal cells in vivo. We found that the rate of postmitotic cells is higher for matrix cells than or striosomal cells. We labeled a consistent number of matrix cells with two systemic injections of the DNA base analog BrdU performed, 12hrs apart, at peak time for matrix cell production (embryonal day 15, E15). We found that BrdU-labeled matrix cells slices harvested at E17 cultured for times ranging from 0, 12, 24hrs and 2-5 days progressively migrate from the subventricular zone (SVZ) deeply into the striatum (Fig.18 A, B, C). To test whether the shift in position was due simply to a passive flattening of the cultures, we selectively labeled a subgroup of the matrix cells that, in vivo, is localized near the germinal layer, and then cultured the slices for 5 days. We found that most of the labeled cells were still in the VZ, SVZ and medial striatum (Fig.18 D and E). These results strongly indicate that striatal neurons do migrate in cultured embryonal slices thus suggesting that intact nigrostriatal afferents are not necessary for striatal cell migration from the germinal layer to the target structure.

As further control on the survival and differentiation process of the matrix cells during the process of cell migration, we stained slices with the neurodifferentiation marker, microtubule associated protein-2 (MAP-2). In all times intervals, characterized to test cell migration, healthy slices showed an intense and intricate network of MAP-2-positive

dendrites indirectly suggesting a high rate of neuronal survival. MAP-2 pattern of expression detected in the organotypic slices was similar to the one detected in vivo, suggesting that the organotypic slice system does not dramatically affect the differentiation process of striatal neurons (Fig.18 F).

Tyrosine-hydroxylase positive cells detected in 5 days cultured organotypic slices

To transect the nigrostriatal afferents innervating from the substantia nigra, fetal brains were sliced in coronal sections. Nigrostriatal dopaminergic terminals were visualized by the immunodetectable tyrosine hydroxylase (TH) enzyme. TH -positive terminals were detected in slice immediately fixed and processed for TH immunoreactivity. After 3 days in culture TH immunostaining appeared in granules and puncta, presumably representing degenerating terminals. However, we found in the striatum of slices cultured for 5 days few scattered tyrosine-hydroxylase (TH) positive cells. The origin of the TH-positive cells is uncertain.

BrdU-positive striosomal neurons aggregate in distinguishable clusters in only few prenatal organotypic slices

In order to assess the intrinsic potential of the isolated embryonic striatum to develop its striosome and matrix pattern organization, we have labeled striosomal cells with BrdU and tracked the compartment formation. We found, by in vivo studies, that, in the mouse, striosomal neurons start to become postmitotic at E11. Already at E13 striosomal production start to be intermixed with the beginning of matrix cell production. Thus, to avoid contamination between the two striatal populations that could mask the detection of the striosomes, we chose to perform one injection of BrdU at E11.5. We harvested the slices at E16 or E17, when the process of striosome aggregation is on the

edge of starting in the caudal ventrolateral striatum. Indeed in some cultures, clusters of BrdU-labeled cells were present, other than ventrolaterally, however the result was not consistent. To test whether the presence of the dopaminergic afferents would increase the frequency of compartment formation, we cut off horizontal slices to obtain the substantia nigra and the striatum in the same slice. It was in these cultures that the most cunning clusters appeared. However, also with this approach only in a limited number of slices, clusters of BrdU-labeled cells were visible. Unfortunately, however it is still unclear whether or not compartmental organization can form *in vitro*. It is possible that striosomes were indeed clustering in the slices but due to the non complete labeling of the striosomal population they were no-detectable. However, in P2 slices striosomes were clearly detectable even if BrdU injection was performed with a similar protocol.

Clusters immunolabeled by the transcription factor, putative striosomal marker, Fra are detected in organotypic slices cultured for 5 days

In an attempt to selectively mark striosomal neurons and avoid the drawback encountered using BrdU, we decided to use a phenotypic marker of striosomal neurons. We further tried to follow cluster formation using the immunodetectable transcription factor Fra as a putative marker of striosomal cells. By our previous characterization in vivo, we knew that at E17 Fra expression is dispersed throughout the striatum. Only few aggregating clusters may appear ventrolaterally in the very caudal of the striatum. Indeed, E17 slices cultured for 5 days showed FRA-positive patches in the medial and rostral striatum not detectable in E17 slices cultured for only 3 days (Fig.19 A, B). Puzzling, we were not able to observe Fra-positive clusters ventrolaterally. Whether or not the Fra-positive clusters observed are striosomal patches is not clear. To check whether FRA selectively labels striosomal cells in the culture system, we stained E18 and P0 slices cultured for 0hr up to 3-5 days. A clearly non-uniform "patchy" pattern of FRA staining,

which fairly closely resembles the in vivo pattern, was evident (Fig.19 C, D). As further attempt to check whether the Fra-positive patches detected were striosomes, we double-labeled with another phenotypic marker for striosomal neurons MAP-2. We found that, as in vivo, the FRA positive patches in the slices are colocalized with MAP-2 positive patches (Fig.19 E). These results suggest that constitutive FRA expression maintain crucial features of its *in vivo* expression even in the organotypic system.

Discussion

Our experiments indicate the organotypic slice culture as a valuable system for studying the cellular mechanisms of cell migration in the prenatal striatum. The phenotypic characterization here reported further suggests that organotypic slices could be used as suitable tool for pharmacologically investigating the gene regulation underlying the prenatal establishment of the striosome/matrix phenotypes. Under the culture conditions we used, slices maintained their three dimensions structure without flattening to a cellular monolayer. Healthy slices manifested, as in vivo, an intricate web of dendritic arborization suggesting that the neurodifferentiation process is in range of normality. Striosomal neurons expressed compartment-predominant antigens in typical patterns.

Striatal cell migration in organotypic slice is due to active cell movements not to passive shifts in positions

Our results establish that matrix cells do migrate from the germinal zone into the striatum in organotypic slices cultured for increasing times by active cellular movements. Cell migration in vitro has been tested for cortical neurons in long term roller tube cultures (Roberts and al., 1993). However, long term roller tube cultures loose their three dimensions structure becoming almost a cellular monolayer thus leaving the possibility that passive movements, due to the flattening of the culture, may contribute to the shift in position observed. We discarded the possibility that the shifts in position we observed were simply due to a passive flattening of the cultures by selectively labeling a subgroup of the matrix cells that, in vivo, remains localized near the germinal layer. Even after several days in culture we found that most of the labeled cells did not passively shift into the striatum but instead they were localized near the germinal layer (Fig.18 D and E).

Prenatal organotypic slices retained compartment-predominant expression of the immunodetectable Fra proteins in the typical patterns observed in vivo

By preserving most of the three dimensions infrastructure, organotypic slices represent an in vitro system suitable to analyze the phenotypic striosome/matrix pattern. In E18 and P2 slices cultured from 0hrs to several days the striosomal-predominant expression of the transcription factor Fra observed in vivo is preserved in the in vitro system. Postnatal organotypic rat slices have been utilized to study in vitro the regulation of Fos/Fra gene expression and to investigate the biochemical mechanisms underlying the striosomes and matrix phenotypes (Liu et al., 1995, 1996). Our experiments established the possibility to pharmacologically investigate on the molecular mechanisms orchestrating the early formation of the striosome/matrix phenotypic pattern even before birth. In addition, the expression of Fra proteins detected even after 5 days in culture indirectly suggests that the constitutive regulation of the transcription factor is not dependent on the intact innervation of the afferents.

Tyrosine hydroxylase positive cells are detected in the striatum after several days of culturing

We assessed the degenerating state of the transected dopaminergic nigrostriatal afferents by immunostaining for tyrosine hydroxylase (TH). We found that the dopaminergic terminals after several days of culturing have lost their fibrous aspect instead they appear as granules and puncta. However, in E18 organotypic slices cultured for 5 days we found few TH-positive cells that we didn't detect in vivo. Also in newborn rat slices cultured with the roller tube methodology for at least 3 days, few TH-positive cells have been detected (Ostergaard et al., 1991). Even in cocultures with the substantia nigra, the re-innervation of the dopaminergic afferents does not prevent the appearance of this type of

cells (Ostergaard et al., 1991). The origin of these cells is unclear. They may be no-striatal cells that have migrated from other regions attracted by some diffusible factors or they may be striatal cells which in normal conditions do not express TH or express it only at very low. In culture conditions they may be induced to express or upregulate the expression of the enzyme. In favor of this last hypothesis preliminary evidences have shown in the adult rat striatum and in the monkey caudate nucleus TH-positive cell bodies (Tashiro et al., 1989; Dubach et al., 1987). Whether these cells synthesize dopamine or other cathecholaminergic neurotransmitters is not known. Given the low number and the need of several days to appear it seems unlikely that they could drastically affect the system.

Selective striosome-aggregation in prenatal organotypic slices is a controversy issue

Our intention was to test whether striosomes could emerge from the intermixed stage with the matrix neurons in culture. Previous data were in favor of this hypothesis. Embryonic striata explanted into the eye chamber produced tissue segregated into a single patch and matrix compartment (Johnston et al., 1987). Furthermore, the early-born striosomal neurons reassociated which each other in embryonic striatal reaggregate cultures (Krushel et al., 1989; Krushel and van der Kooy, 1993). It has been further hypothesized that an intrinsic potential of self-adhesion among the striosomal neurons is at the basis of compartment segregation (Krushel et al., 1995). Despite all these evidences we could not obtain detectable clusters of BrdU-labeled striosomal neurons in more than few slices. To investigate whether our difficulties were arising by labeling only a subgroup of the striosomal population, we decided to use a striosomal phenotypic marker that we previously characterized in vivo and in late embryonic slice. We used the expression of the transcription factor Fra. From a diffuse expression throughout the striatum, after 5 days in culture Fra-positive clusters appeared in the medial striatum. However, we couldn't detect clear Fra-positive clusters in the ventro-lateral striatum. In postnatal organotypic slices

acetilcholinesterase (AchE) expression and NADPH-diaphorase expression have seen to pass, as in vivo, from a patchy expression to a more diffuse expression (Ostergaard et al., 1993, 1995). However, these experiments have been performed in a roller tube culture system which as a drawback the flattening of the slice in almost a cellular monolayer. Whether the changes in pattern observed for AchE and NADPH-diaphorase are due to maturation on the phenotypic striosome/matrix pattern or to passive cell diffusion is not clear.

We infer from these results that even if self-adhesion among early born neurons could be an intrinsic property of the striosomal neurons, proper compartment organization may require molecular mechanisms driven by extrinsic afferents.

Figure 18. Active cell migration is observed in healthy prenatal organotypic slices.

A, Photomicrograph of BrdU-labeled matrix cells (2 injections; 50mg/kg; at E15, 10am-10pm) in striatal prenatal organotypic slice fixed immediately after slicing at E17 to define the time 0. BrdU-positive matrix cells are located in the germinal zone. B, After 3 days in culture, matrix cells are detected migrating out the germinal zone into the striatum. C, By 5 days in culture, BrdU-positive matrix cells have clearly invaded the striatum. D, BrdU-labeling of a subgroup of matrix positive cells (1 injection; 100mg/kg; at E17, 3hrs before slicing) that in vivo does not migrate deep into the striatum. The slice has been fixed immediately after slicing to define time 0. BrdU-labeled cells are detected in the germinal zone. E, Even after 5 days in culture, BrdU-positive cells are near the germinal zone showing that this subgroup of matrix cells does not migrate into the striatum also in vitro. Thus the shift in position observed in B and C is not only caused by passive movements due to the flattening of the slice but to active cell migration. F, Healthy slices cultured even for 5 days show an intricate network of dendritic arborization similar to the one detected in vivo. Thus indicating that cell death is a limited phenomen and that the process of neurodifferentiation proceed in the range of normality. CP, caudatoputamen; Ctx, cortex; GZ, germinal zone; V, ventricle. Each scale bar indicates 500 μm.

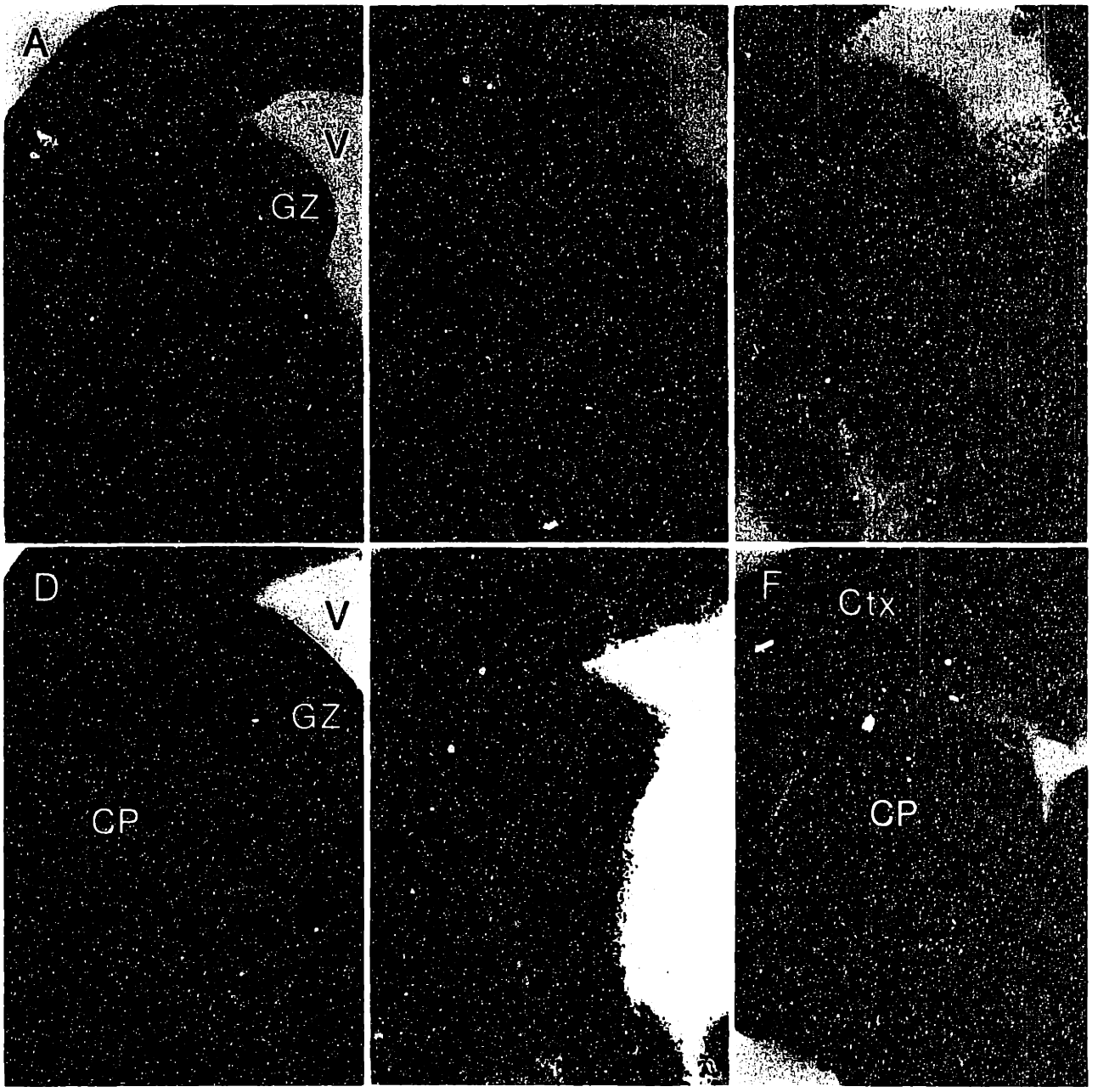
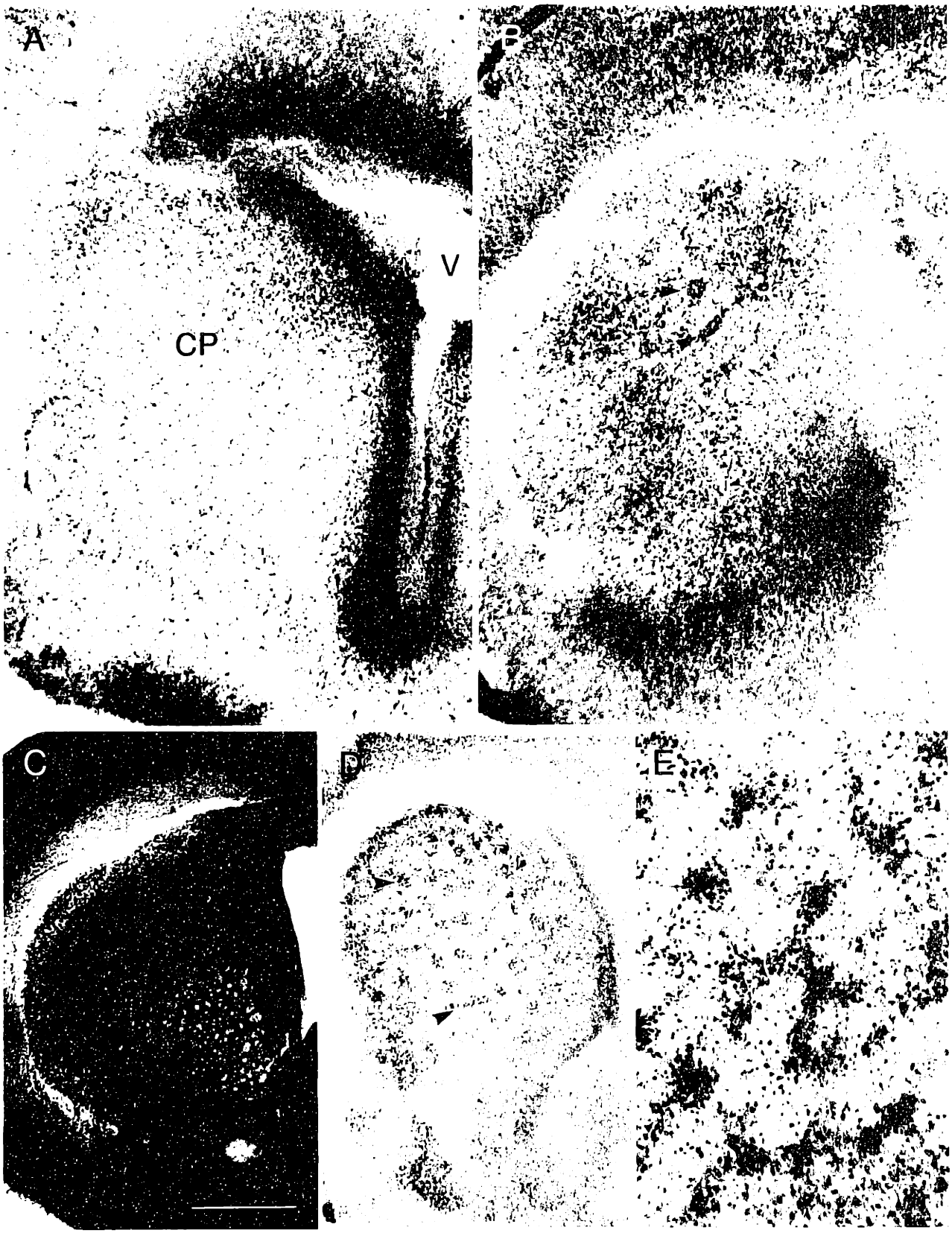

A
V
GZ
D
V
GZ
CP
F
Ctx
CP

Figure 19. Fra-positive clusters emerge in E17 slices from a dispersed distribution after 5 days in culture. Culture system does not disrupt already formed Fra-positive patches. Evidences that Fra-positive clusters are striosomes.

A, Photomicrograph of Fra immunostaining in a E17 striatal slices cultured for 3 days. The distribution of Fra proteins is dispersed throughout the striatum. B, After 5 days in culture, Fra positive clusters start to appear in the medial striatum. Thus suggesting that striosomal neurons could aggregate in vitro giving rise to striosome-like structures. C, Photomicrograph of Fra immunostaining in P0 striatal slice fixed immediatly after slicing. Fra-positive patches are detectable. D, Photomicrograph of Fra staining in a E18 striatal slice cultured for 5 days. Fra-positive patches are not disrupted by the culture system. E, Fra-positive patches, detected in D, appear to be striosomes as they colocalize, as in vivo, with high MAP-2 staining. CP, caudatoputamen; V, ventricle. Scale bar in C indicates 500 μm.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CHAPTER 5

Conclusion

The striatum is formed by two compartments: the striosomes and the matrix. The striosomes appear as clusters of contiguous cells with similar phenotypes embedded into the surrounding matrix. Which are the mechanisms that lead to the formation of the striatal compartments is still not defined. The information required for pattern formation may arise from an intrinsic genetic program acquired at the time of cell determination and parallel mechanisms afferent-dependent may refine the process. To test the contribution of the afferents on striatal development we have used a remote no-invasive pharmacological approach. To test the extent of the genetic component we used an in vitro system where the afferents were transected and only cell-cell short range interactions were spared.

Developmental striatal responsiveness to afferent stimulation in the postnatal striatum

We have focused our investigation primarily on the nigrostriatal dopamine-containing afferents. The afferents from the substantia nigra innervate the striatum very early in development and follow very closely the process of pattern formation. In the adult, it has been established that dopamine-containing afferents are crucial for the proper function of the striatum as their degeneration provoke devastating effects as manifested in Parkinson's disease. We have tested whether the striatal neurons were responsive to dopaminergic stimulation during development. Striatal neurons transduce dopamine action through two classes of receptors, the D1- and the D2-classes of dopamine receptors. Dopamine receptors are intracellularly coupled through G-proteins. D1-class dopamine receptors activate the enzyme adenylyl cyclase, consequently provoking an increase in the

level of the second messenger cAMP. By contrast, D2-class dopamine receptors inhibit the adenylyl cyclase decreasing cAMP levels. cAMP variations, among different effects, cause changes in gene expression. The expression of genes such as Fos, Fra and NGFI-A, members of the IEG family, is sensitive to cAMP regulation. Therefore using Fra, Fos, and NGFI-A expression to monitor changes at single and population level, we tested striatal functional responsiveness to pharmacological dopaminergic stimulation during development. Dopaminergic indirect agonists such as cocaine and amphetamine have as end effect to increase the level of dopamine at the synapses. We found that dopaminergic stimulation with cocaine and amphetamine induces Fos, Fra and NGFI-A proteins above baseline level. Thus indicating that striatal neurons are responsive to dopamine from very early in development. Furthermore we found that dopaminergic stimulation by psychomotorstimulants activates neurons of the striosomal and matrix compartment with a differential profile. In the adult striatum, it has been shown that cocaine and amphetamine have drug specific pattern of IEG induction. Cocaine induces IEG proteins with a distribution mostly even throughout the striatum whereas amphetamine induces IEG proteins with a striosomes-predominant pattern in the rostral striatum (Fig.20). We have found that for a prolonged period of development both psychostimulants induce IEG proteins with similar patterns. During the first postnatal week both cocaine and amphetamine induce Fos, Fra and NGFI-A specifically in the striosomal compartment (Fig.21). The similarity in behavior extends also to the second postnatal week when both psychostimulants induce IEG proteins in the main output nuclei of the striatum, the substantia nigra and the globus pallidus (Fig.21). Only from the third postnatal week, cocaine and amphetamine patterns of induction of IEG proteins start to acquire the distribution typical of adulthood (Fig.21). We have further tested whether striatal neurons were responsive to dopaminergic stimulation even before birth. We found that cocaine administrated through the maternal circulation induces IEG expression in the striatum.

Remarkably the induction is restricted to the developing striosomal compartment. We tested that, from the time of birth, the increase in expression of IEG proteins is mediated by the D1 pathway. Pretreatments with the D1-selective antagonist, SCH23390, block the induction of IEG proteins. By contrast, the pharmacological block of the D2 dopamine receptors by the D2 receptors selective antagonist, eticlopride, induce the expression of IEG proteins. Remarkably, we found that despite the expression of D2 receptors in both compartments the induction of Fos, Fra and NGFI-A proteins is restrained to the striosomal compartment for the first postnatal week.

Only few works have addressed the influence of glutamatergic afferents on striatal development. In the adult striatum, it has been observed a glutamatergic modulation on the dopaminergic signals. We tested whether glutamatergic receptors may modulate dopaminergic signals by pharmacologically blocking the NMDA receptors with MK801. We found that NMDA receptor blockade prevents the induction of IEG proteins by dopaminergic stimulation and partially downregulates the basal level of IEG expression from the first postnatal week. Thus suggesting that the interactions between the dopaminergic and glutamatergic systems start to become effective during early development.

D1 dopamine receptors have during development a striosomal-predominant expression. This specific expression raised the idea that dopamine through D1 receptors may influence the striatal development and in particular the striosomal compartment. To determine how the D1 dopamine receptors may influence the development of the striosomes/matrix pattern, we characterized the striatum of mutant mouse pups engineered to lack the D1 receptor. We found that D1 receptors are necessary for the proper establishment of a phenotypic marker of the striosome/matrix pattern, as shown by the barely detectable detection of dynorphin in the striosomes of the mutant mice. However, the compartmental aggregation is not radically affected by the lack of D1 receptors throughout development. In fact we found several striosome-predominant phenotypic

marker distribution, including Fra and NGFI-A expressions, not affected. These results indicate that striosomes may aggregate virtually in a normal pattern even in absence of D1 receptors. In addition these results establish that Fra and NGFI-A basal expressions are independent of the D1 pathway.

From the evidences collected we propose the presence of two distinct pools of Fos, Fra and NGFI-A proteins. One pool is composed of proteins expressed even after pharmacological D1 and NMDA blockade or D1 genetic engineered absence. We consider this pool composed of constitutive proteins. Another pool is very sensitive to dopaminergic and glutamatergic regulation and we consider composed by proteins which regulation is afferent-dependent. Thus suggesting that intrinsic programs of development may proceed in parallel to dopaminergic and glutamatergic activity-dependent modeling of striatal function.

Developmental phases in the developing striatum revealed by Fos, Fra and NGFI-A basal expression

We found that inducible and constitutive Fos, Fra, and NGFI-A patterns of expression uncover crucial steps of striatal development. During prenatal development Fos, Fra and NGFI-A are confined to the striosomal compartment. This prenatal period could be divided according to IEG expression and striosomal neurons localization into a ventrolateral phase, followed by a dispersed phase and finally by a striosomes phase (Fig.22). In the first phase striosomal neurons and IEG expression are detected in the ventrolateral striatum especially caudally (Fig.22). In the second phase striosomal neurons and IEG expression is detected dispersed throughout the striatum. In the third phase striosomal neurons have finally aggregated in the characteristic patches and similarly IEG expression is detected in patches (Fig.22). The striosomal phase extends also postnatally during the first postnatal week by the end of which the IEG expression looses compartment constraints (Fig.22). Fos and Fra basal expression are confined to the

striosomal compartment. Postnatally Fos and Fra proteins downregulate. Fos is completely undetectable whereas Fra remains weakly expressed near the ventricle and in the fundus striatum. By contrast, NGFI-A expression spreads also to the matrix compartment. As speculation Fra and Fos basal expression may be involved into the regulation of functions specific for striosomal development whereas NGFI-A proteins may be involved in the regulation of functions of general striatal development.

Characterization of a novel phenotypic striosomal marker

In an attempt to investigate whether the emergence of the striosome/matrix pattern was due to a differential stage of neurodifferentiation between the striosomal and matrix neuronal populations, we characterized the expression of the neurodifferentiation marker microtubule-associated protein-2 (MAP-2) during striatal development. MAP-2 is a regulatory protein for dendritic cytoskeleton stabilization (review in Johnson and Jope, 1992). We found that during early development MAP-2 is expressed at low level in both compartments. At the time of striosomal compartment aggregation, we detected high MAP-2 staining selectively in the developing striosomes. We further found that after compartment formation MAP-2 proteins are downregulated in all striatum. By contrast, cortical MAP-2 expression was not subjected to downregulation. We propose that when striosomal neurons are aggregating, they may need a highly stabilized dendritic arborization to counteract the desegregating force exerted by matrix neurons. In favor of this hypothesis is the evidence that high MAP-2 expression is not a general requirement for dendritic maturation because we did not observe this phase during the dendritic maturation of the matrix neurons.

Striatal development in vitro

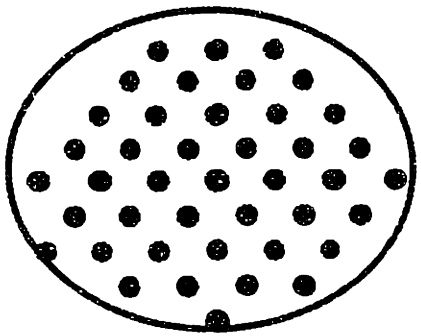
To test the intrinsic potentialities of the embryonic striatum to develop in a system deprived of most long range information we turn to an in vitro approach. We developed an embryonic organotypic culture system. Short term organotypic slices cultures maintain most of the three dimensions structure necessary for cell-cell interactions allowing conditions very close to physiological. We determined that striatal cells actively migrate from the germinal layer into the striatum. We excluded that this result is due to passive movements caused by the flattening of the slices as a subpopulation of striatal cells, which does not migrate in vivo, remains located near the germinal zone even after several days of culturing. We further investigated whether striatal compartments could segregate from the intermixed state without most of the afferents. In cell culture aggregates it has been shown that striosomal cells maintain the cell specific adhesive properties. However, only in few prenatal organotypic slices BrdU-positive striosomal neurons aggregated in distinguishable clusters. To test whether this result was not consistent due to the no complete labeling of the striosomal neurons by BrdU administration, we used the putative phenotypic striosomal marker Fra. Indeed, clusters immunolabeled by the transcription factor Fra were detected to emerge from a dispersed pattern in prenatal organotypic slices cultured for 5 days. Whether or not the Fra-positive clusters observed were striosomes is not clear. However, to check whether Fra expression is confined, as in vivo, in the striosomal cells we characterized slices obtained from brains sliced at ages when striosomes are aggregated. We found that the patchy pattern of Fra immunostaining detected labeled indeed striosomes as Fra expression colocalized with another striosomal phenotypic marker, microtubule associated protein-2. These results suggest that the expression of the constitutive pool of Fra proteins is not dependent on the intact innervation of the afferents. Furthermore these experiments establish the possibility to pharmacologically investigate the molecular mechanisms orchestrating the early formation of the striosome/matrix phenotypic pattern in organotypic slices even before birth.

The in vitro experiments have been performed in collaboration with Dr. Elena Barragan and Ai Yamamoto.

Figure 20. Schematic diagram of the drug-specific patterns of Fos, Fra and NGFI-A inducible proteins detected in the adult striatum.

Drug-specific patterns of Fos, Fra and NGFI-A induction in the adult striatum

cocaine



amphetamine

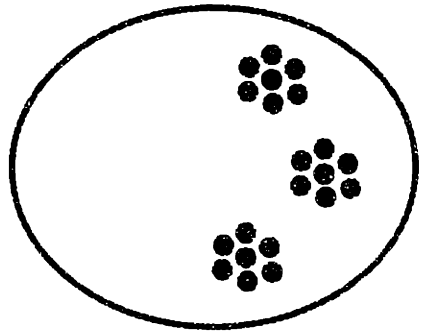


Figure 21. Schematic diagram of the developmental phases of Fos, Fra and NGFI-A inducible proteins detected in the developing striatum.

Developmental phases of Fra, Fos and NGFI-A induction in the developing striatum

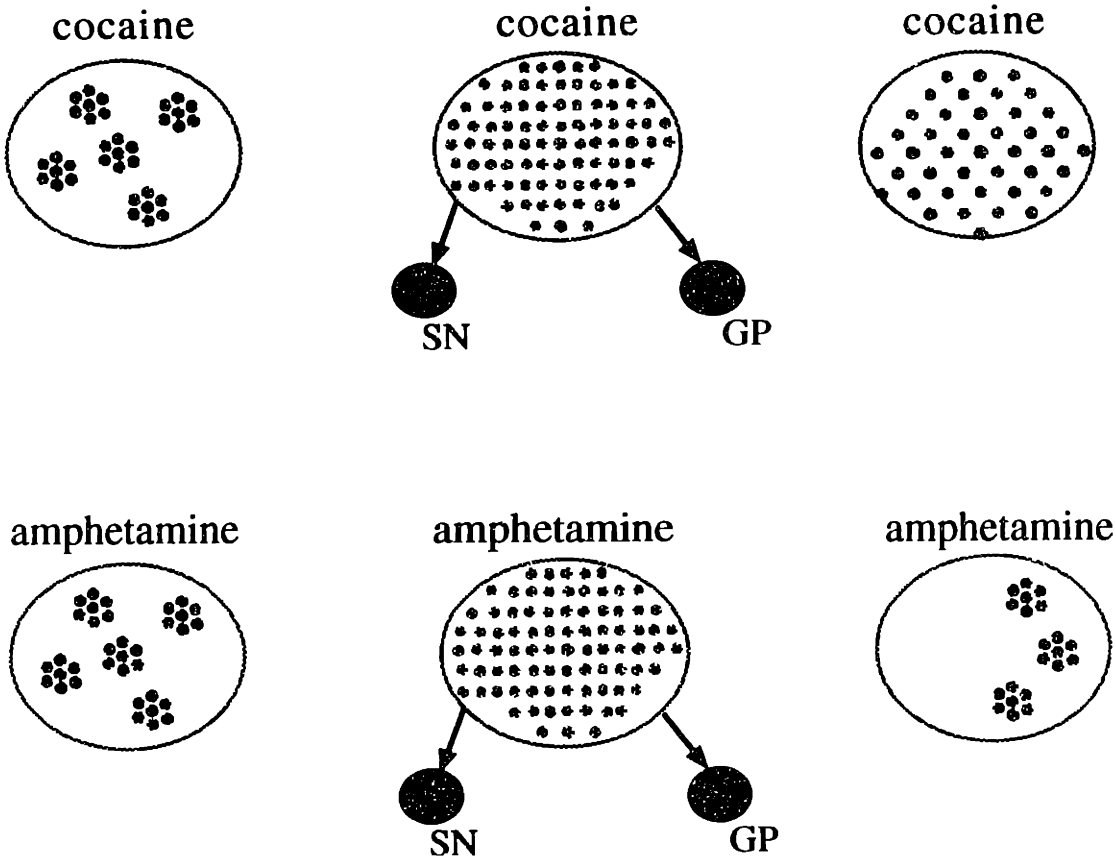
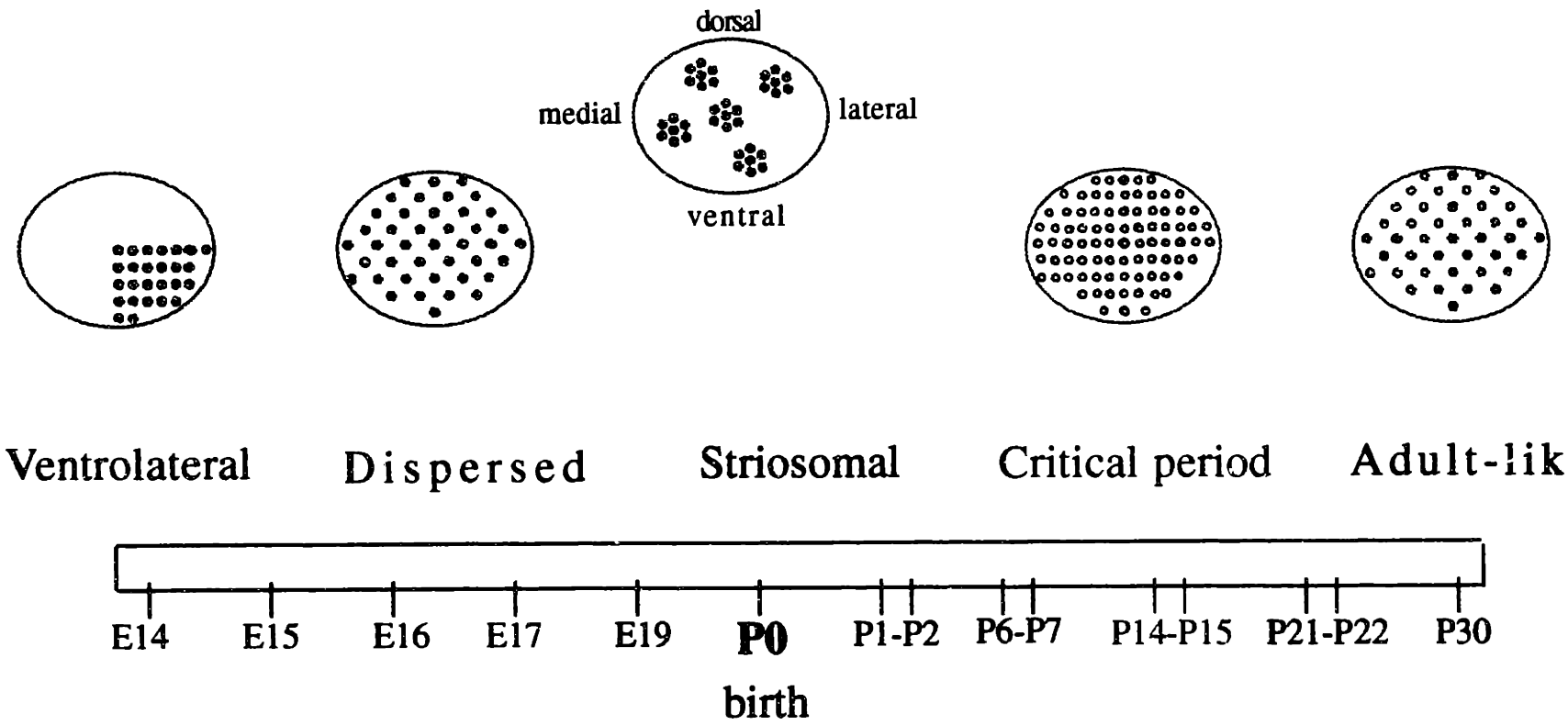


Figure 22. Schematic diagram of the developmental phases revealed by the constitutive expression of Fos, Fra and NGFI-A in the developing striatum.

Developmental phases in the developing striatum revealed by Fos, Fra and NGFI-A expression



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