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Genetic and epigenetic control of midbrain dopaminergic neuron development

CARLA PERRONE-CAPANO[1,2] and UMBERTO DI PORZIO*[1]

[1]*Istituto Internazionale di Genetica e Biofisica, CNR, Naples, Italy and*
[2]*Università di Catanzaro, Facoltà di Farmacia, Roccelletta di Borgia, Italy.*

ABSTRACT The relatively few dopaminergic (DA) neurons in the mammalian brain regulate many important neural functions, including motor integration, neuroendocrine hormone release, cognition, emotive behaviors and reward. A number of laboratories, including ours, have contributed to unravel the mechanisms of DA phenotype induction and maturation and elucidated the role of epigenetic factors involved in specification, development and maintenance of midbrain dopaminergic functions. DA progenitors are first "committed" to give rise to DA neurons by the action of two secreted factors, Sonic hedgehog and fibroblast growth factor 8 (FGF8). Subsequently, the function of selectively activated transcription factors, Nurr1 and Ptx3, is required for the DA final determination. Further development of DA neurotransmission requires specific interactions with the developing target striatal cells, which modulate key DA functions, namely synthesis and uptake of the neurotransmitter. Committed and determined DA neurons express the key genes involved in DA neurotransmission at different times in development. In rodents, synthesis and intracellular accumulation of DA is achieved shortly after expression of *Nurr1*, while the onset of high affinity uptake, responsible for ending the neurotransmission, takes place after a few days. Cell contacts between the presynaptic DA neurons and target striatal neurons are apparently necessary for the fine modulation of DA function, *in vivo* and *in vitro*. Strikingly, the *in situ* maturation and phenotypic specialization of DA neurons grafted into the adult striatum/caudate-putamen parallels the normal development of committed fetal dopamine neurons during neurogenesis. The correct matching between the right presynaptic and postsynaptic neurons is required also for grafted DA cells.

KEY WORDS: *Tyrosine hydroxylase, dopamine transporter, sonic hedgehog, Nurr1, cell communication.*

Introduction

The catecholamine dopamine (DA) in the mammalian brain subserves important regulatory role in many neural functions, including fine motor integration, neuroendocrine hormone release, cognition, emotive behavior, male sexual behavior, reward and memory. The use of various addictive drugs that enhance dopamine neurotransmission lays on the role of DA in hedonistic pleasure. At the biochemical level, DA is synthesized from tyrosine by tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine biosynthesis. Upon release from the presynaptic terminals into the synaptic cleft, DA interacts with postsynaptic D1- or D2-type G-coupled receptors (Missale *et al.*, 1998). DA also activates presynaptic autoreceptors of D2-type which are involved in the regulation of dopamine synthesis, metabolism and release (Elsworth and Roth , 1997; Jones *et al.*, 1999). Neurotransmission is terminated by high affinity uptake into the presynaptic DA fibers of the released DA, through the activity of the dopamine transporter plasma membrane glycoprotein (DAT), a member of the sodium/chloride-dependent neurotransmitter transporter family (Amara and Arriza, 1993; Reith *et al.*, 1997).

In mammals, midbrain DA neurons are located ventrally to form the retrorubral nucleus (A8), the *substantia nigra* (A9, lateral part of the ventral midbrain) and the ventral tegmental area (A10, ventromedial part of the midbrain, VTA) (Nelson *et al.*, 1996). In rodents, neurons arising from the *substantia nigra* project to the dorsal and lateral striatum (corresponding to the *caudate-putamen* in primates) in an orderly medial-to-lateral arrangement to form the ascending nigrostriatal pathway; they receive innervation from

Abbreviations used in this paper: A, anatomical area; AHD2, aldehyde dehydrogenase; DA, dopamine; DAT, dopamine transporter; E, embryonic age; FGF, fibroblast growth factor; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; RXR, receptor for 9-cis-retinoic acid; SHH, Sonic hedgehog; TH, tyrosine hydroxylase; VMAT2, synaptic vesicle monoamine transporter; VTA, ventral tegmental area.

***Address correspondence to:** Dr. Umberto di Porzio. Istituto Internazionale di Genetica e Biofisica, CNR, Via Marconi 10, 80125 Naples, Italy.
TEL. : 39 081 725 7262. FAX: 39 081 593 6123. e-mail: diporzio@iigb.na.cnr.it

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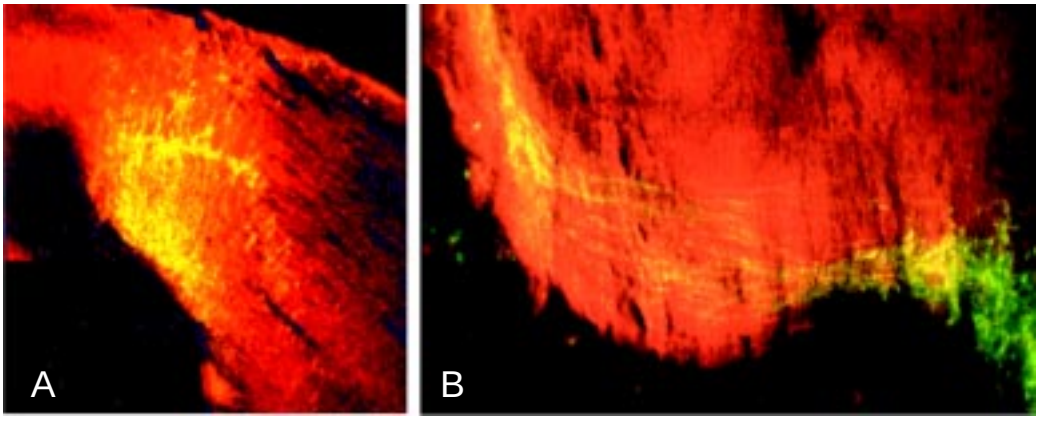

Fig. 1. TH⁺ immunofluorescence in the embryonic mouse midbrain. *Mid-sagittal sections of E15 mesencephalon show TH-like immunoreactive neurons (putative dopaminergic neurons), which form parallel rows of highly fluorescent cells, elongated in a dorso-ventral direction and extending between the floor of the mesencephalon and the ependymal layer of the mesencephalic vesicle (***A***, x 100). Fluorescent fibers corresponding to the developing ascending dopaminergic pathways project in the rostral direction along the anterior part of the mesencephalic flexure (***B***, x 225).*

multiple structures in the diencephalon and telencephalon (Beckstead *et al.*, 1979; van der Kooy, 1979). The nigrostriatal pathway regulates motor control and its degeneration in humans is associated with Parkinson's disease, the syndrome described by the English neurologist in his "Essay on the shaking palsy" in 1817. VTA DA neurons project to the medial and the ventral striatum (*nucleus accumbens* and olfactory tubercles, also known as limbic system) and to the prefrontal cortex to form the mesocorticolimbic pathway. In primates also A9 and A8 DA neurons project to the prefrontal cortex to form a mesofrontal DA system (Williams and Goldman-Rakic, 1998). This pathway is involved in emotional and reward behavior and in motivation (Schultz, 1997). Disturbances in this system have been associated with schizophrenia (although direct evidence is still elusive), addictive behavioral disorders and attention-deficit hyperactivity disorder (Egan and Weinberger, 1996; Swanson *et al.*, 1998). DA also modulates interactions between prefrontal cortex and visual association areas, which are important in visual memory (Williams and Goldman-Rakic, 1995). In addition, dopaminergic neurotransmission is involved in learning and memory dysfunctions associated with traumatic brain injury (Tang *et al.*, 1997). All three dopaminergic mesencephalic nuclei (A8, A9 and A10 region) project towards the hippocampal formation, although the functional significance of the mesohippocampal DA system is still unknown. It has been suggested that this projection could have a role in modulation of memory processes (Gasbarri *et al.*, 1997). In addition to its hippocampal innervation, the retrorubral A8 dopaminergic cell group projects to the *substantia nigra* and VTA and possibly is involved in the coordination of the nigrostriatal and mesolimbic systems (Arts *et al.*, 1996).

The physiological role and clinical relevance of dopaminergic neurons are well recognized. The mechanisms underlying their development have been the object of intense investigation (reviewed in: Perrone-Capano and di Porzio, 1996; di Porzio *et al.*, 1999).

In the *substantia nigra*, DA neurons appear highly polarized, with axons directed rostrally toward the basal ganglia and the frontal cortex and dendrites caudally confined. Studies *in vitro* have shown that mesencephalic or striatal glial cells can differentially dictate the morphology of mesencephalic DA neurons. Glial monolayer from the mesencephalon (where *in vivo* lay DA cell bodies and their dendrites) allows development of highly branched DA neurons and induces dendritic and axonal elongation. In contrast, DA neurons grown on striatal glia (where *in vivo* DA axons sprout) develop a "linear" morphology with a single, thin axon (Denis-Donini *et al.*, 1984). Thus the acquisition of DA neuron polarity could be regulated by glia-neuron interactions. Recent work has begun to shed light also on the molecular signals that confer target specificity to different dopaminergic neurons during development, defining the topographical projections of the nigrostriatal and mesolimbic afferences (Yue *et al.*, 1999). The ephrin receptor EphB1 and its ligand EphrinB2 are candidate guidance molecules for the establishment of these distinct pathways. They are expressed in complementary patterns in the developing midbrain DA neurons and their targets: EphB1 receptors are predominantly expressed in the *substantia nigra*, while their expression is low in the caudate-putamen; on the contrary, the expression of the ligand Ephrin-B2 is low in the VTA, but it is highly expressed in the limbic system. Ephrin-B2 can inhibit neuritic outgrowth and induce degeneration of the *substantia nigra* neurons, but not VTA neurons *in vitro*. Thus, ligand-receptor pair may contribute to the establishment of the distinct neural pathways by selectively inhibiting the neurite outgrowth and cell survival of DA neurons in inappropriate target districts.

The precise anatomical localization and functional differentiation of DA neurons in the mammalian brain is achieved through the action and gradient disposition of various diffusible factors. Data from explant culture studies and biochemical and genetic experiments have demonstrated that the stereotypic location of these neurons along anteroposterior and dorsoventral axes is defined by the integration of two distinct secreted molecules, Sonic hedgehog (SHH) and FGF8, which are necessary and sufficient for the induction of DA neurons (Hynes and Rosenthal, 1999a). Moreover, intersection of SHH and FGF8 signaling is able to specify the location of DA neurons also ectopically along the neural tube (Ye *et al.*, 1998). These experiments indicate that signaling centers and secreted signals establish a functional epigenetic Cartesian grid of positional information in the neural tube, specifying cell fates along the two main axes of this system and inducing multiple classes of neurons according to their position (Hynes and Rosenthal, 1999b).

The inductive signals are thought to activate cascades of other signaling molecules and transcription factors which lead to the final differentiation of DA neurons. Two transcription factors, Nurr1 and Ptx3, expressed at crucial times in differentiating midbrain DA cells, have been recently identified.

An "orphan" member of the steroid-thyroid hormone receptor superfamily, Nurr1, is expressed in the mouse ventral midbrain one

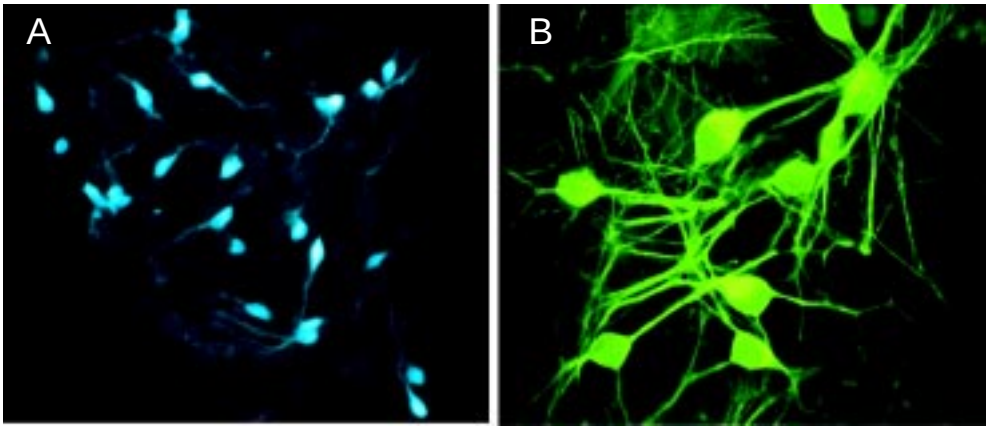

Fig. 2. Dopaminergic neurons enriched in midbrain primary cultures. *Cluster of dopaminergic neurons in two day-old primary cultures of E13 mouse mesencephalon, enriched by fluorescence activated cell sorter (di Porzio et al., 1987) and visualized by formaldehyde-induced catecholamine fluorescence, according to Furness et al., 1977 (***A***, x 180). Cluster of TH+ immunofluorescent neurons from the same preparation as in A in two-week-old cultures (***B***, x 500).*

day before the appearance of TH, and its expression continues in mature dopaminergic neurons during adulthood (Zetterstrom *et al.*, 1997). Absence of Nurr1 in knock out mice leads to agenesis of DA neurons in the midbrain and the consequent lack of striatal dopamine innervation. Interestingly, DA cell groups in the diencephalon (areas A11/A13), as well as DA neurons of the olfactory bulb are not affected in *Nurr1* null mutants and continue to express *TH* at the normal time, thus excluding that Nurr1 directly regulates *TH* gene expression in these neurons (Zetterstrom *et al.*, 1997; Castillo *et al.*, 1998).

Differently from *Nurr1*, which is also expressed in other CNS regions, the expression of the homeodomain gene *Ptx3* remains restricted to the mesencephalic DA system (Smidt *et al.*, 1997). Early commitment of DA cell lineage, as measured by expression of *Ptx3,* is still observed in the absence of Nurr1 (Saucedo-Cardenas *et al.*, 1998). However, these late precursors fail to differentiate and undergo apoptosis, suggesting that Nurr1 may only be responsible for late steps in the differentiation of DA neurons. The consequence of ablating *Ptx3* has not yet been reported and its role in DA neuron specification remains to be clarified. It has been proposed that Ptx3 and Nurr1, although regulated independently, may function in a cooperative manner to regulate factors required for terminal differentiation of midbrain DA neurons. They may act by activating transcription of key genes in DA function, such as TH (Sakurada *et al.*, 1999).

TABLE 1

DEVELOPMENT OF EMBRYONIC TH+ NEURONS FOUR MONTHS AFTER GRAFT IN THE HOST STRIATUM

	MES Cell Grafts		HYP Cell Grafts	
	ACE/MPTP	Unlesioned	ACE/MPTP	Unlesioned
TH+ neurons implanted	12,000		6,400	
TH+ neurons in the host (4 months grafts)	2625+341	2152+280	124+19	147+30
Survival (%)	22	18	2	2
Development (%)*	90	40	<10	<10

*(TH+ neurons that show large cell body, regular nucleus and extended neuritic arborization)

In the last two decades our group has contributed to the studies of midbrain DA circuits establishment and maturation during ontogeny. Our work has been focused mainly on:

- the ontogeny of DA neurons *in vivo* and *in vitro*;
- the onset and modulation of key genes necessary to DA neuron development, *in vivo* and *in vitro*;
- the role of interaction with target neurons in the maturation of DA neuron function, *in vitro* and in graft experiments.

The birth of mesencephalic DA neurons

When are DA neurons born during CNS development? To investigate their ontogeny, immunohistochemistry using antibodies against TH or DA, ^{3}H-thymidine labeling of proliferating DA neuroblasts and sensitive molecular biology techniques have been employed. In the mesencephalon, TH is expressed early during ontogeny (see below). TH has been used as a marker of all catecholaminergic neuroblasts: TH+ cells are considered the precursors of dopaminergic neurons in the midbrain, noradrenergic neurons in the brain stem and adrenergic neurons in the ventral *medulla oblongata*. DA midbrain neuroblasts are generated near the midbrain-hindbrain junction (Voorn *et al.*, 1988) and migrate radially to their final position in the ventral midbrain (di Porzio *et al.*, 1990).

In the mouse mesencephalon, rare and scattered TH+ cells and fibers have been detected starting at embryonic day (E) 9.5 close to the ventricular ependymal layer, suggesting that DA differentiation can occur in early postmitotic neural precursors (di Porzio *et al.*, 1990). Many migrating TH+ cells show already distinct neuronal morphological features. This appears to be a distinct feature of midbrain DA neurons. For instance, in the olfactory bulb *TH* is not expressed until the cells have reached their final destination, the glomerular layer (McLean and Shipley, 1988). TH+ clusters reminiscent of the areas A9 and A10 can be detected at E13 (Berger *et al.*, 1982; di Porzio *et al.*, 1990; Son *et al.*, 1996). At E15 the TH+ cells enlarge between the floor of the mesencephalon at its flexure and the ependymal layer and start to extend neurites toward the telencephalon (Fig. 1).

The sequence of developmental events for mesencephalic dopaminergic neurons is similar in humans and rodents. The duration of the developmental period is, however, significantly protracted in humans: TH+ cells appear in the ventral mesencephalon at 6.5 weeks adjacent to the ventricular zone; their

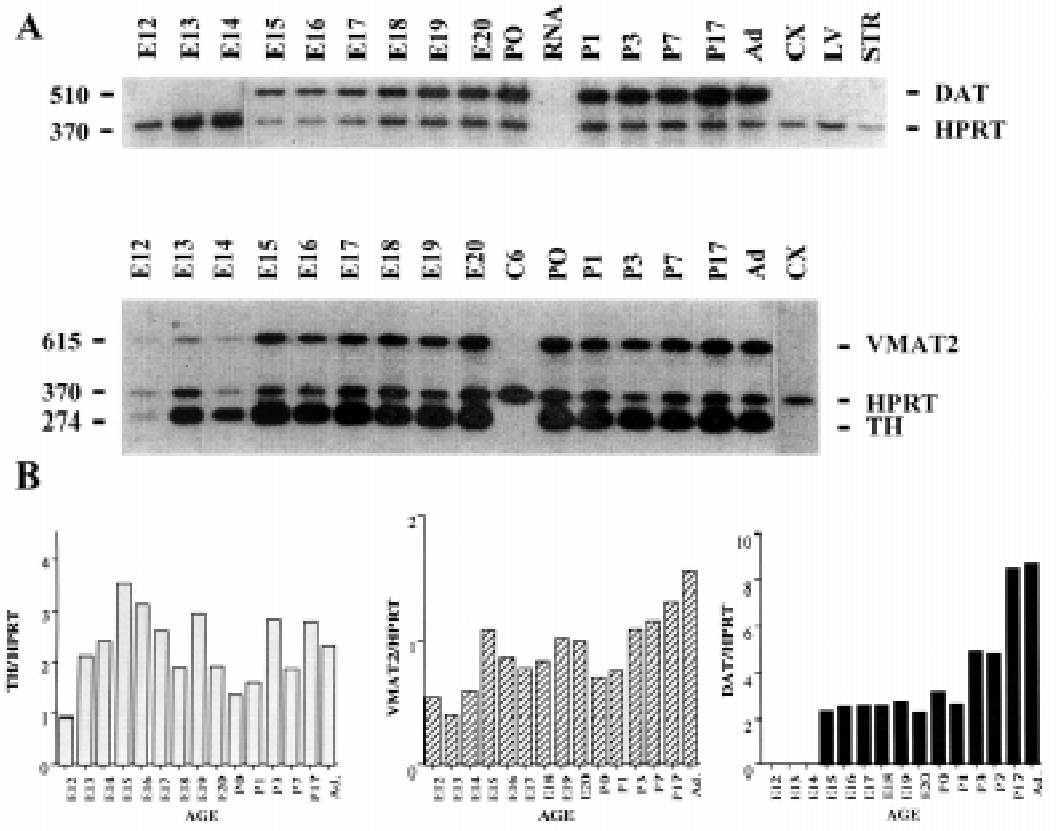

Fig. 3. TH, VMAT2 and DAT gene expression during development of the rat ventral mesencephalon. (A) *Semiquantitative RT-PCR analysis was performed on rat ventral midbrain RNA from various embryonic (E) and postnatal (P) ages. Hypoxanthine phosphoribosyl transferase (HPRT) mRNA was co-reverse transcribed and co-amplified in every reaction and used as an internal standard (Pernas-Alonso et al., 1999). Other RNA samples are: Ad., adult rat ventral mesencephalon; CX, adult parietal cortex; LV, adult liver; STR, E16 striatum; C6, C6 glioma cell line; RNA, non-reverse-transcribed RNA from E20 mesencephalon. Size and identity of the PCR products are indicated on the left and on the right, respectively.* **(B)** *The intensity of radioactive fragments amplified from target transcripts was normalized to that of HPRT, allowing a relative quantification of tyrosine hydroxylase (TH/HPRT), vesicular monoamines transporter (VMAT2/HPRT) and dopamine transporter (DAT/HPRT).*

ventral migration begins at 6.7 weeks and TH^+ neurites are seen initially in the developing *putamen* at 9.0 weeks (Freeman *et al.*, 1991).

Nurr1 gene expression in developing DA neurons

As outlined in the introduction, Nurr1 is an essential transcription factor for the final differentiation and survival of midbrain DA neurons (Zetterstrom *et al.*, 1997; Castillo *et al.*, 1998). Its gene expression is activated soon after determination of these neurons and is maintained in the adult life. We found that in the rat ventral midbrain, *Nurr1* gene expression shows a 10 fold increase between E13 and E15, in a crucial time for the maturation and organization of the *substantia nigra* and VTA (Da Pozzo *et al.*, 1999; Perrone-Capano *et al.*, 2000, and unpublished results).

Nurr1 can heterodimerize with the receptor for 9-cis-retinoic acid (RXR), activating transcription in response to RXR ligands (Forman *et al.*, 1995; Perlmann and Jansson, 1995; Maruyama *et al.*, 1997). Moreover, *Nurr1* is an immediate early gene rapidly induced by electrical activity in cell lines and in the adult brain (Law *et al.*, 1992; Xing *et al.*, 1997). These observations prompted us to study the expression of *Nurr1* in rat mesencephalic primary cultures and its modulation by epigenetic and intrinsic factors. Preliminary results show that expression of *Nurr1* is plastic and can be upregulated, at least *in vitro*, by depolarization and by retinoids (Da Pozzo *et al.*, 1999; Perrone-Capano *et al.*, 2000, and unpublished observations). A role for retinoids in the differentiation of DA neurons is suggested by the early expression of the retinoic acid-generating enzyme aldehyde dehydrogenase (AHD2) in the somata of embryonic nigral neurons and in their axons projecting to the striatum (McCaffery and Drager, 1994). Altogether, these observations suggest that Nurr1 could be the point of convergence between retinoic and activity-dependent signaling pathways, thus playing a key role not only in differentiation, but also in maturation and maintenance of DA neural circuits (Eichele, 1997).

Specification and expansion of DA neurons *in vitro*

Given the potential therapeutical usefulness of midbrain DA neurons, their enrichment has been a goal of researchers in this field for many years. It has been rarely achieved, or only partially, by sophisticated dissection techniques (Shimoda *et al.*, 1992), or cell sorting of embryonic DA neurons (Fig. 2) (di Porzio *et al.*, 1987). More recently, following the discovery of stem cells in the embryonic and adult CNS (McKay, 1997; Temple and Alvarez-Buylla, 1999), a number of cytokines and growth factors have been used to expand DA neuron precursor population *in vitro* (Ling *et al.*, 1998; Engele and Bayatti, 1999). However, a physiological role of these factors *in vivo* has not been demonstrated. These new approaches have raised the possibility to generate unlimited numbers of DA neurons to be grafted in patients with Parkinson's disease (Svendsen *et al.*, 1997; Studer *et al.*, 1998).

Fibroblast growth factor 2 (FGF2) , also known as basic FGF, acts as mitogen for neuronal precursors in fetal rat mesencephalic cultures and delay their differentiation (Bouvier and Mytileneou, 1995). Interleukin 1 causes a significant increase in the number of TH^+ cells dissociated from embryonic rat midbrain and expanded in the presence of epidermal growth factor (Ling *et al.*, 1998). Recently, it has been shown that the overexpression of *Nurr1*, in

conjunction with factors derived from midbrain type 1 astrocytes, induces an immortalized multipotent neural stem cell line derived from mouse cerebellum to differentiate into DA neurons (Wagner *et al.*, 1999). These results highlight the crucial role of Nurr1 in the determination of the DA phenotype and indicate that this transcription factor and nuclear receptor turns on the expression of relevant genes which make the stem cells responsive to still unidentified factors secreted by the appropriate glial cells.

In our laboratory, we have used the combined action of growth and inductive factors to stimulate mesencephalic neuroblast proliferation and to expand *in vitro* the number of committed DA neurons. We found that SHH enhances DA differentiation in neuronal precursors dissociated from fetal rat mesencephalon (E12-13) and expanded in the presence of FGF2, as monitored by high affinity DA uptake, immunocytochemistry and detection of specific transcripts (Da Pozzo *et al.*, 1999 and unpublished observations). This culture system is currently used by our group to study the cellular and molecular mechanisms underlying DA neuron commitment and differentiation, and the role of Nurr1 in these events.

Maturation of the dopaminergic phenotype

The activation of genes involved in DA neurotransmission takes place at different time in development under the control of various environmental cues, following the early commitment of DA neuroblasts. It is plausible that autocrine, paracrine, glial-mediated and target-derived trophic factors are required to achieve maturation and survival/maintenance of postmitotic DA neurons. Putative dopaminotrophic factors and their role in the pathogenesis and potential treatment of DA- associated neurological diseases, have been extensively reviewed in the course of the last years (Engele and Bayatti, 1999, Bradford *et al.*, 1999) and will not be discussed here.

Monitoring the expression of various genes and their function during the embryonic development of the mesencephalon, we have shown that DA synthesis, storage and high- affinity uptake develop asynchronously (Perrone-Capano and di Porzio, 1996). Amongst the various specific dopaminergic markers, TH mRNA appears early (is already present at E12) (Fig. 3). TH activity can be detected early by the accumulation of DA into mesencephalic neurons (Fiszman *et al.*, 1991). Like *TH*, also the synaptic vesicle monoamine transporter gene (*VMAT2*) is expressed early, several days before the establishment of nigrostriatal DA neurotransmission (which in rodents occurs at E15-16) (Fig. 3). VMAT2 activity is readily necessary to accumulate DA into presynaptic vesicles even before DA release and function, probably to reduce DA oxidation and its putative neurotoxic effects. VMAT2 belongs to the family of vesicular neurotransmitter transporters which allow storage of monoamines into dense core vesicles in most aminergic neurons using an electrochemical gradient (Amara and Arriza, 1993).

Interactions of presynaptic midbrain DA neurons with striatal target neurons play an important role in modulating key aspects of midbrain DA neurotransmission, namely DA synthesis and DA uptake, mediated by TH and DAT, respectively (Prochiantz *et al.*, 1979; di Porzio *et al.*, 1980; Perrone-Capano *et al.*, 1996). DAT is also the site of action of various psychostimulant drugs and neurotoxins. *DAT* gene inactivation in transgenic mice has confirmed unequivocally DAT's physiological role: homozygous *DAT* null mice show spontaneous hyperlocomotion due to protracted persistence of DA in the extracellular space and are insensitive to the action of amphetamine, cocaine and the specific DA toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Giros *et al.*, 1996; Gainetdinov *et al.*, 1997).

In cells acutely dissociated from the embryonic rat ventral mesencephalon, measurable DA is detected as early as E12.5 and its concentration increases sharply at E16, reaching a plateau before birth. In the striatum, DA is first detected at E16, suggesting that DA nigral fibers reach their target tissue at this embryonic age (Fig. 4A), in accordance with morphological data showing the arrival of the first TH^+ and $AHD2^+$ axons to the striatum at that age (Berger *et al.*, 1982; Fiszman *et al.*, 1991; McCaffery and Drager, 1994). In contrast to the early appearance of endogenous DA levels in the mesencephalon (Fig. 4A), specific high-affinity DA

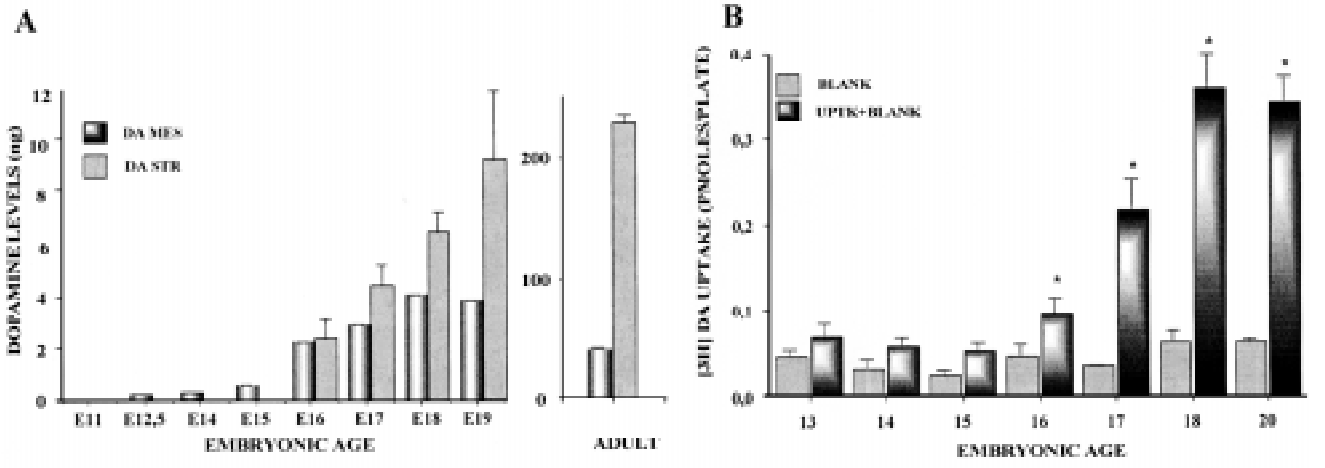

Fig. 4. Onset of DA accumulation and DA uptake in the developing rodent ventral midbrain. *DA levels were determined by high performance liquid chromatography in rat embryonic and adult ventral mesencephalon (MES) and striatum (STR). (***A***).* ^{3}*H-DA high-affinity uptake was measured during embryonic development using 50 nM labeled dopamine at 37 °C or at 4 °C (blank) in cells acutely dissociated from rat MES. (***B***). Bars represent the mean values ± SEM of separate experiments performed in triplicates. Asterisks represent p< 0.05 between uptake values at 37 °C and at 4 °C (ANOVA, Scheffé F test).*

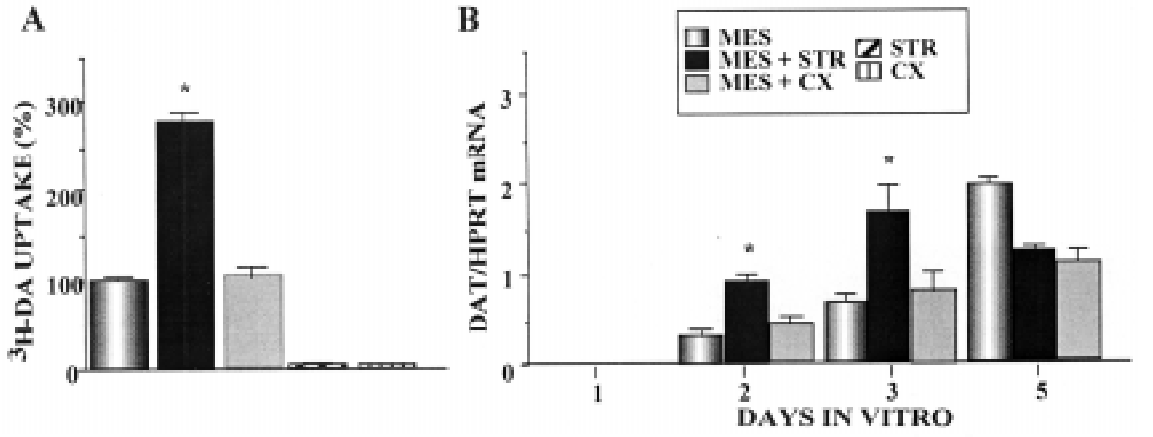

Fig. 5. Target striatal cells increase dopamine transporter function and gene expression. (A) *^{3}H-DA uptake was measured in one week-old primary cultures generated from rat E13 ventral mesencephalon alone (MES) or cocultured with target E16 striatal (MES + STR) and non-target E16 cortical (MES + CX) cells; striatal (STR) and cortical (CX) cultures show no uptake above background level.* **(B)** *Dopamine transporter (DAT) mRNA was measured in E13 mesencephalic (MES) primary cultures and cocultures with E16 striatal (MES + STR) or cortical (MES + CX) cells. Semi-quantification of DAT transcripts was achieved by an RT-PCR assay and normalized to hypoxantine-phosphoribosyl-transferase (HPRT) mRNA levels. Bars represent the mean values ± SEM of separate experiments performed in triplicates. Asterisks represent $p< 0.05$ between cocultures and control mesencephalic cultures (ANOVA, Scheffé F test).*

uptake in rat ventral mesencephalon *in vivo* is found only at E16: it increases sharply thereafter, reaching a plateau before birth (Fig. 4B; Fiszman *et al.*, 1991). Thus, the onset of DA uptake seems concomitant with the arrival of DA fibers to the striatum .

Consistent with the late appearance of the high-affinity DA uptake, *DAT* gene transcript is detected during the ontogeny of rat ventral mesencephalon around E15 (Fig. 3; Perrone-Capano *et al.*, 1994). In order to assess whether the onset of DA uptake could depend on interaction of DA neurons with striatal target cells we used an *in vitro* approach. *In vitro* studies are possible since DA neurons appear to fully maturate in primary cultures, according to functional, biochemical and morphological parameters, including synthesis, storage, release, uptake of the neurotransmitter (Daguet *et al.*, 1980; Choi *et al.*, 1993; Perrone-Capano *et al.*, 1999) and synapse formation (van den Pol *et al.*, 1986). We have shown that the levels of *DAT* gene transcripts and the number of uptake sites are selectively increased in rodent E13 mesencephalic DA neurons *in vitro* after addition of E16 striatal cells in coculture (Fig. 5; Daguet *et al.*, 1980; Perrone-Capano *et al.*, 1996). In contrast, *DAT* expression is not modulated by striatal neurons when cocultures are established using E16 mesencephalic neurons (Perrone-Capano *et al.*, 1996). The latter observation suggests that mesencephalic DA neurons respond to specific target influences within a restricted developmental window. Up-regulation of DAT mRNA level by striatal cells seems to require direct cell interactions since in culture target cells are ineffective when separated from mesencephalic cells by a porous barrier, which impairs direct cell contacts allowing only diffusion of soluble molecules (Perrone-Capano *et al.*, 1996). Interestingly, the still unidentified non-diffusible "signals" derived from target striatal cells must be specific since non-target CNS cells fail to stimulate DA uptake or *DAT* gene expression (Perrone-Capano *et al.*, 1996) (Fig. 5). It is worth noticing that the effects of striatum in cocultures on DAT mRNA takes place in the first 3 days *in vitro*, while that on the DAT function (with increased number of uptake sites) is most effective after 8 days *in vitro* (Prochiantz *et al.*, 1979; di Porzio *et al.*, 1980). In synthesis, *DAT* gene expression in developing mesencephalic DA neurons is conditioned by a specific cellular environment and probably requires continuous stimuli mediated by specific and direct cell interactions.

The early events, and the time course of specific gene onset, the time of action of putative trophic factors as well as the role of striatal target cells in rodent DA neuron development are summarized in the diagram in Fig. 6.

Degeneration of DA neurons and restoration of DA function

The striatum thus seems to regulate, both *in vivo* and *in vitro*, the maturation of the dopaminergic function during early and late development and could be involved in regulation of developmental DA neuron death and survival of DA neurons in postnatal life. The latter hypothesis is supported by a wealth of lesion and transplantation studies showing that the extent of striatal lesions during development can determine the magnitude of DA neuron loss in the *substantia nigra* (Macaya *et al.*, 1994) and that the striatum can sustain maturation, axonal growth and survival of grafted embryonic mesencephalic DA neurons (Constantini and Isacson, 1999). Graft studies have been performed in animal models of Parkinson disease, generated by surgical or neurotoxic lesions. The neurotoxin MPTP, originally discovered as a contaminant of illicit drug of abuse in young parkinsonian patients, elicits in humans and monkeys a severe extrapyramidal syndrome similar to idiopathic Parkinson's disease (Davis *et al.*, 1979; Langston *et al.*, 1983). MPTP neurotoxicity requires its enzymatic transformation into 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase followed by its concentration into target cells, the DA neurons. The mesencephalic glial cells from the mouse embryo take up MPTP *in vitro*, transforming it into MPP^+, and release it into the culture medium. Mesencephalic neurons in culture manifest a high-affinity uptake mechanism for MPP^+, similar to that for DA, since both molecules use DAT to enter DA neurons (Schinelli *et al.*, 1988). Thus MPTP is a specific dopaminergic neurotoxin, since DAT is expressed only in DA neurons. It induces alterations of the dopamine pathways also in rodents, although less effectively than in primates. In order to study embryonic DA neuron development into mature host, we have used mice treated with MPTP (whose toxicity was enhanced by acetaldehyde pretreatment) to lesion bilaterally the *substantia nigra* (Zuddas *et al.*, 1990; di Porzio and Zuddas, 1992). In the mouse, about 20-25% of E13 DA mesencephalic

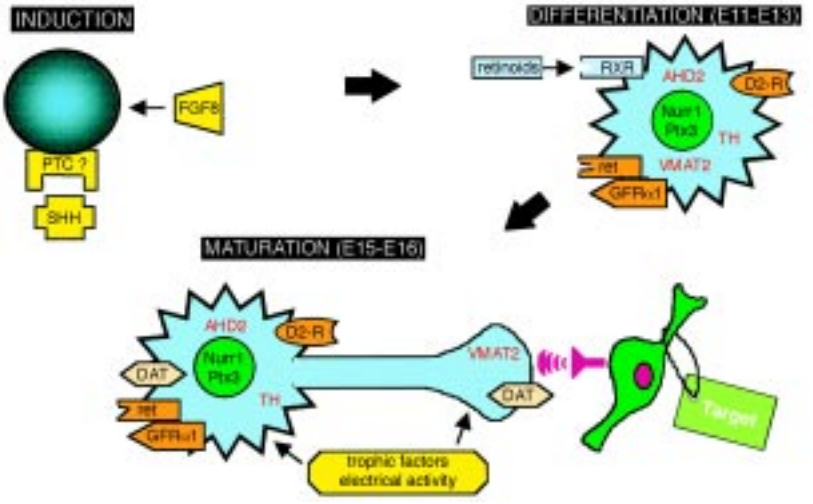

Fig 6. Tentative model and time course for DA neuron development. *The diagram summarizes the early events and the molecules involved in rodent midbrain DA neuron development. The onset of key genes is outlined, as well as the putative intervention of trophic factors and the influence of electrical activity and striatal target cells (Target). Upon induction by Sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8) and their interaction with specific receptors (patched, PTC, the putative SHH receptor and FGF receptors) DA neuroblasts are committed and differentiation events take place with the onset of specific transcription factors (Nurr1 and Ptx3) and receptors (retinoid X receptors, RXR; the receptor tyrosine kinase c-Ret (ret) which, together with GFRα1 forms the receptor complex for the glial cell line-derived neurotrophic factor, GDNF; and D2-type dopamine receptors, D2-R). During this time period, genes specific for DA functions are activated (retinoic acid-generating enzyme aldehyde dehydrogenase, AHD2; synaptic vesicle monoamine transporter, VMAT2; tyrosine hydroxylase, TH). Only after interaction with striatal target cells and/or cell activity, does dopaminergic maturation occur, with functional onset of the dopamine transporter, DAT. Putative dopaminotrophic factors intervene at various developmental stages. E, embryonic age in days.*

neurons implanted in the denervated host striatum showed a long term survival and formed a dense network of fibers which established functional reinnervation. Functional recovery was observed already a few weeks after implantation, and was maintained for the entire life of the animal. On the contrary, implants of embryonic hypothalamic neurons, which normally do not innervate the striatum, showed little or no survival in the above experimental paradigms and no functional recovery (Table 1). Similarly, DA neurons from the arcuate nucleus, noradrenergic neuroblasts from the *locus coeruleus* and cortical neurons were unable to restore function in rat denervated striatum (Hudson *et al.*, 1994). The overall DA fiber outgrowth of mesencephalic DA neurons grafted into the striatum was significantly reduced if the endogenous DA innervation was left intact (Table 1), suggesting that the striatum normally innervated by the DA fibers is an unfavorable environment for the neuritic outgrowth of the implanted fetal DA neurons, perhaps due to competition with the endogenous DA fibers for "target sites". Similar results were obtained in parkinsonian and hemi-parkinsonian rhesus monkeys implanted in the medial caudate nucleus with dissociated cells from embryonic monkey ventral mesencephalon, where a functional recovery was observed already few weeks after grafting and was sustained for up to 7 months (when the animals were sacrificed) (Bankiewicz *et al.*, 1990). In the grafted caudate nucleus, a sprouting of endogenous DA fibers possibly derived from the mesolimbic projection to the striatum was also observed ventral to the implant. These DA fibers probably sprouted from the VTA, which was spared both in mice and in monkeys treated with MPTP under our experimental conditions. The endogenous sprouting suggests that the implanted embryonic DA neurons or the lesion produced in the caudate by the implantation procedure can elicit the caudate to release still unidentified factors responsible for the formation of the new dopaminergic neuronal processes (Bankiewicz *et al.*, 1990; Liberatore *et al.*, 1999).

Altogether, these findings suggest that in the nigrostriatal system a proper matching between target and presynaptic elements is required for the maturation of embryonic dopaminergic neurons, during embryogenesis and when grafted in hosts with lesioned nigrostriatal system, where they can achieve functional restoration.

Concluding remarks

One of the most fascinating questions in developmental neurobiology is how neurons are specified, acquire their peculiar characteristics and find their correct connections to form functional circuits. The development of different subsets of neurons involves the expression of a program intrinsic to the each cell type and the response to extrinsic environmental influences represented by soluble factors and interactions with other cells. In the last years, the development of the midbrain dopaminergic system has been studied extensively in many laboratories, including our. It shows how complex and varied neural functions subserved by a small group of neurons can be achieved trough the correct interplay of genetic and epigenetic cues. New tools such as gene targeted-disruption techniques, cell lines obtained from CNS and neural stem cells are currently being used to unravel the molecular mechanisms underlying DA functions. The identification of molecules and cellular interactions involved in the development of the mammalian DA circuits allows pharmacological manipulations of DA neurons that can be used in future clinical application to achieve functional restitution to patients with dopamine neuron dysfunction and degeneration.

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