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Nitrinergic Neurons in the Developing and Adult Human Telencephalon: Transient and Permanent Patterns of Expression in Comparison to Other Mammals

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ABSTRACT A subpopulation of cerebral cortical neurons constitutively express nitric oxide synthase (NOS) and, upon demand, produce a novel messenger molecule nitric oxide (NO) with a variety of proposed roles in the developing, adult, and diseased brain. With respect to the intensity of their histochemical (NADPH-diaphorase histochemistry) and immunocytochemical (nNOS and eNOS immunocytochemistry) staining, these nitrinergic neurons are generally divided in type I and type II cells. Type I cells are usually large, intensely stained interneurons, scattered throughout all cortical layers; they frequently co-express GABA, neuropeptide Y, and somatostatin, but rarely contain calcium-binding proteins. Type II cells are small and lightly to moderately stained, about 20-fold more numerous than type I cells, located exclusively in supragranular layers, and found almost exclusively in the primate and human brain. In the developing cerebral cortex, nitrinergic neurons are among the earliest differentiating neurons, mostly because the dominant population of prenatal nitrinergic neurons are specific fetal subplate and Cajal-Retzius cells, which are the earliest generated neurons of the cortical anlage. However, at least in the human brain, a subpopulation of principal (pyramidal) cortical neurons transiently express NOS proteins in a regionally specific manner. In fact, transient overexpression of NOS-activity is a well-documented phenomenon in the developing mammalian cerebral cortex, suggesting that nitric oxide plays a significant role in the establishment and refinement of the cortical synaptic circuitry. Nitrinergic neurons are also present in human fetal basal forebrain and basal ganglia from 15 weeks of gestation onwards, thus being among the first chemically differentiated neurons within these brain regions. Finally, a subpopulation of human dorsal pallidal neurons transiently express NADPH-diaphorase activity during midgestation. *Microsc. Res. Tech. 45:401–419, 1999.*

INTRODUCTION

Nitric oxide (NO) is a novel messenger molecule with a variety of proposed roles in developing, adult, and diseased brain (Edelman and Gally, 1992; Gally et al., 1990; Holscher, 1997; Iadecola, 1993; Kandel and O'Dell, 1992; Yun et al., 1996; Zhang and Snyder, 1995). The NO is produced by several isoforms of the enzyme nitric oxide synthase (NOS), and nitrinergic neurons, i.e., NO-producing neurons, in the brain constitutively express both the neuronal (nNOS) and endothelial (eNOS) isoform of this enzyme (for review, see Yun et al., 1996; Zhang and Snyder, 1995). The activation of both NOS isoforms requires the influx of calcium ions, usually upon the activation of glutamate NMDA-receptors, as well as the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate (Yun et al., 1996). This last feature offers an unique opportunity to use NADPH-diaphorase (NADPH-d) histochemistry as a reliable and selective marker for visualization of nitrinergic neurons, at least in the aldehyde-fixed brain tissue (Bredt et al., 1991; Buwalda et al., 1995; Dawson et al., 1991; Hope et al., 1991; Huang et al., 1993; Kharazia et al., 1994; Matsumoto et al., 1993b; Schmidt et al., 1992; Spessert and Layes, 1994; Tracey et al., 1993; Vaid et al., 1996). While NADPH-d reaction visualizes neurons containing different isoforms of NOS, subpopulations of nitrinergic neurons containing specific NOS isoform can be visualized immunocytochemically by means of the appropriate monoclonal and/or polyclonal antibodies directed against nNOS (Bredt et al., 1991; Northington et al., 1996; Schmidt et al., 1992; Vaid et al., 1996) or eNOS (Dinerman et al., 1994; Northington et al., 1996; Vaid et al., 1996). Therefore, in the following text we will describe neurons displaying NADPH-d reactivity as NADPH-d cells, and neurons expressing specific isoform of NOS as either nNOS- or eNOS-positive cells.

On the basis of available evidence, it seems that NO is involved in all major histogenetic events leading to

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the establishment of functional neuronal circuits, i.e., in proliferation, differentiation, axon outgrowth, synaptogenesis, activity-dependent refinement of synaptic circuits, and programmed cell death. For example, NO mediates the switch from proliferation to cytostasis during the terminal differentiation of neuronal precursor cells (Peunova and Enikolopov, 1995), rapidly and reversibly inhibits growth of neurites of rat dorsal root ganglion neurons in vitro (Hess et al., 1993), stimulates synaptic vesicle exocytosis (Meffert et al., 1994) and alters the synaptic protein interactions that regulate neurotransmitter release and synaptic plasticity (Meffert et al., 1996), and induces apoptosis in primary cultures of cerebral cortical neurons (Palluy and Rigaud, 1996). In the developing visual system, NOS is transiently expressed in target neurons innervated by glutamatergic afferent axons during the remodelling of synaptic circuits (Cramer et al., 1995; González-Hernández et al., 1993; Günlük et al., 1994; Williams et al., 1994; Wu et al., 1994; Zhang et al., 1996; for review, see Cramer and Sur, 1996). In the developing cerebellar cortex, the NOS expression reveals architectonic compartments related to the ingrowth of afferent fibers (Schilling et al., 1994; Yan et al., 1993). In the olfactory system, NO mediates the formation of synaptic connections (Roskams et al., 1994), and in the developing hippocampus NO induces calcium-independent neurotransmitter release in a population of synapses at all stages of maturation (Sporns and Jenkinson, 1997). Some recently published results, however, have shown that the virtual absence of NOS activity fails to prevent the formation of ocular dominance columns in the ferret visual cortex or cortical barrels in the mouse somatosensory cortex (Finney and Shatz, 1998). Whereas the findings of Finney and Shatz (1998) strongly suggest that the NO is unlikely to be essential for the patterning of modular features of thalamocortical connections within the given cortical area, they do not exclude the possible involvement of NO in the patterning of other features of thalamocortical connections, such as appropriate areal targeting during the earlier developmental stages.

Moreover, on the basis of its capability for volume transmission (Zhang and Snyder, 1995) and its probable involvement in long-term potentiation and long-term depression in cerebral cortex and hippocampus (O'Dell et al., 1991; Schuman and Madison, 1991; Shibuki and Okada, 1991), it has been suggested that NO acts as a retrograde messenger in various forms of synaptic plasticity (Kandel and O'Dell, 1992), by linking the space and time in both developing and adult brain (Edelman and Gally, 1992; Gally et al., 1990).

Such findings and considerations, combined with the availability of histochemical and immunohistochemical markers, have served as an impetus for the bourgeoing interest in the involvement of nitrinergic neurons in cerebral cortical development and function. These studies are opening new vistas in developmental neurobiology of the cerebral cortex, especially if we focus on the expression of NOS protein in transient populations of specific fetal cortical neurons, such as Cajal-Retzius and subplate neurons, as well as on developmental periods characterized by ingrowth and remodelling of various classes of cortical afferents.

The purpose of this review is fourfold: (1) to summarize the available evidence on morphological types of nitrinergic neurons and their laminar and areal distribution in the adult cerebral cortex; (2) to discuss the available evidence on the developmental appearance and distribution of nitrinergic neurons in the neocortical anlage; (3) to present the available data on prenatal development of nitrinergic neurons in human basal forebrain and basal ganglia; and (4) to offer a brief description of the major findings of our ongoing and comprehensive studies of nitrinergic neurons in the prenatal human telencephalon.

In the mammalian brain, the neocortical developmental anlage consists of three major architectonic compartments: the marginal zone, the cortical plate, and the subplate zone (for review, see Kostović and Judaš, 1994, 1995; Kostović and Rakic, 1990). In the adult brain, the marginal zone has been transformed into the cortical layer I, the fetal cortical plate gives rise to cortical layers II to VI, and the extensive population of interstitial neurons residing in the subcortical white matter represents the remnant of the fetal subplate zone (Kostović and Rakic, 1990). Therefore, we find it useful to review the available evidence on nitrinergic neurons in developing and adult cortex with respect to the following three major compartments: (1) the marginal zone and the layer I; (2) the subplate zone and the population of adult cortical interstitial cells; and (3) the cortical plate and cortical layers II to VI. Furthermore, we will review the evidence concerning two specific problems: (1) the expression of NADPH-d activity and NOS-proteins in the population of principal (pyramidal) neurons of the cerebral cortex, and (2) tissue processing conditions relevant for the visualization of nitrinergic neurons.

EXPRESSION OF NADPH-D ACTIVITY AND NOS-PROTEINS IN THE CORTICAL LAYER I AND DEVELOPING MARGINAL ZONE

According to most studies, there are no NADPH-d and/or NOS-positive neurons in the neocortical layer I of the adult rat (Bredt et al., 1991; Dawson et al., 1991; Hedlich et al., 1990; Valtschanoff et al., 1993a; Vincent and Kimura, 1992), cat (Mizukawa et al., 1989), monkey (Aoki et al., 1993; Cipolloni and Pandya, 1991; Dombrowski and Barbas, 1996; Hashikawa et al., 1994; Yan et al., 1996b), and human brain (DeFelipe, 1993; Egberongbe et al., 1994; Kowall and Beal, 1988; Lüth et al., 1994; Norris et al., 1996; Unger and Lange, 1992). On the other hand, plexus or band of thin, long, beaded NADPH-d tangential fibres is often described in layer I of the rat (Valtschanoff et al., 1993a), monkey (Gabbott and Bacon, 1996a; Hashikawa et al., 1994; Sandell, 1986), and man (DeFelipe, 1993; Fischer and Kuljis, 1994; Lüth et al., 1994). In layer I of the adult monkey neocortex, NADPH-d fibres frequently contacted small blood vessels that penetrated the cortex from the pial surface (Yan et al., 1996b). These fibres also display area-specific pattern of distribution in adult monkey frontal cortex: NADPH-d fibres in the central part of layer I are particularly prominent in eulaminate neocortical areas 8 and 46, less prominent and diffuse in layer I of limbic areas, and sparse or absent in olfactory frontal cortex (Dombrowski and Barbas, 1996).

However, some authors have observed NADPH-d cells in the layer I. In the visual cortex of the adult rhesus monkey, Sandell (1986) described few NADPH-d cells that send most of their processes down in layers II and III instead of branching within the layer I. According to Fischer and Kuljis (1994), layer I of the adult human cortex does not contain NADPH-d Cajal-Retzius cells, but harbors few small NADPH-d interneurons with few short and thin processes. Finally, two types of NADPH-d cells have been described in the layer I of the medial prefrontal cortex in both monkey (Gabbott and Bacon, 1996a,b), and rat (Gabbott and Bacon, 1995): NADPH-d Cajal-Retzius cells are located immediately beneath the pial surface and have long, thick, and horizontal aspiny dendrites but no observable axons, whereas other NADPH-d cells are located deep within the layer I and have a spray of descending beaded processes ramifying in layers II and superficial part of the layer III.

In the developing neocortex of rats and mice, nitrinergic neurons are usually not observed in the marginal zone (MZ), i.e., the developing layer I (Bravo et al., 1997; Derer and Derer, 1993; Iwase et al., 1998; Tomić et al., 1994; Van Eden et al., 1996); they were also not observed in the neocortex of neonatal and early postnatal kittens (Riche et al., 1995) and fetal sheep (Northington et al., 1996). However, Yan et al. (1994) reported that NADPH-d cells were occasionally found in the layer I of the rat neocortex from the second postnatal day onwards, whereas Bredt and Snyder (1994) have described NADPH-d and nNOS-positive Cajal-Retzius cells in the rat neocortical marginal zone already at embryonic days E14 and E15, and stressed that these are the earliest appearing nitrinergic neurons in the neocortical anlage. Finally, NADPH-d neurons of the marginal zone were reported in all published studies on developing human brain. They were observed from 15 weeks of gestation onwards in the human fetal hippocampal formation (Yan and Ribak, 1997), and from 17 weeks of gestation onwards in developing human neocortex (Yan et al., 1996a). Although one report claims that human fetal Cajal-Retzius cells display a transient NADPH-d reactivity exclusively at 20 weeks of gestation, but not at 19 or 21 weeks of gestation (Meyer and González-Hernández, 1993), already in 1966 Duckett (quoted in Pearse, 1967) has demonstrated that Cajal-Retzius cells are NADPH-d during the last 4 months of gestation in humans, which closely corresponds to the findings of Yan et al. (1996a), as well as our own observations (see below).

EXPRESSION OF NADPH-D ACTIVITY AND NOS-PROTEINS IN NEURONS OF THE FETAL SUBPLATE ZONE AND IN ADULT SUBCORTICAL INTERSTITIAL NEURONS

The presence of a significant population of nitrinergic neurons within the subcortical white matter has been reported in almost all relevant studies of the adult mammalian neocortex; however, estimates of their number vary widely, and only a few studies offer detailed descriptions of their morphology and neurochemical nature. In the neocortex of the adult rat, NADPH-d cells in the subcortical white matter were described as either bipolar or multipolar (Gabbott and Bacon, 1995; Meyer et al., 1991), mostly bipolar (Hedlich et al., 1990; Valtschanoff et al., 1993a), predominantly multipolar (Moro et al., 1995; Vincent and Kimura, 1992), aspiny (Vincent and Kimura, 1992), or sparsely spinous (Gabbott and Bacon, 1995; Hedlich et al., 1990), with dendrites penetrating into deep cortical layers (Hedlich et al., 1990) whereupon their higher order processes became spine bearing (Gabbott and Bacon, 1995), and representing about 30% of all cortical NADPH-d cells (Gabbott and Bacon, 1995); according to Meyer et al. (1991), about 70% of interstitial white matter cells project to the overlying cortex, and contain NADPH-d activity. A large number of intensely stained NADPH-d cells with three to six long, prominent, and varicose processes is also present in the subcortical white matter of the adult cat (Mizukawa et al., 1988a,b, 1989).

In the adult monkey brain, interstitial NADPH-d cells are very numerous and predominantly located within the first 100–200 μm of the white matter underlying the neocortex (Gabbott and Bacon, 1996a,b; Sandell, 1986; Yan et al., 1996b), but occur also in deeper parts of the white matter (Aoki et al., 1993; Dombrowski and Barbas, 1996; Hashikawa et al., 1994). These cells represent a very substantial portion of total cortical NADPH-d neurons: over 50% in the monkey auditory cortex (Cipolloni and Pandya, 1991), and about 40% in the monkey medial prefrontal cortex (Gabbott and Bacon, 1996b). In the monkey prefrontal cortex, NADPH-d interstitial cells were abundant in the white matter below the eulaminate areas where the intracortical distribution of NADPH-d cells is low, and they were comparatively sparsely distributed beneath olfactory and limbic cortices where the intracortical distribution of NADPH-d cells is high (Dombrowski and Barbas, 1996).

In the adult human cortex, the proportion of interstitial NADPH-d cells in total population of cortical NADPH-d neurons is even more impressive (DeFelipe, 1993; Egberongbe et al., 1994; Lüth et al., 1994; Unger and Lange, 1992); according to various estimates, they represent from 60 to 87% of all cortical NADPH-d cells (Fischer and Kuljis, 1994; Kowall and Beal, 1988; Norris et al., 1996). The interstitial NADPH-d cells were variably described as mostly bipolar (Norris et al., 1996), aspiny multipolar (Egberongbe et al., 1994; Kowall and Beal, 1988; Meyer et al., 1992), sparsely spinous horizontally oriented large cells with oval somata, somatic spines, and filopodia-like appendages (Lüth et al., 1994), or densely spiny cells with elongated multipolar somata oriented in parallel to cortical-white matter border (Fischer and Kuljis, 1994). Their dendrites and axons form a dense network of NADPH-d fibres in the subcortical white matter and some processes reach infragranular as well as supragranular cortical layers (Fischer and Kuljis, 1994; Meyer et al., 1992). However, according to Meyer et al. (1992), only about 3% of all interstitial white matter cells (and about 30% of nonpyramidal white matter cells) in the adult human neocortex express NADPH-d activity, suggesting that they might be a dominant population of cortical NADPH-d cells, but only a small population of interstitial cells. It should be noted that the distribution of interstitial NADPH-d cells is altered in the brains of schizophrenic patients (Akbarian et al., 1993a,b; Gentleman et al., 1995), and that the density of neurons having detectable levels of nNOS mRNA was

significantly and specifically decreased in the white matter underlying the frontal cortex of Alzheimer's disease patients (Norris et al., 1996).

Interstitial cells of the adult subcortical white matter are remnants of the fetal subplate zone (for review, see Kostović and Judaš, 1995; Kostović and Rakic, 1990). In some studies of developing rodent neocortex, NADPH-d subplate neurons were not described (Giuili et al., 1994; Iwase et al., 1998; Tomić et al., 1994), and Bredt and Snyder (1994) even explicitely stated that embryonic rat subplate zone is completely devoid of NOS-immunoreactivity and NOS-positive cells. However, Bravo et al. (1997) observed very few NADPH-d cells in the subcortical white matter of the early postnatal rat somatosensory cortex, whereas Yan et al. (1994) described many NADPH-d cells in the subcortical white matter of developing rat neocortex during the first postnatal week. In the developing mouse neocortex, NADPH-d cells appeared within the subplate zone already by embryonic day E16 and remained the only contingent of cortical NADPH-d cells until the second postnatal day (Derer and Derer, 1993). In the cerebral cortex of the fetal sheep (in which gestation lasts about 145 days), nNOS-positive neuropil and cells are present in the subplate zone already at embryonic day E60 (Northington et al., 1996). NADPH-d subplate neurons are also numerous in the cortex of neonatal kittens, but their number decreases dramatically during the first two postnatal weeks (Riche et al., 1995). Finally, our own observations (see below), as well as the findings of Yan et al. (1996a) and Yan and Ribak (1997), clearly demonstrate that NADPH-d neurons in the human fetal cortex appear already at 15 weeks of gestation and that NADPH-d subplate cells represent the most prominent population of nitrinergic cortical neurons during the whole prenatal development.

EXPRESSION OF NADPH-D ACTIVITY AND NOS-PROTEINS IN CORTICAL LAYERS II TO VI AND IN THE DEVELOPING CORTICAL PLATE

Layers II to VI of the adult neocortex develop from the fetal cortical plate, and three types of NADPH-d/NOS-staining deserve description in this cortical compartment: (1) NADPH-d individual neurons, (2) NADPH-d/NOS-positive individual fibres, forming intracortical plexuses, and (3) NADPH-d/NOS-positive diffuse background staining, forming characteristic neuropil bands in some parts of the cortex. Furthermore, one should consider the laminar distribution of intracortical nitrinergic neurons as well as their classification on the basis of the intensity of staining, dendritic and axonal morphology, presence or absence of dendritic spines, and other neurochemical features.

First of all, it should be noted that, on the basis of staining intensity, nitrinergic intracortical neurons can be subdivided in two basic types, described as type I and type II cells by Yan et al. (1996b). Type I cells are intensely stained and completely visualized, in a Golgi-like manner, and usually larger; type II cells are small, lightly to moderately stained, and incompletely visualized by either histochemistry or immunocytochemistry, so that only their somata and proximal dendrites (but not axons!) are visible. This distinction between type I and type II cells is very important for the following reasons:

(1) Whereas type I cells are found scattered in low numbers throughout all cortical layers (see below), type II cells are 20- to 24-fold more numerous than type I cells (Aoki et al., 1993; Yan et al., 1996b), and they are located predominantly in supragranular layers (Aoki et al., 1993; Gabbott and Bacon, 1996a,b; Hashikawa et al., 1994; Norris et al., 1996; Sandell, 1986; Yan et al., 1996b). Except for two recent reports on adult rat neocortex (Gabbott and Bacon, 1995; Moro et al., 1995), type II cells were observed exclusively in the cerebral cortex of monkeys (Aoki et al., 1993; Gabbott and Bacon, 1996a,b; Hashikawa et al., 1994; Sandell, 1986; Yan et al., 1996b) and humans (Lüth et al., 1994; Norris et al., 1996). This suggests that cortical nitrinergic neurons may have different roles in different species, as well as significantly different roles in supragranular vs. infragranular cortical layers.

(2) As type II cells are poorly visualized, cell counts and morphological descriptions of intracortical nitrinergic neurons are based almost exclusively on type I cells. This fact is easily neglected, and has two unwelcome consequences. First, all cortical nitrinergic neurons are generally regarded as local circuit neurons, whereas they pertain almost exclusively to type I cells. Second, it is generally regarded that nitrinergic neurons represent only about 1% of all cortical neurons: 1% (Dawson et al., 1991) or 1–2% (Bredt et al., 1991) in the whole rat cortex, respectively; 0.5–2% in the rat somatosensory cortex (Valtschanoff et al., 1993a); 0.83% in the rat medial prefrontal cortex (Gabbott and Bacon, 1995); and only 0.25% in the monkey medial prefrontal cortex (Gabbott and Bacon, 1996b). Note that these estimates also pertain exclusively to type I cells. If type II cells are indeed about 20-fold more numerous than type I cells (see above), nitrinergic neurons, at least in the primate and human cortex, would represent a substantial 20% of all cortical neurons. Finally, as will be described below, there is growing evidence that principal cortical (pyramidal) neurons also contain both nNOS and eNOS.

In many previous studies, only type I cells were described in the neocortex of adult rats (Aoki et al., 1997; Bredt et al., 1990, 1991; Dawson et al. 1991; Dun et al., 1994a; Hedlich et al., 1990; Leigh et al., 1990; Rhrich-Haddout et al., 1997; Rodrigo et al., 1994; Valtschanoff et al., 1993a; Vincent and Kimura, 1992), cats (Kuchiiwa et al., 1994; Mizukawa et al., 1988a,b, 1989), monkeys (Cipolloni and Pandya, 1991), and humans (DeFelipe, 1993; Egberongbe et al., 1994; Kowall and Beal, 1988; Unger and Lange, 1992). In the cortex of rodents and carnivora, type I cells were variably described as large multipolar cells (Mizukawa et al., 1989; Southam and Garthwaite, 1993; Vincent and Kimura, 1992), predominantly bipolar in supragranular and mostly multipolar in infragranular layers (Moro et al., 1995; Valtschanoff et al., 1993a). Type I cells in the neocortex of monkeys and humans are usually described as a variable mixture of multipolar, bipolar, and bitufted neurons (Aoki et al., 1993; Cipolloni and Pandya, 1991; DeFelipe, 1993; Dombrowski and Barbas, 1996; Egberongbe et al., 1994; Fischer and Kuljis, 1994; Hashikawa et al., 1994; Kowall and Beal, 1988; Lüth et al., 1994; Sandell, 1986; Unger and Lange, 1992; Yan et al., 1996b). However, in the most

detailed morphological studies to date, Gabbott and Bacon described seven classes of cortical NADPH-d cells, including Cajal-Retzius cells, interstitial cells, and intracortical bitufted, bipolar, multipolar, and "pyramid-like" cells in the medial prefrontal cortex of both rat (Gabbott and Bacon, 1995) and monkey (Gabbott and Bacon, 1996a,b).

Type I cells were also variably described as exclusively aspiny neurons (Bredt et al., 1991; Cipolloni and Pandya, 1991; Dawson et al., 1991; DeFelipe, 1993; Dun et al., 1994a; Egberongbe et al., 1994; Hashikawa et al., 1994; Kowall and Beal, 1988; Mizukawa et al., 1989; Mufson et al., 1990; Rhrich-Haddout et al., 1997; Sandell, 1986; Sobreviela and Mufson, 1995; Vincent and Kimura, 1992), or as sparsely spinous neurons (Hedlich et al., 1990; Lüth et al., 1994; Valtschanoff et al., 1993a; Yan et al., 1996b). According to detailed studies of Gabbot et al., the majority of NADPH-d cells in rat and monkey frontal cortex have low to moderate numbers of dendritic spines over second and higher order dendrites (Gabbott and Bacon, 1995, 1996a,b; Gabbott et al., 1995). According to Fischer and Kuljis (1994), all NADPH-d cells in layers I to VI of the human cortex were aspiny, but interstitial NADPH-d cells in the white matter were densely spiny stellate cells, and these spiny stellate cells were specifically affected in patients with motoneuron disease plus dementia as well as Alzheimer's dementia (Kuljis and Schelper, 1996). Aoki et al. (1993, 1997) have presented electron-microscopical evidence on NOS-positive dendritic spines in the neocortex of both rat and monkey. Finally, spine-like protrusions (spicules) have been described even on somata of NADPH-d cells (Gabbott and Bacon, 1996a,b; Hedlich et al., 1990; Lüth et al., 1994; Yan et al., 1996b).

The data on the laminar distribution of type I cells in the rat cortex are highly variable, as follows: they are most numerous in layers II and III (Hedlich et al., 1990; Valtschanoff et al., 1993a), they are scarce in layers II/III and most numerous in layers V/VI (Rhrich-Haddout et al., 1997), they are concentrated in layer VI and white matter (Moro et al., 1995), and they are most numerous in mid- to lower layer V (Gabbott and Bacon, 1995). Note that in all cases the peak distribution of type I cells in rodent cortex is described as unimodal. However, laminar distribution of type I cells in monkey and human cortex is generally described as bimodal, with one peak in layers II/III (Aoki et al., 1993; Dombrowski and Barbas, 1996; Fischer and Kuljis, 1994; Hashikawa et al., 1994; Kowall and Beal, 1988; Sandell, 1986; Unger and Lange, 1992; Yan et al., 1996b), and another peak variably located in layer VIb (Aoki et al., 1993), deep infragranular layers (Dombrowski and Barbas, 1996), layer VI and white matter (Hashikawa et al., 1994), layers V, VI, and white matter (Fischer and Kuljis, 1994), or exclusively in white matter (Lüth et al., 1994; Yan et al., 1996b). However, some authors describe only one peak located in deep cortex and white matter (DeFelipe, 1993; Egberongbe et al., 1994), or point out areal differences in the laminar distribution of NADPH-d cells (Cipolloni and Pandya, 1991). For example, NADPH-d cells in some auditory areas of the monkey cortex are located predominantly in infragranular layers, whereas in other auditory areas they are about equally numerous in supra- and infragranular layers (Cipolloni and Pandya, 1991). It is generally agreed that type I cells are the least numerous in cortical layer IV (Aoki et al., 1993; Cipolloni and Pandya, 1991; Fischer and Kuljis, 1994; Lüth et al., 1994; Sandell, 1986).

A moderately dense network of numerous fine and less numerous thick, highly varicose NADPH-d fibers has been commonly described in all cortical layers throughout all neocortical areas; however, clear areal differences in density and the pattern of distribution of this network have been described in the neocortex of the adult monkey (Hashikawa et al., 1994). The exact origin of most of of these fibers is unknown, but they are probably of both intrinsic and extrinsic origin (for review, see Iadecola, 1993). Namely, a number of NADPH-d fibres of unidentified origin cross the cortex-white matter boundary (Sandell, 1986), and some thick NADPH-d fibres can be followed from the white matter into the layer IV, showing a dense plexus and being connected with pericellular NADPH-d baskets in layers IV to VI (Lüth et al., 1994). NADPH-d and NOS-positive fibres often form perivascular fibre networks (DeFelipe, 1993) and contact intracortical blood vessels (Estrada et al., 1993; Moro et al., 1995; Regidor et al., 1993b; Schottler et al., 1996; Yan et al., 1996b; Yan and Ribak, 1997). Tangential NADPH-d fibres in layer I have been usually interpreted as monoaminergic (DeFelipe, 1993; Lüth et al., 1994), and those forming pericellular baskets as probably serotoninergic (DeFelipe, 1993; Gabbott and Bacon, 1996a,b; Lüth et al., 1994).

In contrast to the rich network of intracortical NADPH-d fibres, axons of individual NADPH-d and/or NOS-positive cortical neurons are often poorly visualized, and therefore rarely described; for the comprehensive review of axonal morphology of individual NADPH-d cells, the reader is reffered to detailed studies of Gabbot and Bacon (1995, 1996a,b).

The neurochemical nature of NADPH-d and NOS-positive cells has been investigated in a number of co-localization studies. In the cortex of rats and humans, type I cells colocalize with somatostatin and neuropeptide Y (Dawson et al., 1991; Kowall and Beal, 1988; Unger and Lange, 1992), and rat cortical type I cells also contain GABA (Hedlich et al., 1990; Valtschanoff et al., 1993a), but not parvalbumin (Hedlich et al., 1990). In general, very few (about 1%) cortical type I cells also contain either parvalbumin, calbindin, or calretinin (Dun et al., 1994a,b; Gabbott and Bacon, 1995, 1996a,b). About 80% of type I cells in the rat cortex contain GABA, but they represent less than 2% of total GABAergic population (Gabbott and Bacon, 1995). In the monkey cortex, only 58% of type I cells and just 9% of interstitial NADPH-d cells also contain GABA (Yan et al., 1996b). On the other hand, all type II cells also contain GABA or calbindin, but not parvalbumin, suggesting that type II cells, at least in the primate cortex, represent a subpopulation of calbindin-containing GABAergic interneurons (Yan et al., 1996b). It is interesting to note that interstitial NADPH-d cells in the white matter of rat cortex (Valtschanoff et al., 1993a) and monkey auditory cortex (Cipolloni and Pandya, 1991) do not contain GABA.

In the cortex of adult rat, NMDA-R1 subunit of glutamate receptors frequently colocalizes with nNOS at both pre- and postsynaptic sites and in dendritic

spines (Aoki et al., 1997), and the majority of cortical and hippocampal NOS-positive neurons predominantly express GluR1 and GluR4 subunits of glutamate AMPA-receptors, but very low to undetectable levels of GluR2 subunit (Catania et al., 1995). Although such pattern of expression of GluR subunits is a common characteristic of all cortical interneurons, this finding suggests that cortical NOS-positive cells contain calcium-permeable AMPA-receptors (Catania et al., 1995). Furthermore, NOS-positive neurons in rat brain express more NMDA receptor mRNA than NOS-negative neurons (Price et al., 1993). About 10% of rat cortical type I cells also contain muscarinic receptors, as revealed by monoclonal antibody M35 raised against the epitope present on all (m1–m5) muscarinic receptor subtypes (Moro et al., 1995). However, about 70% of interstitial NADPH-d cells in the white matter of monkey and human neocortex also contain muscarinic m2-receptors, and approximately 90% of these NADPH-d cells were rich in acetylcholinesterase (Smiley et al., 1998).

In contrast to data on nitrinergic cells and fiber network, the data on neuropil staining in the adult cortex are relatively scarce. For example, Gabbott and Bacon (1995) have described three defined bands of diffuse NADPH-d staining located in layers II, upper V, and deep V, respectively, of the rat medial prefrontal cortex. In the monkey prefrontal cortex, a dense band of diffuse background NADPH-d activity was found in the superficial and deep olfactory cortex, as well as in the indusium griseum (Dombrowski and Barbas, 1996). In the human visual cortex, diffuse background NADPH-d activity is especially high in cortical layer IV, and sharply decreases at the area 17/18 border (Lüth et al., 1994). In the primary visual cortex of the monkey, both NADPH-d activity (Sandell, 1986) and nNOS-immunoreactivity (Aoki et al., 1993) of the neuropil coincided with intensely stained cytochrome-oxidase neuropil bands, and were similarly modulated by monocular deprivation (i.e., displayed ocular-dominant columns activated by the intact eye in monocularly deprived monkeys). Moreover, over 80% of NOS-positive profiles in layer 4C were axon terminals, and some of NOS-positive axons were heavily myelinated, suggesting that these might be extrinsic NOS-positive axons originating from thalamus (Aoki et al., 1993). In the rat cortex, Bredt et al. (1991) have also noted that cortical neuropil is enriched in NOS-protein and NADPH-d staining, but devoid of nNOS mRNA, suggesting that NOS protein has been transported to nerve fibers distant from its site of synthesis.

Several developmental studies in rodents have demonstrated that the expression of NOS-activity and NOS mRNA begins already in the embryonic cerebrum (Brenman et al., 1997; Brien et al., 1995; Ma et al., 1991; Ogura et al., 1996; Samama et al., 1995). In fact, there is a transient overexpression of NOS protein and mRNA (Bredt and Snyder, 1994; Giuili et al., 1994), followed by a progressive decrease of expression during the early postnatal development (Bredt and Snyder, 1994; Giuili et al., 1994; Northington et al., 1996; Riche et al., 1995; Yan and Ribak, 1997) as well as the changes in subcellular distribution of cytosolic and particulate isoforms of NOS (Matsumoto et al., 1993a). These findings suggest that nitric oxide probably plays a significant role in the development of cortical circuitry. While type I nitrinergic neurons appear relatively early in the developing cortex of non-primate mammals (Bredt and Snyder, 1994; Derer and Derer, 1993; Iwase et al., 1998; Northington et al., 1996; Riche et al., 1995; Terada et al., 1996; Uehara-Kunugi et al., 1991; Yan et al., 1994) and are present in the developing human cortex already during the first half of gestation (Yan and Ribak, 1997; Yan et al., 1996a), type II nitrinergic neurons in the primate cortex appear late, during the last weeks of gestation (Yan and Ribak, 1997; Yan et al., 1996a).

NADPH-D ACTIVITY, nNOS, AND eNOS IN CORTICAL PYRAMIDAL NEURONS

The existence of NADPH-d and/or NOS-positive pyramidal neurons in the subiculum of the adult rat hippocampal formation is relatively uncontroversial. For example, most neurons in the inner part of the subicular pyramidal layer in the adult rat are NADPH-d positive (Vincent and Kimura, 1992), nNOS-positive (Valtschanoff et al., 1993b; Dinerman et al., 1994), and only a few of these neurons contain GABA (Valtschanoff et al., 1993b). According to Vaid et al. (1996), rat subicular pyramidal neurons are NADPH-d positive and contain both nNOS and eNOS; nNOS-positive pyramidal neurons are concentrated mainly in the most superficial cell layers of the adult rat subiculum (Lin and Totterdell, 1998), and in the rat ventral subiculum, NADPH-d activity and nNOS-immunoreactivity are present preferentially in those pyramidal neurons with the regular spiking phenotype (Greene et al., 1997).

On the other hand, the existence of NADPH-d and/or NOS-positive pyramidal neurons in the remaining parts of the hippocampal formation, and especially in the neocortex, is a highly controversial topic.The findings of most earlier studies have led to the conclusion that there are no NADPH-d or NOS-positive pyramidal neurons in the hippocampus of adult rat (Bredt et al., 1990, 1991; Dawson et al., 1991; Hope et al., 1991; Kato et al., 1994; Schottler et al., 1996; Valtschanoff et al., 1993b; Vincent and Kimura, 1992), cat (Mizukawa et al., 1989), or primates (Mufson et al., 1990; Sobreviela and Mufson, 1995). Moreover, after the targeted disruption of the nNOS gene, no NADPH-d staining or nNOS-immunoreactivity is detected in the hippocampus of the mutant mice (Huang et al., 1993). However, findings of more recent studies suggest that the expression of NOS in hippocampal pyramidal neurons might be area-specific and different in different species. For example, in the hippocampus of adult rat, NADPH-d activity was present in pyramidal neurons of the CA1 field (Dinerman et al., 1994; Endoh et al., 1994; Ikeda et al., 1996; Southam and Garthwaite, 1993; Vaid et al., 1996; Wallace and Fredens, 1992), but absent in pyramidal neurons of the CA3/4 field (Ikeda et al., 1996) and abruptly lost at the CA1/CA2 boundary (Endoh et al., 1993). Pyramidal cells of the CA1 field are also nNOS-positive (Wendland et al., 1994), and express nNOS mRNA in the adult rat hippocampus (Endoh et al., 1994) and in rat hippocampal pyramidal neurons in culture (Chiang et al., 1994).

On the other hand, Schmidt et al. (1992) have described some nNOS-positive pyramidal neurons in the adult rat CA2 field, whereas some other researchers have stressed that pyramidal neurons in the adult rat

CA1 field are consistently nNOS-immunonegative (Dinerman et al., 1994; Lin and Totterdell, 1998; O'Dell et al., 1994). In the adult human hippocampus, a subpopulation of pyramidal cells in the fields CA2, CA3, and CA4, but not in the field CA1, were moderately nNOS-positive and the cell staining abruptly terminated at the border to the CA1 field (Egberongbe et al., 1994). These discrepancies can be at least partly resolved by the recent finding that CA1 pyramidal neurons express eNOS instead of nNOS (Dinerman et al., 1994; O'Dell et al., 1994; Vaid et al., 1996). Furthermore, it has been suggested that the visualisation of NADPH-d activity and NOS-immunoreactivity in hippocampal pyramidal neurons is very sensitive to the type of tissue fixation (Dinerman et al., 1994; Wendland et al., 1994) and the type of histochemical preprocessing (Greene et al., 1997; Vaid et al., 1996).

With respect to the neocortex, most researchers have found no NADPH-d or NOS-positive pyramidal neurons in adult rat (Aoki et al., 1997; Moro et al., 1995; Vincent et al., 1994), cat (Mizukawa et al., 1988a,b, 1989), monkey (Aoki et al., 1993; Cipolloni and Pandya, 1991; Dombrowski and Barbas, 1996; Gabott and Bacon, 1996a,b; Hashikawa et al., 1994; Sandell, 1986; Yan et al., 1996b) and human (DeFelipe, 1993; Egberongbe et al., 1994; Fischer and Kuljis, 1994; Kowall and Beal, 1988; Kuljis and Schelper, 1996; Lüth et al., 1994; Norris et al., 1996; Unger and Lange, 1992). However, it should be noted that, according to Valtschanoff et al. (1993a), very few pyramidal-shaped neurons in layer V as well as some pyramidal-shaped neurons with ascending axons in layer VI of the adult rat neocortex were NADPH-d positive; but these neurons were described as small and most of them did not exhibit dendritic spines (Valtschanoff et al., 1993a). In layer V of the monkey primary visual cortex, Sandell (1986) has noted that giant pyramidal cells of Meynert occassionally contained sufficient NADPH-d activity to distinguish them from the surrounding neuropil. In the electron-microscopical studies of NOS-positive neurons in the adult neocortex of rat (Aoki et al., 1997) and monkey (Aoki et al., 1993), it has been noted that approximately 30 to 75% of nNOS-positive profiles were spinous. Judging from that prevalence of spinous labeling, it is likely that at least some of the labeled spines belong to spiny neurons, i.e., the spiny stellate and pyramidal neurons, even if their perikarya and proximal dendrites contain levels of nNOS that are too low to be detectable by light microscopy (Aoki et al., 1993, 1997). Another observation indicative of the presence of nNOS in non-GABAergic, most likely glutamatergic axon terminals, is that nNOS-positive terminals occasionally formed asymmetric axo-spinous junctions (Aoki et al., 1997).

Finally, Gabbott and colleagues have described NADPH-d "pyramidal-like" neurons of both normal and inverted-pyramidal morphology and very similar to a "true" Golgi-impregnated pyramidal neurons. Such cells were most common in rat cortical area 24b (Gabbott and Bacon, 1995), and in layers II and III of the monkey area 24c (Gabbott and Bacon, 1996a). However, on the basis of their low dendritic spine densities, combined peculiarities of apical and basal dendritic morphology, and the origin, trajectories and branching patterns of their axons, Gabbott et al. have concluded that these "pyramidal-like" cells are in fact nonpyramidal neurons representing extremes of a morphological spectrum (Gabbott and Bacon, 1995, 1996a,b; Gabbott et al., 1995).

On the other hand, Wallace et al. (1995) have found that in the aged human primary motor cortex (but not in adjacent postcentral, medial parietal, or cingulate cortex!), 5 to 80% of giant pyramidal Betz cells are NADPH-d and NOS-positive. Furthermore, pyramidal neurons in layers V and VI (also mostly Betz cells) display a weak to moderate NADPH-d staining in the motor cortex of children with variety of severe neurological infections (Wallace et al., 1996). It has, therefore, been suggested that human neocortical pyramidal neurons may start expressing nNOS as a response to damage or age-related stress and that the NO released from these cells may have a neuroprotective role (Wallace et al., 1995, 1996).

The results of some experimental studies on adult rat cerebral cortex have led to a similar conclusions. For example, experimental lesions of the adult rat neocortex can induce transient expression of NADPH-d staining (Kitchener et al., 1993) and nNOS-immunoreactivity (Wallace et al., 1995) in layers V and VI pyramidal neurons in the vicinity of the lesion site, as well as a widespread bilateral expression of NADPH-d staining in pyramidal neurons of neocortical layer V and hippocampal CA1 and CA2 subfields (Divac et al., 1993; Regidor et al., 1993a). Moreover, global cerebral ischemia leads to the temporary induction of NADPH-d activity in rat hippocampal CA1, but not CA3, pyramidal neurons (Kato et al., 1994).

If we consider the studies of nitrinergic neurons in the developing cerebral cortex, the evidence presented in studies published to date is also equivocal. For example, no NADPH-d pyramidal neurons have been found in the developing cortex of mouse (Derer and Derer, 1993; Giuili et al., 1994), rat (Bravo et al., 1997; Iwase et al., 1998; Tomić et al., 1994; Van Eden et al., 1996), cat (Riche et al., 1995), sheep (Northington et al., 1996), and human (Yan et al., 1996a). On the other hand, already in 1966, Duckett (quoted in Pearse, 1967) described NADPH-d Betz cells in the layer V of the cerebral cortex in an 18-week-old human fetus, and Yan et al. (1994) have found some weakly NADPH-d pyramidal-like cells in infragranular layers of the developing postnatal rat neocortex. Moreover, in the prenatal rat cerebral cortex, the entire cortical plate stained prominently for nNOS from embryonic day E15 to E19 (Bredt and Snyder, 1994). This suggests that at least some cortical pyramidal neurons transiently express nNOS, because efferent corticothalamic fibres in the intermediate zone were also nNOS-positive at the same developmental period (Bredt and Snyder, 1994). The nNOS-staining of the cortical plate began to decline at birth and largely vanished by the end of the second postnatal week; furthermore, this transient expression of nNOS appeared to be confined to neurons of the cortical plate (Bredt and Snyder, 1994). Transient expression of NADPH-d activity has been recently described in pyramidal neurons of the prenatal human hippocampal formation (Yan and Ribak, 1997). As we will describe below, transient expression of NADPH-d activity is also a prominent feature of pyramidal neurons in restricted regions of the developing human neocortex.

From the above considerations, one can conclude that cortical pyramidal neurons (and probably other cortical nitrinergic neurons) can express nNOS, eNOS, or both. This expression of NOS-proteins (and, consequently, NADPH-d activity) is most probably area-specific, species-specific, significantly affected by fixation and processing conditions of the tissue, and, at least in some cell populations, developmentally regulated. To resolve these complex issues, one should first obtain answers to the following questions: What kind of fixation and tissue processing is optimal for the visualization of NADPH-d activity and eNOS- or nNOS-immunoreactivity? Are there differences in subcellular localization and sensitivity to differential tissue processing of eNOS and nNOS? Is NADPH-d activity reliable marker for the presence of both eNOS and nNOS under all tissue processing conditions? Are requirements for the visualization of nNOS, eNOS, and NADPH-d activity different in developing vs. adult cortex? We briefly review the available evidence concerning these topics in the following section.

TISSUE PROCESSING CONDITIONS AND THE VISUALIZATION OF nNOS, eNOS, AND NADPH-D ACTIVITY IN THE DEVELOPING AND ADULT CEREBRAL CORTEX

The histochemical NADPH-d staining is based on the NADPH-dependent reduction of nitro blue tetrazolium (NBT) to yield insoluble blue formazans detectable by light microscopy at sites of NADPH-d activity (Beesley, 1995; Thomas and Pearse, 1961, 1964). Brain NOS proteins contain highly conserved consensus sequences for binding of NADPH (Yun et al., 1996; Zhang and Snyder, 1995) as well as catalytic NADPH-d activity (Schmidt et al., 1992). However, NADPH is an unspecific co-substrate for a number of brain enzymes which can also display NADPH-d activity (Grozdanovic and Gossrau, 1995; Kemp et al., 1988; Kuonen et al., 1988). The activity of NOS proteins represents only a fraction of total NADPH-d activity (Tracey et al., 1993) and, in crude supernatant fractions of brain homogenates, a correlation between NOS and NADPH-d can only be demonstrated after treatment with aldehyde fixatives (Matsumoto et al., 1993b). Fortunately, it seems that, after the aldehyde fixation, NADPH-d staining in the brain represents solely the activity of NOS proteins (Dawson et al., 1991; Dinerman et al., 1994; Grozdanovic et al., 1995; Hope and Vincent, 1989; Hope et al., 1991; Matsumoto et al., 1993b; Nakos and Gossrau, 1994; Weinberg et al., 1996; Wörl et al., 1994). Therefore, it is generally accepted that neuronal NADPH-d is a nitric oxide synthase, and that NADPH-d histochemistry provides a specific histochemical marker for neurons producing nitric oxide (Bredt et al., 1991; Dawson et al., 1991; Hope et al., 1991). However, it should be noted that minor inconsistencies in the co-localization of NADPH-d activity and NOS-immunoreactivity were noted in the olfactory bulb (Spessert and Layes, 1994; Spessert et al., 1994), the suprachiasmatic nuclei of rat and mouse (Wang and Morris, 1996), and in a negligible subpopulation of cerebral cortical neurons (Kharazia et al., 1994). Furthermore, Buwalda et al. (1995) presented evidence that aldehydes, rather than to progressively suppress NOS-unrelated enzymes, differentially elicit NADPH-d activity in some groups of neurons while leaving NOS-immunoreactivity unaffected.

The NADPH-d staining is not affected by the method of tissue sectioning, e.g., vibratome vs. cryostat (Vaid et al., 1996), but is influenced by preprocessing tissue incubation procedures, e.g., by sucrose incubation during cryoprotection (Vaid et al., 1996). The intensity of NADPH-d staining might also be affected by the pH value because the maximal rate of NADPH-d activity and formazan production in brain extracts has been observed at pH 8.5 (Kuonen et al., 1988), and similar findings were reported for histological sections of the rat spinal cord (Blottner and Baumgarten, 1995).

Fixation conditions clearly affect the sensitivity but not the selectivity of the NADPH-d staining (Rothe et al., 1998; Spessert and Layes, 1994). The best quality of NADPH-d staining was achieved by fixative containing 3% paraformaldehyde and 0.1% glutaraldehyde (Greene et al., 1997), or after the perfusion fixation with 4% paraformaldehyde and 0.4% glutaraldehyde (Rothe et al., 1998). The intensity of NADPH-d staining was substantially decreased by elevating glutaraldehyde concentrations (Rothe et al., 1998; Spessert and Layes, 1994) and prolongation of the postfixation time (Rothe et al., 1998), or by addition of lysine/sodium periodate to the fixative (Spessert and Layes, 1994). However, some authors have found that eNOS-associated NADPH-d staining is more robust with glutaraldehyde fixatives (Dinerman et al., 1994), whereas others reported that nNOS-associated NADPH-d activity is highly resistant to both formaldehyde and glutaraldehyde fixation (Weinberg et al., 1996).

The use of the detergent Triton X-100 also enhances the NADPH-d staining in the neural tissue (Nichols et al., 1992; Rothe et al., 1998; Würdig and Wolf, 1994) and elevates the production of NBT-derived formazan in biochemical studies as well (Kuonen et al., 1988). However, high Triton concentrations as well as the long-term exposure to Triton X-100 can nearly abolish the staining (Fang et al., 1994).

It seems that eNOS is a membrane-bound protein, because its activity is found predominantly in the particulate supernatant fraction (Förstermann et al., 1991a), its deduced amino acid sequence shows a consensus sequence for N-terminal myristoylation (Lamas et al., 1992), and mutation of the N-terminal myristoylation site converts eNOS from a membrane-bound to a soluble protein (Sessa et al., 1993). On the other hand, initial biochemical studies indicated that brain nNOS is mainly a soluble, cytosolic enzyme (Bredt and Snyder, 1990; Förstermann et al., 1991b; Ohshima et al., 1992). However, at least a part of NADPH-d activity (Kuonen et al., 1988) and up to 60% of nNOS activity (Hecker et al., 1994) were reported to be membrane-bound. Furthermore, several studies have shown that at the subcellular level the NADPH-d activity was localized predominantly to intracellular membrane portions (Calka et al., 1994; Faber-Zuschratter and Wolf, 1994; Rothe et al., 1998; Tang et al., 1995; Wolf et al., 1992, 1993), and that the addition of Triton X-100 led to a striking diminution in membrane staining in favor of the formation of cytosolic formazan granules (Wolf et al., 1992, 1993; Würdig and Wolf, 1994). Whereas the nNOS-immunoreactivity in some ultrastructural studies was seen mainly in the cytosol

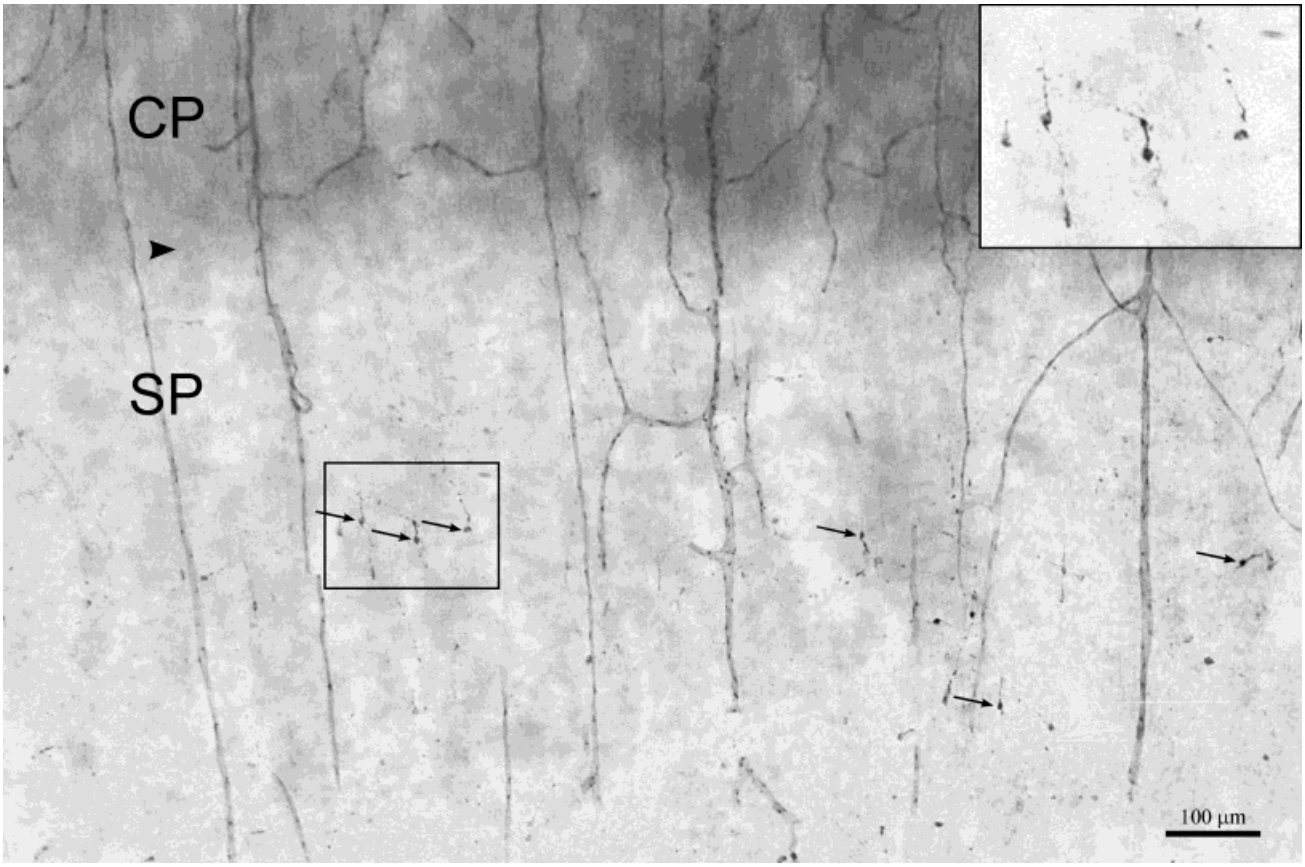

Fig. 1. In a 15-week-old human fetus, the neocortical plate (CP) displays a diffuse NADPH-d staining of the background neuropil, but contains no NADPH-d cells. However, NADPH-d cells are already present within the subplate (SP) zone (arrows; see also zoomed **inset**). At this developmental stage, nitrinergic neurons are already relatively numerous in the subplate zone of the middle third of the cerebral hemisphere (topologically corresponding to basal ganglia levels), and occasionally observed in the rostral part of the frontal pole, but they are still absent in the occipital subplate zone. Note that NADPH-d histochemistry clearly visualizes cerebral blood vessels. Large arrowhead marks the border between the cortical plate and the subplate zone. Scale bar = 100 µm.

(Rothe et al., 1998; Wang and Morris, 1996), others reported that nNOS is a predominantly membrane-bound protein (Hecker et al., 1994; Rodrigo et al., 1997). These inconsistencies can be only partially resolved by assuming that the membrane-bound NADPH-d activity is related to the particulate eNOS, especially when taking into account that a particulate nNOS form has been isolated from the rat cerebellum (Hiki et al., 1992) and that nNOS contains a peptide sequence that mediates its interactions with other membrane-bound proteins and/or its insertion into endocellular membranes (Brenman et al., 1996; Hendricks, 1995). Translocation from membrane to cytosol, accompanied by alterations of enzyme activity, has already been described for eNOS (Michel et al., 1993).

In conclusion, fixation and tissue preprocessing conditions, as well as the use of detergents, may cause methodological errors in localizing NADPH-d activity and nNOS- or eNOS-immunoreactivity. For example, aldehydes cause protein linkage that may affect enzyme activity and thereby influence its histochemical properties; aldehyde bonds can also prevent the binding of antibodies to their epitopes. The use of detergent Triton X-100 may cause detachment of eNOS from membranes and thus suppress its NADPH-d activity, although the eNOS might still be demonstrable immunocytochemically (Dinerman et al., 1994). On the other hand, sucrose incubation may preserve the viability of the active form of eNOS, which is a myristoylated, predominantly membrane-associated enzyme that is more active when it is membrane-associated (Busconi and Michel, 1993; Michel et al., 1993; Pollock et al., 1991), thereby enabling the NADPH-d histochemical reaction to proceed (Vaid et al., 1996). Sucrose incubation also markedly increased the degree of neuronal eNOS, but not nNOS, immunoreactivity (Vaid et al., 1996). Similarly, in frozen sections of 4% paraformaldehyde, but not gultaraldehyde, fixed brains, specific inhibitors of NOS activity, prevented the NADPH-dependent conversion of NBT to formazan (Blottner and Baumgarten, 1995). Obviously, these methodological issues deserve further detailed studies.

Finally, the existence of additional NOS isoforms, especially if transiently expressed in the developing brain, might significantly influence the interpretation of histochemical and immunocytochemical findings. Structural diversity of nNOS mRNA has been recently described in the nervous system of mice (Ogura et al.,

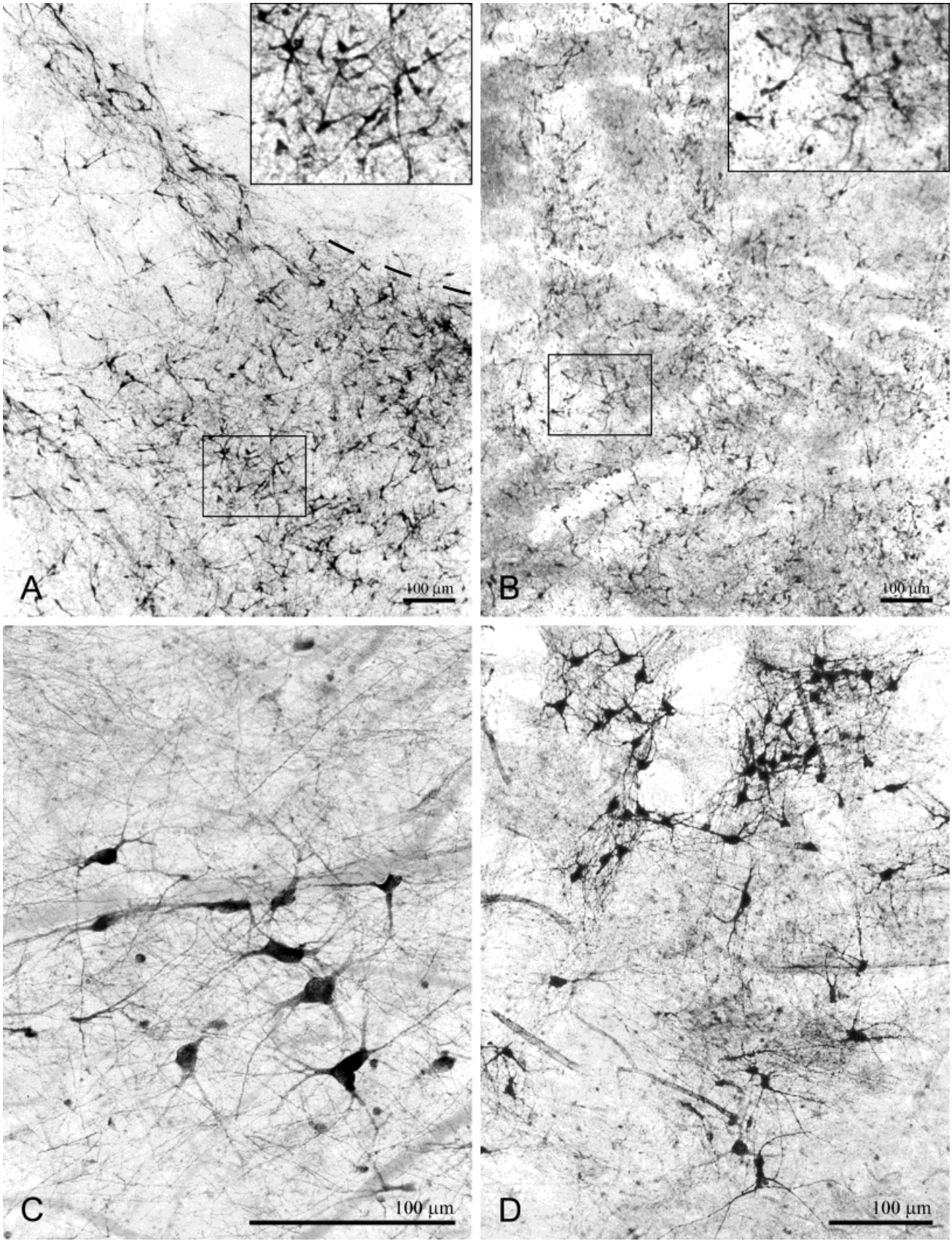

Fig. 2. NADPH-d neurons of the basal forebrain (**A,C**) and basal ganglia (**B,D**) in the 15-week-old (A,B) and 37-week-old (C,D) human fetus. Note that NADPH-d cells are very numerous and intensely stained in a Golgi-like manner in both basal forebrain (A) and putamen (B) of a 15-week-old fetus, but undergo extensive dendritic elaboration (C,D) towards the end of gestation period. Dashed line in A marks the border between the ventral globus pallidus (above the line) and the central part of the basal forebrain complex (below the line). C displays a cluster of large multipolar NADPH-d cells in the central part of the basal nucleus of Meynert, while B,D display NADPH-d neurons in the central part of the putamen. Note that clustering of NADPH-d striatal neurons is better pronounced in newborn (D) than in early fetus (B). Scale bars = 100 µm.

1993) and in human neuroblastoma cell lines (Fujisawa et al., 1994). One type of human nNOS mRNA showed a 315-bp inframe deletion from the entire nNOS cDNA (Fujisawa et al., 1994) and, in mice, the region deleted corresponds precisely to two exons of the mouse nNOS gene, thus suggesting that two forms are produced by alternative splicing (Ogura et al., 1993) and that the structural diversity in human and mouse nNOS may be associated with functional diversity (Fujisawa et al., 1994). Brenman et al. (1997) recently described at least six distinct alternatively spliced molecular species of nNOS mRNA, producing nNOS proteins of differing enzymatic characteristics and structural features. One of these isoforms is fully active, but mislocalized protein, lacking a major protein-protein interaction domain (PDZ domain) responsible for targeting nNOS to synaptic membranes (Brenman et al., 1997). Moreover, the expression of this nNOS-beta isoform was developmentally regulated and significantly enhanced in the embryonic mouse brain (Brenman et al., 1997).

DEVELOPMENT OF NITRINERGIC NEURONS IN THE PRENATAL HUMAN TELENCEPHALON

The data on nitrinergic neurons in the developing human brain are very scarce. As already noted, the only detailed reports are those of Yan and Ribak (1997) on the developing human hippocampal formation, of Yan et al. (1996a) on the developing prefrontal cortex, and the study of Meyer and González-Hernández (1993) dealing exclusively with NADPH-d Cajal-Retzius cells.

Several years ago, we initiated a comprehensive study of nitrinergic neurons in prenatal human telencephalon. We have analyzed 19 brains of human fetuses, ranging in age from 15 to 37 weeks of gestation, which are part of the extensive Zagreb neuroembryological collection (Kostović et al., 1991). All specimens were obtained from routine autopsies with approval of the Institutional Ethical Committee. The brains were fixed in 4% paraformaldehyde solution, buffered with 0.1 M PBS (pH = 7.4), for a period of 24–48 hours, and then cut in several coronal blocks. These blocks of tissue were cryoprotected by immersion in a graded series of sucrose solution (concentrations 5 to 30%) at 4°C, and then cut on the Cryostat (Leitz, Nussloch, Germany). Cryostat sections, 40–50 µm thick, were stained according to the standard direct NADPH-d protocol (Ellison et al., 1987). Briefly, the freshly prepared incubation solution consisted of 50 ml of 0.1 M PBS (pH = 8.0) with 1 ml of 0.8% Triton X-100 (Sigma, St. Louis, MO), 1 mM beta-NADPH (Sigma, St. Louis, MO), and 0.8 mM nitro-blue tetrazolium (NBT, Sigma). Free-floating or slide mounted sections were incubated 3 to 7 hours at 37°C, and the reaction was terminated by transfer of stained sections into the 0.1 M PBS. The sections were then rinsed with distilled water, mounted, dried overnight, dehydrated in a graded series of ethanol, briefly cleared with xylol, and coverslipped by using the Permount medium (Fisher, Pittsburgh, PA). The specificity of histochemical reaction was confirmed by omitting either NADPH or NBT from the incubation solution (sections treated in this way remain completely unstained).

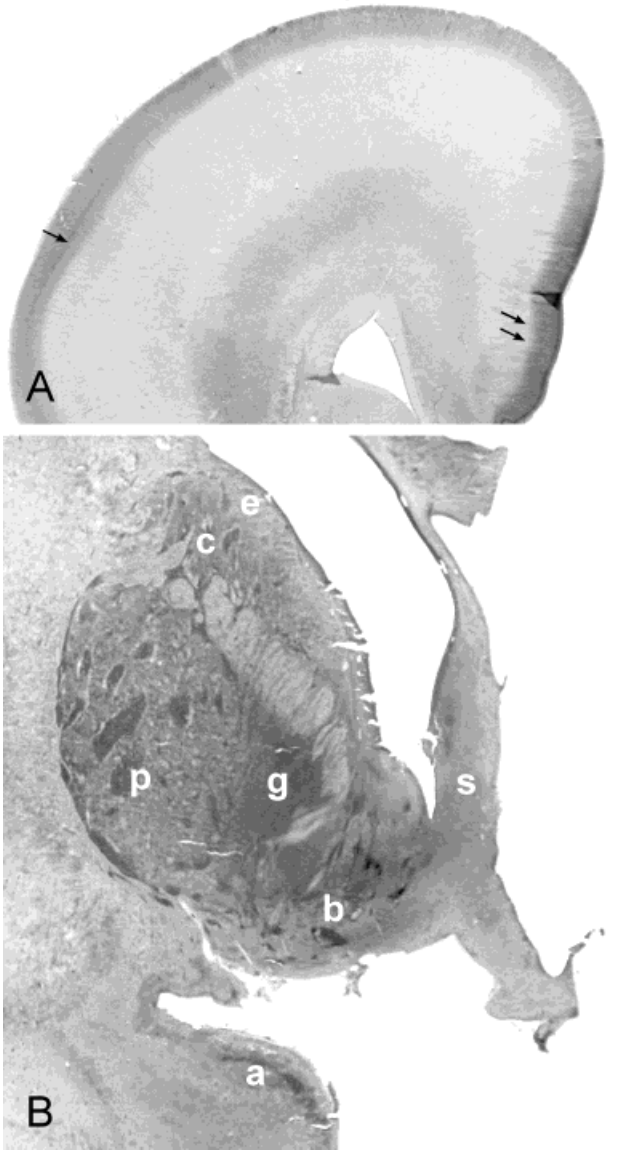

Fig. 3. Differences in the NADPH-d staining intensity delineate different regions of the neocortical anlage and specific compartments of the basal ganglia and basal forebrain region in a 18-week-old (A) and 24-week-old (B) human fetus. **A:** Already at 18 weeks of gestation, NADPH-d staining within the cortical plate displays clear regional differences in the cortical anlage. Whereas anterior cingulate and adjacent medial frontal cortex (two arrows) display strong NADPH-d staining with especially prominent superficial dark NADPH-d reactive band, the opercular frontal cortex displays a prominent band of increased NADPH-d activity in the middle of the cortical plate (single arrow), and the intervening part of the dorsolateral and dorsal frontal cortex displays a homogeneous NADPH-d staining of the cortical plate. For details, see text. **B:** In a 24-week-old fetus, very strong NADPH-d staining of the neuropil clearly delineates putamen (p), caudate nucleus (c), globus pallidus (g), and parts of the amygdala (a). Note the very weak NADPH-d staining of the ganglionic eminence (e), clear compartmentalization of NADPH-d neuropil into islands and matrix in caudate (c) and putamen (p), and patches of the increased NADPH-d activity distributed throughout the neighbouring basal forebrain (b) and septal (s) region. Magnification, 5× in A and 10x in B.

The results of these studies were already briefly reported (Judaš et al., 1995; Judaš and Kostović, 1997; Sajin et al., 1993; Sestan and Kostović, 1994; Sestan et al., 1994, 1998) and are the subject of several currently submitted publications. Therefore, here we can only briefly summarize the major findings of our research. In

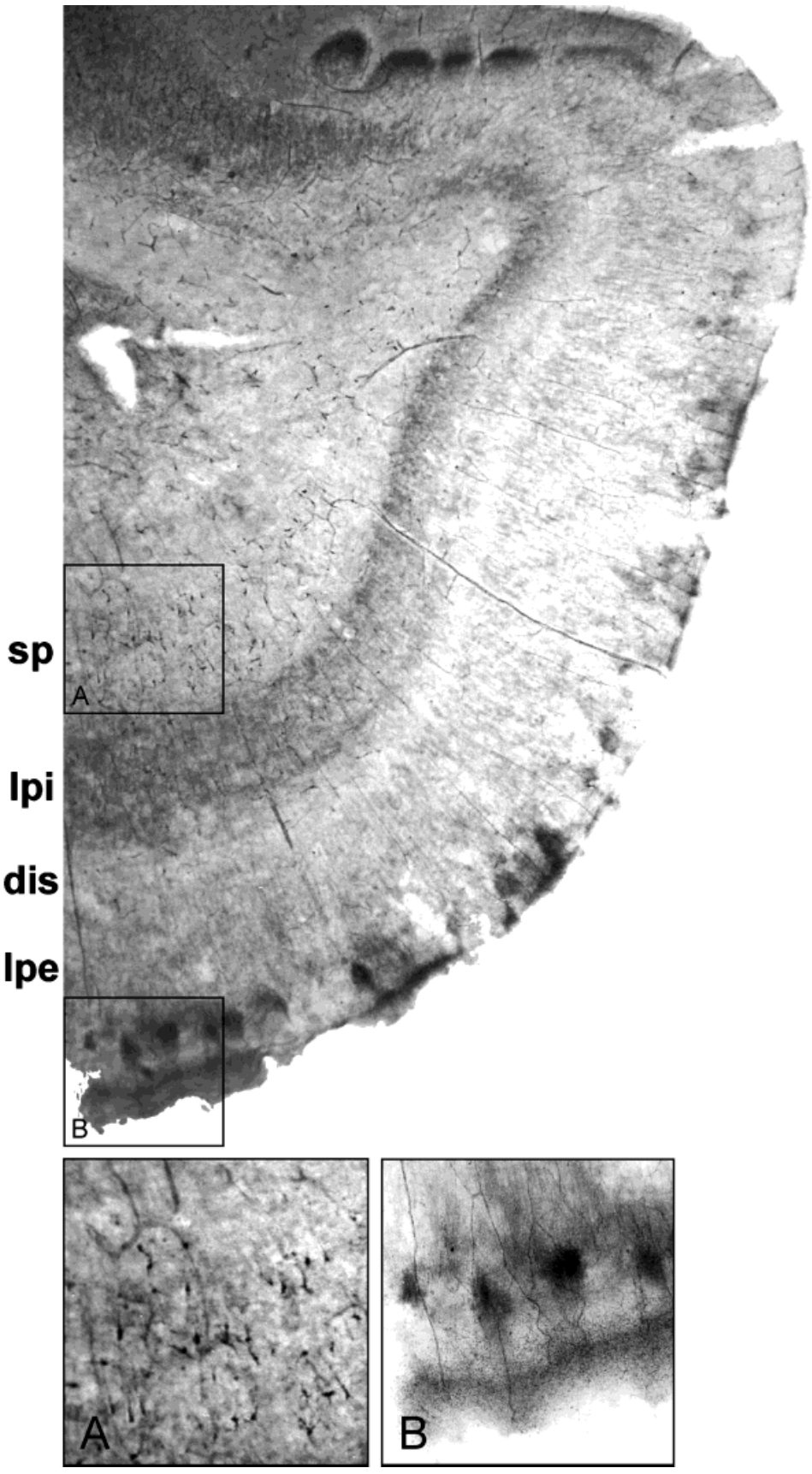

Fig. 4. The differences in NADPH-d neuropil staining clearly delineate major laminar and modular compartments of the developing entorhinal cortex in a 23-week-old human fetus: lamina principalis externa (lpe), lamina dissecans (dis), lamina principalis interna (lpi), and the subplate zone (sp). The entorhinal subplate zone (frame and **inset A**), as well as the lamina principalis interna (lpi), contain numerous intensely stained NADPH-d neurons. Note that intensely NADPH-d stained patches in the superficial cortical plate (frame and **inset B**) correspond to developing entorhinal pre-alpha islands. Magnification, 40×.

the youngest specimen available, i.e., in the 15-week-old fetus, NADPH-d neurons are already present in the developing subplate zone (Fig. 1), basal forebrain (Fig. 2A,C), and basal ganglia (Fig. 2B,D). So, nitrinergic neurons are present and numerous in the human fetal telencephalon already during the first half of gestation and, in a 15-week-old fetus, these neurons are among the best differentiated postmitotic neurons in the whole telencephalon. Furthermore, strong background neuropil staining is present in basal ganglia (Fig. 3B) and the cortical plate (Figs. 1, 3A, 4; see also Fig. 6A).

The advanced maturation of human fetal basal forebrain neurons was already described by means of conventional Nissl staining and acetylcholinesterase histochemistry (Kostović, 1986). However, Golgi-like NADPH-d staining of these cells enabled us to show that, although these neurons appear early, their extensive dendritic differentiation occurs only during the last third of gestation (compare Fig. 2A and C). Furthermore, human fetal basal forebrain contains both large and small NADPH-d neurons, suggesting that at least some magnocellular cholinergic basal forebrain neurons transiently produce nitric oxide during the fetal and perinatal development (Grizelj et al., 1998). This finding is important because previous studies of the adult human basal forebrain stressed the fact that only small and medium-sized non-cholinergic basal forebrain neurons express NADPH-d activity, while magnocellular cholinergic neurons do not contain that marker (Ellison et al., 1987; Geula et al., 1993).

With respect to the dendritic differentiation, the situation is very similar in neurons of the human fetal basal ganglia: nitrinergic neurons are present and numerous in the caudate and putamen of the 15-week-old fetus, but display prominent dendritic development and clustering of somata only towards the end of the prenatal period (compare Fig. 2B and D). Clustering of the neuropil NADPH-d activity into island and matrix compartments is also characteristic for the developing human basal ganglia (Sajin et al., 1993). Nitrinergic neurons of the amygdala display similar morphology and the developmental profile as those in caudate and putamen (not shown).

While adult monkey and human globus pallidus does not contain nitrinergic neurons and displays very weak NADPH-d activity of the background neuropil (Egberongbe et al., 1994; Hashikawa et al., 1994), the globus pallidus of human fetuses transiently displays very strong neuropil NADPH-d activity (Judaš and Kostović, 1997; see Fig. 3B). Moreover, a subset of dorsal pallidal neurons, situated predominantly in the medial pallidal segment, transiently express NADPH-d activity between 15 and 24 weeks of gestation (Judaš and Kostović, 1997).

The nitrinergic neurons of the neocortical anlage appear first in the subplate zone, at 15 weeks of gestation (Fig. 1) and maybe even one or two weeks earlier (younger specimens were not available for this study). During the following few weeks, their number progressively increases, and from about 20 weeks of gestation onwards, NADPH-d subplate cells are the most numerous and most conspicious nitrinergic neurons of the neocortical anlage. However, their morphology significantly changes during the last trimester of gestation (Fig. 5). Similar changes in the morphology of nitrinergic subplate neurons were noted in the developing cortex of cat (Riche et al., 1995) and sheep (Northington et al., 1996).

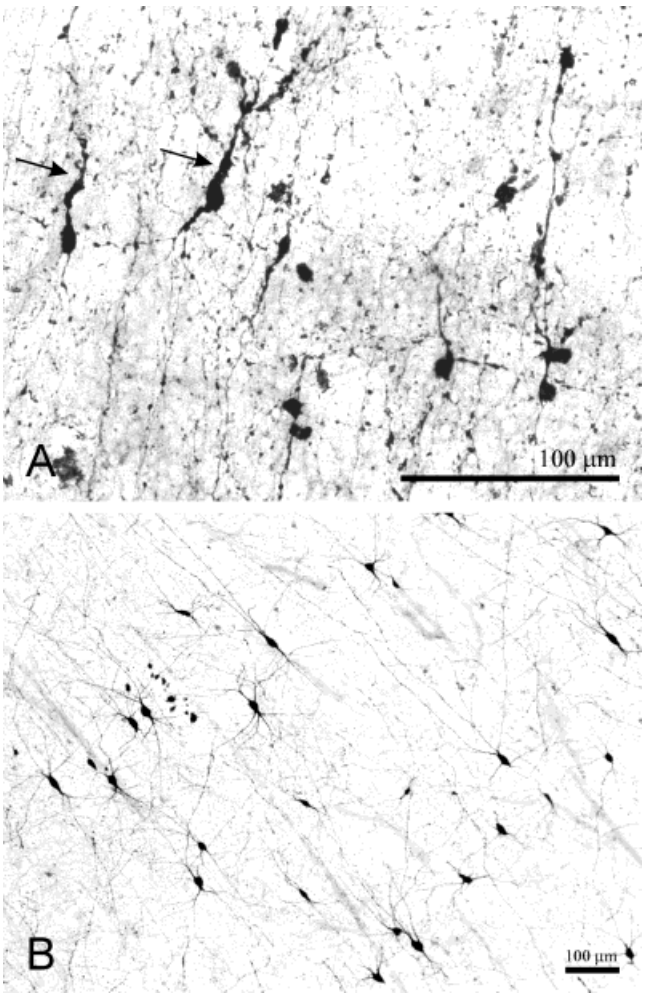

Fig. 5. The morphology of NADPH-d subplate neurons significantly changes from midgestation to the newborn period. **A:** In a 21-week-old fetus, subplate neurons display an intense NADPH-d reactivity and frequently have unusual morphological features, such as grossly distended proximal dendrites (arrows). The network of beaded NADPH-d fibers within the subplate zone is only moderately pronounced. **B:** In the newborn, subplate neurons are still very numerous, display a variety of morphological shapes, and have very long and extensively branched dendrites, which, together with NADPH-d fibers of unidentified origin, form a prominent network of NADPH-d processes. Scale bars = 100 µm.

NADPH-d positive Cajal-Retzius cells were observed in the neocortical marginal zone from 16/17 weeks of gestation onwards (not shown), and isolated NADPH-d Cajal-Retzius cells can be found even in the newborn (Fig. 6B). This finding is at variance with results of Meyer and González-Hernández (1993), but closely agrees with findings of Yan et al. (1996a) and Yan and Ribak (1997).

Within the cortical plate, first type I NADPH-d cells appear only after 17 weeks of gestation. At first, these cells are limited to the deepest tier of the cortical plate and then progressively appear in its middle and superficial parts, so that by 24 weeks of gestation they are

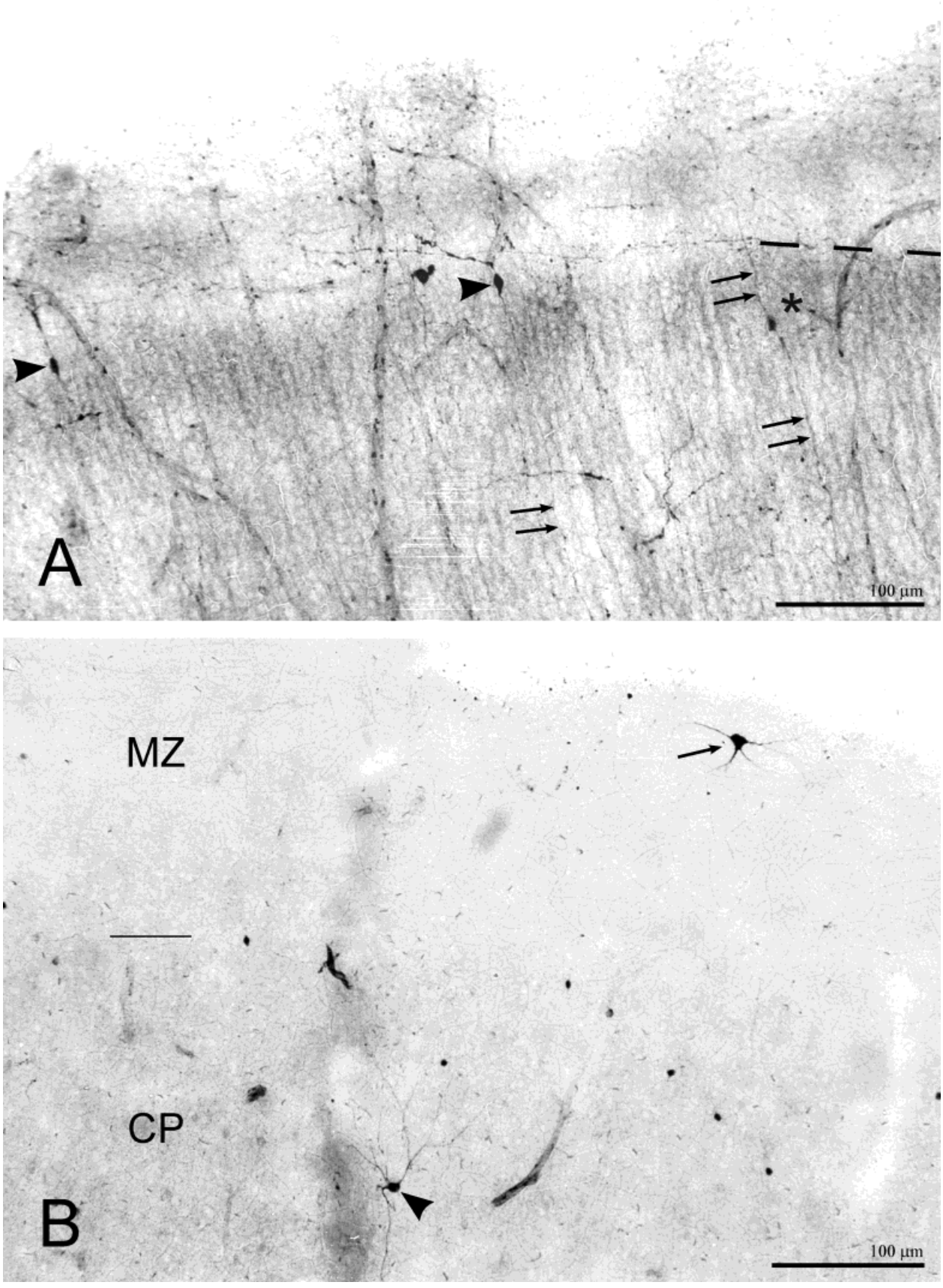

Fig 6.

present throughout the full thickness of the cortical plate. However, migratory-like NADPH-d neurons can be observed in the middle of the cortical plate already at 18 weeks of gestation, and between 20 and 23 weeks of gestation they are present in its most superficial part (Fig. 6A). As far as we know, this is the first description of migratory-like NADPH-d neurons in the developing neocortex of any mammalian species. Finally, a subpopulation of cortical pyramidal neurons express moderate to strong NADPH-d staining in a regionally specific manner (Sestan et al., 1998). In some cortical regions, these pyramidal neurons were present only in the superficial part of the cortical plate, whereas in others they were present in its middle part (Sestan et al., 1998); NADPH-d pyramidal cells were never noted in temporal and occipital cortex. Another early and regionally-specific feature of NADPH-d staining in the developing neocortex is the presence of intensely stained neuropil band in the most superficial part of the cortical plate. Such a band was observed in anterior cingulate cortex (Fig. 3A), and within the large part of the developing frontoparietal region (Fig. 6A), but was absent in temporal and occipital cortex.

Key novel findings of our studies of nitrinergic neurons in the human fetal telencephalon can be summarized as follows:

(1) A subset of principal neocortical (pyramidal) cells displays NADPH-d activity very early, before the end of the first half of gestation (Sestan et al., 1998);

(2) Differences in NADPH-d staining of cells and neuropil of the cortical plate are clearly visible by 18 weeks of gestation, suggesting that regional differentiation of the fetal cortical plate begins before the ingrowth of specific thalamocortical afferents (Sestan et al., 1998; see also Fig. 3A);

(3) NADPH-d positive migratory-like neurons can be observed within the subplate zone and the cortical plate of 18- to 23-week-old fetuses, but not in older specimens, thus suggesting that a subset of late migrating neurons may express NOS (see Fig. 6A);

(4) Whereas the adult primate basal forebrain contains small to medium-sized nitrinergic neurons that are not cholinergic (e.g., Ellison et al., 1987; Geula et al., 1993), human fetal basal forebrain contains both small and very large NADPH-d cells, thus suggesting that at least a subset of fetal magnocellular basal forebrain cholinergic neurons transiently express NOS (see Fig. 2A,C);

(5) A subset of human dorsal pallidal neurons transiently express NADPH-d activity from 15 to 24 weeks of gestation (Judaš and Kostović, 1997).

To the best of our knowledge, none of these findings have been previously reported for the fetal telencephalon of any mammal, the only exception being the report of Yan and Ribak (1997) on human fetal hippocampal formation concerning NADPH-d positive pyramidal neurons. However, these authors described NADPH-d positive pyramidal neurons in archicortex and mesocortex (Yan and Ribak, 1997), whereas our findings relate to the neocortical NADPH-d positive pyramidal neurons.

CONCLUSIONS

The evidence discussed in this review as well as our own investigations of the development of nitrinergic neurons in the human telencephalon suggest the following general conclusions. The cerebral cortex of adult mammals contains a population of morphologically and neurochemically, and thus probably functionally, diverse nitrinergic neurons. These neurons constitutively express nNOS, eNOS, and most probably other, still incompletely characterized isoforms of NOS proteins. Whereas type I nitrinergic neurons are exclusively local circuit neurons that often contain GABA, but rarely contain calcium-binding proteins, type II nitrinergic neurons represent much more extensive and still poorly characterized cellular population. We have shown that some fetal neocortical pyramidal neurons also express light to moderate NADPH-d activity in somata and proximal dendrites (Sestan et al., 1998) and thus may be regarded as a subset of type II neurons, at least in the human fetal brain. All nitrinergic neurons express a variety of neurotransmitter receptors and thus are exposed to the regulatory influence of most major neurotransmitter systems. The fact that type II nitrinergic neurons have been to date described almost exclusively in primate cortex suggests that the functional role of cortical nitrinergic neurons may significantly differ in different species and in infragranular vs. supragranular cortical layers. Nitrinergic neurons are among the earliest differentiating neurons in the developing cerebral cortex. The expression of specific NOS isoforms as well as their subcellular localization are both developmentally regulated. This, together with the documented transient overexpression of NOS in both the neuropil and specific neuronal populations of the developing cortex, suggests that nitric oxide may play a significant role in the initial establishment and subsequent refinement of the cortical synaptic circuitry. Finally, the demonstration of specific NOS isoforms in specific populations of developing and adult cortical neurons is critically dependent on tissue processing conditions and the choice of fixative. Despite the numerous published studies, these issues are still not resolved in a fully satisfactory manner.

Fig. 6. NADPH-d positive cells in the superficial part of the cortical plate (CP) and the marginal zone (MZ) of a 22-week-old fetus (A) and the newborn infant (B). The border between MZ and CP is marked with dashed line in A, and solid line in B. Note that diffuse neuropil staining, as well as the staining of cortical blood vessels, is much stronger in fetal (A) than in newborn (B) neocortex. **A:** The superficial part of the cortical plate in a 22-week-old fetus contains NADPH-d migratory-like young postmitotic neurons (asterisk) with leading and trailing processes (arrows), and young postmitotic neurons (arrowheads) whose apical dendrites penetrate into the marginal zone and just begin to differentiate. Note the dark band of NADPH-d staining in the most superficial part of the cortical plate, at the border with the marginal zone. **B:** Intensely NADPH-d stained interneuron (arrowhead) in the superficial part of the cortical plate (CP), and similar NADPH-d cell (arrow) in the superficial part of the marginal zone in the newborn cortex. Scale bars = 100 μm.

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