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Increasing proportions of tyrosine hydroxylase-immunoreactive interneurons colocalize with choline acetyltransferase or vasoactive intestinal peptide in the developing rat cerebral cortex

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Abstract

Cortical interneurons are critical for information processing, and their dysfunction has been implicated in neurological disorders. One subset of this diverse cell population expresses tyrosine hydroxylase (TH) during postnatal rat development. Cortical TH-immunoreactive neurons appear at postnatal day (P) 16. The number of TH cells sharply increases between P16 and P20 and subsequently decreases to adult values. The absence of apoptotic markers in these cells suggests that the reduction in cell number is not due to cell death but is due to a decline in TH production. Cortical TH cells lack all additional catecholaminergic enzymes, and many coexpress GABA and calretinin, but little else is known about their phenotype or function. Because interneurons containing choline acetyltransferase (ChAT) or vasoactive intestinal peptide (VIP) share characteristics with cortical TH neurons, the coexpression of TH with ChAT or VIP was examined throughout the neocortex at P16, P20, and P30. The proportions of TH cell profiles double-labeled for ChAT or VIP significantly increased between P16 and P30. Based on their proximity to blood vessels, intrinsic cholinergic and VIPergic cells have been hypothesized to regulate cortical microcirculation. Labeling with the gliovascular marker aquaporin-4 revealed that at least half of the TH cells were apposed to microvessels at these ages, and many of these cells contained ChAT or VIP. Cortical TH neurons did not coproduce nitric oxide synthase. These results suggest that increasing proportions of cortical TH neurons express ChAT or VIP developmentally and that a subset of these TH neurons may regulate local blood flow.

Keywords

Catecholamine; L-DOPA; Cholinergic; Aquaporin-4; Interneuron; Neocortex

1. Introduction

In the mammalian cerebral cortex, interneurons play a key role in the modulation and integration of information processed by the cortical circuitry (Markram et al., 2004). In

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contrast to the more abundant projection neurons, which are excitatory glutamatergic pyramidal cells, local-circuit cortical interneurons are predominantly non-pyramidal, inhibitory gamma aminobutyric acid (GABA)-releasing cells. Numerous subgroups of interneurons have been identified based on their neurochemical, morphological, and electrophysiological properties (Ascoli et al., 2008). For example, three nonoverlapping subsets of GABAergic interneurons have been identified in the rodent based on the presence of the calcium-binding proteins parvalbumin or calretinin or the peptide somatostatin (Kubota et al., 1994; Gonchar and Burkhalter, 1997; Xu et al., 2004). Exploration of cortical interneuron diversity and the developmental processes that yield the repertoire of phenotypes is critical because interneuron dysfunction has been implicated in several neurodevelopmental disorders (Benes and Berretta, 2001; Reynolds et al., 2002; Levitt et al., 2004; Lewis et al., 2005; Daskalakis et al., 2007; Di Cristo, 2007; Rajkowska et al., 2007; Gonzalez-Burgos and Lewis, 2008). Here we examine a subset of cortical interneurons in the developing rat that produce tyrosine hydroxylase (TH), the first, rate-limiting catecholaminergic biosynthetic enzyme. Little is known about the neurochemical phenotype and function of these cells despite their potential clinical relevance (Fukuda et al., 1999; Marui et al., 2003).

Cortical TH-immunoreactive (IR) neurons are present in the rodent (Berger et al., 1985; Kosaka et al., 1987a;1987b; Vincent, 1988; Satoh and Suzuki, 1990; Asmus et al., 2008), cetacean (Hof et al., 1995), non-human primate (Weihe et al., 2006; Raghanti et al., 2009), and human (Gaspar et al., 1987; Hornung et al., 1989; Kuljis et al., 1989; Trottier et al., 1989; Fukuda et al., 1999; Ikemoto et al., 1999; Benavides-Piccione and DeFelipe, 2003; Marui et al., 2003; Benavides-Piccione and DeFelipe, 2007; Raghanti et al., 2009). The majority of these cells in the rodent are small and fusiform, and many colocalize with GABA or the GABA biosynthetic enzyme, glutamic acid decarboxylase (GAD), suggesting that they are interneurons. Many cortical TH-IR neurons in the rat colocalize with calretinin but not with parvalbumin or somatostatin (Asmus et al., 2008). The coexpression of calretinin indirectly suggests that these TH-containing cells migrate from the caudal ganglionic eminence (CGE) during embryonic development (Wichterle et al., 2001; Nery et al., 2002; Xu et al., 2004; Butt et al., 2005; Fogarty et al., 2007; Wonders et al., 2008). Cortical TH-IR cells remain enigmatic, however, because of their species-specific laminar distribution, absence of additional catecholaminergic properties, and atypical developmental characteristics described below. Moreover, the complete transmitter phenotype expressed by these cells is not fully understood.

In the rat neocortex, TH-IR neurons were observed mainly in layer II/III (Berger et al., 1985; Kosaka et al., 1987a;1987b; Asmus et al., 2008), whereas in the mouse (Satoh and Suzuki, 1990), hamster (Vincent, 1988), and human (Gaspar et al., 1987; Hornung et al., 1989; Kuljis et al., 1989; Trottier et al., 1989; Ikemoto et al., 1999; Benavides-Piccione and DeFelipe, 2003; Marui et al., 2003; Benavides-Piccione and DeFelipe, 2007; Raghanti et al., 2009), TH-IR neurons were seen predominantly in layers V and VI. These TH cells are outside the classically-defined catecholaminergic cell groups (Hokfelt et al., 1984), and they lack all catecholaminergic biosynthetic enzymes subsequent to TH, including aromatic amino acid decarboxylase (AADC), suggesting that L-DOPA is their end-product (Berger et al., 1985; Gaspar et al., 1987; Ikemoto et al., 1999; Weihe et al., 2006). Cortical TH-IR neurons, therefore, may be classified as “postclassical dopaergic” cells (Weihe et al., 2006; Ugrumov, 2009).

During postnatal rodent development, the observable number of cortical TH-IR cells dramatically rises and falls (Berger et al., 1985; Satoh and Suzuki, 1990; Asmus et al., 2008). In the rat, these cells are first observed at P16, they peak in number at P18 and P20, and then decline to adult values between P24 and P40 (Asmus et al., 2008). Cortical TH-IR

cell profiles did not exhibit morphological characteristics or molecular markers (fragmented DNA, active caspase-3) indicative of programmed cell death at any of the ages examined between P20 and P40, suggesting that the reduction in the number of cell profiles is not due to cell death but rather is due to a decline in the detectable level of TH in these cells.

Of the numerous neurochemical markers described in different interneuron subgroups, two in particular are found in cells that closely resemble cortical TH-IR neurons. The acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT) and vasoactive intestinal peptide (VIP) are present in subpopulations of small, bipolar/fusiform, predominantly GABAergic interneurons located mainly in layer II/III of the rodent cortex (Fuxe et al., 1977; Emson et al., 1979; McDonald et al., 1982; Eckenstein and Thoenen, 1983; Houser et al., 1983; Connor and Peters, 1984; Eckenstein and Baughman, 1984; Levey et al., 1984; Morrison et al., 1984; Houser et al., 1985; Parnavelas et al., 1986; Kosaka et al., 1988; Peters and Harriman, 1988; Zilles et al., 1991; Chedotal et al., 1994; Gilmor et al., 1996; Kawaguchi and Kubota, 1996; Bayraktar et al., 1997; Cauli et al., 1997; Cha et al., 1997; Porter et al., 1998; Schafer et al., 1998; Bhagwandin et al., 2006; David et al., 2007; von Engelhardt et al., 2007; Consonni et al., 2009). Immunohistochemical analyses of the rat cortex reveal that ChAT and VIP are present predominantly in distinct neuronal populations; only 19-34% of the VIP-IR neurons contained ChAT immunoreactivity (Chedotal et al., 1994; Bayraktar et al., 1997).

The current study tested the hypothesis that cortical TH-IR cells acquire a cholinergic or VIPergic phenotype during postnatal development. The developmental coexpression of TH and either ChAT or VIP was investigated by double-immunolabeling for these markers at P16, P20 and P30. Previous research has shown that intrinsic cortical ChAT- and VIP-containing cell bodies and axons are closely associated with blood vessels, and these cells have been hypothesized to regulate regional blood flow (Eckenstein and Baughman, 1984; Chedotal et al., 1994; Fukuyama et al., 1996; Cauli et al., 2004; Consonni et al., 2009; Cauli and Hamel, 2010). Moreover, dopamine has been shown to regulate cortical microcirculation (Krimmer et al., 1998). The proximity of TH-IR somata and cortical blood vessels was examined by immunostaining for aquaporin-4 (AQP4), a channel protein present in the astrocytic endfeet that surround central nervous system (CNS) microvessels (Tait et al., 2008). The coproduction of ChAT or VIP in the TH-IR cells associated with blood vessels was also examined. Finally, because a subset of cortical interneurons in the rat (Kubota et al., 1994; Gonchar and Burkhalter, 1997), mouse (Lee and Jeon, 2005) and human (Benavides-Piccione and DeFelipe, 2003) are known to produce the neuronal isoform of the enzyme nitric oxide synthase (nNOS), the coexpression of TH and nNOS was examined.

The primary objectives of the present study were to gain a more thorough understanding of the neurochemical phenotype of developing cortical TH-IR neurons by examining the coexpression of ChAT, VIP, or nNOS and to begin to address a potential function of these cells by assessing their association with cortical microvessels.

2. Results

The morphology, cytoarchitectural distribution and relative number of TH-IR neuron profiles in the cerebral cortex were similar to the characteristics of these cells reported previously in the rat (Berger et al., 1985; Kosaka et al., 1987a; 1987b; Asmus et al., 2008). Cortical TH-IR cell profiles were typically small and fusiform, were found throughout the neocortex predominantly in layer II/III, and were more numerous at P20 than at P16 and P30. Likewise, the shape and distribution of cortical ChAT-, VIP-, and nNOS-IR cell profiles matched previous descriptions.

To investigate the coexpression of TH and ChAT in cortical neurons, sections of brains at three age intervals were double-immunolabeled for these enzymes. An average of 182, 203, and 170 TH-IR cell profiles were examined per brain at P16, P20, and P30, respectively. The total number of cortical TH-IR cell profiles that were counted to determine colocalization with ChAT at each of these ages was 910, 814, and 682, respectively. Many cortical TH-IR cells contained ChAT immunoreactivity (Fig. 1). The average percentages of TH-IR cell profiles that were immunoreactive for ChAT at P16, P20, and P30 were 36%, 47%, and 53%, respectively (Fig. 2). Statistical analysis showed significant differences in the proportions of double-labeled cell profiles over time (1-way ANOVA; $P = 0.0071$; Table 1). Post-hoc analysis (Tukey's HSD) demonstrated a significant increase in the proportion of TH and ChAT double-labeled cell profiles between P16 and P30 ($P < 0.05$).

Double-immunolabeling for TH and VIP similarly revealed that many cortical TH-IR neuron profiles were immunoreactive for VIP (Fig 3). An average of 145, 201, and 141 TH-IR cell profiles were examined per brain at P16, P20, and P30, respectively. The total number of TH-IR cell profiles that were counted to determine coexpression with VIP at each of these ages was 728, 804, and 564, respectively. The average percentages of TH-IR cell profiles that contained VIP immunoreactivity at P16, P20, and P30 were 49%, 44%, and 66%, respectively (Fig. 4). These proportions significantly increased over time (1-way ANOVA; $P = 0.0006$), with post-hoc analysis (Tukey's HSD) showing significant differences in the proportions of TH and VIP double-labeled cell profiles between P30 and both P16 and P20 ($P < 0.05$; Table 2).

To examine the proximity of cortical TH-IR neuron profiles and blood vessels, sections from brains at the three age classes were double-labeled for TH and AQP4, which delineates the CNS vasculature (Tait et al., 2008). 763 total TH-IR cell profiles were sampled with at least 226 cells sampled at each age. Blood vessels outlined by AQP4 immunoreactivity were richly distributed through all layers of the cerebral cortex, running in all planes (Fig. 5). Cortical TH-IR cell profiles were commonly observed in close apposition to AQP4-highlighted microvessels (Fig. 5A-C). Approximately 50% of the TH-IR cell profiles were apposed to microvessels at each of the three age classes, with negligible differences between the ages (49% at P16, 55% at P20, and 50% at P30).

To assess the coexpression of ChAT or VIP in the microvessel-associated TH-IR cells, sections from brains at the three age classes were labeled for AQP4, TH, and either ChAT or VIP. For analysis of TH and ChAT coexpression, at least 52 TH-IR neuron profiles that were apposed to cortical blood vessels were sampled at each of the age classes (164 total cells). For examination of TH and VIP coproduction, at least 52 TH-IR cell profiles apposed to microvessels were also sampled at each of the age classes (158 total). Many TH-IR neurons apposed to microvessels were colabeled for either ChAT or VIP (Fig. 5D-F). The percentages of microvessel-associated TH-IR cells that were colabeled for ChAT were 16%, 47%, and 54% at P16, P20, and P30, respectively. The percentages of microvessel-associated TH-IR cells coexpressing VIP were 50%, 54%, and 57% at P16, P20 and P30, respectively.

The coproduction of TH and nNOS in cortical neurons was also investigated. All three ages were represented, and more than 200 total cortical TH-IR neuron profiles were examined. As reported previously in the rodent (Kubota et al., 1994; Gonchar and Burkhalter, 1997; Lee and Jeon, 2005), the profiles of nNOS-IR somata were typically round, multipolar and larger than the TH-IR cell profiles. Colocalization of TH and nNOS was never seen in cortical cell profiles at any age examined (Fig. 6).

3. Discussion

Our results demonstrate that subsets of cortical interneurons which produce TH during postnatal rat development coexpress ChAT or VIP but not nNOS. Many of the cortical TH-IR neurons are apposed to cortical microvessels, and a subset of these microvessel-associated TH-IR cells coproduce ChAT or VIP. Taken together with earlier findings, these results lend important insights into the phenotype and possible function of this subset of cortical interneurons.

Previous work in a variety of species, including rats, mice, hamsters, cetaceans, non-human primates and humans (Berger et al., 1985; Kosaka et al., 1987a;1987b; Vincent, 1988; Satoh and Suzuki, 1990; Hof et al., 1995; Weihe et al., 2006; Benavides-Piccione and DeFelipe, 2007; Asmus et al., 2008; Raghanti et al., 2009; Ugrumov, 2009), demonstrated the presence of TH-IR interneurons in the cerebral cortex that are outside the classical catecholaminergic cell groups (Hokfelt et al., 1984). Neither endogenous catecholamines nor other catecholaminergic biosynthetic enzymes have been observed in these cells (Berger et al., 1985; Gaspar et al., 1987; Vincent, 1988; Satoh and Suzuki, 1990; Ikemoto et al., 1999; Weihe et al., 2006). These results place cortical TH-IR cells as part of a growing list of “postclassical” monoenzymatic TH cells found in a variety of brain regions (Weihe et al., 2006; Ugrumov, 2009). At least some of these cells appear to be capable of synthesizing L-DOPA as an end-product, though they lack any known transmitter secretory mechanisms. One postulated function of “dopaergic” (TH-only) cells is that they supply L-DOPA to neighboring monoenzymatic AADC-containing neurons, which may then synthesize dopamine, as observed in the hypothalamic arcuate nucleus of developing rats (Ugrumov et al., 2004). On the other hand, Misu and colleagues (2003) presented evidence that L-DOPA itself is released in a neurotransmitter-like fashion and may serve as a signaling molecule. Monoenzymatic TH cells are seen in a variety of regions of the developing brain, often displaying only transiently detectable levels of TH (Weihe et al., 2006; Ugrumov, 2009). The TH-IR cells of the cerebral cortex, as in other brain regions, may be seen more frequently during development because the production of L-DOPA plays a role in the establishment of cortical circuitry and/or because these are developing cells en route to their mature phenotype.

The complete repertoire of neurotransmitter markers expressed by cortical TH-IR neurons in the developing and adult rat is incompletely understood. Many cortical TH-producing cells were previously shown to express a GABAergic phenotype, suggesting that these cells are a subset of inhibitory interneurons (Kosaka et al., 1987a; Asmus et al., 2008). Here we showed that subsets of cortical TH-IR cells coexpressed ChAT or VIP. The proportions of TH-IR cell profiles that colocalized with these markers increased significantly between P16 and P30. One explanation for our data is that subsets of TH-IR cells postnatally acquire a transmitter phenotype that includes ChAT or VIP production. If no change in the transmitter phenotype of these cells occurs during development, similar proportions of TH-IR cell profiles that coexpress ChAT or VIP would be expected across the ages examined here. It is possible that the increase in the proportion of TH-IR cell profiles that coexpress ChAT or VIP is due to the decrease in the relative number of TH-IR profiles after P20; the TH-IR cell profile number drops by approximately one-half between P20 and P30 (Asmus et al., 2008). However, if this is the case, a significant change in the proportion of coexpressing cells would be expected between P16 and P20, during which the relative number of TH-IR profiles approximately triples, but no difference was noted between these ages. An alternative explanation is that there is a reduction in TH production in TH-IR cells that do not coexpress ChAT or VIP. In this scenario, however, a similar percentage of colocalization would be expected at P16 and P30, ages at which the relative numbers of TH-IR cell profiles do not differ. Finally, it is possible that a combination of these two

explanations accounts for our data. Future studies are required to distinguish between these interpretations. Interestingly, cortical interneurons in a variety of species display plasticity in their neurochemical properties. For example, the number of cortical ChAT-IR neurons in the mouse peaks during postnatal development (Consonni et al., 2009), and transitory production of various peptides and calcium-binding proteins is seen in the cortical interneurons of a variety of mammals (Del Rio et al., 1991; Wahle, 1993; Alcantara et al., 1996; Schierle et al., 1997). The factors that regulate the development of cortical interneuron phenotypes is an area of ongoing study (Wonders and Anderson, 2006).

In the current report, we consider the cortical TH-IR neurons that coproduce ChAT or VIP as distinct subsets based on previous reports showing that ChAT-IR and VIP-IR cells are predominantly non-overlapping populations (Chedotal et al., 1994; Bayraktar et al., 1997). These studies indicate, for example, that one-third or fewer of the VIP-IR neurons contain ChAT immunoreactivity. It is possible that a small subset of TH-IR cells coexpresses both ChAT and VIP. Future triple-labeling would be required to confirm this. However, the colocalization of these three markers in a small subset of cortical TH-IR cells would not change the conclusions reached here.

The colocalization of VIP in a subset of TH-IR cells reported here, together with the previous finding that TH and calretinin are frequently coexpressed in cortical cells (Asmus et al., 2008), indirectly supports the hypothesis that at least some TH-IR interneurons migrate developmentally from the CGE. Calretinin is a calcium-binding protein that is present in CGE-derived cells (Wichterle et al., 2001; Nery et al., 2002; Xu et al., 2004; Butt et al., 2005; Fogarty et al., 2007; Wonders et al., 2008). Miyoshi and colleagues (2010) recently demonstrated that many VIP-producing interneurons are derived from the CGE in the mouse. Furthermore, many cortical VIP- and ChAT-coexpressing cells in the rat contain calretinin (Cauli et al., 1997; Porter et al., 1998).

Acetylcholine and VIP are vasoactive in the CNS, and intrinsic cholinergic or VIPergic somata and axons are in close proximity to cortical blood vessels (Eckenstein and Baughman, 1984; Chedotal et al., 1994; Fukuyama et al., 1996; Cauli et al., 2004; Consonni et al., 2009; Cauli and Hamel, 2010). Therefore, these subsets of interneurons have been hypothesized to play a role in neurovascular coupling. We addressed whether cortical TH-IR cells were adjacent to blood vessels and whether any of the microvessel-associated TH-IR cells also contained ChAT or VIP. Cortical microvessels were delineated by immunostaining for AQP4, a channel protein found in the astrocytic endfeet surrounding the CNS vasculature (Tait et al., 2008). This technique was used similarly by Consonni et al. (2009) to show that ChAT-IR cells were frequently apposed to microvessels in the mouse cerebral cortex. Here we report that approximately half of the TH-IR cell profiles were apposed to AQP4-highlighted blood vessels at all ages examined, with similar proportions across ages. Moreover, multiple labeling for the three markers, AQP4, TH, and either ChAT or VIP, revealed that many of the microvessel-associated TH-IR cells coproduced ChAT or VIP at the three age classes. The reported percentages of TH-IR cell profiles apposed to microvessels may be underestimates. TH-IR cell profiles at the edge of the section may be apposed to microvessels in the adjacent section. A stereological approach might yield a higher percentage. However, the purpose of this part of our study was to assess whether any cortical TH-IR cells were associated with microvessels as a first step toward understanding the potential function of these cells. To determine absolute proportions of TH-IR cell profiles apposed to blood vessels and to provide evidence for direct contact of TH/ChAT- or TH/VIP-coexpressing axonal processes with blood vessel walls, future studies should employ stereology and electron microscopy.

Considering the previous evidence that ChAT- and VIP-producing interneurons regulate cortical blood flow (Eckenstein and Baughman, 1984; Chedotal et al., 1994; Fukuyama et al., 1996; Cauli et al., 2004; Consonni et al., 2009; Cauli and Hamel, 2010), our data indirectly support a role for the TH/ChAT- and TH/VIP-copositive cells that were associated with cortical microvessels in neurovascular coupling. The potential function of the L-DOPA produced by these cells is unclear. Notably, blood vessel endothelial cells and cultured astrocytes contain AADC (Hardebo et al., 1980; Juorio et al., 1993), raising the possibility that the L-DOPA released by cortical TH-IR interneurons is converted to dopamine by endothelial cells and/or astrocytes (Ugrumov, 2009). Dopamine has been shown to regulate cortical microcirculation (Krimer et al., 1998).

Major changes in cortical remodeling occur during postnatal development in the rat. Cholinergic axons innervate the cortex, for example, predominantly during the first two postnatal weeks, but axon growth continues beyond this time period in some cortical areas (Mechawar and Descarries, 2001). Vascular sprouting occurs through the third postnatal week (Rowan and Maxwell, 1981; Michaloudi et al., 2006), and blood vessel growth influences gliogenesis during this period (Zerlin and Goldman, 1997). The production of TH, and therefore L-DOPA, which peaks during the third postnatal week, may play a role in the organization of cortical circuitry and/or in the regulation of blood flow during this time of rapidly changing metabolic needs. It is also possible that the arrival of nascent cortical fibers induces the reduction in TH production in cortical cells after P20 as these cells acquire their mature transmitter phenotype.

Previous work showed that a subset of rat cortical interneurons produces nNOS (Kubota et al., 1994; Gonchar and Burkhalter, 1997). Moreover, some cortical nNOS-IR cells in the mouse coexpress calretinin (Lee and Jeon, 2005), similar to the cortical TH-IR cells in the rat (Asmus et al., 2008). Our results strongly suggest, however, that TH and nNOS are present in distinct subgroups of interneurons. We observed no colocalization of TH and nNOS in cortical neurons at any age studied. Moreover, the majority of nNOS-IR cells were dissimilar from TH-IR neurons in their morphology and laminar distribution. This lack of colocalization with nNOS provides another example of differences in the characteristics of cortical TH-producing cells between species. In the human, approximately one-fourth of cortical TH-IR neurons contains nNOS (Benavides-Piccione and DeFelipe, 2003). The species-specific neurochemical properties and laminar distribution observed in cortical TH-IR neurons raise interesting questions regarding the unique phylogenetic pattern and function of these cells in different species (Benavides-Piccione and DeFelipe, 2007; Raghanti et al., 2009).

A more complete understanding of the neurochemical characteristics of cortical TH-producing interneurons in the rat is emerging. Previous studies demonstrated that these nonpyramidal cells are seen more frequently during postnatal development, and many appear to be GABAergic, calretinin-containing cells, suggesting that they are inhibitory interneurons that migrate from the CGE during embryonic development (Berger et al., 1985; Kosaka et al., 1987a; 1987b; Asmus et al., 2008). Here we show that concurrent with the postnatal increase and subsequent decrease in the detectable levels of TH, the proportions of cortical TH-IR cells that coexpress ChAT or VIP increase. Many of these TH-IR cells are apposed to cortical microvessels, and a substantial subset of these microvessel-associated cells coproduce ChAT or VIP, indirectly supporting a role for these cells in neurovascular coupling. Loss of TH-IR neurons in the human cortex was noted in dementia (Marui et al., 2003) and Parkinson's disease (Fukuda et al., 1999). Furthermore, aberrant interneuron development has been linked to several major neurological disorders, including epilepsy, schizophrenia, bipolar disorder, and depression (Benes and Berretta, 2001; Reynolds et al., 2002; Powell et al., 2003; Levitt et al., 2004; Lewis et al., 2005; Garbelli et al., 2006;

Daskalakis et al., 2007; Di Cristo, 2007; Rajkowska et al., 2007; Gonzalez-Burgos and Lewis, 2008; Barinka et al., 2010). Further characterization of the subpopulation of cortical interneurons that produces TH will provide insight into the functions of these cells across species and is warranted because of their potential clinical relevance.

4. Experimental Procedure

4.1 Animals and Tissue Processing

Timed-pregnant Sprague-Dawley rats and pups (Zivic Laboratories, New Castle, PA) were maintained and handled according to guidelines set by the Centre College Institutional Animal Care and Use Committee and the National Institutes of Health. Each litter was housed with its mother until weaning at P21. The day of birth was considered P0. Male and female rats were used at all ages sampled; no sex-related differences have been observed in the properties of cortical neurons examined here.

Based on previous results (Asmus et al., 2008), we focused our analysis on three key ages: P16, when cortical TH-IR cell profiles first appear; P20, when these cell profiles peak in number; and P30, when the number of these cell profiles significantly declines. Rats at P16 (n=5), P20 (n=4), and P30 (n=4) were deeply anesthetized and transcardially perfused with 325-400 ml (increasing volume with age) of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed in the paraformaldehyde solution at room temperature for one hour, then cryoprotected in 20% sucrose in 0.1 M phosphate buffer containing 0.1% sodium azide at 4°C for at least 24 hours. Brains were cryosectioned in the coronal plane at 14 µm, and non-consecutive sections at approximately 100 µm intervals were mounted onto gelatin-coated slides.

4.2 Immunohistochemistry

Sections were double- or triple-labeled by preparing cocktails of primary antibodies raised in different species and diluted in 0.01 M phosphate buffer, 2.9% NaCl, 0.3% Triton, 0.1% sodium azide, and 2% bovine serum albumin. Overnight incubation with primary antibodies was followed by incubation for 1-2 hours with a cocktail of species-specific secondary antibodies conjugated to distinct fluorochromes. Each incubation step was followed by rinses in potassium phosphate-buffered saline (KPBS), and all incubations were conducted at room temperature. Finally, sections were coverslipped with glycerol/KPBS (1:1).

One of two TH antibodies (rabbit polyclonal anti-TH, 1:200, Pel-Freez Biologicals, Rogers, AR, or mouse monoclonal anti-TH, 1:200, ImmunoStar, Hudson, WI) was used in combination with one or more of the following antibodies: goat polyclonal anti-ChAT (1:100, Millipore, Temecula, CA), rabbit polyclonal anti-VIP (1:100, ImmunoStar), rabbit polyclonal anti-nNOS (1:500, Millipore), or rabbit polyclonal anti-AQP4 (1:200 for double-labeling, 1:750 for triple-labeling, Millipore). The secondary antibodies used were Cy2- or DyLight 488-conjugated donkey anti-rabbit, DyLight 488- or Rhodamine Red-X-conjugated donkey anti-goat, and Rhodamine Red-X-conjugated donkey anti-mouse (all 1:50 except DyLight 488-conjugated secondaries 1:100, Jackson ImmunoResearch, West Grove, PA). Control experiments confirmed that secondary antibodies bound only to the appropriate primary antibodies.

4.3 Data collection and analysis

Sections were analyzed and images captured and annotated using a Zeiss Axioskop 2 Plus fluorescence microscope equipped with an AxioCam MRc5 CCD digital camera operated with AxioVision software (Carl Zeiss, Thornwood, NY). Photomicrographic images were

cropped, lettered and merged using Adobe Photoshop CS3 software (Adobe Systems, Seattle, WA).

Cortical neuron profiles were scored positively for TH, ChAT, VIP, and nNOS immunoreactivity if they conformed to the following criteria: 1) cell profiles were consistent with the patterns of immunoreactivity (cellular distribution and morphology) described in the literature for these markers; 2) at least one extension off of a clearly defined cell body could be observed. Cell profiles immunoreactive for TH alone or double-labeled were counted at 400X magnification in sections through the neocortex, from the cingulate cortex medially to the rhinal fissure laterally. Colocalization of TH with ChAT, VIP, or nNOS was identified by the precise overlap of cell body immunofluorescence in the same focal plane. The use of confocal microscopy was not necessary to determine colocalization because individual cell body profiles were identified easily in the 14 μ m sections. This relatively thin section thickness greatly reduced the possibility of counting two overlaying cells as a single double-labeled cell. Nonadjacent sections separated by at least 84 μ m were analyzed, which eliminated the possibility of counting the same cell twice.

The percentages of cortical TH-IR neuron profiles that colocalized with ChAT or VIP were averaged at each of the three age classes, and these values were converted to ratios. The JMP software package (version 8, SAS Institute, Cary, NC) was used for all statistical analyses. A one-way ANOVA was used to test differences among mean ratios of TH-IR profiles that colocalized with ChAT at the three age intervals, and Tukey's HSD (Honestly Significant Difference) Test was used for post-hoc comparisons. This statistical analysis with post-hoc comparisons was also implemented to test differences among mean ratios of TH-IR profiles that colocalized with VIP at the three ages examined.

The proximity of TH-IR somata with cortical microvessel walls was assessed by double-labeling for TH and AQP4. Five brains at P16 were examined, and three brains at both P20 and P30 were analyzed. At least 226 TH-IR neuron profiles were sampled at each of the age classes (763 total TH-IR cell profiles), and the proportions of profiles closely apposed to AQP4-labeled blood vessels were determined. The percentages of microvessel-associated TH-IR cell profiles that coexpressed ChAT or VIP were determined by triple-labeling sections from at least two brains at each of the three age classes. For each marker (ChAT or VIP) at least 52 TH-IR neuron profiles that were apposed to cortical blood vessels were sampled at each of the age classes (164 total for ChAT and 158 total for VIP). Blood vessels were identified with AQP4, visualized with a DyLight 488-conjugated secondary antibody. Microvessel-associated neurons were double-labeled for TH and either ChAT or VIP, visualized as described above with Rhodamine Red-X-and DyLight 488-conjugated secondaries. The identification of three markers using two fluorochromes was successful because the markers were present in distinguishable cell types: TH, ChAT and VIP were present in interneurons and AQP4 was observed in astrocytic endfeet surrounding microvessels (Tait et al., 2008).

For analysis of TH and nNOS colocalization, three brains at each age were examined. At both P16 and P20, more than 50 cortical TH-IR neuron profiles were counted, and at P30 more than 100 profiles were analyzed.

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Figure 1.

Colocalization of ChAT and TH in cortical interneurons. Photomicrographs of the same field of cerebral cortex at P20 showing a neuron profile (arrows) double-labeled for ChAT (A) and TH (B). Panel C is an overlay of the two images demonstrating the colocalization of these markers in the same cell profile. ChAT-IR, TH-negative cell profiles and fibers (A) and TH-IR, ChAT-negative fibers (B) were seen in this field as expected. Scale bar = 20 μ m.

Figure 2.

The proportion of cortical TH-IR neuron profiles that colocalized with ChAT increased significantly during postnatal development (1-way ANOVA, $P = 0.0071$). Each bar (with $\pm$ standard deviations) represents the average proportion of TH-IR cell profiles colocalized with ChAT. Post-hoc analysis (Tukey's HSD) demonstrated significant differences between P16 and P30 ($P < 0.05$). Bars with different letters indicate significant differences among age classes.

Figure 3. Colocalization of VIP and TH in cortical interneurons. Photomicrographs of the same field of cerebral cortex at P16 showing a neuron profile (arrows) double-labeled for VIP (A) and TH (B). Panel C is an overlay of the two images demonstrating the colocalization of these markers in the same cell profile. Scale bar = 20 μ m.

Figure 4.

The proportion of cortical TH-IR neuron profiles that colocalized with VIP increased significantly during postnatal development (1-way ANOVA, $P = 0.0006$). Each bar ($\pm$ standard deviations) represents the average proportion of TH-IR cell profiles colocalized with VIP. Post-hoc analysis (Tukey's HSD) demonstrated significant differences between P30 and both P16 and P20 ($P < 0.05$). Bars with different letters indicate significant differences among age classes.

Figure 5.

Cortical neuron profiles coexpressing TH and VIP were frequently apposed to blood vessels. A-C: Photomicrographs of the same field of cerebral cortex at P30 showing numerous microvessels outlined by AQP4 immunoreactivity (A) and a TH-IR neuron profile (arrow) adjacent to a microvessel (B). Panel C is an overlay of the two images, demonstrating the close proximity of the TH-IR cell profile and the blood vessel. D-F: Photomicrographs of the same field of cerebral cortex at P30 showing a VIP-IR neuron profile (arrow) apposed to an AQP4-highlighted blood vessel (D). Both VIP and AQP4 were visualized using DyLight 488 (green). The VIP-IR cell profile in D is colabeled for TH (arrow; E). Panel F is an

overlay of the two images, showing that the microvessel-associated cell profile was colabeled for VIP and TH. Scale bars = 20 μm .

Figure 6. nNOS was not colocalized with TH in cortical interneurons. Photomicrographs of the same field of cerebral cortex at P16 showing two nNOS-IR cell profiles (A) and one TH-IR cell profile (B). Panel C is an overlay of the two images, showing that these markers are not colocalized in the same cell profiles.

Table 1

ANOVA summary statistics for TH and ChAT colocalization

Source of variation	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Age	2	0.0655	0.0327	8.4341	0.0071*
Error	10	0.0388	0.0038		
Total	12	0.1043			

Comparisons for all pairs (Tukey's HSD)

Level	Level	Difference	Standard error dif	Lower confidence limit	Upper confidence limit	p-Value
P30	P16	0.1670	0.0418	0.0524	0.2817	0.0065*
P20	P16	0.1097	0.0418	-0.0048	0.2243	0.0605
P30	P20	0.0573	0.0440	-0.0634	0.1781	0.4260

Table 2

ANOVA summary statistics for TH and VIP colocalization

Source of variation	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Age	2	0.1133	0.0567	17.2633	0.0006*
Error	10	0.0328	0.0033		
Total	12	0.1461			

Comparisons for all pairs (Tukey's HSD)					
Level	Level	Difference	Standard error dif	Lower confidence limit	Upper confidence limit
P30	P20	0.2234	0.0405	0.1124	0.3344
P30	P16	0.1779	0.0384	0.0726	0.2833
P16	P20	0.0455	0.0384	-0.0599	0.1508
					0.4890