MOUSE CORTICAL CHOLINERGIC NEURONS: ONTOGENY OF
PHENOTYPES IN VIVO AND IN VITRO

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Coiculescu, Olivia Elena, Mouse cortical cholinergic neurons: Ontogeny of phenotypes in vivo and in vitro. Doctor of Philosophy (Biology), August 2005, 78 pp., 3 tables, 18 figures, references, 104 titles.

The development of cholinergic neurons in mouse frontal cortex was studied both in vivo and in vitro by immunocytochemistry with an antibody to choline acetyltransferase (ChAT), the enzyme responsible for acetylcholine synthesis. While cortical cholinergic neurons have previously been characterized in rat cortex, up until very recently, intrinsic cortical cholinergic neurons were considered to be absent in mouse, and little is known about their development or phenotypic characteristics. The present study found no ChAT-positive neurons in mouse frontal cortex on postnatal day 0 (P0, the day of birth). On P7 there were few, faintly stained, ChAT-positive neurons. The numerical density of ChAT-positive neurons increased substantially with age, from none on P0, to 9.2 ± 1.4 on P7, to 14.8 ± 0.9 on P16, and 41.6 ± 3.9 in adulthood. Considering that the numerical density of total neurons decreases during this postnatal period, the data represent a marked developmental increase in the percentage of cholinergic neurons. The development of cholinergic neurons showed very similar timelines in rat and mouse frontal cortex.

Cultures prepared from mouse frontal cortex on embryonic day 16 were maintained for 25, 76, or 100 days in vitro (div). The percentage of ChAT-positive neurons was considerably higher than in vivo, ranging from a mean 28% to 31% across the three age (div) groups. With increasing age of the cultures, the numerical density of total neurons and ChAT-positive neurons decreased while the percentage of ChAT-
positive neurons did not change significantly. These observations suggest some temporal stability in the cultures.

Using dual immunofluorescence, ChAT-positive neurons were tested for colocalization with GAD or TH. The majority of ChAT-positive neurons colocalized with GAD, both in vitro and in vivo. However, ChAT did not colocalize with TH, either in vitro or in vivo. Our comparison of intact frontal cortex and cultures suggest that while the percentage of cholinergic neurons was greater in the cultures, the cholinergic neurons developed phenotypic similarities in vitro and in vivo.
ACKNOWLEDGMENTS

The author gratefully acknowledges the many people who have given their encouragement, assistance and support. These include her advisor, Dr. Jannon Fuchs, Committee members Dr. Guenter Gross, Dr. Harris Schwark, Dr. Lynda Uphouse and Dr. Robert Benjamin, and her family and friends. The author thanks Dr. Guenter Gross for his generous gift of frontal cortex cultures.
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ABBREVIATION LIST

ACh…………………………………………acetylcholine
AChE…………………………………………acetylcholinesterase
ChAT…………………………………………choline acetyltransferase
DAB…………………………………………3,3’-diaminobenzidine
DAPI…………………………………………4’, 6-diamidino-2-phenylindole
div…………………………………………….days in vitro
DOPA…………………………………………dihydroxyphenylalanine
E…………………………………………….embryonic day
GABA……………………………………….gamma-aminobutyric acid
GAD…………………………………………glutamic acid decarboxylase
IR……………………………………………immunoreactivity
ISH………………………………………….in situ hybridization
NF…………………………………………..neurofilament
P…………………………………………….postnatal day
PBS…………………………………………phosphate buffered saline
SEM………………………………………...standard error of mean
TH…………………………………………...tyrosine hydroxylase
VACHT……………………………………..vesicular acetylcholine transporter
INTRODUCTION

Significance of the Cholinergic System

The study of the central cholinergic system, one of the main neurotransmitter systems that regulates brain activity, developed greatly during 1970s and 1980s, related to concomitant progress from studies on brain plasticity and Alzheimer’s disease. When the focus on the main pathogenic elements in Alzheimer’s disease switched from acetylcholine (ACh) to amyloid precursor protein and β-amyloid deposition, the interest in cholinergic regulation of cortical events decreased for almost two decades. The study of cholinergic neurons regained its importance in the past few years when the two theories on Alzheimer’s disease pathogeny were combined in a new model.

Besides the relation to Alzheimer’s disease (Coyle et al., 1983; Mesulam, 1986; Bartus et al., 1999), central cholinergic neurons are also involved in Huntington’s disease (Ferrante et al., 1987). In both degenerative diseases the disturbance of the central cholinergic system is not clearly understood and does not appear to be closely related to the etiology of these diseases, but rather to the development of their clinical symptoms. A number of other disorders have been associated with disturbances of the cholinergic system. For example, the decrease of choline acetyltransferase (ChAT) activity in brain has been associated with schizophrenia (Karson et al., 1996) and sudden infant death syndrome (Mallard et al., 1999).

Cholinergic neurons play an important role in activities such as sustained attention (Sarter et al., 2001), learning, memory (Blokdland, 1996), arousal, and movement (Steketea, 2003). The main sources of acetylcholine in cortex are axons of cholinergic basal forebrain neurons and intrinsic cortical cholinergic neurons (Semba,
2004). An additional source of cortical acetylcholine is provided by projections of the cholinergic neurons in the mesopontine tegmentum, but they are limited to the medial prefrontal cortex (Semba, 2004). About 70% of the cortical ChAT in rat seems to come from basal forebrain (Eckenstein and Baughman, 1984), and most of the cognitive functions of cortical acetylcholine mentioned before are thought to be based on this diffuse system of cholinergic afferents coming from basal forebrain (Sarter et al., 1997; Semba 2004).

**Description of Choline Acetyltransferase**

Choline acetyltransferase (ChAT) is the enzyme responsible for the biosynthesis of acetylcholine. ChAT mediates a single step reaction involving the transfer of an acetyl group from acetyl coenzyme A to choline at the synaptic endings of cholinergic neurons. Acetyl coenzyme A is produced from glucose in the mitochondrial compartment in mammals. Intracellular choline is believed to be taken up from the extracellular fluid by a sodium-dependent, hemicolinium-3-sensitive, high-affinity choline transporter, CHT1 (Okuda et al., 2003). Choline also may be obtained by phospholipases from membrane phosphatidylcholine (Tucek, 1985). Choline uptake is believed to be the rate-limiting step in acetylcholine synthesis (Okuda et al., 2003). After synthesis, acetylcholine is transported into synaptic vesicles by a vesicular acetylcholine transporter (VACHT) and is released into the synaptic cleft where it activates nicotinic and muscarinic receptors in the pre- and post-synaptic membranes. Acetylcholine is hydrolyzed into choline and acetate by acetylcholinesterase (AChE). The processes described are summarized in Figure 1.
Distribution of Central Cholinergic Neurons

The existence of cholinergic neurons in cortex was a matter of controversy in the early 1980’s. Studies using immunocytochemistry did not show the presence of ChAT-positive neurons in cortex of rat (Sofroniew et al., 1982; Armstrong et al., 1983), cat (Kimura, 1991), or monkey (Lewis, 1991). These results could be explained by the staining methodology used: Armstrong et al. (1983) and Lewis (1991) used AB8, a monoclonal ChAT antibody (Levey et al., 1983) demonstrating a staining pattern that differed from those using other monoclonal ChAT antibodies (see Table 1). Sofroniew et
al. (1982) used PAP (peroxidase-antiperoxidase) method, which is less sensitive than
the avidin-biotin labeling technique used currently (Eckenstein and Thoenen, 1983).

Table 1. Summary of studies that used monoclonal ChAT antibodies in mammalian
cortex. Table continues on the next page.

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>SOURCE</th>
<th>SPECIES</th>
<th>REFERENCES</th>
<th>CHAT-POSITIVE NEURONS IN CORTEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB8 ChAT</td>
<td>Levey</td>
<td>Sprague-Dawley Rat</td>
<td>Armstrong et al., 1983</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cynomolgus Monkey</td>
<td>Lewis, 1999</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cat</td>
<td>Kimura, 1991</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wistar Rat</td>
<td>Nishimura et al., 1988</td>
<td>Both bipolar and pyramidal shaped neurons</td>
</tr>
<tr>
<td>Monoclonal Rat anti-bovine ChAT</td>
<td>Eckenstein et al., 1982</td>
<td>Long Evans Rat</td>
<td>Sofroniew et al., 1982</td>
<td>None (used PAP)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wistar Rat</td>
<td>Eckenstein and Thoenen, 1983</td>
<td>Layers 2-6 bipolar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Long Evans rat</td>
<td>Eckenstein and Baughman, 1984</td>
<td>Layers 2-6 (mostly 2-3) bipolar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sprague-Dawley Albino Rat</td>
<td>Dori and Parnavelas, 1989</td>
<td>After P11 layers 2-3 bipolar</td>
</tr>
<tr>
<td></td>
<td>Boehringer Mannheim</td>
<td>Cynomolgus Monkey</td>
<td>Campbell, 1987</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C57Bl/6j Mouse</td>
<td>Schambra et al., 1989</td>
<td>E14-P2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C57Bl/6j Mouse</td>
<td>Schambra et al., 1989</td>
<td>E14-P2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wistar Rat</td>
<td>Le Jeune et al., 1991</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human</td>
<td>Benagiano, 2003</td>
<td>Parietal cortex</td>
</tr>
</tbody>
</table>
Besides the studies using monoclonal antibodies (Table 1), some researchers used polyclonal antibodies. Table 2 summarizes a few of the studies using the Chemicon polyclonal antibody used in the present study. Comparing polyclonal with monoclonal ChAT antibodies as well as with the newest VACHT antibody, Rico and Cavada (1998) showed that the polyclonal ChAT antibody yields the best cell body staining while monoclonal ChAT antibody gives less morphological details; the VACHT antibody, whose staining pattern is markedly granular and does not stain dendrites, consequently gives the lowest cellular detail (Ardvisson et al., 1997).

Some studies have verified the identification of neurons by ChAT immunostaining with polyclonal antibodies by parallel in situ hybridization for ChAT mRNA (Schäfer et al., 1998; Kasashima et al., 1999), see Tables 2 and 3.
Table 2. Summary of studies that used polyclonal ChAT antibodies in mammalian tissue.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ANTIBODY</th>
<th>REFERENCE</th>
<th>TISSUE</th>
<th>OBSERVATIONS</th>
</tr>
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<tbody>
<tr>
<td>C57BL/6 Mouse</td>
<td>Goat anti-ChAT</td>
<td>Berger-Sweeney et al., 2001</td>
<td>Medial Septum</td>
<td>1:250 DAB and fluorescence 60 μm sections</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N. Basalis Magnocellularis</td>
<td></td>
</tr>
<tr>
<td>Wistar Rat</td>
<td>Goat anti-ChAT</td>
<td>Van Vulpens and Van Der Kooy, 1996</td>
<td>Striatum</td>
<td>1:500 40-hour incubation 40-50 μm sections</td>
</tr>
<tr>
<td>Long Evans Rat</td>
<td>Goat anti-ChAT</td>
<td>Birthelmer et al., 2003</td>
<td>Medial Septum</td>
<td>1:200 18-hour incubation 60 μm sections</td>
</tr>
<tr>
<td>Macaca Nemestrina</td>
<td>Goat anti-ChAT</td>
<td>Rico and Cavada, 1998</td>
<td>Thalamus</td>
<td>PAP staining colocalizes with monoclonal ChAT antibody and with VACHT antibody</td>
</tr>
<tr>
<td>Wistar Rat</td>
<td>Rabbit anti-ChAT</td>
<td>Schäfer et al., 1998</td>
<td>Cortex</td>
<td>1:1000 small bipolar, all layers, mostly 2-3, identified in parallel: both VACHT and ChAT antibody and mRNA</td>
</tr>
<tr>
<td>Human</td>
<td>Rabbit anti-ChAT</td>
<td>Kasashima et al., 1999</td>
<td>Cortex</td>
<td>double study with in situ hybridization</td>
</tr>
</tbody>
</table>

The use of more specific ChAT antibodies (Semba, 2004) and improved staining techniques allowed the identification of ChAT immunoreactive neurons in the cerebral cortex of the rat (Eckenstein and Baughman, 1984; Eckenstein and Thoenen, 1988;
Houser et al., 1983; Ichikawa et al., 1986; Kosaka et al., 1988; Nishimura, 1988; Levey et al., 1984; Umbriaco et al., 1994), cat (Avendaño et al., 1996; Stichel et al., 1987), fetal monkey (Hendry et al., 1987), and adult human (Kasashima et al., 1999 and Oda, 1999). Unlike in the rat, cat and monkey cortex, ChAT immunoreactive cells were said to be absent in adult mouse cortex (Kitt et al., 1994), and only recently they were identified in parietal cortex by Descarries et al. (2005).

The cholinergic neurons reported in rat are small, fusiform or bipolar neurons, present in layers 2-6 throughout all areas of the cortex, with highest density in layers 2 and 3 (Eckenstein and Thoenen, 1983; Eckenstein and Baughman, 1984; Houser et al., 1982; Houser et al., 1985; Chedotal et al., 1999). Nishimura (1988) showed the presence of pyramidal-like neurons in rat frontal cortex. In his study, cholinergic neurons are distributed over all cortical layers except layer 1, with these differences between layers: pyramidal ChAT-positive neurons are present only in layer 5 and bipolar neurons are present mostly in layers 4 and 6. However this study used the AB8 antibody (Levey et al., 1983), which resulted in a staining pattern different from other antibodies and yielded results inconsistent with other studies (see Table 2).

Unlike rat cortex, where, with the exception of the Nishimura study, most of the ChAT-positive neurons are described as small and bipolar, in human cortex most of the cholinergic neurons are medium-sized or large pyramidal neurons. These neurons are located predominantly in layers 3 and 5, with a higher density in the motor and secondary sensory areas compared to other cortical areas. In humans non-pyramidal cholinergic neurons are observed in the superficial layer of the cingulate gyrus and in the parahippocampus (Oda, 1999).
Recently, \textit{in situ} hybridization has been applied to detect ChAT mRNA in neurons for identifying cholinergic neurons. Some \textit{in situ} hybridization studies fail to identify cholinergic neurons in rat cortex (Oh et al., 1992; Ichikawa et al., 1997) while others, using a long exposure time (3 months versus 2 months, see Table 3), show a small number of faintly labeled neurons distributed throughout the rat cortex, primarily in layers 2 and 3 (Lauterborn et al., 1993; Ibanez et al., 1991, Schäfer et al., 1998). The apparent discrepancy among these results may possibly reflect differences in threshold sensitivity of detection systems used by different studies (i.e., isotopic versus non-isotopic localization of the hybridization probe). As seen in Table 3, all studies using radiolabeled riboprobes identified the presence of cholinergic neurons in cortex, while the digoxigenin-labeled riboprobes yielded negative results, suggesting that the isotopic technique may have been a more sensitive technique than the digoxigenin method.

Neurons with ChAT mRNA are present also in human cerebral cortex, most of them being medium-sized pyramidal neurons in layer 3, with several large neurons specifically in layer 5 of motor cortex (Kasashima et al., 1998). They colocalize with ChAT immunoreactivity (Kasashima et al., 1999), but fewer neurons are identified by \textit{in situ} hybridization in comparison with ChAT immunocytochemistry studies, suggesting a low ChAT synthesis in neocortical cholinergic neurons (Kasashima et al., 1999). A summary of \textit{in situ} hybridization studies is presented in Table 3.
Table 3. *In situ* hybridization studies of cholinergic neurons in cerebral cortex-ISH=*in situ* hybridization, IR=immunoreactivity.

<table>
<thead>
<tr>
<th>REFERENCE</th>
<th>SPECIES</th>
<th>TYPE OF RIBOPROBE</th>
<th>OBSERVATION-CORTEX</th>
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</thead>
<tbody>
<tr>
<td>Lauterborn et al., 1993</td>
<td>Sprague-Dawley Rat</td>
<td>35S label 3-month exposure better</td>
<td>Faint hybridization, mainly layers 2, 3</td>
</tr>
<tr>
<td>Ichikawa et al., 1997</td>
<td>Sprague-Dawley Rat</td>
<td>Digoxigenin</td>
<td>No ISH-neurons in cortex ChAT-positive (IR) cells in layers 2-6</td>
</tr>
<tr>
<td>Oh et al., 1992</td>
<td>Sprague-Dawley Rat</td>
<td>Digoxigenin</td>
<td>No ISH-neurons in cortex</td>
</tr>
<tr>
<td>Butcher et al., 1992</td>
<td>Sprague-Dawley Rat</td>
<td>Digoxigenin</td>
<td>No ISH-neurons in cortex</td>
</tr>
<tr>
<td>Ibanez et al., 1991</td>
<td>Sprague-Dawley Rat</td>
<td>35S label 3-month exposure</td>
<td>Moderate hybridization</td>
</tr>
<tr>
<td>Kasashima et al., 1998</td>
<td>Human</td>
<td>35S label 3-month exposure</td>
<td>layers 3 and 5, motor cortex</td>
</tr>
<tr>
<td>Schäfer et al., 1998</td>
<td>Wistar Rat</td>
<td>35S label</td>
<td>Small bipolar through all the layers mostly 2-3 identified in parallel with VACHT and ChAT IR and VACHT ISH</td>
</tr>
<tr>
<td>Kasashima et al., 1999</td>
<td>Human</td>
<td>35S label 3-month exposure</td>
<td>ISH colocalize with ChAT IR, but fewer were identified with ISH than IR</td>
</tr>
</tbody>
</table>

The chemical phenotypes of cortical intrinsic cholinergic neurons differ from those in basal forebrain (Eckenstein and Baughman, 1984; Chedotal et al., 1994), as indicated by the presence of colocalization of ChAT-positive neurons with vasointestinal peptide (VIP) only in cortical neurons. This difference suggests that the cortical
cholinergic neurons can develop independently and create their own local circuitry (Eckenstein and Baughman, 1984). This colocalization is compatible with the fact that ChAT and VIP axons are both closely associated with intracortical blood vessels; indeed, it has been proposed that the main function of both acetylcholine and VIP is to regulate cerebral blood flow (Chedotal et al., 1994)
Development of Cholinergic Neurons

Historically, the main tool for the study of the developing cholinergic system was initially neurochemistry (i.e., tissue enzyme activity of both ChAT and AChE measured in dissected regions of cortex), while morphological details could be obtained by histochemical visualization of AChE fibers and neurons. Later, development of ChAT immunocytochemistry offered a better approach for the identification, localization and description of cortical cholinergic neurons. Still, most studies of postnatal development of intrinsic cortical cholinergic neurons have been done on rats. To substantiate our studies on postnatal development of cortical cholinergic neurons in mouse cortex, we also did parallel studies in rat to compare with results described in the literature.

Development of cortical cholinergic neurons in rat.

Neurochemistry studies.

All studies of ChAT activity in homogenized tissue from developing rat cortex report low enzymatic activity during the first postnatal week, increasing steadily in all layers starting with the early part of the second postnatal week, and continuing to increase over the subsequent two weeks (Coyle et al., 1976, McDonald et al., 1987, Virgili et al., 1990, Zahalka et al., 1993). However, these
studies could not differentiate between extrinsic sources of ChAT (basal forebrain projections) and intrinsic sources (cortical cholinergic neurons).

Acetylcholinesterase (AChE) has low levels early in the postnatal period but increases faster than ChAT, attaining 80% of the adult level by P14 (Virgili et al., 1990; Coyle et al., 1976). However the relevance of AChE results is limited by the expression of this degradative enzyme in non-cholinergic neurons.

**ChAT immunocytochemistry studies.**

Whereas the change in ChAT activity during postnatal development of rat cortex is consistent among reports, studies using ChAT immunocytochemistry reached two different conclusions. Dori and Parnavelas, (1989) reported that cholinergic neurons develop relatively late postnatally in the rat cerebral cortex, mainly from the later part of the second week. On the other hand, Mechavar and Descarries (2001) found that cholinergic neurons develop earlier, and are present in all cortical areas starting with postnatal day 4 (P4) where P is the day of birth.

Dori and Parnavelas (1989) showed ChAT-positive cells are present in the rat developing occipital cortex as early as embryonic day 17 (E17) and their presence is maintained during prenatal development, but their number reduced suddenly to none at birth. In their study ChAT-positive neurons could not be identified in postnatal life until P11 when very few cells are observed (2-3 per section, faintly stained). ChAT-positive neurons are present mostly in layers 2-4,
with few in layer 5. Their number as well as the intensity of staining increases gradually in the second and third postnatal weeks, reaching adult levels at the end of the third week.

Mechawar and Descarries (2001) supported an earlier development of cholinergic neurons in rat cortex using a more specific ChAT antibody (Semba, 2001). Their study showed that in the rat frontal, parietal and occipital areas, cholinergic neurons develop early and rapidly: at P0 there are no ChAT-positive neurons, at P4 few faintly stained interneurons are visible in frontal, parietal and occipital cortex; at P8 all cortical layers in these regions display many strongly ChAT-positive neurons and their number increases from P8 to P16, showing at P32 the same distribution as in adult brain.

Differences in these ChAT immunocytochemistry results could be related to the use of different ChAT antibodies or to the section thickness. The antibody used by Mechawar and Descarries is more specific (Semba, 2001) compared to that used by Dori and Parnavelas. Dori and Parnavelas used very thick sections (100 µm) for embryonic and early postnatal ages (age range not specified) and 40 µm sections for late postnatal and adult brains, while Mechawar and Descarries used 50 µm sections for all ages. The thick sections are disadvantageous because the antibody may not have penetrated the whole tissue and cells are more difficult to delineate, possibly contributing to the lack of identification of ChAT-positive neurons.
Development of cortical cholinergic neurons in mouse.

Neurochemistry studies.

Similar to the rat studies, ChAT activity in postnatal mouse cortex studies showed very low ChAT activity until P6, increasing steadily to 40% of adult activity at P18 and reaching adult levels at the end of second postnatal month (Höhmann et al., 1985; 1988). Unilateral lesion of basal forebrain neurons on P1 reduces ChAT activity in homogenized ipsilateral frontoparietal cortex to 10% at P4-5, 50% at P7-8, and 60% at P16. These differences are reduced at P30 (Höhmann et al., 1988). The lesion of basal forebrain innervation showed concurrent development of both basal forebrain and intrinsic cortical sources of ChAT in cortex.

ChAT immunocytochemistry studies.

In mouse cortex, ChAT-positive cells appear as early as E14 in germinal zones. On E16 they are present in the prelimbic cortex and parietal cortex where they are still observed on P2 (Schambra et al., 1989). Schambra et al. (1989) did not study the development of cholinergic neurons beyond P2.

While ChAT neurochemistry studies show a parallelism between the development of rat and mouse cortex (ChAT levels are very low on the first postnatal week and increase steadily over the next 2 postnatal weeks to the adult
level), the immunocytochemistry studies are not as consistent: there are two
different opinions regarding the initiation of ChAT-positive neurons development
in rat cortex, and in mouse the postnatal development was not studied beyond
P2.

The lack of knowledge of the postnatal development of cholinergic
neurons in mouse cortex is surprising given the potential usefulness of
genetically engineered mice for elucidating the physiology and behavioral
functions of cholinergic neurons. Consequently, an extensive study of the
localization and the development of intrinsic mouse cortex cholinergic neurons
emerges as a necessary starting-point for further studies of their physiological
and behavioral functions using genetic or pharmacological manipulation of this
neuronal population.

Cholinergic Neurons in Cultures

The presence of cholinergic neurons in dissociated cortex cultures was
studied in rat cortex cultures with AChE staining (Mesulam and Dichter, 1981).
Their results showed an average of 15% AChE-positive neurons in culture but
the results are biased by the known fact that AChE is not specific for cholinergic
neurons (Levey et al., 1984; Le Jeune et al., 1991).

In mouse cortex, cholinergic neurons are present starting with E14
(Schambra et al., 1989). Consequently ChAT-positive neurons should be present
in murine cortex cultures obtained from embryonic dissociated cortical tissue at
this age. The presence of cholinergic neurons in culture was indicated by the ChAT chemistry (Boespflug and Swaiman, 1986) and by the pharmacology and toxicology studies of murine frontal cortex cultures that demonstrate indirectly the presence of cholinergic neurons in the cultures (Keefer et al., 2001). Keefer and his coworkers (2001) showed that murine dissociated frontal cortex culture respond to the application of atropine (non-specific muscarinic antagonist), curare (nicotinic receptors antagonist) and eserine (AChE inhibitor), revealing the presence of ACh receptors and of functional cholinergic synapses in these cultures.

ChAT activity in murine cortex cultures parallels maturation and development of neurons (Boespflug and Swaiman, 1986): the measured level of ChAT activity in whole culture reaches a peak in the first 3 weeks in vitro, when the number of total neurons in culture is also the highest, and decreases after this period in parallel with decreasing neuronal population. However, Boespflug and Swaiman (1986) did not determine whether this change in the ChAT level is caused by a decrease in number of cholinergic neurons or by a decrease of ChAT production by a constant number of cholinergic neurons. This question could be answered by a study of the morphology of cholinergic neurons in cultures and the influence of culture age on the number of cholinergic neurons in cultures.
Colocalization with Other Neurotransmitters

Previous studies showed a tendency of cholinergic neurons to colocalize with neuropeptides and other neurotransmitters. A number of other neurotransmitters and neuromodulators colocalize with acetylcholine (Tohyama et al., 1998): enkephalin (superior olivary nucleus, sympathetic preganglionic neurons), substance P (laterodorsal tegmental nucleus, sympathetic postganglionic neurons), galanin (basal nucleus of Meynert), CGRP (motor neurons, superior olivary nucleus), GABA (superior olivary nucleus), somatostatin, neurotensin and ACTH (sympathetic preganglionic neurons), neuropeptide Y and cholecystokinin (parasympathetic preganglionic neurons). Acetylcholine colocalizes with VIP in cerebral cortex neurons (Eckenstein, et al., 1984) and parasympathetic postganglionic neurons (Tohyama et al., 1998).

Considering the fact that the ChAT-positive neurons described in cortex are mostly bipolar (a subclass of interneurons, most of which are GABAergic), several studies tested whether ChAT is present in interneurons.

The possible colocalization of GABA with a cholinergic marker, using AChE histochemistry and GABA autoradiography, was suggested previously in rat dissociated cortex cultures derived from E16 embryos (Mesulam and Dichter, 1981). GABA was also shown to colocalize with AChE in rat cortex, using histochemistry staining for AChE and immunocytochemistry for GABA (Hallanger et al., 1986). These studies, however, based their results on the assumption that AChE is a good marker for cholinergic neurons. However AChE is typically
localized postsynaptically to cholinergic terminals where it hydrolyzes acetylcholine released at the synapse, so GABAergic cortical neurons that colocalize AChE may in fact receive cholinergic afferents, but may not be cholinergic (Hallanger et al., 1986).

Previous studies on the colocalization of ChAT with glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, are not consistent: some of them showed a strong colocalization between cholinergic and GABAergic markers, while others could not find any colocalization. In the rat cerebral cortex, Kosaka et al. (1988) reported that half of the cholinergic neurons are also immunoreactive for either GABA or GAD. Bayraktar et al. (1997) found a higher percentage (88%) of ChAT-immunoreactive neurons costaining for GABA, while Gritti et al. (1993) did not find ChAT and GAD colocalization. The differences between these studies could be due to procedural differences. To colocalize ChAT with GAD in cortex, Kosaka et al. (1988) and Bayraktar et al. (1997) used diaminobenzidine (DAB) immunohistochemistry on alternate sections stained for either GAD or ChAT, and compared paired surfaces of adjacent sections. Gritti and coworkers (1993) sequentially processed the same sections with DAB and benzidine dihydrochloride. Dual immunofluorescence might be more definitive in colocalizing ChAT with GAD by reducing the uncertainty inherent in aligning paired surfaces of adjacent sections.

It would also be interesting to know if ChAT is present in catecholaminergic neurons in the developing central nervous system.
In early neuroblasts from whole chick embryo on E3, ChAT colocalizes with tyrosine hydroxylase (TH), the enzyme that catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA) in catecholamine neurons (Kentroti et al., 1995). Colocalization of ChAT with TH was found also in neurons of the dorsal motor nucleus of the vagus (Manier et al., 1987; Armstrong et al., 1990). VAChT, another marker for cholinergic neurons, colocalizes with TH in rat embryonic neurons of both parasympathetic and sympathetic lineages (Schäfer et al., 1997). During development, the autonomic nervous system shows a phenotypic switch. Studies on rat sympathetic neurons in vivo (Landis and Keefe, 1983) and in vitro (Wolinsky and Patterson, 1983) provided evidence that mature cholinergic neurons arise from previously noradrenergic neurons.

Immunocytochemistry studies suggested that TH colocalizes with GABA phenotype in postnatal developing rat olfactory bulb (Kosaka et al., 1987) and in adult human cortex (Trottier et al., 1989). The function of the TH-positive neurons in cortex is not well understood, but they seem to be implicated in the neurogenic control of cerebral arterioles and capillaries (Matsuyama et al., 1985). The cerebral arterioles and capillaries have dual central innervation, aminergic and cholinergic (Itakura et al., 1977). The ChAT colocalization with TH has not been studied in the cortex.
RESEARCH OBJECTIVES

The objectives of the present study are to compare the phenotypic development of cholinergic neurons in the mouse frontal cortex \textit{in vivo} and in dissociated neuronal culture.

The Presence of Cholinergic Neurons in Mouse Frontal Cortex Cultures

There is little information on the temporal stability of neurotransmitter phenotypes in cultures, yet the reliability of electrophysiological and pharmacological studies of neuronal cultures depends on temporal stability. The presence of cholinergic neurons in the murine frontal cortex cultures used in this study was demonstrated indirectly through pharmacological influences on spontaneous spiking pattern of networks of cultured neurons on the microelectrodes array (Keefer et al., 2001). The purposes of my study were to determine whether such neurons are present using ChAT immunohistochemistry and to study the influence of culture age, measured as days \textit{in vitro} (div), on the composition of neuronal networks, especially the percentage of cholinergic neurons.
The Presence of Cholinergic Neurons in the Frontal Cortex of Postnatal Mouse

In contrast to the studies on the cortex of other experimental animals, the presence of cholinergic neurons in the postnatal development of mouse cortex has not yet been reported. An aim of my study is to characterize the postnatal development of ChAT-positive neurons in mouse frontal cortex and to compare it with frontal cortex cultures.

Colocalization of ChAT with GAD and TH in Developing and Adult Mouse Cortex and in Frontal Cortex Cultures

Colocalization of ChAT with GAD was shown in the rat cortex, but the published results are inconsistent. The antibody specificity and the staining methodologies could be reasons for the differences reported in the literature. Colocalization of TH and ChAT was established in sympathetic neurons from cervical ganglia in vitro and in vivo and in cultured neuroblasts from chick embryos. VAChT, another marker for cholinergic neurons, colocalizes with TH in rat embryonic autonomic neurons. No data are available regarding the colocalization of ChAT and TH in the cortex. Using dual immunofluorescence, our study aimed to obtain more details about the morphology of cholinergic neurons in vivo and in vitro and to examine the postnatal development of colocalization in cholinergic neurons.
MATERIALS AND METHODS

Tissue Processing

The present study was carried out on 12 albino/ICR mice and 8 Long-Evans rats (Harlan, Indianapolis, IN). For microscopy, three mice and two rats per age (P0, P7, P16, adult) were deeply anesthetized with urethane (0.01 ml/g, i.p.), and perfused through the heart with 25-50 ml ice-cold saline solution (0.9%, NaCl) followed by 100-150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, kept at 4°C. Immediately after, the brains were dissected out and postfixed for 2 hours in 4% paraformaldehyde. The brain was kept in 30% sucrose until it sank and was then cut into 40-µm thick transverse sections with a cryostat microtome (Microm, Heidelberg, Germany). Sections were processed for ChAT, GAD and TH immunocytochemistry as described below.

Frontal Cortex Cultures

Frontal cortex cultures were obtained courtesy of Dr. Guenter Gross from the Center of Network Neuroscience, University of North Texas. Cortical tissues from E16 mice were dissociated mechanically and enzymatically (papain), and were seeded onto coverslips whose surface was previously prepared by flaming, polylysine and laminin to improve cell adhesion. The cultures were maintained in Dulbecco's modified minimal essential medium (D-MEM, HyClone, Logan, UT).
supplemented with 5% horse serum (Gibco™, Invitrogen Corporation, Carlsbad, CA) and 5% fetal calf serum (Gibco™) and 1% B27 supplement (Gibco™) in a 90% air/10% CO₂ atmosphere. No antibiotics/antimitotics were used. Cultures were "fed" twice a week with 50% volume exchanges of D-MEM containing 5% horse serum and 1% B27 supplement. Cultures maintained under these conditions can remain spontaneously active and pharmacologically responsive for more than 6 months (Gross, 1994).

Immunocytochemistry

ChAT immunocytochemistry.

The following protocol for ChAT immunostaining was used for mouse and rat, for both in vitro and in vivo studies (Mechawar and Descarries, 2001). In brief, the sections were first incubated in a blocking solution of phosphate buffered saline (PBS) containing 10% (8% for cultures) normal mouse serum (Vector, Burlingame, CA) and 0.3% (0.1% for cultures) Triton X-100, for 30 minutes at room temperature. Sections were incubated in the same solution containing 1-2 μg/ml (1:1000-1:500 dilution) of polyclonal goat antibody against ChAT (AB144P, Chemicon, Temecula, CA) in darkness for 48 hours at 4°C, followed by three 15-minute rinses with PBS. The sections were then incubated in a 1:1000 dilution of biotinylated mouse antigoat antibody (Jackson ImmunoResearch, West Grove, PA) in PBS containing 10% (8% for cultures)
normal mouse serum for 1 hour at room temperature, followed by three 15-minute rinses with PBS. The avidin-biotin complex procedure (ABC Kit, Vectastain Elite; Vector, Burlingame, CA) was then applied (1 hour), followed by three 15-minute rinses with PBS and a 4-minute incubation in a 0.05% solution of 3, 3'-diaminobenzidine (DAB; DAB kit, Sigma, St.Louis, MO) with 1% NiSO₄ or NiCl₂ in PBS to which 0.04% H₂O₂ had been added. In control sections, the primary or secondary antibodies were omitted, which completely abolished the immunostaining.

All sections were mounted onto gelatin-coated slides, dehydrated in graded ethanols, placed into xylenes, and coverslipped with DPX (EM Sciences, Washington, PA). Cultures were coverslipped using an aqueous mounting medium (Aquamount, Vector, Burlingame, CA). For immunofluorescence the secondary antibody incubation was followed by incubation with 1:1000 avidin-bound fluorescein (Vector, Burlingame, CA) for 1 hour in darkness at room temperature. Sections were then mounted on gelatin-coated slides with ProLong mounting medium (Molecular Probes, Eugene, OR). Slides were kept in darkness at 4° C.

GAD immunocytochemistry.

For identification of GABAergic neurons we used GAD₆₅ antibody, which requires the same fixation protocol as the ChAT antibody (Bayraktar et al., 1997) and can be used in combination with ChAT in the same tissue.
Cultures were sequentially incubated at room temperature in: 1) a blocking solution of PBS containing 10% normal horse serum (Vector, Burlingame, CA), and 0.3% (0.1% for cultures) Triton X-100 for 30 minutes; 2) the same solution containing 1 µg/ml (1:1000 dilution) of mouse monoclonal antibody against GAD$_{65}$ (Developmental Studies Hybridoma Bank, Iowa City, IA) for 48 hours, and 3) a 1:1000 dilution of biotinylated horse antibody (Vector, Burlingame, CA), in PBS containing 10% normal horse serum for 1 hour. This was followed by incubation in 1:1000 avidin-bound Texas Red (Vector, Burlingame, CA) for 1 hour. Between the incubations, 15-minute rinses in PBS were applied as described in the previous protocol. For sections we used mouse on mouse kit (M.O.M.$^{™}$) from Vector, Burlingame, CA, catalog number BMK-2202 as follows: 1) 30-minute incubation in 0.3% Triton X; 2) 1-hour incubation in M.O.M.$^{™}$ Mouse Ig Blocking Reagent; 3) 30-minute M.O.M.$^{™}$ Diluent solution; 4) overnight incubation in 1:100 or 1:500 primary antibody in M.O.M.$^{™}$ Diluent; 5) 30-minute incubation in M.O.M.$^{™}$ biotinylated Anti-Mouse IgG reagent followed by incubation in 1:1000 avidin-bound Texas Red for 1 hour. After each incubation, 15-minute rinses in PBS were applied as described in the previous protocol. The immunostained sections were mounted on gelatin-coated slides using ProLong (Molecular Probes, Eugene, OR) and were kept at 4°C.
TH immunocytochemistry.

Both sections and cultures were sequentially incubated at room temperature in: 1) a blocking solution of PBS containing 10% normal goat serum (Vector, Burlingame, CA) and 0.3% (0.1% for cultures) Triton X-100, for 30 minutes; 2) the same solution containing 1 µg/ml (1:1000-dilution) rabbit polyclonal antibody against tyrosine hydroxylase (AB152, Chemicon, Temecula, CA) for 48 hours, and 3) a 1:1000 dilution of biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) in PBS containing 10% normal goat serum for 1 hour. This was followed by the avidin-biotin complex procedure (ABC Kit, Vectastain Elite; Vector, Burlingame, CA) (1 hour), and a 4-minute incubation in a 0.05% solution of 3,3′-diaminobenzidine (DAB; Sigma, Saint Louis, MO) with nickel ammonium sulfate intensification, prepared in PBS to which 0.04% H$_2$O$_2$ had been added. After each incubation, 15-minute rinses in PBS were applied as described in the previous protocol.

For immunofluorescence, we follow the first two steps described above: The sections were sequentially incubated at room temperature in: 1) a blocking solution of PBS containing 10% normal goat serum (Vector, Burlingame, CA) and 0.3% (0.1% for cultures) Triton X-100, for 30 minutes; 2) the same solution containing 1 µg/ml (1:1000-dilution) of rabbit polyclonal antibody against tyrosine hydroxylase (AB152, Chemicon, Temecula, CA) for 48 hours. Then sections were incubated for 1 hour in 1 µg/ml AlexaFluor 546-bound goat anti-rabbit antibody (1:1000) (Jackson ImmunoResearch, West Grove, PA). Tissue was
mounted on gelatin-coated slides with ProLong (Molecular Probes, Eugene, OR). Slides were kept at 4°C in darkness.

Dual staining.

For the demonstration of colocalization of ChAT and GAD or TH, both cultures and cortex sections were reacted as described previously for ChAT, then were blocked, and then were reacted for GAD or TH.

Immunofluorescence stained tissue was co-stained for nuclei using either (1) 2.5 µg/µl Hoeschst 33258 (Sigma, St.Louis, MO), 5 minutes in darkness, at room temperature, followed by three 15-minute PBS rinses (Xia, 1995), or (2) 1:1000 propidium iodide, 5-minute incubation in darkness at room temperature, followed by three 15-minute PBS rinses (Xia, 1995).

Cultures were costained for neurofilament as follows: the cultures were sequentially incubated at room temperature in: 1) a blocking solution of PBS containing 10% normal horse serum (Vector, Burlingame, CA) and 0.3% Triton X-100, for 30 minutes; 2) the same solution containing 1.5 µl/ml (1:750-dilution) of rabbit polyclonal antibody against neurofilament (catalog number AB1983, Chemicon, Temecula, CA) for 48 hours, and 3) a 1:1000 dilution of biotinylated horse anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) in PBS containing 10% normal horse serum for 1 hour. The secondary antibody incubation was followed by incubation with avidin bound fluorescein for 1 hour at
room temperature. Tissue was then mounted on gelatin-coated slides with ProLong (Molecular Probes, Eugene, OR). Slides were kept in darkness at 4°C.

For tissues or cultures immunostained with antibodies tagged with DAB peroxidase, Nissl counterstaining was used. The dried slides were bathed in decreasing concentrations of alcohol (100%, 90%, 70% and 50%) for 2 minutes each, followed by a brief dip in distilled water, then in cresyl violet for 2 minutes or until an appropriate staining intensity was reached. The staining was followed by a distilled water rinse and dehydration in increasing concentrations of alcohol. Slides were then taken through xylenes and coverslipped with DPX mountant.

Data Analysis

ChAT, GAD, and TH were colocalized using a double immunofluorescence technique. The immunofluorescence sections were analyzed using a Zeiss Axioscope 2 microscope™ (Carl Zeiss, Inc., http://www.zeiss.de/micro) and Axiocam HS camera™ (http://www.zeiss.de/us/micro/home.nsf), and the data were recorded using Openlab 3.1.7.® (http://www.improvision.com) software on a MacIntosh® operating system (http://www.apple.com), all courtesy of Dr. Pam Padilla, UNT.

The DAB peroxidase stained tissue was analyzed using a Nikon microscope (http://nikonusa.com), and a camera lucida was used to draw the neurons. The drawings were analyzed using Adobe Photoshop®
(http://adobe.com) and Scion (NIH) software on a Microsoft \textsuperscript{®} operating system (http://www.microsoft.com). The images were recorded with a Sony digital camera and downloaded to Adobe Photoshop.

For \textit{in vitro} studies, data were collected from 10 cultures and statistical analysis was performed using SPSS \textsuperscript{®} (http://www.spss.com/) and Excel software. For \textit{in vivo} studies, data were collected from 10 sections from 3 brains for each age. Adobe Photoshop and Scion software were used to calculate the density of ChAT-positive neurons, defined as the number of ChAT-positive neurons per mm\textsuperscript{2} of tissue. Statistical analysis was performed using SPSS and Excel software.

Means and standard error of mean were calculated and statistical analyses were performed using one-way analyses of variance (ANOVAs and Tukey post hoc tests) and Pearson correlation tests.
RESULTS

Choline Acetyltransferase in Frontal Cortex Culture

ChAT-positive neurons in mouse frontal cortex culture.

The ABC-DAB peroxidase stained frontal cortex cultures were observed using light microscopy at 100X, 200X, and 400X total magnification. The neuronal cell bodies were drawn using a camera lucida at 200X magnification. For each culture, I counted 20 areas (except culture number 1 where 46 areas were counted) of 2.5 mm$^2$ each. The areas counted were selected randomly from regions that lack cell clusters. The spatial coordinates of each counted area were recorded to prevent the recounting. The use of NiSO$_4$ intensification made ChAT-positive neurons readily apparent as dark-brown stained cells. Morphological criteria were used for the identification of non-stained neurons (Fig. 2).

The neurons drawn were counted and their numbers were statistically analyzed. The variables measured were: (1) number of ChAT-positive neurons per mm$^2$; (2) number of non-stained neurons per mm$^2$; (3) total number of neurons per mm$^2$ (sum of previous variables); (4) percentage of neurons that were ChAT-stained per mm$^2$ (number of ChAT-positive neurons X100/total number of neurons).
Figure 2. Immunocytochemistry identifies ChAT-positive neurons in frontal cortex culture. Note ChAT-positive neurons (dark brown) and ChAT-negative cells (light yellow). Observe the intensely stained perykaria and processes.

To analyze the morphology of ChAT-positive neurons, I used triple immunostaining with neurofilament antibody, a specific marker for neurons (Karlsson et al., 1989), and DAPI, specific for nuclei (see Fig. 3).
Figure 3. Frontal cortex cultures: triple immunofluorescence: ChAT (red), neurofilament (NF; green), DAPI (blue). Observe that ChAT (red) is present only in neurons, identified by NF (green). Regions where NF and ChAT colocalize appear yellow. Some neurons did not stain for ChAT (green). All cell nuclei stained for DAPI (blue).

Neurofilament staining shows the presence of neurons as shown by dual immunofluorescence with propidium iodide, a stain for nuclei (see Fig. 4).
Figure 4. Frontal cortex culture; double immunofluorescence: nuclei (red), neurofilament (green). Note the presence of neurons, identified by neurofilament staining (double labeled cells), and glial cells (red).

The cholinergic neurons could be classified morphologically in two categories: 1) large pyramidal-like neurons and 2) fusiform, multipolar and bipolar neurons as depicted in Figure 2. Both types of cells were neuronal as demonstrated by colocalization with neurofilament antibody (see Fig. 3).
Differences between individual cultures.

The number of ChAT-positive neurons per mm$^2$ and the total number of neurons per mm$^2$ were significantly different across cultures ($p<0.001$ for both variables). However, the percentage of ChAT-positive neurons did not differ significantly across cultures, $p=0.064$ (Fig. 5).

![Graph A](image1.png)

![Graph B](image2.png)
Figure 5. A. Total number of neurons and ChAT-positive neurons per mm$^2$ in each culture. B. Percentage of ChAT-positive neurons per mm$^2$ in each culture (Mean ± SEM).

It is important to identify the factors responsible for the differences between cultures. All cultures were harvested from E15 mice, so the age of the embryos could not be evaluated as a factor that might influence the morphology of the cultures. The dissection of frontal cortex and the seeding were performed by a single technician, Anthony Curran, M.S., who used the same seeding and feeding methodology for all cultures during the time interval of our study. We performed histology on some of the brains after he dissected the cortex and we verified that only the cortex was used for culturing, without contamination from striatum. The seeding dates for the cultures were as follows: 10/12/01 for culture 1; 10/19/01 for culture 6; 03/08/02 for cultures 2, 3, 4 and 5 and 05/31/02 for cultures 7, 8, 9, 10. Data analysis did not show a relation between a specific seeding date and the variables analyzed. Consequently, the age of the cultures (days in vitro) was studied to determine if this represented a significant factor influencing the morphology of cultures.
The effect of culture age (div) on the number of cholinergic neurons.

There were 4 cultures at 25 div (cultures 7, 8, 9, and 10), 4 cultures at 76 div (cultures 2, 3, 4 and 5) and 2 cultures at more than 100 div (cultures 1 and 6).

The total number of neurons per mm$^2$ decreased significantly with age ($p<0.05$; see Fig. 6A.). The number of ChAT-positive neurons per mm$^2$ also decreased significantly with age ($p<0.05$; see Fig. 6B). The percentage of ChAT-positive neurons did not vary significantly across different age groups ($p=0.199$; see Fig. 6C).
Figure 6. The effect of cultures age (div) on: A. Total number of neurons per mm$^2$; B. Number of ChAT-positive neurons per mm$^2$ and C. Percentage of ChAT-positive neurons. Shown are the Mean ± SEM.

These decreases in total number of neurons per mm$^2$ and ChAT-positive neurons per mm$^2$ with age of culture and the relatively constant percentage of
ChAT neurons across ages suggest that there may be a correlation between the ChAT-positive neurons and total neurons. The decrease in number of cholinergic neurons may parallel the decrease in the size of neuronal population.

Correlation between ChAT-positive neurons and total number of neurons at different culture ages.

As shown in Figure 7, for each age group (div), there was a significant correlation between the number of ChAT-positive neurons per mm$^2$ and the total number of neurons per mm$^2$ at all ages (25 div: $r=0.895$, $p<0.01$, $n=81$; 76 div: $r=0.661$, $p<0.01$, $n=81$; 100 div: $r=0.881$, $p<0.0$, $n=66$, Pearson correlation test; $n$ represents number of fields of view analyzed per age group).
Figure 7. Relation between the number of ChAT-positive neurons and total number of neurons per mm$^2$ at A. 25 div; B. 76 div; and C. 100 div. Each point represents data from one field of view (2.5 mm$^2$). The numbers of cultures studied were 4 (7, 8, 9 and 10) for 25 div, 4 (cultures 2, 3, 4 and 5) for 76 div, and 2 (cultures 1 and 6) for 100 div.
Across cultures the percentage of ChAT-positive neurons was significantly correlated with total number of neurons at 25 div ($r=0.435$, $p<0.01$, $n=81$), but not at 76 div ($r=-0.11$, $p=0.327$, $n=81$) or at more than 100 div ($r=0.183$, $p=0.142$, $n=66$), as shown in Figure 8.
Figure 8. Relation between percentage of ChAT-positive neurons and total number of neurons per mm² at A. 25 div; B. 76 div; and C. 100 div. Each point represents data from one field of view (2.5 mm²). The numbers of cultures studied were 4 (cultures 7, 8, 9 and 10) for 25 div, 4 (cultures 2, 3, 4 and 5) for 76 div, and 2 (cultures 1 and 6) for 100 div.
Colocalization in cultures

In frontal cortex cultures, dual fluorescence immunostaining showed that ChAT and GAD colocalized in some neurons, as illustrated in Figure 9.

A.

B.
Figure 9. Frontal cortex cultures; triple immunofluorescence labeled for ChAT (green), GAD (red), and DAPI (blue). Some neurons were only ChAT-positive (green), some were only GAD-positive (red) and some neurons showed colocalization of ChAT and GAD (yellow). All cell nuclei stained for DAPI (blue).

While GAD-only labeled neurons were small, bipolar neurons, (see Fig. 9 C), the neurons showing colocalization of GAD and ChAT included both pyramidal, large neurons and fusiform, small neurons. They appeared to be distributed uniformly over the surfaces of the three cultures studied.

In contrast, there was no colocalization of ChAT and TH in frontal cortex cultures, as seen in Figure 10.
Figure 10. Frontal cortex cultures; A. triple immunofluorescence labeled ChAT (green), TH (red), and DAPI (blue), B. dual immunofluorescence labeled ChAT (green), TH (red). No colocalization was observed. All cells’ nuclei stained for DAPI (blue).
TH-positive neurons had small cell bodies that were almost round and had very long processes extending over the culture surface. There were very few in each culture (between 130 and 150 TH-positive neurons per culture, 3 cultures studied, see Fig. 11).

Figure 11. TH immunostaining in a frontal cortex culture. Note TH-positive neurons (dark brown) and TH-negative cells (light yellow). Observe the intensely stained perykaria and processes.
ChAT-Positive Neurons in Developing Frontal Cortex

Cholinergic neurons in developing mouse frontal cortex.

Our study showed well-stained ChAT-positive neuronal cell bodies in mouse cortex. The staining intensity was comparable to that of cholinergic neurons in mouse striatum and in rat cortex and striatum from the present study. In the developing mouse brain at P0 there were no ChAT-positive neurons, and few cholinergic fibers. At P7, faintly stained cholinergic neurons were observed and their number and intensity of staining increased considerably by P16, with maximum number and intensity in adult brain.

In contrast to a previous study (Kitt et al., 1994) in which no ChAT-positive neurons were observed in the adult mouse cortex, this study showed ChAT-positive neurons in the adult mouse frontal cortex, as shown in Figure 12.
Figure 12. ChAT-positive neurons in mouse frontal cortex (A), and mouse striatum (B). ChAT DAB-peroxidase staining with Nissl counterstain.

The majority of observed ChAT-positive neurons were small, fusiform neurons, bipolar and multipolar, with dendrites oriented perpendicularly to the cortical surface, as illustrated in Figure 13. At all ages studied, ChAT-positive neurons were located in mostly in supragranular layers (layers 2-3) and in layer 5.

Figure 13. Mouse frontal cortex ChAT DAB-peroxidase immunostaining with Nissl counterstain. ChAT-positive neurons are bipolar and multipolar.
Cholinergic neurons in developing rat frontal cortex.

ChAT staining was performed in mouse and rat brains at postnatal ages P0, P7, P16 and adult. At P0 there were no cholinergic neurons, just cholinergic fibers; at P7 ChAT-positive neurons were present. They continued to be present at P16 and adulthood. A useful reference for the staining specificity and intensity was to compare neurons in cortex with those in the striatum, which were strongly ChAT-positive in our study (see Fig. 14) and in others (Aznavour et al., 2003). The rat striatum (Aznavour et al., 2003) has ChAT-positive neurons at P0, with small cell bodies and short dendrites confined to the outer half of the neostriatum. Postnatally, their number decreases along with increases in both cell body size and length of dendrites.
Figure 14. ChAT-positive neurons in A. rat frontal cortex, and B. rat striatum. ChAT DAB-peroxidase staining.

In rat, as in mouse, ChAT-positive neurons were small, bipolar and multipolar neurons with neuronal processes oriented perpendicularly to the cortical surface, as illustrated in Figure 15.

At P7, ChAT-positive neurons were located in layers 2-3. At P16 and in adult brain, the number of bipolar ChAT-positive neurons increased in layers 2-3 and they were also found in layer 5 with few in layer 6.

Figure 15. Rat frontal cortex stained with ChAT DAB-peroxidase, Nissl counterstain. ChAT-positive neurons are bipolar and multipolar at P7, P16 and adult brains.

Quantitative analysis of ChAT-positive neurons in mouse cortex.

The number of ChAT-positive neurons per mm$^2$ of the section was determined in 40 µm-thick light microscopy sections as described below. Samples consisted of all ChAT-positive cell bodies located through all layers of frontal cortex in 3-4 sections from each of 3 different animals for each age group.
Somal profiles were drawn with a camera lucida at a total magnification of 100X. The areas of the cortical sections drawn were measured using Adobe Photoshop and Scion (NIH) software. The ChAT-positive cell bodies were counted and their numerical density (the number of ChAT-positive neurons per mm$^2$) was calculated for P7, P16 and adult brains. For each of these ages there were no significant differences across sections, as seen in Figure 16.

Some variability could be caused by differences in the stain intensity on different staining days and by differences in perfusion. However, perfusion strength did not appear to affect the stain intensity at any age group studied. For example, at P0 and P7 the tissue was more fragile compared with the other ages, which was likely due to lighter perfusions, but striatal neurons were strongly ChAT-immunopositive at all ages (see Figs. 12 and 14).
Figure 16. The numerical density of ChAT-positive neurons was not significantly different across the mouse frontal cortex sections at A. P7 (one way ANOVA, p=0.058), B. P16 (p=0.060) or C. adult (p=0.935). Values shown are Mean ± SEM.
The numerical density of ChAT-positive neurons, defined as number of ChAT-positive neurons per mm$^2$, increased with age from no cholinergic neurons at P0 to 9.2 ± 1.4 ChAT-positive neurons /mm$^2$ at P7, to 14.8 ± 1 ChAT-positive neurons /mm$^2$ at P16 (not significantly greater compared to P7, p=0.137). The density of ChAT-positive neurons was significantly greater in adult brain, with 41.6 ± 3.9 ChAT-positive neurons/mm$^2$ (p<0.001; Fig. 17). The number of cholinergic neurons per unit of area in the adult mouse frontal cortex is compatible with previous studies of rat neocortex (see Discussion).

Figure 17. Increase in numerical density of ChAT-positive neurons with age. The number of fields of view (n) were n>>20 for P0, n=72 for P7, n=95 at P16 and n=64 for adult brain. Three brains were used for each age group. (Mean ± SEM).
Does a developmental increase in the density of cholinergic neurons mean that the percentage of cholinergic neurons also increased? The interpretation of this change depends on developmental changes in the numerical density of all neurons.

During postnatal development there is a decrease in the numerical density of neurons in the neocortex. The decrease in numerical density in mouse cortex calculated as density of neurons per unit of volume (Leuba et al., 1977), parallels age-related decreases in the numerical density in rat (Micheva and Beaulieu, 1995). The temporal similarity between development of cortex in mouse and in rat is depicted in Figure 18.

**Figure 18.** Postnatal changes in the numerical density of neurons in rat and mouse cortex based on published data. For rat cortex, the total numerical density was counted through the whole cortical depth (Micheva and Beaulieu, 1995). For mouse frontal cortex, layer-by-layer counts were published by Leuba et al. (1977), and the averages shown in this figure were weighted according to laminar
thickness of each age. The lines of best fit for the two graphs were calculated using Microsoft Excel (for mouse, the formula was $y = 520881x^{-0.3819}$ with $R^2 = 0.7$ and for rat $y = 381068x^{-0.4647}$ with $R^2 = 0.9$).

Considering the density of ChAT-positive neurons relative to the developmental decline in the density of total neurons (Fig. 19), it is apparent that the percentage of neurons that are cholinergic increases considerably during postnatal development.

Figure 19. The developmental trend in numerical density of ChAT-positive neurons (mouse) relative to the developmental changes in total neuron density (mouse and rat). Represented are the average numerical density of ChAT-positive neurons for each studied age and the total neuronal numerical density as shown in Figure 18.
Colocalization of Neurotransmitters in Developing Mouse Frontal Cortex

ChAT and GAD colocalization in developing mouse frontal cortex.

Dual immunofluorescence showed colocalization of ChAT with GAD in mouse frontal cortex at P7, P16 and adult (see Fig. 20).
Figure 20. Mouse frontal cortex dual immunostained for ChAT (green) and GAD (red) on P0 (A), P7 (B), P16 (C) and adult (D). GAD-positive neurons (red) were observed in all layers. There were ChAT-positive neurons and fibers (green) on P7, P16 and adult brain and ChAT-positive fibers on P0.
ChAT and TH colocalization in developing mouse frontal cortex.

Dual immunostaining did not show colocalization of ChAT with TH in mouse frontal cortex at any of the postnatal ages studied, as illustrated in Figure 21.
Figure 21. Mouse frontal cortex dual stained for ChAT (green) and TH (red) at P0 (A), P7 (B) and (E), P16 (C) and (F), and adult (D). There were ChAT-positive neurons and fibers (green) on P7 and P16, and ChAT positive fibers on P0. TH-positive neurons (red) were usually found in the deep layers, 5 and 6.

ChAT and TH were not colocalized in cortical neurons at any of the ages studied.
DISCUSSION

Cholinergic Neurons are Present in Mouse Frontal Cortex in Vivo

Historically, the use of immunohistochemistry for ChAT was an important step in the study of the cholinergic system in cortex. Early studies used acetylcholinesterase (AChE) as a marker, but this is not specific for cholinergic fibers and neurons. Levey et al. (1984) found no cells double labeled for ChAT and AChE in rat cortex and concluded that cortical cholinergic cells are not visualized by AChE histochemistry. This result was confirmed by another study (Le Jeune et al., 1991), which also showed that cortical cholinergic neurons stain for ChAT and not for AChE. Immunostaining for ChAT seems to offer greater morphological details compared with another marker, VAChT, used more recently for cholinergic neurons. While both markers stain the same neurons, ChAT produces a uniform staining of the cell bodies and processes, and VAChT staining is more granular and does not extend into axons (Rico and Cavada, 1998).

The controversy among previous studies regarding the presence of cholinergic neurons in cortex seems to be related to the variety of ChAT antibodies used (summarized in Table 1). Differences in the specificity of antibodies might have contributed to the variation seen among these studies, especially for neurons with low enzyme content, as may be the case with cortical cholinergic neurons (Semba, 2001).
The polyclonal antibody (Chemicon) used in our study shows a good staining intensity in basal forebrain, thalamus and cortex (for references, see Table 3). Our staining results were in agreement with developmental results on rat cortex (Mechawar and Descarries, 2001) and yielded similar quality of staining of striatal neurons in both mouse and rat. The morphology and distribution of ChAT-positive neurons described in rat frontal cortex by this study were similar to those described in other studies (Mechawar and Descarries, 2001; Houser et al., 1985; Schäfer et al., 1998, Chedotal et al., 1994). Therefore, although published descriptions were unavailable for mouse neocortex, it is likely that we correctly identified ChAT-positive neurons in both mouse and rat frontal cortex.

The differences in methods used to count ChAT-positive cell make it difficult to precisely compare our values with those in literature. In adult mouse frontal cortex we found an average of 41.6 ± 3.9 ChAT-positive neurons per mm$^2$. In a different species, rat, Chedotal et al. (1994) found 9.7 ChAT-positive neurons/10$^7$µm$^3$ (all layers, frontoparietal and cingulated cortex) while Houser et al. (1985) reported 11.94 ChAT-positive neurons/10$^7$µm$^3$ in motor cortex and 12.2 ChAT-positive neurons/10$^7$µm$^3$ in sensory cortex, based on an unweighted average of ChAT-positive neuron density for each layer. Dekker and Thal (1993), by counting 5 areas of 1 mm$^2$ each (40 µm thick sections) found, also in rat a density of 39.9 ± 5.1 ChAT-positive neurons/ mm$^2$, comparable with our results for adult mouse brain (41.6 ± 3.9). None of these studies used a stereological method to count cells, and their counts were for the purpose of
comparing the relative number of ChAT-positive neurons across areas or, as in our situation, across different studied ages, rather than to determine their absolute number. Following the calculation technique used by Houser et al. (1985) for 40 µm thick sections, we determined the volume of the sampled regions by multiplying each area by 40 µm. In this way we obtained 10.4 ChAT-positive cells/10^7 µm^3, very close to previously reported densities (Chedotal et al., 1994, Houser et al., 1985). While there is still controversy regarding the relative merits of two-dimensional versus three-dimensional methods of cell counting, both seem to be able to provide estimates of the total number and numerical density of neurons in cortex (Benes and Lange, 2001). However, we performed two-dimensional cell counting and prefer to express our results per unit of surface (mm^2), rather than to introduce the bias involved in calculating the volume.

While the presence of ChAT-positive intrinsic neurons in cerebral cortex was demonstrated in mouse and rat, the functions of these neurons are still unclear. They seem to innervate the intracortical vascular bed (Chedotal et al., 1994), but this is just one of their roles. Le Jeune et al. (1991) consider ChAT-positive neurons likely to be involved in local cortical circuitry. Nishimura et al. (1988) speculate that the axons of ChAT-positive cells project to the contralateral hemisphere.
Boespflug et al. (1986) used a biochemical assay, to show the presence of ChAT activity in dissociated mouse cortex cultures, but they did not observe individual ChAT-positive neurons in these cultures. In the present study, an average of 29.0 ± 9.0 % of observed neurons in frontal cortex cultures were ChAT-positive. This is much larger than the 15% that had AChE activity in rat dissociated cortex cultures by Mesulam and Dichter (1981). The difference in these numbers, however, may be partially due the more specific method of identifying cholinergic neurons used by our study (ChAT immunocytochemistry), as compared with AChE histochemistry used in the previous study.

The number of cholinergic neurons per mm$^2$ decreased with the age of the cultures (see Fig. 6A), as did the total number of neurons per mm$^2$ (Fig. 6B), while the percentage of cholinergic neurons did not change significantly with the age of the culture (Fig. 6C). The decrease in the number of total neurons and ChAT-positive neurons might be related either to astrocytes that promote both the detachment of intrinsic CNS neurons (Gilad et al., 1988) and the decrease of ChAT activity as measured biochemically in rat cortex cultures (Hayes et al., 1991) or to apoptosis that are more evident in earlier cultures compared with late culture (Xu et al., 2004), or, possibly, to both factors. Recent evidence (Shute et al., 2005) demonstrated that astrocytes could be the source of a pro-apoptotic signal to neurons in vitro.
In mouse cortex cultures, astrocytes develop after 5 div and increase progressively in both size and number after 21 div, presumably causing both a reduction in number of neurons and a paucity of neurites (Boespflug et al., 1986). The mechanisms proposed for astrocytic down-regulation of the number of neurons, and presumably including cholinergic neurons, are: (1) physical interference with fiber growth of cholinergic neurons and (2) blockage of neuron-to-neuron interactions (Hartikka et al., 1988). Leprince et al. (1989) also reported effects of astrocytes on neural development, suggesting that astrocytes release a low molecular weight molecule that causes neuron cell death in rat cerebellar and hippocampal cultures. However, recent studies using pure neuronal cultures (less than 1% astrocytes) contradict these results by reporting a favorable action of astrocytes on the development of cultures. Astrocytes have been shown to induce synapse formation and maturation of neurons in vitro (Nägler et al., 2001; Ullian et al., 2001; Fields and Stevens-Graham, 2002, Pfiegler and Barres, 1997) and may provide some positive, supporting factor for cholinergic neurons (Nelson et al., 1997).

Development of Cholinergic Neurons in Mouse Frontal Cortex

Our study demonstrated that cholinergic neurons in mouse frontal cortex developed in the postnatal period with a timeline comparable to that in rat. At birth (P0) there were no cholinergic neurons visible in the frontal cortex in either mouse or rat, and the development of ChAT-positive neurons during the first
three postnatal weeks was similar in both species. In our study, ChAT-positive neurons were identified for the first time at P7, and their number and staining intensity increased subsequently with age. The numerical density of ChAT-positive neurons was highest in the adult brain. Our results concur with biochemical studies showing that ChAT activity levels were very low until P6, thereafter increasing steadily to adult levels (Höhmann et al., 1985; 1988). However, measurements of ChAT activity levels in whole pieces of cortex cannot differentiate between ChAT from intrinsic cholinergic neurons and ChAT innervation from external sources such as basal forebrain.

During postnatal development of mouse frontal cortex, the numerical density of ChAT-positive neurons, measured as number of ChAT-positive neurons per mm$^2$, did not increase significantly between P7 and P16, but increased significantly between P16 and adulthood (see Fig. 17). These results are not dissimilar from previous biochemical measurements in mouse cortex showing no measurable ChAT activity levels until P6 followed by a steady increase in ChAT levels reaching 40% of adult activity by P18 and adult levels at the end of second postnatal month (Höhmann and Ebner, 1985).

Our study is the first to characterize the postnatal development of intrinsic cholinergic neurons in mouse cortex. The only ChAT immunohistochemistry study on developing mouse cortex did not study the postnatal development beyond P2 and shows ChAT-positive cells as early as E14 in germinal zones; on E16 they are present on the prelimbic cortex and parietal cortex where they are still observed on P2 (Schambra et al., 1989).
In parallel with the mouse study, we did a rat study primarily to substantiate our staining methodology. Our observations in the rat frontal cortex were comparable in some respects to those of Mechawar and Descarries (2001) where cholinergic neurons develop early and rapidly. On P0 there are no ChAT-positive neurons, and on P4 few faintly stained interneurons are visible. On P8 all cortical layers display many strongly ChAT-positive neurons, and their number increases with age to P16 showing at P32 the adult distribution. However, Mechawar and Descarries (2001) described the presence of ChAT-positive neurons over all cortical layers of developing and adult rat cortex, while we observed developmental changes in laminar distribution of ChAT-positive neurons. On P7 in both mouse and rat cortex ChAT-positive cells were restricted to layers 2-3, while at P16 and in adult brains ChAT-positive neurons were present mainly in layers 2-3, but also in layer 5 with few in 6.

As shown in Figure 18, the postnatal development of rat and mouse neuron density shows parallel developmental patterns in cortex. Thus it is reasonable to compare the postnatal development of cholinergic neurons in rat and mouse using the same time-line for both.
Colocalization Studies

In our frontal cortex cultures, ChAT colocalized with GAD but not with TH. In our in vivo study dual-labeled neurons appeared to include fusiform, bipolar, multipolar, and pyramidal-type form. The only attempt to colocalize ChAT with GABA-phenotypes *in vitro* was done by Mesulam and Dichter (1981) using AChE histochemical staining and GABA autoradiography. However, AChE is not useful as cholinergic marker (as discussed previously).

Our *in vivo* observations showed also ChAT and GAD colocalization, and did not show ChAT colocalization with TH. Some immunocytochemical studies report colocalization of GABA and ChAT, at least in rat cortex, where 50% (Kosaka et al., 1988) to 88% (Bayraktar et al., 1997) of ChAT-positive neurons contain GABA. On the other hand, Gritti et al. (1993) could not find any colocalization in rat cortex. The immunocytochemistry techniques used in these studies were either diaminobenzidine (DAB) immunohistochemistry for both GABA and ChAT with examination of paired surfaces of adjacent sections (Kosaka et al., 1988, Bayraktar et al., 1997), or sequential processing of the same sections with DAB and benzidine dihydrochloride (BDHC) as described by Gritti et al. (1993). The difference between the investigations is difficult to explain; most likely it is due to technical differences, such as the use of different ChAT and GABA antibodies.

Dual immunofluorescence is recognized as a better way to colocalize two different antigens, and our study offered this advantage. The ChAT antibody
used here exhibited the same pattern of immunostaining for cholinergic neurons in rat brain as described in other published results. We also used a GAD antibody that shows a better specificity for identification of GABAergic neurons as compared to GABA antibodies (Okamura et al., 1989).

The different morphology of TH-positive versus ChAT-positive neurons as well as their different distribution in cortex supports the lack of colocalization of TH with ChAT. Regarding TH-positive neurons, our study described both in vitro and in vivo a completely different morphology compared to ChAT-positive neurons. TH-positive neurons appeared to be stellate with multiple long processes. In contrast, ChAT-positive neurons were either bipolar or pyramidal shaped in cultures, and bipolar, multipolar and pyramidal-like in frontal cortex. In mouse cortex, TH-positive neurons are located predominantly in deep layers, mostly layers 5 and 6 (Satoh and Suzuki, 1990), while ChAT-positive neurons were distributed predominantly in layers 2-3 and 5. About 30-50% of cultured neurons in dissociated cortex cultures express a GABA phenotype (Mesulam and Dichter, 1981). The number of TH-positive neurons was much lower (less than 150 neurons per culture) (our observation) while Iacovitti and his coworkers (1987) reported, at 21 div, an average of only 20 TH-positive neurons per culture.

In conclusion, our study represents an important step in understanding the development of cortical cholinergic neurons. While these neurons are well characterized in rat cortex by both ChAT and VACht immunocytochemistry and in situ hybridization, little is known about the presence, development and morphological aspects of these neurons in mouse cortex. For a long interval of
time, intrinsic cortical cholinergic neurons were considered absent in mouse (Kitt et al., 1994) and just recently their presence was confirmed (Descarries et al., 2005). Using ChAT immunocytochemistry technique, our study demonstrated the existence of ChAT-positive neurons in mouse frontal cortex and in dissociated frontal cortex cultures. Furthermore, we studied the postnatal development of these neurons in mouse cortex. In mouse frontal cortex ChAT-positive neurons were identified for the first time at P7 and their numerical density increased with age reaching the highest density in adult brain. *In vitro*, our research showed a constant percentage of ChAT-immunostained neurons for the ages studied. We colocalized ChAT with GAD in both cultures and cortex, while ChAT and TH did not colocalize in both *in vitro* and *in vivo*. 
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