Differential Expression of Acetylcholinesterase in the Developing Barrel Cortex of Three Rodent Species

Acetylcholinesterase (AChE) is transiently expressed in several immature axon systems. Its presence in developing thalamocortical afferents has led to the use of enzyme histochemistry to visualize this axon system in rats. Because of the spatiotemporal distribution of the enzyme in the rat neocortex, it has been suggested that AChE plays a role in the establishment of thalamocortical connectivity.

We show here that AChE is distributed in a pattern that is markedly different in SI cortex of rats as compared to that of mice and hamsters. In rat pups, AChE-rich patches are distributed in a vibrissa-related array in the SI cortex soon after birth, whereas regions of cortex that lie between individual patches, and between rows of patches, are impoverished in the enzyme. In contrast, sections through flattened cortices from PND3 and older mice and hamsters reveal lightly stained, AChE-positive spots in the center of barrel cores, while barrel walls remain devoid of AChE; septa that divide individual barrels are densely enzyme positive. Differences in laminar localization of the enzyme for all three species are also visible.

In the thalamus of postnatal rats, both the ventral posterior medial (VPM) and ventral posterior lateral (VPL) nuclei express AChE, correlating with the presence of enzyme-containing patches throughout the barrelfield cortex. In the other two rodents, however, the enzyme is present in VPL but not in VPM, despite the fact that in these species the cortical barrels associated with both thalamic nuclei have very little of the enzyme. Thus, the relationship between the distribution of AChE in nuclei of the thalamic ventrobasal complex and the presence of AChE in the terminals of their cortical axons in the barrelfield is not consistent across different rodent species.

Our results call for caution in the use of AChE histochemistry as a universal marker for immature thalamocortical axons, and challenge the generality of currently hypothesized roles for this transiently expressed enzyme during the development of the rodent thalamocortical projection.

Acetylcholine esterase (AChE), an enzyme commonly known for its function in degrading the neurotransmitter acetylcholine, is also localized in regions of the developing nervous system that are not necessarily associated with cholinergic transmission (Kostovic and Goldman-Rakic, 1983; Kostovic and Rakic, 1984; see reviews by Robertson, 1991; Robertson and Yu, 1993). More than a decade ago, Kristt (1979, 1983, 1989) showed that high levels of AChE are present in the ventrobasal thalamus and barrel cortex of perinatal rats and that the enzyme is transiently distributed in whisker-related cortical patches during development. He also demonstrated that this patchy distribution of the enzyme is transient, and is no longer detectable in the adult animal. Since Kristt's original work, more sensitive histochemical methods for visualizing the presence of AChE have been introduced (Bear et al., 1985). However, in relation to the detailed study of thalamocortical afferentation, the newer techniques have primarily made use of the rat as an animal model. For instance, Robertson and his colleagues have shown that in the developing rat neocortex, the primary visual, auditory, and somatosensory areas are AChE positive, as are many of the thalamic nuclei that project to them (Robertson et al., 1989, 1991; Kageyama et al., 1990; but see Kristt and Waldman, 1982; Robertson et al., 1989). Localization of the enzyme to the projection fields of thalamocortical axons, as well as the marked decrease in cortical enzyme levels following lesions of thalamic nuclei, indicate that much of the AChE in cortical layers III-IV in the rat derives via thalamic afferents (Robertson et al., 1988a,b). This is further supported by ultrastructural studies showing that AChE is present in the granular endoplasmic reticulum of thalamic neurons and that the enzyme is localized in the cortical neuropil, on the extracellular phase of membranous profiles surrounding putative thalamic axons (Robertson and Yu, 1993). A recent report also documents an early expression of the enzyme in thalamic sensory nuclei, at times when these nuclei are just beginning to form and as their constituent cells are starting to emit axons towards the neocortex (Schlaggar et al., 1993).

Collectively, these reports have led to the suggestion that AChE has an important role in the formation of thalamocortical connectivity (Robertson, 1987, 1991; Robertson and Yu, 1993). However, for any such role to be generally true, one would expect that the enzyme have a similar distribution in all species that share a similar cortical organization. For example, if AChE has a specific function in the development of connectivity in the barrel field cortex, the overall pattern of enzyme distribution should be the same in the primary somatosensory cortex of rats, mice, and hamsters, three rodent species in each of which barrels develop within the first few days after birth. In order to test whether this is so, we undertook a comparison of the maturational expression of AChE in the SI cortex of these three rodent species with a view towards highlighting the similarities or differences in the patterns of enzyme distribution amongst them. We show that AChE is expressed in a pattern that is similar in the mouse and hamster, but that enzyme distribution in the rat barrel field cortex is markedly different. Moreover, the spatial distribution of the enzyme in the ventral posterior thalamic nucleus (VP) of the three rodents does not bear a consistent relationship to whether or not AChE-positive patches overlie cortical barrels associated with its medial (VPM) and lateral (VPL) subdivisions. Our results indicate that the roles suggested for AChE during the development of connectivity in the rat somatosensory cortex cannot be generalized to other rodent species without significant remediation.

Materials and Methods

Data reported in this study were collected from five litters of mice (CD-1; Charles River Laboratories), four litters of rats (Sprague-Dawley; Taconic Farms, Germantown, NY, and Harlan, IN), and six litters of hamsters (Syrian golden; MIT breeding colony) as well as from two adult animals of each species. Day of insemination (plug date, sperm positivity, timed breeding) was designated as E0. The day of birth is designated as PND0 for all animals.

Pups aged PND 0, 1, 2, 3, 5, 8, and 10, as well as adult animals were overdosed with sodium pentobarbital and perfused transcardially with cold heparinized saline, followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brains were removed and post-

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fixed overnight at 4°C. Cortices from two or more animals at each
time point were removed, flattened between glass slides, and cry-"oprotected. The tissue was cut parallel to the pial surface at a thick-"ness of 60 μm. Forebrains from other animals (at all time points)"were cut in the coronal plane. All sections were mounted on gelatin-subbed slides and air dried.

We followed the protocol for AChE histochemistry described in
Bear et al. (1985), implementing two modifications: (1) the preincu-"bation step involving iso-OMPA to block nonspecific staining was"omitted (such an omission has little effect on the pattern of staining;"Schlaggar, personal communication); and (2) we used a longer time("18–20 h; Schlaggar et al., 1993) for immersing sections in the sub-"strate incubation solution containing 0.002 m copper sulfate, 0.115%"acetyltiocoholine iodide, 0.078 m magnesium chloride, 0.002 m cop-"per glycine, 24% sodium sulfate, in 0.05 m maleate buffer (at 37°C,"pH 6.0). Slides were transferred to 20% and 10% solutions of sodium"sulfate for 5 min and 1 min, respectively, washed in distilled water,"and developed for 1–10 min in 4% ammonium sulfide in 0.1 m phos-"phate buffer and perchloric acid (pH 6.0). After washing, tissues were"fixed in 4% buffered paraformaldehyde (20 min), rinsed again, toned"with 0.2% gold chloride (5 min), immersed in 5% sodium thiosulfate"(5 min), and after a final rinse, the sections were coverslipped using"a glycerol jelly as the mounting medium. Selected coronal sections"had coverslips removed after the AChE staining pattern was photo-
graphed, and the tissue was counterstained with cresyl violet to de-
lineate cortical laminar boundaries.

For Dil labeling of thalamocortical axons, paraformaldehyde-fixed"brains from mice aged PND 2, 3, 4, and 5 were used. The brain was"blocked transversely just rostral to the pretectum, to expose the di-
encephalon from its caudal aspect. A small crystal of Dil (1,1-dioc-
tadecyl 3,3,3-tetramethylindocarbocyanine perchlorate; Molecular"Probes, Eugene, OR) was placed in the ventrobasal complex. The"brains from mice aged PND 2, 3, 4, and 5 were used. The brain was"blocked transversely just rostral to the pretectum, to expose the di-
cencephalon from its caudal aspect. A small crystal of Dil (1,1-dioc-
tadecyl 3,3,3-tetramethylindocarbocyanine perchlorate; Molecular"Probes, Eugene, OR) was placed in the ventrobasal complex. The"tissue was kept in 4% paraformaldehyde at 37°C for about 2 weeks.

AChE-positive axons in the mouse and hamster are, for the most part, confined to the white matter; a few"barrels associated with whiskers and peri-oral sinus hairs re-
main AChE negative throughout development, as do more
dorsomedial and anterior barrels, associated with the paws. Supragranular layers have low levels of enzyme during early postnatal life but a transient increase in staining is seen in these"laminae between PND 5 and 8 in the mouse and between"PND 8 and 10 in the hamster (Figs. 2, 4); in hamsters, this"enzyme expression subsides thereafter, whereas in mice, the"supragranular layers retain a relatively high level of AChE into"adulthood.

Although layer V in rats becomes increasingly more AChE"positive and layer VI remains low in AChE, the pattern of enzyme"localization in infragranular layers is somewhat more complex in the other two rodents. In the mouse, a dense band of AChE staining located at the border of layers IV/V (just below where cytoarchitectonic the barrels form) becomes more pronounced during the second postnatal week; over time, this band gets thinner but it is still definable in adult-
hood (Fig. 2, AD M). Also, in contrast to the rat, layer VI is"densely stained throughout development in the mouse, but is"much less so in the adult. Enzyme localization in the hamster"shares features with both rats and mice. As for the mouse, a
dense AChE-containing band is present in the lower part of"layer IV/upper layer V during the first postnatal week in ham-
sters. However, in contrast to the mouse, all of layer V be-
comes AChE positive after PND8 in the hamster. Thus, overall,"the adult mouse exhibits high levels of AChE in the upper"cortical layers, whereas in the mature hamster the enzyme is"present mostly in the deep layers.

Sections Parallel to the Pial Surface
Periphery-related patterns in SI cortex can be more clearly visualized in sections that are cut tangential to the cortical surface (Fig. 5). In rats, AChE-containing patches overlie the"barrels, whereas regions between individual barrels and be-
 tween whisker-related rows, as also the agranular cortex sur-
rounding the barrels, do not express the enzyme. In mice and hamsters, AChE staining in layer IV gives an overall negative image of that seen in rats: for the most part, barrels are low in"enzyme, whereas the septae and agranular cortical areas become progressively more AChE positive over time, thus out-
lining individual barrels (Fig. 5). (Note, however, that the"bands of staining along septae surrounding each barrel in the"mouse and hamster are much thinner than the enzyme-neg-
ate regions between barrels in the rat.) In mouse SI cortex,"faint spots of AChE staining are present in barrel cores as of"PND5 (Fig. 5). Similar spots can also be identified in the ham-
ster, but they are much lighter and are difficult to document photographically. They are just barely visible in Figure 5 (P10 H).

Comparison of Pattern Formation by Thalamocortical Axons Seen with Dil Labeling and with AChE Staining in the Mouse Cortex
A possible explanation for the differences in enzyme staining patterns between the mouse (or hamster) and the rat is that in the strain (CD1) of mice that we use the thalamocortical projection may develop along a significantly different schedule than that reported for rats, or from the time course already
Figure 1. AChE staining patterns in the barrelfield cortex of the developing mouse (M, left column), rat (R, middle column), and hamster (H, right column). On the day of birth, an enzyme-positive band is present in the lower part of the cortical plate in rats (PO R). Over the next few days, this band becomes segregated into patches (P3 R, between arrowheads). The equivalent region in the mouse and hamster cortex is impoverished in AChE (regions between arrowheads in left column and right column). At progressively older ages the patches in layer IV of the rat cortex become less distinct as does the band of low enzyme expression in the mouse and hamster cortex. See text for detailed description of laminar differences between the three rodent species. All micrographs are oriented with dorsal to the left and lateral at the top. Scale bar, 500 μm.
Figure 2. See Figure 1 caption.
described for Swiss Webster mice (Senft and Woolsey, 1991). To check for this, we labeled thalamic efferents in CD1 mice by placing a crystal of DiI in the ventrobasal thalamic nucleus of postnatal animals. On PND2, DiI-labeled thalamocortical axons already delineate a face representation area, and over the next couple of days, the patterned representation of whisker-related rows, and then of whisker-specific patches, can be identified (data not shown). On PND4, the thalamocortical projection pattern is adult like (cf. Senft and Woolsey, 1991; Agmon et al., 1993). DiI-labeled patches occupy a larger extent of each barrel than do the AChE spots; evidently, the thalamic afferents are not confined only to barrel cores (where enzyme spots are present), but cover the entire tangential extent of individual barrels.

AChE Staining in the Ventrobasal Complex of the Thalamus
The ventral posterior thalamus of all rodents is divided into VPM, which receives trigeminal input, including sensory information from the vibrissa pad, and VPL, which receives in-
Figure 4. High-magnification photomontages to compare AChE staining patterns in the barrel cortex of PND5 mouse (A), rat (B), and hamster (C). Coronal sections, same as those shown in Figure 3. Photomontages from mouse, rat, and hamster are aligned such that the bottom of layer IV aligned is at approximately the same level. Note the lack of enzyme-containing patches in layer IV of mouse and hamster. Laminar borders are indicated on each montage (compare lower magnification views in Fig. 2). wm, cortical white matter. Scale bar, 500 µm.

Discussion

Our results document that a complex expression of AChE prevails in the developing somatosensory cortex of rodents and that specific differences exist in the laminar and tangential localization of the enzyme in neonatal mice and hamsters versus that in rats. The issue under debate concerns the extent to which such comparisons can elucidate the role of AChE in the developing cortex.

The central trigeminal system, in particular that aspect of

put from dorsal column nuclei. In the perinatal rat, AChE staining is very dense in both of these cell groups (Fig. 6; see also Kristt, 1983; Robertson et al., 1989; Schlaggar et al., 1993). Barreloids (the whisker-representation modules of the diencephalon) in the rat thalamus can be identified with enzyme histochemistry after PND3 (arrowheads, Fig. 6). Correlated with this staining pattern, the vibrissa-specific barrels (associated with VPM), as also the nonwhisker barrels (associated with VPL) are rich in AChE. In contrast, in both the mouse and hamster, VPM is impoverished in AChE at all postnatal ages and VPL contains high levels of the enzyme (Fig. 6; see also, Robertson et al., 1989), despite the fact that cortical barrels associated with both nuclei have low levels of enzyme (data not shown).
it which subserves the neural integration of whisker-related activities, is highly specialized in all three rodent species included in this survey. Members of each species exhibit whisking behavior, and each whisker is represented by a modular organization of afferents, postsynaptic cells, glia, and extracellular matrix elements along the trigeminal neuraxis (which includes the brainstem trigeminal nuclei, ventrobasal thalamus, and primary somatosensory cortex) (reviewed in Steindler et al., 1989; Woolsey, 1990; Jhaveri and Erzurumlu, 1992; Schlaggar and O'Leary, 1993). In the cortex, thalamic afferents segregate into whisker-specific patches before the barrels develop; these events occur during early postnatal life, even though the gestation times are quite different in each of these species (15.5 d postconception for hamsters, 19 d for mice, and 21–22 d for rats). It is possible that extra-uterine activation of vibrissae is necessary for the final consolidation of the cortical pattern.

In both mice and rats there is clear evidence that glutamate/aspartate, and not acetylcholine, is the neurotransmitter which subserves the thalamocortical connections (Ottersen et al., 1983; Ottersen and Storm-Mathisen, 1984; Agmon and O'Dowd, 1992; Kharazia and Weinberg, 1994). Choline acetyltransferase (the enzyme necessary for synthesis of acetylcholine) is not detectable in sensory thalamic nuclei of immature or adult rats (Brownstein et al., 1975; Hoover et al., 1978; Armstrong et al., 1983; Hauser et al., 1983) lending support to the claim that thalamocortical projection neurons are not cholinergic. Yet during development, these thalamic nuclei stain intensely for AChE (the enzyme known to be involved in the hydrolysis of acetylcholine) (Kristt, 1983; Robertson et al., 1989; Schlaggar et al., 1993). The expression of AChE in immature thalamic neurons, its presence in the cortical projection zones of these nuclei, and its virtual disappearance from thalamo-recipient zones of the neocortex after

Figure 5. Patterning of periphery-related modules in the flattened SI cortex of mouse, rat, and hamster (each animal on ages PND 5, 8, and 10) as revealed by AChE histochemistry. In all three rodents the aggregation of layer IV cells into barrels has already occurred by this time. AChE-positive patches occupy barrel centers in the rat (middle column, R). In the mouse (left column, M), barrels are for the most part devoid of the enzyme, except for light AChE-containing spots visible over barrel cores. Similar spots are present in the hamster (right column, H) but are much lighter (see, e.g., P10 H). In both mice and hamsters, dense staining is present along septae separating individual barrels. Scale bar, 500 μm.
Figure 6. AChE staining in the ventrobasal complex of PND5 mouse (A), rat (B), and hamster (C). In the rat, both components (VPM and VPL) of the ventrobasal complex are AChE positive; rows of barreloids can be identified (arrowheads), although individual whisker-specific patches are not clear in this section. In the other two species, AChE-positive barreloids (arrowheads) are visible in VPL (asterisk, but VPM remains devoid of enzyme. IgD, dorsal nucleus of the lateral geniculate body. Scale bar, 500 μm.

diencephalic lesions all indicate that much of the AChE localized in developing layers III/IV of sensory neocortex derives primarily from neurons in the thalamus. (Although cells of the basal forebrain are a rich source of acetylcholine to the cortex, their axon terminals are diffusely distributed over the cortex during development, and have a complementary pattern of termination relative to the thalamic projections in the mature animals) (Robertson et al., 1988b). These observations have led to the suggestion that AChE of thalamic origin may serve an important function in the establishment of synaptic connectivity between thalamus and cortex.

AChE as a Marker for Developing Thalamocortical Axons

A comparison of the cortical pattern obtained by labeling thalamic axons with Dil versus that derived by AChE staining jeopardizes the validity of using AChE histochemistry to visualize thalamocortical afferents in all rodents. In the rat, virtually all of the AChE in layers III/IV of the developing cortex obtains from thalamic cell groups (reviewed in Robertson and Yu, 1993), and in fact, AChE histochemistry, is commonly used to identify the distribution of thalamocortical axons. There is good congruence between the areal spread of thalamic axons, as seen with Dil labeling (Erzurumlu and Jhaveri, 1990), and enzyme-positive patches visualized with AChE histochemistry (Schlaggar and O'Leary, 1993), confirming that enzyme histochemistry provides a valid technique for visualizing thalamic axons in the developing rat. Not so for the mouse (or hamster). Dil application in the ventrobasal thalamus of the mouse (present study, also, Senft and Woolsey, 1991) discloses an organization of thalamocortical projections that resembles that seen in the rat, whereas AChE histochemistry reveals a markedly different pattern, demonstrating that enzyme histochemistry does not target (all) thalamocortical afferents. The AChE-positive spots in barrel centers of mice (and of hamsters) could indicate that only a subset of thalamic fibers express the enzyme, but the staining of septae in these two species suggests that at the very least, AChE in layer IV is also expressed in elements other than in ventrobasal fibers. Further experiments involving thalamic lesions in mice or hamsters are needed to show which, if any, aspects of the histochemical reaction products in the cortex have their origin in diencephalic cell groups for these species. Such a species difference in enzyme distribution has also recently been reported by Rhoades et al. (1994).

Kristt and Waldman (1982) have also shown a difference in the pattern of AChE staining in the barrelfield of (Swiss Webster) mice, as compared to that in rats. However, in their mice, lightly stained AChE-positive fibers are present in layer IV of the mouse cortex prior to PND5, and an increase in AChE activity occurs in barrel hollows around PND11. Over the next week, this AChE expression becomes limited to the superficial part of layer IV in their mice, whereas in the deeper layers only the septae are enzyme positive. This course of events differs in several aspects from that documented in our results (Figs. 1, 2, 4, 5) and raises the possibility of strain-specific variations in the distribution of the enzyme.

Putative Role(s) for the Transiently Expressed AChE in Developing Thalamocortical Pathways

Because of its known function in the breakdown of acetylcholine at the neuromuscular junction and at other cholinergic synapses of the central nervous system, AChE has been the subject of many studies; its genetics, biochemistry, and pharmacology have been intensely explored, and much information is available with regard to its physiological function (e.g., see Massoulia et al., 1991; Juliano and Jacobs, 1995). However, despite the enormous amount of effort focused on the study of this molecule, its function during the time it is transiently expressed along noncholinergic pathways remains an enigma. As a result of the work of Kristt and co-workers (1979, 1983, 1982), and more recently from Robertson and colleagues (1991, 1993), interest has converged specifically on the potential role of the enzyme during the development of

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that the mechanisms by which thalamocortical connectivity thalamocortical projections in sensory systems. In a recent review, Robertson and Yu (1993) advanced three hypotheses with regard to how AChE might contribute to the establishment of thalamocortical connectivity. Below we discuss these three hypotheses in the context of our comparative observations on the developing rodent trigeminal system.

AChE serves to destabilize temporary connections formed by thalamic axons with subplate cells, thereby allowing them to continue growing into their target cells in layer IV and establish appropriate synapses (Robertson and Yu, 1993). We cannot directly address this possibility, since we do not know whether there is a differential expression of the enzyme in thalamic axons of the three rodent species as these axons are coursing through the subplate. Nonetheless, the persistent expression of AChE in thalamic axons throughout at least the first 2 weeks of postnatal life begs an alternative or additional role for the enzyme in the immature barrel cortex.

In sum, the hypotheses advanced by Robertson and Yu are attractive, but the differential enzyme localization in the three rodent species indicates that either AChE has functions relevant to species other than those proposed, or that its role(s) in the development of thalamocortical connectivity is distinctly different in rats versus mice and hamsters. The former possibility is worth considering further. If we are to accommodate the differential distribution of the enzyme in each of the three species examined here with a universal role of AChE in cortical development, it is necessary to find an aspect of barrel cortex organization that is manifest during early postnatal life, and that is similar (though not the same) in mice and hamsters, but is uniquely different in rats. Within this framework, it should be noted that comparative studies on the structure of the cytoarchitectonic aggregates that comprise cortical barrels do show species-specific differences; barrels in the mouse have cell-sparse cores (hollows), cell-dense walls, and distinct septae; in the rat, barrel cores (centers) are cell dense, and in the hamster neither barrel cores nor barrel walls show distinct cellular densities (Welker and Woolsey, 1974; Rice et al., 1985). Thus, the presence of AChE-positive patches in the rat, the enzyme-containing septae and barrel cores in mice, and the low level of enzyme expression in hamster barrels may somehow relate to the way in which granule cells become clustered in the immature SI cortex of these three species. Such a relationship would necessitate that AChE be able to mediate cell-cell adhesions. There is mounting evidence from developmental studies that lends credence to this possibility. For instance, cholinesterases appear in well-defined areas of prechordal mesoderm, characteristic of cholinergic innervation of the developing neuroepithelium during embryogenesis; AChE as well as butyrylcholinesterase are expressed by postmitotic neurons of the early neural tube, prior to the time any synapse-related function can be attributed to the enzymes (Drews, 1975; Miki and Mizoguti 1982; Layer, 1983; Mizoguti and Miki, 1985; Layer et al., 1987; Krejci et al., 1991; Schlaggar et al., 1993). AChE is transiently expressed in non-neural embryonic tissues such as the lens and the chondrogenic core of the embryonic chick limb bud, disappearing after the end of organogenesis (Drews and Drews, 1973; Layer and Sporns 1987). And finally, the presence of a carbohydrate epitope resembling that on HNK-1 (a cell-adhesion protein) has been reported for some forms of AChE from electric organs (Bon et al., 1987). Taken together, these points suggest that the enzyme may, indeed, have a function related to adhesive cell-cell interactions.

Whether this is after all the function subserved by the transiently expressed AChE in the immature barrel cortex can only be determined by further experimentation. Such a possibility is, however, supported by the results of Hohmann et al. (1991a,b) that the cytoarchitecture of the developing cor-

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Notes

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