Age-Related Loss of the AMPA Receptor Subunits GluR2/3 in the Human Nucleus Basalis of Meynert

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Magnocellular cholinergic neurons in the basal forebrain have long been recognized as vulnerable to the pathology of Alzheimer’s disease. Despite numerous anatomical, pharmacological, behavioral, and physiological investigations of these neurons the cellular mechanism that underlines their selective vulnerability remains unclear. As part of an ongoing investigation into the molecular mechanism(s) underlying neuronal vulnerability in Alzheimer’s disease and normal aging, we employed immunocytochemical techniques and examined the cellular localization of the α-amino-3-hydroxy-5-methyl-4-isoaxolepropionate (AMPA) glutamate receptor subunits GluR1 and GluR2/3 in the basal forebrain of eight nondemented elderly human subjects (66–102 years). For each case we observed GluR1-positive magnocellular cells darkly labeled within all main divisions of the basal forebrain (Ch1–Ch4). Double-labeling immunohistochemical techniques confirmed that the overwhelming majority (94%) of these neurons were also positive for the p75NGFr antibody, thus substantiating the cholinergic nature of these neurons. In contrast, GluR2/3 immunolabeling upon magnocellular neurons was relatively faint or nonexistent. The latter observations were most apparent in cases of advanced age and in the posterior part of the nucleus basalis of Meynert (NBM) (i.e., Ch4). In addition to the eight elderly subjects, we examined GluR1 and GluR2/3 immunostaining in the NBM of five younger cases, 5, 33, 36, 47, and 48 years of age. Although practical considerations limited our observations to the Ch4 region, we observed both GluR1 and GluR2/3 labeling upon NBM neurons in this latter region. On average, the distribution of labeled cells and intensity of immunoreaction were comparable between GluR1 and GluR2/3. The presence of GluR2/3- and GluR1-labeled neurons in the Ch4 region of younger cases but primarily GluR1 in cases of advanced age suggests an age-related decrease in GluR2/3. Functionally, the loss of GluR2 from the AMPA receptor complex results in ion channels highly permeable to Ca$^{2+}$. These alterations in cation permeability of the AMPA receptor together with the occurrence of a number of other intrinsic and extrinsic events (i.e., decrease Ca$^{2+}$-binding protein) likely contribute to the vulnerability of these neurons in aging and in AD.

Key Words: glutamate; excitotoxic; AMPA; receptor; cholinergic; basal forebrain.

INTRODUCTION

In human brain, different populations of neurons are known to be selectively vulnerable to pathologic insults. For example, in Alzheimer’s disease (AD), cholinergic neurons of the magnocellular basal complex (MBC) are severely affected and undergo marked atrophy as well as cell death (47). During normal aging it too has been reported, although the topic remains controversial, that these cells degenerate and/or die, yet to a lesser extent than in AD (27, 24, 22, 8, 41). Whereas the exact mechanism underlying the selective vulnerability of these neurons in AD and possibly in the aged brain remains unknown, it has been hypothesized that the overstimulation of specific glutamate receptors may contribute to neuronal cell death via a glutamate receptor-mediated rise of intracellular Ca$^{2+}$. In support of a role for glutamate are studies that have shown that cholinergic cells of the basal forebrain are particularly susceptible to the excitotoxic effects of selective glutamate agonists (45). For example, whereas infusions of NMDA destroy mainly neurons in the dorsal and ventral pallidum, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) preferentially affects cholinergic basal forebrain neurons (33). Consequently, AMPA has been widely used as a potent excitotoxin to produce lesions of the basal forebrain.

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An essential feature of excitotoxic-mediated cell death is the destabilization of intracellular Ca\textsuperscript{2+} homeostasis, due to increased flow of Ca\textsuperscript{2+} through the ion channel (32). Notably, Ca\textsuperscript{2+} permeability of AMPA-gated receptor channels depends upon the subunit composition of the assembled glutamate receptor (14). These receptors are composed of different combinations of GluR1–4 (GluRA–D) receptor subunits (6, 20). The GluR2 receptor subunit has a dominant role in determining divalent ion permeability of the receptor channel. Specifically, AMPA receptors containing the GluR2 subunit are characterized by low Ca\textsuperscript{2+} permeability, whereas those composed of GluR1, GluR3, and/or GluR4 are highly Ca\textsuperscript{2+} permeable. Recently, it has been hypothesized that a downregulation/loss of the GluR2 subunit may contribute to neurodegenerative changes associated with various pathologic conditions (3). In support of this hypothesis, a number of investigators have reported the selective loss of GluR2 mRNA and/or protein following brain ischemia (34, 36, 12), following epileptic seizures (37, 10, 38, 5), and in AD (4, 19, 42). Of importance, the loss of the GluR2 receptor subunit in vivo is functionally significant, resulting in an enhanced AMPA-evoked rise in intracellular Ca\textsuperscript{2+} (12). Alterations in the composition of AMPA receptors have also been demonstrated during development. For example, Pellegrini-Giampietro et al. (35) suggested that levels of Ca\textsuperscript{2+}-permeable AMPA receptors may be regulated by a developmental "switch," which turns GluR2 expression "on" or "off" during normal brain development. In support of this notion are studies which employed in situ hybridization techniques in rat brain and demonstrated that following early development, Ca\textsuperscript{2+}-permeable AMPA receptors are turned off (35). Whether additional switches occur during brain maturation and aging is unclear.

In our previous study, we demonstrated that cholinergic magnocellular neurons within the posterior nucleus basalis of Meynert (NBM) of aged humans are characterized by an abundance of GluR1 receptor subunits, whereas GluR2/3 subunits are sparse (18). Notably, in most if not all other brain regions GluR2/3 is predominantly expressed upon the soma of neurons, thus making NBM neurons unique with respect to their phenotypic expression of AMPA receptor subunits. It has been our hypothesis that a loss of the GluR2 receptor subunit, and consequently an enhanced Ca\textsuperscript{2+} flow through the AMPA receptor channels, may result in a dysequilibrium of intracellular Ca\textsuperscript{2+} homeostasis within aged NBM neurons, thus rendering them more vulnerable to excitotoxic or other pathologic insults. In the present study, we investigate the immunohistochemical localization of AMPA receptor subunits throughout the entire basal forebrain complex, including septal/diagonal band nuclei. In AD, it is has been documented in a number of reports that neurons in the posterior NBM are more vulnerable and undergo a greater extent of cell atrophy and death than neurons in the anterior NBM (9, 44, 30, 8, 41). In the present study, we sought to provide a molecular correlate to account for the regionally selective vulnerability of NBM neurons. Specifically, we investigated whether regions deficient in GluR2/3-positive neurons correspond to regions of increased vulnerability (i.e., neurons in the Ch4 region would express lower levels of GluR2/3 than neurons in the Ch1/Ch2 region). In addition, to further assess whether the paucity of GluR2/3-labeling observed in NBM of elderly individuals is an age-related phenotype or characteristic of these neurons throughout life, we examined the NBM of brains from young and middle-aged subjects.

**MATERIALS AND METHODS**

**Nomenclature.** Primate studies by Mesulam et al. (28) provided the basic nomenclature for cytoarchitectonic and histochemical descriptions of the human basal forebrain (40, 29). The cholinergic basal forebrain system is a complex anatomical structure comprising four distinct cell groups designated Ch1–4. The medial septal nucleus (Ch1) is located most rostrally, followed by the vertical (Ch2) and the horizontal (Ch3) limb of the diagonal band of Broca. The most posterior complex (Ch4) embodies magnocellular neurons within the NBM. The Ch4 includes the anterior portion, subdivided into medial (Ch4am) and lateral (Ch4al) parts, intermediate ventral (Ch4iv) and dorsal (Ch4id) subdivisions, and a posterior portion (Ch4p). Cholinergic neurons within the Ch4 project to cerebral cortex, whereas more anterior portions project mainly to the hippocampus (28).

**Human subjects.** Postmortem brain tissue was obtained from 13 nondemented subjects who were free of neurologic or psychiatric illness. They included 8 elderly subjects ranging in age from 66 to 102 years and 5 younger cases ages 48, 47, 36, 33, and 5 years. For case details see Table 1.

**Immunocytochemistry.** Tissue preparation and general immunocytochemistry procedures have been described in detail (16, 17). In brief, blocks of tissue containing the basal forebrain were cut in a coronal plane and fixed in 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB; pH 7.4) for 24–48 h at 4°C. Tissue blocks were subsequently cryoprotected in graded sucrose concentrations (10–30%) in PB for several days. Forty-micrometer-thick tissue sections were cut on a sliding microtome and stored in a cryoprotectant solution at −20°C. Sections were immunostained using polyclonal antibodies produced against synthetic peptides corresponding to sequences specific to the AMPA glutamate receptor subunits GluR1 and GluR2/3 (Chemicon, Temecula, CA). Methods used in the production and purification of these antibodies have been previously described (46). Free-floating tis-
sue sections were incubated overnight with primary antibodies (1:1000 dilution) at 4°C. Next day, sections were treated for 1 h with goat anti-rabbit biotinylated IgG diluted 1:200 in Tris-buffered saline with 1% goat serum and one additional hour with avidin–biotin peroxidase complex. The antigen–antibody reaction was visualized after treating sections with imidazole acetate buffer (IAB; pH 9.6) containing 0.05% diaminobenzidine, 2.5% nickel ammonium sulfate, and 0.005% hydrogen peroxide. Sections were rinsed in IAB (pH 7.4) and mounted from PB onto gelatinized glass slides, coverslipped, and examined with standard bright-field microscopy. As a control for nonspecific staining, sections were incubated either with initial incubation medium minus the primary antibody or with preimmune sera and were otherwise processed as described. In no instance were immunolabeled elements observed in tissue sections processed by the latter procedure. For the two primary antibodies near-adjacent sections were immunostained simultaneously in order to control for variability in staining. In addition, for each case at least one section was stained for Nissl substance to determine the cytoarchitectural boundaries of the basal forebrain according to the description by Saper and Chelimsky (40). Furthermore, representative sections were double-labeled using the GluR1 and antibodies against human nerve growth factor (NGF) receptor p75 (generously provided by Dr. Mark Bothwell) in order to confirm the cholinergic nature of NBM neurons. The double-labeling protocol using two different chromagens (diaminobenzidine and benzidine dihydrochloride, yielding a homogeneous brown and granular blue reaction product, respectively) has been previously described (19). In an effort to assess the extent to which cells were double-labeled with GluR1 and p75NGFr we examined representative sections from the Ch4 region and counted the number of GluR1-positive cells, p75NGFr-labeled neurons, and cells labeled by both antibodies. Due to the limited number of tissue sections available for this study it was not possible to employ stereological methods. Therefore, the numbers provided should not be viewed as the total number of single and/or double-labeled cells but rather as an approximation of the extent to which cells expressed one or both of the antibodies.

## RESULTS

**Double-immunolabeling of aged magnocellular basal forebrain nuclei using antibodies against GluR1 and p75NGFr.** Within the MBC, distinct cellular groups were easily delineated using antibodies against GluR1. Notably, the distribution and cellular morphology of these neurons were comparable to those of p75NGFr-positive cells as identified in this study as well as in previous investigations (30). In order to confirm the colocalization of GluR1 and p75NGFr within single neurons we performed double-immunolabeling experiments using antibodies against both receptor proteins. Clusters of double-labeled magnocellular neurons were observed in the septum/diagonal band area (Fig. 1) as well as in the three subdivisions of the NBM (Fig. 2). Within all subdivisions of the MBC the overwhelming majority of neurons were double-labeled (i.e., 94%). In contrast, cells labeled with either GluR1 or p75NGFr were nearly equal in number, with each accounting for approximately 3% of the neuronal population. Notably, cells single-labeled for GluR1 tended to be smaller than those labeled for p75NGFr or double-labeled for p75NGFr/GluR1. Although additional double-labeling studies are necessary to confirm the transmitter phenotype of these latter neurons, their relatively small size suggests that they may represent GABAergic interneurons. The present study also confirms that double-labeled cells were observed largely within the MBC, thus reflecting the relatively restricted distribu-

### TABLE 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Brain weight (grams)</th>
<th>PMI (hours)</th>
<th>Cause of death</th>
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<tr>
<td>nbm1</td>
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<td>5</td>
<td>1050</td>
<td>22</td>
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<td>1125</td>
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<td>M</td>
<td>36</td>
<td>1260</td>
<td>8</td>
<td>Cardiac failure</td>
</tr>
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<tr>
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<td>1175</td>
<td>19</td>
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<tr>
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<td>1.5</td>
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<td>1.40</td>
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tion of p75NGFr neurons within these latter regions. In contrast, GluR1-labeled neurons were more widely distributed and were observed in a number of nuclei outside of the MBC, including the striatum and globus pallidus (Fig. 4). While the vast majority of the neurons in the MBC were double-labeled, the intensity of immunolabeling varied considerably from neuron to neuron with a few neurons expressing low levels of immunoreactivity, the vast majority of cells moderate levels of staining, and a few cells high levels.

In the septal/diagonal band area double-labeled cells were abundantly observed (Fig. 1). These neurons displayed labeling for GluR1 and p75NGFr within the soma as well as proximal and distal processes. In the septal/diagonal band area we also observed a number of small, presumably GABAergic interneurons, labeled with GluR1 but not p75NGFr (Fig. 1).

Within more posterior aspects of the MBC, neurons double-labeled for GluR1 and p75NGFr were abundant (Fig. 2). In those portions containing the highest density of cells (i.e., Ch4a and Ch4i), we observed a surrounding network of fibers double-labeled with both antibodies (Figs. 2A and 2B). In the most caudal portion of the MBC double-labeled neurons were also observed although the density of cells was less than in the Ch4a and Ch4i (Fig. 2C).

Morphological characterization of GluR1-labeled neurons in the aged basal forebrain. Darkly stained neurons were observed in all divisions of the MBC (Figs. 3A, 3C, 3E, and 3G). Most rostrally, the vertical limb of Ch2 contained scattered immunoreactive neurons of diverse morphology, embedded in a dense fibrous network and merging ventrolaterally with a more densely populated horizontal portion (i.e., Ch3) of this nucleus (Fig. 3A). Neuronal perikarya were characterized by intense cytoplasmic staining, unstained nuclei, and multiple processes which were immunoreactive in proximal and distal portions (Fig. 3B). Within the Ch4a, we often observed two clusters of GluR1-positive neurons, medial and lateral, separated by blood vessels (Fig. 3C). The lateral portion showed the most dense cellular distribution of the whole basal forebrain (Fig. 3D). GluR1-positive magnocellular neurons appeared similar in this portion, having mainly an oval shape, with a dark cytoplasmic staining. This area was also characterized by the most dense matrix of GluR1-positive processes. In the Ch4i, GluR1-immunoreactive cells were also clustered in two main groups, one ventromedial, and the other dorsolateral adjacent to the anterior commissure (Fig. 3E). Numerous GluR1-positive cells were also seen along the margins of the anterior commissure. In this latter area, we observed populations of magnocellular neurons with either an elongated bipolar or an oval shape and with a considerably less dense network of GluR1-positive processes compared to more anterior subdivisions (Fig. 3F). In the Ch4p subdivision, at the level of the descending limb of the anterior commissure (Fig. 3G), we observed a population of GluR1-positive magnocellular neurons with staining characteristics comparable to those within the Ch4i (Fig. 3H).

Morphological characterization of GluR2/3-labeled neurons in the aged basal forebrain. As the result of relatively low levels of GluR2/3 immunostaining upon magnocellular basal forebrain neurons, it was difficult to delineate the boundaries of specific nuclei using this...
antibody (Figs. 4A, 4C, 4E, and 4G). At high magnification, it appeared that the intensity of immunolabeling was progressively diminishing in a rostrocaudal direction, although due to the relatively low levels of peroxidase reaction product it was not feasible to provide rankings of the staining intensity (Figs. 4B, 4D, 4F).

**FIG. 2.** A series of photomicrographs representing three main levels of the nucleus basalis: Ch4a (A), Ch4i (B), and Ch4p (C). Medial is to the left, dorsal is to the top. Shown on the left side are low-power photomicrographs of tissue sections double-immunolabeled with GluR1 (brown) and NGFr (granular blue) antibodies. Whereas the GluR1 immunostaining is widely distributed throughout the brain, NGFr-positive cells are restricted to the three subdivisions of the NBM (blue arrows). High-power photomicrographs containing double-labeled cells from corresponding low-power tissue sections are shown on the right. While almost all NBM neurons are double-labeled, there is a great variability with respect to the intensity of immunoreaction for the two antibodies, particularly NGFr. Scale bars, 1 cm for the low-power and 50 μm for the high-power photomicrographs.
FIG. 3. Low-power photomicrographs of coronal sections through the Ch2 (A), Ch4a (C), Ch4i (E), and Ch4p (G) of a 76-year-old female. Medial is to the left, dorsal is to the top. Tissue sections were immunostained with the GluR1 antibody. Darkly stained basal forebrain neurons easily demarcate each division. Large arrows point at the regions from which representative neurons are shown in the corresponding high-power photomicrographs (B, D, F, H). In the Ch2 region, vertically oriented cells merge into the more densely populated ventral portion of the diagonal band nucleus (A). GluR1-positive cells within this region show greatest morphologic diversity (B). Within the substantia innominata Ch2 neurons are scattered among NBM neurons of the medial Ch4a (small open arrow), whereas the densely populated lateral Ch4a (large arrow) is shown lateral to the blood vessels (C). A small solid arrow points to a group of immunoreactive cells which may correspond to Mesulam's Ch3. The lateral Ch4a contains mostly a homogeneous population of GluR1-positive NBM neurons embedded in a dense network of immunoreactive fibers (D). The Ch4i is divided into intermedioventral and intermediodorsal regions (E). Note that numerous displaced NBM neurons line the ventral and dorsal edge of the anterior commissure. More caudally, a group of neurons extends from the Ch4p (arrow) into the external medullary lamina that separates the putamen from the globus pallidus (G). Ventrally, a dense fibrous GluR1 immunoreactivity is found in the central nucleus of the amygdala. As shown in more anterior divisions, NBM neurons within the Ch4i and Ch4p show intense GluR1 immunoreactivity of their cell bodies and processes (F, H). Scale bar, 2 mm for low-power and 100 μm for high-power photomicrographs.
GluR2/3 immunostaining was most prominent in the Ch1/Ch2 area, where it labeled with a moderate intensity the vast majority of magnocellular neurons and a dense dendritic network (Figs. 4A and 4F, and 4H). Nevertheless, in this same region we also observed a number of cells displaying either very low levels of immunoreactivity or no immunoreactivity at all. Unlabeled neurons were observed most readily using No-
Distribution of GluR1- and GluR2/3-positive elements within structures adjacent to the NBM. In contrast to the NBM, the putamen was relatively free of GluR1 labeling except for some isolated cells displaying moderate labeling of their soma and most proximal dendrites (Fig. 5A). Rather, in the putamen we observed an abundance of GluR2/3-labeled cells (Fig. 5B). These latter cells were medium in size and characterized by a round unstained nucleus, surrounded by a darkly labeled cytoplasm. The neurons were multipolar, thus contributing to a dense matrix of GluR2/3 labeling. The globus pallidus contained numerous cells immunoreactive to GluR1 and GluR2/3. These cells had oval or polygonal somata with long thin processes. Whereas these neurons were lightly to moderately immunoreactive to GluR1 (Figs. 5C and 5E), they were intensely labeled with GluR2/3 antibodies, particularly in the internal globus pallidus (Figs. 5D and 5F). In the central nucleus of the amygdala, we observed a dense matrix of GluR1-labeled fibers within which were embedded a number of intensely stained cells (Fig. 5G). This nucleus also displayed an abundance of darkly GluR2/3-labeled neurons embedded in a network of moderately stained fibers (Fig. 5H).

GluR1 and GluR2/3 immunolabeling in the NBM of young cases. Due to the limited availability of tissue from young subjects, the distribution pattern of GluR1- and GluR2/3-immunoreactive elements was examined only in the Ch4a of the five younger cases. These cases on average showed a pattern of immunolabeling which was comparable with both antibodies. For example, in the 5-year-old case we observed an abundance of GluR1- and GluR2/3-labeled neurons (Figs. 6A and 6B). This case also displayed a moderate degree of neuropil staining. While a similar pattern of immunoreactivity was observed for the remaining four cases, the level of neuronal staining was notably higher for both GluR1 and GluR2/3 compared with the 5-year-old, while the neuropil appeared more lightly stained (Figs. 6C and 6D). This pattern of immunolabeling was distinct from aged cases in which neurons were darkly stained for GluR1 but either unstained or lightly stained for GluR2/3.

**DISCUSSION**

In our previous study, we described the distribution and cellular localization of AMPA receptor subunits GluR1 and GluR2/3 within the posterior NBM (i.e., Ch4p) of aged humans (18). The present report is based upon examination of not only a greater sample size of aged cases, but also the complete rostrocaudal extension of the MBC cholinergic system and the NBM of five younger cases.

In all the subdivisions of the aged MBC, GluR1-positive neurons were distributed in a pattern consistent with previously published descriptions of hyperchromic magnocellular neurons (13) and acetylcholinesterase- or choline acetyltransferase- (ChAT) positive cholinergic cells (40, 29) within the human MBC. Nissl counterstaining of GluR1-immunostained tissue sections demonstrated that within this region all hyperchromic magnocellular, presumably cholinergic, neurons were GluR1-positive. These results are corroborated by similar observations in the monkey (23). GluR1-positive magnocellular neurons were also observed extending into the internal and external medullary laminae and the anterior commissure. In addition, a number of smaller, presumably noncholinergic cells were GluR1 labeled. In contrast to studies in nonhuman primates, we were not able to clearly discern Ch1 from Ch2 neurons and therefore refer to the most rostral portion of the MBC as the Ch1/Ch2 region. In addition, as described by Hedreen et al. (13), the Ch3 nucleus is not readily identifiable in humans and therefore is not described as an isolated entity.

It has been previously shown that antibodies against the low-affinity p75NGF receptor provide an excellent marker of cholinergic neurons in the human MBC (30). In the present work, our double-immunolabeling studies demonstrated that virtually all p75NGF-positive cells were also GluR1-positive, thus providing us with an additional level of confidence concerning the cholinergic nature of GluR1-positive MBC neurons. Within all nuclear groups, GluR1- and p75NGFr-labeled neurons were similarly distributed. Likewise, both antibodies labeled neuronal somata as well as proximal and distal dendrites. In addition, throughout the neuropil GluR1- and p75NGFr-labeled fibers were observed. Often these fibers formed a dense network which surrounded labeled perikarya. Despite the fact that the vast majority of neurons in the MBC were double-labeled for GluR1 and p75NGFr, we also observed a small number of GluR1-positive cells within the MBC which appeared to lack p75NGFr immunoreactivity. That these latter observations reflect the true biology and are not the result of any technical issues is supported by previous studies of the human MBC.
which found that not all hyperchromic magnocellular neurons express NGF receptors (1) or stain positively for ChAT (29). In the present study, we do observe some variability in the intensity of p75NGFr-labeled neurons within the MBC of elderly subjects. At present it is unclear whether these findings represent a biologically significant downregulation of the receptor within a subpopulation of MBC neurons or reflect technical...
issues such as accessibility of the primary antibody to the antigenic site. In a recent report by Salehi et al. (39) the authors present in their Fig. 2 data supporting marked intersubject variation in the mean value of total area of p75 staining per cell. Notably, this intersubject variability could not be attributed to age, postmortem interval, fixation time, or pH of the cerebrospinal fluid (i.e., an indicator of agonal state). The authors, however, do observe a marked diminishment of p75 labeling in the MBN of AD patients compared with controls.

Using antibodies against GluR2/3/4c, Martin et al. (23) found that in the MBC of adult monkey the staining pattern of these latter subunits was less robust than for GluR1. Likewise, we observe in aged humans that GluR1 consistently and robustly labeled neurons and fibers while the intensity of GluR2/3 labeling was more variable, including many unstained magnocellular neurons. Of importance, numerous GluR2/3-positive neuronal bodies and fibers were regularly observed within adjacent limbic and cortical structures, supporting the notion that the absence of this staining in the MBC is not due to technical considerations. Within the MBC, the pattern of GluR2/3 immunolabeling varied among different nuclear groups. For example, the Ch1/Ch2 region was characterized by the most intensely labeled GluR2/3-positive perikarya and processes. In contrast, in the NBM and particularly the Ch4p region the overwhelming majority of magnocellular neurons were either lightly labeled or unstained. In interpreting the significance of these latter findings it is important to consider that a number of studies support the view that in AD (9, 44, 30) and in normal aging (8) the extent of cell loss and neuronal atrophy is much less in the anterior MBC compared to the posterior MBC. Although the majority of these latter studies did not use unbiased stereological methods to arrive at their respective conclusions they do, nevertheless, suggest that within the MBC there exists an anterior/posterior gradient with respect to neuronal vulnerability (i.e., neurons in the anterior MBC are less vulnerable than neurons in the posterior MBC). The present study suggests that underlying this selective vulnerability is the differential expression of various glutamate receptor subunits. For example, in the MBC of elderly individuals GluR1 was observed labeling neurons throughout all regions (i.e., Ch1–Ch4). In contrast, GluR2/3 labeling was observed largely in the anterior aspects of the

FIG. 6. High-power photomicrographs of adjacent tissue sections representing the Ch4i of a 5-year-old (A, B) and Ch4i of a 33-year-old (C, D). Tissue sections were immunostained with GluR1 (A, C) and GluR2/3 (B, D) antibodies. Note that NBM neurons are GluR1- and GluR2/3-positive. This staining pattern is in marked contrast to the NBM of elderly individuals within which neurons are largely GluR1-positive. Scale bar, 300 μm.
MBC with few if any labeled neurons in the posterior MBC. The presence of GluR1 and GluR2/3 upon magnocellular neurons in the anterior MBC but largely GluR1 in the posterior MBC suggests that neurons within the anterior and posterior MBC may respond differently following glutamate receptor activation. Specifically, the absence of the GluR2 subunit from AMPA receptors in the posterior MBC suggests that the ion channel of the assembled receptor is permeable to Ca$^{2+}$. In contrast, neurons in the anterior MBC likely express AMPA receptors assembled from the GluR1 and GluR2/3 receptor subunits and thus maintain a relatively low permeability to Ca$^{2+}$. The absence of the GluR2 subunit from the AMPA receptor and the increased flow of calcium through the ion channel have been hypothesized to contribute to neurodegenerative changes associated with various pathologic conditions including stroke (34, 36, 12), epileptic seizures (37, 10, 38, 5), and AD (4, 19, 42). That the destabilization of intracellular levels of Ca$^{2+}$ may contribute to the vulnerability of magnocellular neurons in the posterior MBC is supported by the work of Wu and Geula (48) who demonstrated an age-related reduction in calbindin D$_{28k}$ (CalBP) labeling within the MBC of elderly individuals. Moreover, the reduction in CalBP was greatest in the posterior MBC and least in the anterior MBC. CalBP is a member of the calcium binding proteins and is found in the MBC of primates but not rodents (15, 11). Functionally, calbindin acts to buffer excess Ca$^{2+}$ levels within neurons and thus protects cells against the deleterious effects of high concentrations of intracellular Ca$^{2+}$. That AMPA receptors in the posterior MBC may be more permeable to Ca$^{2+}$ compared to those in the anterior MBC together with a marked loss of CalBD in the posterior MBC and the resultant decrease in the cells ability to buffer intracellular calcium provide the first molecular correlate to account for the enhanced vulnerability of neurons in the posterior MBC in normal aging (8) and AD (9, 30, 44). Conversely, neurons in the anterior MBC may be less vulnerable because AMPA receptors are less permeable to calcium and they maintain a greater ability to buffer intercellular calcium.

Of significance, our study of different aged subjects suggests that the paucity of GluR2/3 staining, particularly in the posterior MBC, in the elderly may be the result of an age-dependent downregulation of this receptor subunit. For example, of those cases studied below the age of 50 each displayed robust GluR1 and GluR2/3 labeling of NBM neurons. Although our sample size was limited, it is of importance that in the younger subjects GluR2/3 immunoreactivity was consistently observed in the NBM (i.e., a region most severely affected in aging and AD). Although we were not able to examine the anterior aspects of the MBC in younger subjects, in interpreting our data it is important to bear in mind that the presence of at least some GluR2/3-labeled neurons in the anterior MBC of elderly patients supports their presence in younger cases. In this regard, it is also interesting that the loss of CalBD is likewise age-dependent, occurring largely in cases 70 years and older (48). Thus, not only do our data provide a molecular correlate to account for the increased vulnerability in the posterior MBC but they also provide a plausible explanation to account for a putative increased vulnerability with advancing age (8).

In apparent contradiction to our findings is an in situ hybridization study reporting that in aged human subjects (mean age 67.5 ± 12.4 years) NBM neurons expressed mRNAs for GluR1, GluR2, and GluR3 (43). While the latter study examined subjects ranging in age from 46 to 95 years, no mention was made as to whether any age-related differences in mRNA expression were observed. Moreover, the data presented in Fig. 2 of Tomiyama et al. (43) indicate that only the anterior portion of the NBM (Ch4a) was examined. As stated previously, we also observe staining for GluR1 and GluR2/3 within the Ch4a region, particularly in cases below 50 years of age.

While the precise mechanism regulating GluR2 expression remains to be determined, Pellegrini-Giampietro et al. (35) have suggested that neuronal GluR2 expression may be developmentally turned on or off, regulating levels of Ca$^{2+}$-permeable AMPA receptors. In support of this notion are studies demonstrating that in rat brain alterations in GluR2 gene expression early in development switched off Ca$^{2+}$-permeable AMPA receptors so that their number steadily declined with increasing age (35). Likewise, in the NBM it may be reasonable to consider whether with advancing age these receptors undergo a comparable switch, this time, however, resulting in an increase in Ca$^{2+}$-permeable AMPA receptors. At the present time additional research is needed to determine whether such events are tenable, including identification of those factors that may activate this switch and initiate the cascade of pathologic events leading to cell death.

When considering various pathologic events affecting the vulnerability of MBC neurons in AD it is important to recall the studies of Arendt et al. (2) suggesting that β-amyloid (Aβ) deposition, which is a hallmark pathologic event of AD, is often observed in the MBC, while immediately adjacent structures are far less affected (31). Contributing to these latter observations is the demonstration that in aged humans magnocellular cholinergic neurons express Aβ mRNA, an expression that is increased severalfold in AD (7). Notably, Aβ has been demonstrated in vitro to potentiate neuronal vulnerability to glutamate receptor-mediated activation (21, 25, 26). While the precise mechanism underlying this potentiation remains to be determined it is likely to involve the destabilization of intracellular Ca$^{2+}$ (25). In this regard, it is of particular interest that
MBC neurons may already be at risk for maintaining homeostatic levels of intracellular Ca\(^{2+}\). Thus, the contributors to neuronal vulnerability in the MBC may well be established long before the onset of AD and subsequently exacerbated with, for example, the deposition of A\(\beta\).

In summary, our data support an age-related decrease of GluR2/3, but not GluR1, immunolabeling in the MBC. Moreover, this decrease is most pronounced in the posterior MBC. While the age of onset for this decrease is, in all likelihood, substantial (i.e., spanning many years), it appears to begin in the fifth or sixth decade of life. That the decrease in the GluR2/3 subunit may lead to AMPA receptors with greater permeability to calcium provides one means whereby these cells may be more vulnerable following glutamate receptor activation. Moreover, the finding that these cells simultaneously appear to be losing their ability to buffer excess intracellular Ca\(^{2+}\) provides further insight into the molecular mechanism underlying the selective vulnerability of MBC neurons.

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